COMBINING ANTI-MIR-155 WITH CHEMOTHERAPY FOR THE TREATMENT OF LUNG CANCERS

Katrien Van Roosbroeck¹,a, Francesca Fanini²,a, Tetsuro Setoyama¹,a, Cristina Ivan³,⁴, Cristian Rodriguez-Aguayo¹,³, Enrique Fuentes-Mattei¹, Lianchun Xiao⁵, Ivan Vannini², Roxana S. Redis¹, Lucilla D’Abundo¹,⁶, Xinna Zhang³,⁴, Milena S. Nicoloso⁷, Simona Rossi¹, Vianey Gonzalez-Villasana¹,³,⁸, Rajesha Rupaimoole⁴, Manuela Ferracin⁶, Fortunato Morabito¹⁰, Antonino Neri¹¹, Peter P. Ruvolo¹², Vivian R. Ruvolo¹², Chad V. Pecot¹², Dino Amadori², Lynne Abruzzo¹⁴, Steliania Calin¹⁵, Xuemei Wang⁵, M. James You¹⁵, Alessandra Ferrajoli¹², Robert Orlowski¹⁶, William Plunkett¹, Tara M. Lichtenberg¹⁷, Ramana V. Davuluri¹⁸, Ioana Berindan-Neagoe¹⁹,²⁰, Massimo Negrini⁶, Ignacio I. Wistuba¹³,²¹, Hagop M. Kantarjian¹², Anil K. Sood³,⁴, Gabriel Lopez-Berestein¹,³, Michael J. Keating¹², Muller Fabbri²²,², George A. Calin¹,³,¹²,²

Affiliation
1 Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA
2 Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) S.r.l. IRCCS, Unit of Gene Therapy, Meldola (FC) 47014, Italy
3 Center for RNA Interference and Non-Coding RNAs, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA
4 Department of Gynecologic Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA
5 Department of Biostatistics, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA
6 Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara 44121, Italy
7 Division of Experimental Oncology 2, CRO, National Cancer Institute, Aviano 33081, Italy
8 Departamento de Biologia Celular y Genetica, Universidad Autonoma de Nuevo Leon, 66450 San Nicolas de los Garza, Nuevo Leon, Mexico
9 Department of Experimental, Diagnostic and Specialty Medicine – DIMES, University of Bologna, Bologna 40126, Italy
10 Department of Onco-Hematology, A.O. of Cosenza, Cosenza 87100, Italy
11 Department of Clinical Sciences and Community Health, University of Milano and Hematology, Ospedale Policlinico, Milano 20122, Italy
Keywords: microRNA, miR-155, TP53, lung cancer, leukemia

Financial Support: This work was supported in part by a Developmental Research Award by Leukemia SPORE P50 CA100632. Dr Calin is The Alan M. Gewirtz Leukemia & Lymphoma Society Scholar. Work in Dr. Calin’s laboratory is supported in part by the National Institutes of Health/National Cancer Institute (NIH/NCI) grants 1UH2TR00943-01 and 1 R01 CA182905-01, the UT MD Anderson Cancer Center SPORE in Melanoma grant from NCI (P50 CA093459), Aim at Melanoma Foundation and the Miriam and Jim Mulva research funds, the UT MD Anderson Cancer Center Brain SPORE (2P50CA127001), a Developmental Research award from Leukemia SPORE, a CLL Moonshot Flagship project, a 2015 Knowledge GAP MDACC grant, an Owens Foundation grant, and the Estate of C. G. Johnson, Jr., Dr. Fabbri is a St.
Baldrick Foundation’s Scholar and is supported by the Concern Foundation, Hyundai Hope of Wheels, STOP Cancer, Alex’s Lemonade, the William Lawrence & Blanche Hughes Foundation, the Jean Perkins Foundation, the Nautica Malibu Triathlon Funds, the award number P30CA014089 from the National Cancer Institute at the National Institutes of Health, the Hugh and Audy Lou Colvin Foundation, and by a Shirley McKernan donation. Dr. Van Roosbroeck was a Henri Benedictus Fellow of the King Baudouin Foundation and the Belgian American Education Foundation (B.A.E.F.). Dr. Berindan-Neagoe was partially financed by a POSCCE grant (709/2010) entitled Clinical and Economical Impact of Proteome and Transcriptome Molecular Profiling in Neoadjuvant Therapy of Triple Negative Breast Cancer (BREASTIMPACT). Drs. Negrini, Neri and Morabito are partially funded by Associazione Italiana per la Ricerca sul Cancro (the Italian Association for Cancer Research (AIRC), “Special Program Molecular Clinical Oncology - 5 per mille” n. 9980, 2010/15). Dr. Fortunato is also supported by the AIRC “Innovative immunotherapeutic treatments of human cancer” n.16695, 2015/18. Part of this work was also supported by National Cancer Institute at the National Institutes of Health (grant number U54 CA151668) and by the Betty Anne Asche Murray Distinguished Professorship (Dr. Sood).

Correspondence
George A. Calin, M.D., Ph.D., Departments of Experimental Therapeutics and Leukemia, Unit 1950, The University of Texas MD Anderson Cancer Center, 1881 East Road, Houston, TX 77054; Telephone: (713)-792-5461; Fax: (713)-745-4526; E-mail: gcalin@mdanderson.org

Muller Fabbri, M.D., Ph.D., Departments of Pediatrics and Molecular Microbiology & Immunology, Children’s Hospital Los Angeles, University of Southern California, 4650 Sunset Blvd, Mailstop # 57, Los Angeles, CA 90027; Telephone: (323)-361-8920; Fax: (323)-361-4902; E-mail: mfabbri@chla.usc.edu

Conflict of Interest Disclosure: The authors declare no competing financial interests.

Word Count: 6,430
Number of Figures and Tables: 4 Figures and 1 Table
Number of References: 66
ABSTRACT

Purpose
The oncogenic miR-155 is upregulated in many human cancers and its expression is increased in more aggressive and therapy resistant tumors, but the molecular mechanisms underlying miR-155-induced therapy resistance are not fully understood. The main objectives of this study were to determine the role of miR-155 in resistance to chemotherapy and to evaluate anti-miR-155 treatment to chemosensitize tumors.

Experimental Design
We performed in vitro studies on cell lines to investigate the role of miR-155 in therapy resistance. To assess the effects of miR-155 inhibition on chemoresistance, we used an in vivo orthotopic lung cancer model of athymic nude mice, which we treated with anti-miR-155 alone or in combination with chemotherapy. To analyze the association of miR-155 expression and the combination of miR-155 and TP53 expression with cancer survival, we studied 956 patients with lung cancer, chronic lymphocytic leukemia and acute lymphoblastic leukemia.

Results
We demonstrate that miR-155 induces resistance to multiple chemotherapeutic agents in vitro, and that downregulation of miR-155 successfully resensitizes tumors to chemotherapy in vivo. We show that anti-miR-155-DOPC can be considered non-toxic in vivo. We further demonstrate that miR-155 and TP53 are linked in a negative feedback mechanism, and demonstrate that a combination of high expression of miR-155 and low expression of TP53 is significantly associated with shorter survival in lung cancer.

Conclusions
Our findings support the existence of a miR-155/TP53 feedback loop, which is involved in resistance to chemotherapy and which can be specifically targeted to overcome drug resistance, an important cause of cancer-related death.
TRANSLATIONAL RELEVANCE

Resistance to therapy is an important issue in the treatment of cancer, responsible for many cancer-related deaths. Despite decades of research into overcoming this resistance, only modest advances have been made and the resistance mechanisms remain poorly understood. This is the first report of a miR-155/TP53 negative feedback mechanism, in which there is a direct targeting of TP53 by miR-155, and which is involved in the resistance to multiple chemotherapeutic drugs used in the treatment of lung cancer and leukemias. The finding that treatment with anti-miR-155 can reverse chemoresistance in vivo and that anti-miR-155-DOPC is not toxic in vivo supports a potential clinical use of anti-miR-155 therapy in human clinical trials of various cancer types as an addition to current chemotherapy regimens in order to overcome cancer-enacted resistance mechanisms.
INTRODUCTION

Resistance to therapy is the leading cause of failure to respond to chemotherapeutic drugs that leads to the high mortality in cancer (1, 2). Despite decades of research, only modest advances have been made in developing strategies to overcome resistance (3). The addition of non-coding RNAs (ncRNAs) to the ever-expanding set of genes deregulated in cancer (4, 5) offer the opportunity to deeper understand these mechanisms and the hope to eradicate chemoresistance. Non-small cell lung cancer (NSCLC) and chronic lymphocytic leukemia (CLL) are the most frequent adult solid and hematological malignancies in the Western world, respectively (2), and resistance to therapy is a very significant medical issue in these patients. Virtually all NSCLC patients will eventually develop resistance to the chemotherapeutic agents they are exposed to (6), and all CLL patients requiring treatment, including the standard of care chemotherapy-based fludarabine, cyclophosphamide and rituximab (FCR) treatment, are expected to relapse (7). The poorest prognosis CLL subgroup is characterized by deletions of chromosome 17p (del17p), the genomic locus of TP53, having an overall survival of less than 2 years (8, 9). The tumor suppressor gene TP53 is frequently deleted or mutated in human cancers and is involved in the development of drug resistance by cancer cells (10).

MicroRNAs (miRNAs) are small ncRNAs that regulate the expression of protein coding genes (11). MiR-155 is a well-known oncogenic miRNA, which is upregulated in a wide variety of human cancers (12, 13), especially in more aggressive and therapy resistant tumors (14, 15). For example, we identified a signature of deregulated miRNAs in patients with CLL and 17p deletion, versus patients with normal genotype, having good prognosis (16). In the 17p deletion group, miR-155 was the most upregulated miRNA (16). Moreover, we and others have demonstrated that miR-155 has prognostic significance in multiple types of tumors, including leukemia (17, 18) and lung cancer (19, 20).
Overexpression of miR-155 has been associated with drug resistance in several human cancers, including breast cancer, B-cell lymphoma and colon cancer (14, 21, 22), but the molecular mechanisms through which miR-155 increases cancer cell resistance to treatment are not fully understood. Therefore, the main objectives of this study were to determine the molecular mechanism through which miR-155 induces resistance to chemotherapy and to evaluate anti-miR-155 treatment to chemosensitize tumors. We demonstrate that overexpression of miR-155 induces resistance to chemotherapy, which can be reversed upon miR-155 inhibition. We show that anti-miR-155-DOPC does not induce adverse events and can be considered non-toxic in vivo. We further identify a miR-155/TP53 negative regulatory feedback loop, which affects the development of cancer drug resistance. The inverse expression correlation between miR-155 and TP53 transcripts is additionally supported by survival data from four lung cancer cohorts, in which we show that high expression of miR-155 and low expression of TP53 are associated with shorter survival, further confirming the involvement of miR-155 in TP53-mediated resistance mechanisms.

**MATERIALS AND METHODS**

**Patient samples**

The origin of all patient datasets is presented in Table 1. The total number of patients included in the survival analyses was 956. Both analyzed CLL subgroups were previously described: CLL-NEJM (23) and CLL-Clin Cancer Res (24). Clinical characteristics of both CLL cohorts are can be found in Supplementary Table S1.

Twenty-four NSCLC samples were collected at the Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Italy (NSCLC-Italy), 58 lung adenocarcinoma samples were collected at The University of Texas MD Anderson Cancer Center (lung adenocarcinoma-MDACC), and 52 ALL samples were collected at MDACC (ALL-MDACC).
Clinical characteristics of both lung cancer datasets and the ALL dataset can be found in Supplementary Tables S2 and S3, respectively. All patients provided written informed consent prior to inclusion in the study, and collection of the samples was approved by the institutional review board at each institution (IRST Srl IRCCS, and MDACC). All the work described has been carried out in accordance with the Declaration of Helsinki. In addition, the TCGA datasets for lung adeno-carcinoma (n = 343) and lung squamous cell carcinoma (n = 192) were downloaded from the data portal at https://tcga-data.nci.nih.gov/tcga (currently https://gdc.cancer.gov) and survival analysis was performed (Table 1).

Cell culture, transfection and treatment

Cell lines

Lung cancer cell lines A549, H460, H2009 and H1299, and leukemia cell lines REH and JM1 were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and cultured following the recommendations in the Product Information Sheet (ATCC). REH cells with TP53 knockdown (REH shp53) were generated by retroviral transduction with the gene-specific shRNA transfer vector pMKO.1 puro p53 shRNA 2 (plasmid 10672, Addgene, Cambridge, MA), as previously described (25). REH cells transfected with the empty vector pMKO.1 puro GFP shRNA (REH wt) were used as negative controls. Cell lines were authenticated via STR DNA fingerprinting and tested for mycoplasma contamination with the MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland) at the time they were cultured for the experiments performed in frame of this research.

miRNA mimics/inhibitor transfection

Transfections were performed with 100 nM of the precursor molecules (hsa-miR-155-5p pre-miRNA precursor or pre-miRNA precursor negative control #1), 200 nM of the miRVana inhibitors (miRVana hsa-miR-155 inhibitor and miRVana inhibitor negative control #1), and
Lipofectamine 2000 reagent or Lipofectamine RNAiMAX reagent, respectively (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. MiRNA transfection efficiencies were evaluated by qRT-PCR.

miR-155 lentivirus infection

pMIRNA1 – miR-155 and pMIRNA1 – Empty Vectors were obtained from System Biosciences (Mountain View, CA), and viral particles were produced according to the manufacturer’s instructions. A549, REH wt, REH shp53 and JM1 cells were infected with the miR-155 lentivirus with an efficiency of approximately 50% as determined by GFP measurement by flow cytometry. Empty lentivirus (LDEV, lentivirus empty vector) was used as a negative control for the experiments.

Drug treatment

Cisplatin (CDDP) was obtained from the oncology pharmacy of MDACC and S.r.l. IRCCS as an aqueous solution at a concentration of 1mg/ml corresponding to 3.3 mM. Exposures ranged from 0.01 to 10 µM. Doxorubicin (hydrochloride) was purchase as a powder from Sigma-Aldrich (St. Louis, MO) and resuspended in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to a stock concentration of 1.78 mM. Exposures ranged from 0.01 to 0.5 µM.

RNA extraction and quantitative real-time PCR

RNA was isolated with Trizol (Life Technologies), or with mirVana™ miRNA Isolation Kit (Ambion, Life Technologies), according to the manufacturer’s instructions. MiR-155 expression was analyzed with TaqMan miRNA Assays (Life Technologies). cDNA was synthesized using gene-specific stem-loop reverse transcription primer and the TaqMan microRNA reverse-transcription kit (Life Technologies). Real-Time qRT-PCR was carried out in an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Life Technologies) or on a Bio-
Rad CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories). Experiments were performed in triplicate and normalized to RNU44, U6 or RNU6B. Relative expression levels were calculated with the comparative Ct method (ΔΔCt).

**Protein extraction and Western blotting**

Protein extraction and Western blotting were performed as previously described (26, 27). The following primary antibodies were used: anti-Vinculin clone FB11 mouse monoclonal antibody (Biohit, Sartorius, Goettingen, Germany), anti-Human/Mouse/Rat p53 goat polyclonal antibody (R&D Systems, Minneapolis, MN), anti-p21WAF1 Ab-3 (Clone DCS-60.2) mouse monoclonal antibody (Lab Vision, Thermo Fisher Scientific), anti-p21 Waf1/Cip1 (DCS60) mouse monoclonal antibody (Cell Signaling) and anti-TP53DINP1 rabbit polyclonal antibody (OriGene, Rockville, MD). Western blots were quantified with Photoshop CS6 (Adobe, Mountain View, CA).

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) was performed with the EZ-ChIP Chromatin Immunoprecipitation kit (EMD Millipore, Billerica, MA) and the rabbit polyclonal antibody p53 (FL-393) (Santa Cruz Biotechnology) according to the manufacturer’s instructions. The TP53 binding sites in miR-155 (TP53 BS1/2 and TP53 BS3) were amplified with RedTaq DNA polymerase (Sigma-Aldrich) using the primers below, and analyzed on a 2% agarose gel.

1. **TP53 BS1/2 ChIP FW**
   5’ – GATCAAAAGATTCTCACCTGGG – 3’
2. **TP53 BS1/2 ChIP RV**
   5’ – ATCTGAACTACCTGGTCAGCTGT – 3’
3. **TP53 BS3 ChIP FW**
   5’ – AGCAGGGTAAATAACATCTGACAGC – 3’
4. **TP53 BS3 ChIP RV**
   5’ – CATATGGAGGAAGAACAGGCTTGG – 3’

**Luciferase assay and mutagenesis**
Identification of putative TP53 binding sites within a 10 kb genomic region surrounding miR-155 was performed by a TP53 binding site prediction program, which we developed earlier (28, 29). The identified TP53 binding site BS3 downstream of the miR-155 gene was cloned in the pGL4.23 (luc2/minP) vector (Promega, Madison, WI) with the following primers (underlined are the added restriction sites):

TP53 BS3 FW 5’ – GCGGTACCGGGAAACTGAAAGGCTATGAA – 3’
TP53 BS3 RV 5’ – GCGCTAGCCCATATGGAGGAAGAAAC – 3’

A549 cells were seeded at 50,000 cells/well in 24-well plates and co-transfected with the pGL4.23 vector containing the predicted TP53 binding site, the pCMV6-XL5-TP53 expressing vector (OriGene) and the pGL4.74 (hRluc/TK) vector containing renilla luciferase. Twenty-four hours after transfection, the samples were analyzed with the Dual-Luciferase Reporter Assay System (Promega) in a Glomax 96 Microplate Luminometer (Promega) as described in the manufacturer’s manual. Mutagenesis of the TP53 recognition sequence BS3 was performed with the QuickChange XL Site-Direct Mutagenesis Kit (Stratagene, Agilent Technologies, Santa Clara, CA), which deleted a portion of the TP53 consensus sequence, according to manufacturer’s instructions, and with the following primers:

TP53 BS3-DEL FW 5’ – CATATTTGAAATGTCTAGGTTCAAGTTCAATAGCTTAGCC – 3’
TP53 BS3-DEL RV 5’ – GGCTAAGCTATTGAACTTGAACCTAGACATTTCAAATATG – 3’

Identification of putative miR-155 binding sites in TP53 was performed with RNAhybrid (v2.2) (30). The identified miR-155 binding sites in TP53 (in coding sequence, BS-CDS; in 3'UTR, BS-UTR) were cloned in a pGL3 Control vector (Promega) with the following primers (underlined are the added restriction sites):

miR-155 BS-CDS FW 5’ – GGACTAGTCATGAGCGCTGCTCAGATAG – 3’
miR-155 BS-CDS RV 5’ – TCCCCGCGGGCCCATGCAGTAAGCCAAGA – 3’
miR-155 BS-UTR FW 5’ – GGACTAGTAAAGAAATCTCACCCCATC – 3’
miR-155 BS-UTR RV 5’ – TCCCCGCGGAAGGCTGACATGTAAGCCAAGA – 3’
H1299 and H460 were seeded and transfected as mentioned above. Luciferase assays and mutagenesis of the identified binding sites were carried out as mentioned above. The following primers, which deleted the miR-155 binding site, were used:

- **miR-155 BS-CDS-DEL FW**
  5’ – GGTCTGGCCCCTCCTCAGCATTTGCGTGTGGAGTATTTGG – 3’
  
- **miR-155 BS-CDS-DEL RV**
  5’ – CCAAATACTCCACACGCAAATGCTGAGGAGGGCCAGACC – 3’

- **miR-155 BS-UTR-DEL FW**
  5’ – GAGACTGGGTCTCGCTTTGTGATCTTGGCTTACTGCAGCC – 3’

- **miR-155 BS-UTR-DEL RV**
  5’ – GGCTGCAGTAAGCCAAGATCACAAAGCGAGACCCAGTCTC – 3’

**Drug resistance assays**

**MTT-based in vitro toxicology assay**

Five thousand cells treated with different drug concentrations were plated in a 96-well plate (4 replicates per condition). After 72 hours, the MTT-based *In Vitro* Toxicology Assay (Sigma-Aldrich) was carried out according to the manufacturer’s instructions. Proliferation was analyzed by measuring absorbance at 580 nm with a SpectraMax Plus MicroPlate Reader (Molecular Devices, Sunnyvale, CA).

**In vitro cell growth assays**

Twenty-four hours after transfection with anti-miR-155-5p precursor or negative control, A549 cells were treated with 5 µM CDDP for 6h followed by a 24, 48, 72 and 96h washout. JM1 stable clones (JM1-LVEV and JM1-LVEV) were treated with 1 µM CDDP for 6h followed by a 24, 48, 72 and 96h washout, while REH stable clones (REH wt-LVEV, REH wt-155LV, REH shp53-LVEV and REH shp53-155LV) were treated with 0.1 µM doxorubicin for 1h followed by a
24, 48, 72 and 96h washout. Cells were counted 24, 48, 72, and 96h after treatment with the Trypan Blue exclusion assay. Experiments were carried out in triplicates and minimum two independent experiments were performed.

In vitro proliferation assay

Twenty-four hours after transfection with hsa-miR-155 inhibitor or negative control, H2009 cells were treated with 10 µM CDDP for 6h followed by a 24, 48 and 72h washout. Cell proliferation was assessed by the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer’s instructions.

Clonogenic assay

A549 cell stably infected with empty lentivirus (A549-LVEV) or with miR-155 overexpressing lentivirus (A549-155LV) were untreated (negative control) or treated with 5 µM CDDP for 6h. Twenty-four hours after treatment, cells were trypsinized and 1,000 cells were plated in triplicates in 60 mm dishes. After 10 days, colonies were fixed with 80% methanol, stained with 0.25% 1,9-dimethyl-methylene blue in 50% ethanol (Sigma-Aldrich), and individual colonies were counted.

In vivo orthotopic mouse models

All mice used in this study were housed and maintained according to guidelines set by the American Association for Accreditation of Laboratory Animal Care and the US Public Health Service policy on Human Care and Use of Laboratory Animals. The mouse study was approved and supervised by The University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee, which adheres to the ARRIVE guidelines for in vivo experiments. The number of mice was determined based on previous experience with these kind of orthotopic mouse models (31-33), as well as on the power calculations that a group size of 10 would give
80% power to detect changes of 1.686 and 1.638 standard deviations or more in a single group when 5, respectively 4, groups were considered (α=0.05). Female athymic nude mice between 6 and 8 weeks of age and with a weight of 20-25 g were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD).

Orthotopic lung cancer model

Control anti-miR (mirVana miRNA inhibitor negative control #1; Life Technologies) or anti-miR-155 (hsa-miR-155 mirVana miRNA inhibitor; Life Technologies) was incorporated into DOPC nanoliposomes for in vivo delivery as previously described (34). The intrapulmonary injections of A549 cells stably infected with either lentivirus containing empty lentiviral vector (A549-LVEV) or with lentivirus containing a miR-155 overexpressing lentiviral vector (A549-155LV) (32). One week after injection, the mice were randomized in 4 (initial experiment) or 5 (second, independent experiment) groups and treatment with CDDP and/or nanoliposomes (either negative control or miR-155 inhibiting) was started. CDDP (160 μg/mouse) was administered i.p. once a week, while liposomal nanoparticles (200 μg/kg) were administered i.v. twice a week. The treatment schedules can be found in Figures 2A and Supplementary Figure S2A.

In the initial experiment, out of 40 mice initially injected with A549 cells, two died of surgery and one died of CDDP toxicity three weeks after start of treatment. In the second, independent experiment, out of 50 mice initially injected with A549 cells, six died of surgery and two died of CDDP toxicity 3-4 weeks after start of treatment. After four, respectively five, weeks of treatment, the mice were sacrificed and analyzed macroscopically as previously described (32).

MiR-155 expression in tissue sections was analyzed by in situ hybridization as previously described (31). Double-digoxigenin (DIG)-labeled locked nucleic acid (LNA) probes for miR-155 (Exiqon, Copenhagen, Denmark) were used.
Cell proliferation, angiogenesis and microvesicle density, and apoptosis were assessed by Ki-67 or CD31 immunostaining, or with the TUNEL assay as previously described (31, 35). Ki-67, CD31 and TUNEL positive cells were counted in three random fields per slide and five slides per group were analyzed at 200x magnification.

The expression of TP53 was determined by immunohistochemical analysis using freshly cut frozen mice tissue. The slides were fixed in cold acetone/acetone + chloroform 1:1/acetone, and blocked with cold-water fish skin gelatin 4% (Electron Microscopy Sciences, Hatfield, PA) in PBS. Slides were incubated overnight at 4°C with primary antibody anti-Tp53 (Cell Signaling), washed with PBS, incubated with the goat anti-rabbit Alexa 594 secondary antibody (The Jackson Laboratory, Bar Harbor, ME), washed and counterstained with Hoechst. The expression of TP53 was counted in three random fields per slide (one slide per mouse, 5 slides per group) at 200x magnification.

**In vivo toxicology assessment of anti-miR-155**

Male CD-1® IGS mice with a weight of 35-40 g were purchased from Charles Rivers and randomized into two groups (anti-miR-NC and anti-miR-155; n=17/group). Liposomal control anti-miR-DOPC (anti-miR-NC-DOPC) and anti-miR-155-DOPC nanoparticles were injected into the respective mice i.v. via tail vein injection at a concentration of 200 µg/kg of body weight. Body weight was measured before and after treatment and was not significantly different between both groups. After 72 hours of treatment, mice were euthanized by exsanguination following IACUC approved protocols. Blood samples and tissues (fixed and embedded in paraffin) were collected at necropsy for further analyses. Blood samples were processed for blood chemistry and hematology. Blood chemistry analyses included blood urea nitrogen content (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine and lactic dehydrogenase (LDH), and were evaluated on an Integra 400 Plus analyzer (Roche Diagnostics, Risch-Rotkreuz, Switzerland). Hematology
analyses consisted of complete blood count (white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin, hematocrit, average volume of RBC (mean corpuscular volume or MCV), average amount of hemoglobin in one RBC (mean corpuscular hemoglobin or MCH), average concentration of hemoglobin in one RBC (MCH concentration or MCHC), red cell distribution width (RDW), platelet and mean platelet volume (MPV)), as well as white blood cells differential count (levels of segmented neutrophils, lymphocytes, monocytes, eosinophils, basophils and large unstained cells (LUC)), and were evaluated on an Avida 120 Hematology System (Siemens Healthineers, Erlangen, Germany). Paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E) for routine histopathology.

To assess serum cytokine levels, mice were treated with single i.v. injections of either anti-miR-NC-DOPC (n=10) or anti-miR-155-DOPC (n=10). Seventy-two hours after injection, serum was collected using cardiac puncture and analyzed with a Luminex assay (MCYTOMAG-70K-PMX/MULTIPLEX map mouse cytokine/chemokine magnetic bead panel, EMD Millipore) detecting 25 pro-inflammatory cytokines (G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, MCP-1, MIP-1α, MIP-1β, MIP-2, RANTES, and TNF-α) using a Luminex 100 system (Luminex, Austin, TX), as previously described (36).

Integrated function and pathway analysis

We retrieved experimentally validated miR-155 targets from the following 4 databases: miRTarBase (http://mirtarbase.mbc.nctu.edu.tw), TarBase (http://diana.imis.athena-innovation.gr/DianaTools/), miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) and miRecords (http://c1.accurascience.com/miRecords/). We restricted ourselves to those targets that were supported by strong experimental evidence, such as reporter assay, Western blot, quantitative PCR and immunoprecipitation. Of the 248 miR-155 targets that were identified, integrated function and pathway analysis was performed using DAVID bioinformatics resources.
We imposed a cut-off of 10% FDR to indicate a statistically significant association between a pathway and the list of mRNA targets of miR-155. The p-value and false discovery rate were generated by a modified Fisher Exact test.

**TCGA data analysis**

Input data were downloaded from the publicly available data portal of The Cancer Genome Atlas Project (TCGA) at https://tcga-data.nci.nih.gov/tcga. Level 3 Illumina RNA-Seq and miRNA-Seq were used for the analysis of mRNA and miRNA expression, respectively. For miRNA-Seq data, we derived the “reads_per_million_miRNA_mapped” values for mature forms of each microRNA from the “isoform_quantification” files. Patient samples with survival data of 0 “days_to_last_follow_up” were excluded. Data for somatic mutations of TP53 in TCGA samples were downloaded from the cBio Portal at http://www.cbioportal.org/public-portal/.

**Statistics**

All patient-related analyses were carried out in the R statistical environment, version 3.0. Survival analyses were performed as previously described (31) with some modifications. Briefly, for each cohort, a relationship between miR-155/TP53 expression and overall survival was assessed as follows. Patients were grouped into percentiles according to miR-155 and TP53 expression. The log-rank test was employed to determine the association between miRNA/mRNA expression and survival. The Kaplan-Meyer method was used to generate survival curves. The p-value and the cut-off to optimally separate the patients in high and low (min p-value) miR-155 and TP53 were recorded. We then considered whether combining inverse expression of miR-155 and TP53 would associate with survival. We used the following procedure. A fixed cut-off for miR-155 together with a fixed cut-off for TP53 splits the cohort in four groups corresponding to low or high miR-155 and low or high TP53 expression. For each pair of cut-offs we contrasted the two groups linked to a negative
association: tumors with high levels of miR-155 and low levels of TP53 versus tumors with low
levels of miR-155 and high levels of TP53. We recorded the best separation obtained (min p-
value) for the pair and noticed that the difference in median survival time between the
two groups contrasted is significantly larger than the difference between the groups classified
into high/low based on the expression of miR-155 or TP53 alone. The relationship between
survival and covariates (miR-155 and TP53 expression levels and available prognostic factors
or other clinical parameters) was examined using a Cox proportional hazard model.

For lung adenocarcinoma cases with miR-155 expression, TP53 mutational status and
survival information available, we checked for a relationship between miR-155 expression, TP53
expression and overall survival in patients with wild-type TP53 and mutated TP53 in a similar
manner as described above. According to the TP53 mutational status, patients were divided into
two groups: (i) those expressing wild-type TP53 (unmutated) or harboring TP53 mutations not
affecting its protein function (according to the IARC TP53 database p53.iarc.fr), and (ii) those
harboring TP53 mutations that affect TP53 protein function (according to the IARC TP53
database p53.iarc.fr). For each group, Kaplan-Meier overall survival curves were generated for
high vs. low miR-155, and high miR-155 and low TP53 vs. low miR-155 and high TP53.

Statistical analysis of the in vitro and in vivo data was carried out with GraphPad Prism 6
software. To verify whether data followed a normal distribution, the Shapiro-Wilk normality test
was performed, and an unpaired t-test (normal distribution) or non-parametric Mann-Whitney-
Wilcoxon test (non-normal distribution) was applied to determine P-values. All tests were two-
sided and P-values <0.05 were considered statistically significant. Statistical significances are
presented as * according to the following scheme: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****,
P < 0.0001.
RESULTS

MiR-155 induces chemoresistance in vitro

We treated three different lung cancer and leukemia cell lines with endogenous levels of miR-155 expression and after miR-155 overexpression (either by miR-155 precursor or miR-155 lentivirus) with chemotherapeutic agents commonly used to treat patients: the lung cancer cell line A549 with cisplatin (CDDP, cis-diamminodichloroplatinum) (6, 37), the acute lymphoblastic leukemia (ALL) cell line REH with doxorubicin (38) and the immunoblastic B-cell leukemia/lymphoma cell line JM1 with CDDP (39). As shown in Figure 1A-B, A549, REH and JM1 cells overexpressing miR-155 showed a significantly better viability and displayed significantly lower chemosensitivity when undergoing treatment with CDDP or doxorubicin than cells expressing normal levels of miR-155. In addition, we performed a clonogenic assay for A549 cells stably overexpressing miR-155 (A549-155LV) and treated with CDDP vs. control cells (A549-LVEV) treated with CDDP. We observed a significant increase in the number of colonies when miR-155 was overexpressed, further demonstrating the chemoresistance induced by miR-155 (Figure 1C). Moreover, when we treated the H2009 lung cancer cell line with miR-155 inhibitor and CDDP, we found that these cells grew significantly less than H2009 cells treated with negative control inhibitor and CDDP (Figure 1D). Of note, when TP53 expression was abolished in REH cells by shRNA treatment, the protective effect to chemotherapeutic agents in cells overexpressing miR-155 disappeared (Figure 1A, middle panel and Figure 1B, middle panel). Finally, no difference in chemosensitivity was observed after fludarabine treatment and miR-155 overexpression in MEC1 and MEC2 cell lines, both of which carry a deletion of the TP53 locus (40) (Supplementary Figure S1). Altogether, these data suggest a role of miR-155 in drug resistance in various types of cancer, including lung cancer and leukemia, for multiple types of chemotherapy.
MiR-155-induced chemoresistance can be reversed *in vivo* by treatment with anti-miR-155-DOPC.

To evaluate the *in vivo* involvement of miR-155 in therapy resistance, we established an orthotopic lung cancer mouse model by intrapulmonary injection of A549-LVEV (control) cells or with A549-155LV (miR-155 overexpressing) cells. Two independent experiments were carried out with four (Supplementary Figure S2) and five (Figure 2) treatment groups, respectively, in which mice were treated with negative control anti-miR (anti-miR-NC) or with anti-miR-155 alone or in combination with CDDP, according to the schedule in Figure 2A and Supplementary Figure S2A. Mice injected with A549-LVEV cells and treated with CDDP and anti-miR-NC showed a decrease in number of tumors, reduced primary tumor size and a reduced aggregate mass of metastases when compared to untreated mice injected with A549-LVEV cells, although this decrease was not significant, indicating that these tumors are sensitive to CDDP, as was expected (Figure 2B-C and Supplementary Figure S2B-D). When miR-155 was overexpressed (through injection of A549-155LV cells), the tumors became resistant to CDDP treatment and the administration of anti-miR-155 alone significantly reduced number of tumors, tumor size and aggregate mass of metastases (Figure 2B-C). In addition, when anti-miR-155 was combined with CDDP treatment, the chemotherapy resistance was almost completely reversed (Figure 2B-C and Supplementary Figure S2B-D). *In situ* hybridization for miR-155 showed an increase of miR-155 expression in miR-155 overexpressing tumors treated with CDDP and anti-miR-NC, and miR-155 levels comparable to or lower than A549-LVEV tumors when miR-155 overexpressing tumors were treated with anti-miR-155 alone or in combination with CDDP (Figure 2D and Supplementary Figure S2E). Immunohistochemistry for Ki-67 (apoptosis), CD31 (angiogenesis) and the TUNEL (apoptosis) assay suggested that miR-155, even in the presence of CDDP, is able to induce cell proliferation and angiogenesis, and reduce apoptosis, effects that are completely abolished when miR-155 is inhibited (Figure 2E and Supplementary Figure S2F). Although treatment
with anti-miR-155 alone resulted in a significant decrease in proliferation and angiogenesis, and
increase in apoptosis, the effects are even more pronounced when anti-miR-155 is combined
with CDDP therapy (Figure 2E). Therefore, the \textit{in vivo} reversion of chemoresistance by anti-
miR-155 administration is consistent and reproducible by independent sets of experiments.

\textbf{Anti-miR-155-DOPC does not induce toxic effects \textit{in vivo}}

To assess the \textit{in vivo} toxicity effects of anti-miR-155-DOPC, we evaluated blood
chemistry, hematology, cytokine production and general histology in mice injected with a single
dose of either anti-miR-NC-DOPC or anti-miR-155-DOPC (Figure 3). Blood chemistry analyses
for metabolites to assess overall tissue damage (Figure 3A) and complete hematology
investigation of WBC, RBC and platelets (Figure 3B-C) showed no significant differences
between both groups, except for the platelet count, which was marginally significantly higher in
anti-miR-155-DOPC treated mice (p=0.0464, Figure 3B). We further performed a cytokine
assay detecting 25 pro-inflammatory cytokines in the serum of mice injected with either anti-
miR-NC-DOPC or anti-miR-155-DOPC. With the exception of IL-12 (p40), IL-17, MIP-1α and
MIP-1β, which showed marginally statistically significant differences, no activation of the
immune system was observed (Figure 3D). Finally, H&E histological analysis showed no
inflammatory changes in brain, heart, kidney, liver, lung and spleen in any of the groups (Figure
3E). We previously demonstrated that DOPC liposomal nanoparticles are not toxic \textit{in vivo} for
doses up to 20 mg/kg for 5 consecutive days (41). These data suggest that the therapeutic
effects observed in our \textit{in vivo} orthotopic mouse model are likely caused by targeting of miR-
155, rather than immune induction, and that anti-miR-155-DOPC can be considered non-toxic in
mice.
Identification of a miR-155/TP53 negative feedback loop

miR-155 is significantly overexpressed in patients with CLL and deletion of 17p, where the genomic TP53 locus resides (16), suggesting that TP53 might suppress the expression of miR-155. To assess this hypothesis, we performed chromatin immunoprecipitation (ChIP) for TP53 in the wild-type ALL cell line REH (REH wt) and showed that TP53 binds to one of three predicted binding sites (BS3) downstream of miR-155 (Figure 4A-B). A luciferase reporter assay for BS3 confirmed that TP53 inhibits the expression of miR-155 through direct binding in the region downstream of miR-155 (Figure 4C). The silencing effect was abrogated when BS3 was mutated, further confirming a direct binding of TP53 to BS3 (Figure 4C). To determine whether miR-155 is involved in a feedback loop, we checked whether overexpression of miR-155 affected TP53 expression. We transfected TP53 wild-type (wt) A549 and H460 cells (42) with miR-155 and observed reduced expression of TP53 protein, as well as of the known miR-155 target TP53INP1 (43, 44) and p21 (Figure 4D). When downregulating miR-155 in the H2009 lung cancer cell line harboring a mutation in TP53 that does not affect the miR-155 binding sites, we observed increased TP53 and p21 protein expression (Figure 4E). A luciferase reporter assay in the TP53 null cell line H1299 for two identified miR-155 binding sites in the 3' untranslated region (3' UTR) of TP53 mRNA (BS-UTR) (Figure 4F) and in the TP53 coding sequence (BS-CDS), respectively, showed a direct binding of miR-155 to BS-UTR (Figure 4G) but not to BS-CDS (data not shown). The silencing effect was abolished when BS-UTR was mutated (Figure 4G), indicating a direct binding of miR-155 to the 3' UTR of TP53. Similar experiments in the TP53 wild-type cell line H460 showed a reduction in luciferase activity as well (Figure 4H). Finally, to assess the effects of miR-155 overexpression on TP53 expression in vivo, we performed TP53 immunostaining on the mouse tumors, and observed decrease in TP53 expression when miR-155 was overexpressed. Treatment with anti-miR-155 alone did not significantly affect TP53 expression, but a combination of anti-miR-155 with CDDP...
resulted in a significant increase of TP53 expression (Figure 2E and Supplementary Figure S2F). Altogether, these in vitro and in vivo data demonstrate a negative feedback loop between miR-155 and TP53, which is involved in resistance to chemotherapy.

To understand the biological significance of the newly identified miR-155/TP53 feedback loop, and to determine how our findings fit in with other known functions and targets of miR-155, we performed integrated function and pathway analysis on 248 experimentally validated miR-155 target genes. Thirteen pathways (Supplementary Table S4) were significantly (p<0.01 and FDR<10%) enriched, the majority of which were related to cancer (pathways in cancer, colorectal cancer, pancreatic cancer), cell growth and death (cell cycle, apoptosis), as well as signal transduction pathways often deregulated in cancer and involved in drug resistance (Wnt signaling pathway, TGF-β signaling pathway, signaling by BMP, signaling by NGF). These pathways closely relate to the roles of miR-155 as an oncogene (45), TP53 as tumor suppressor and apoptosis inducer (10), and our novel findings of a miR-155/TP53 negative feedback loop involved in resistance to therapy.

High expression of miR-155 and low expression of TP53 are correlated with survival

MiR-155 was found to have prognostic impact in patients with various types of cancer (19) including lung cancer (20), leukemia (17, 18), breast cancer (46), renal cell carcinoma (47), glioma (48), colorectal cancer (49) and gallbladder carcinoma (50). We additionally assessed the correlation of miR-155 with survival in two independent and already published CLL cohorts (CLL-NEJM (23) and CLL-Clin Cancer Res (24)), in a new ALL cohort (ALL-MDACC), and in four lung cancer datasets (2 new cohorts, NSCLC-Italy and lung adenocarcinoma-MDACC, and the TCGA cohorts for lung adenocarcinoma and squamous cell carcinoma). To our surprise, we only found a correlation between high expression of miR-155 in the leukemia datasets (Supplementary Figure S3), but not in any of the lung cancer cohorts (Table 1). We previously
showed that a combination of miR-520d-3p and its target EphA2 is a better prognostic factor for ovarian cancer than each gene by itself (31). To investigate whether this is also the case for our newly identified miR-155/TP53 negative feedback loop, we associated miR-155 and TP53 transcript expression with overall survival (OS) and time-to-progression (TTP) in four sets of lung cancer (Table 1). We used OS as a measure of resistance to therapy. In all cohorts, we found a significant decrease in survival when miR-155 expression was high and TP53 mRNA expression was low. This was only true for TP53 mRNA, as no significant associations could be observed in the TCGA lung cancer datasets between miR-155/TP53 protein expression and survival. Unfortunately, no TP53 expression data were available for any of the CLL and ALL datasets.

When TP53 mutation status was considered in the lung adenocarcinoma – TCGA subset, only in cases with unmutated (wild-type) TP53 or with TP53 mutations not affecting its function, high miR-155 expression (Supplementary Figure S4A-B), as well as a combination of high miR-155 and low TP53 expression (Supplementary Figure S4C-D), was significantly associated with shorter OS. Since all tumors in the NSCLC-Italy dataset were selected for having unmutated TP53, the same can be concluded for this dataset. Unfortunately, for the lung adenocarcinoma-MDACC and lung squamous cell carcinoma-TCGA datasets, too few patients were left to perform this analysis and get a reliable significance.

Uni- and multivariate analyses containing the miR-155 and TP53 expression data, several known prognostic factors and available clinical parameters (Supplementary Table S5), as well as hazard ratio (HR) calculations using the estimated parameters from the Cox models (Supplementary Table S6), confirmed that high miR-155 and low TP53 mRNA expression or high miR-155 expression (when no TP53 expression data were available) were independently associated with survival in most datasets (Supplementary Tables S5 and S6). This co-occurrence of high miR-155 expression with low TP53 mRNA expression appears to be
important for predicting survival, as in all analyzed lung cancer datasets, miR-155 expression
and TP53 mRNA expression by itself were not sufficient to be associated with survival.
Interestingly, for the leukemia datasets (in which miR-155 expression alone was significantly
associated with survival), when considering miR-155 as a continuous variable in the univariate
analyses, the significance is lost for all cohorts, except CLL-Clin Cancer Res (Supplementary
Table S5). This further supports our concept that a combination of both miR-155 and TP53
expression represents a better marker to predict survival.

DISCUSSION

Here, we showed for the first time that TP53 and miR-155 are linked in a new feedback
mechanism. Besides miR-155, TP53 has been found to be involved in other miRNA regulatory
loops, for example a regulatory feedback loop between p53, miR-329/300/381/655 and PTTG1
in pituitary tumors (51), a positive feedback loop between miR-192, MDM2 and TP53 in breast
cancer (52), and a feed-forward loop involving miR-17/20a, DAPK3 and TP53 (53). In addition,
TP53 is regulated through many other mechanisms, of which the most important is MDM2,
which blocks the transcriptional activity of TP53 and mediates its ubiquitylation and proteosomal
degradation. In turn, TP53 transactivates MDM2 expression to maintain or increase the levels of
MDM2 as is appropriate (54). Furthermore, TP53 has two family members, TP63 and TP73,
which share significant homology with TP53 and which have several common targets as well as
similar tumor suppressive activities as TP53. All three TP53 family members have been found to
be involved in chemoresistance (reviewed in (55-57)). Moreover, although not yet validated,
mir-155 has predicted target sites in TP63, TP73 and MDM2 as well (miRWalk2.0). This
suggests that the actual involvement of mir-155 in chemoresistance is most likely far more
complicated than the simple mir-155/TP53 feedback mechanism we describe here. To which
extent MDM2 and the TP53 family members TP63 and TP73 are involved in miR-155-mediated chemoresistance warrants further investigation.

We further demonstrated that the miR-155/TP53 feedback loop is involved in resistance to multiple chemotherapeutic drugs used in treatment combinations in lung cancer (6) and leukemia (38, 58). Through miR-155 downregulation in vivo, we successfully resensitized the tumors to chemotherapy, and therefore, this miR-155/TP53 interactor loop could be exploited for miRNA-based therapeutic interventions in cancer patients (59, 60). Others have shown that LNA-based and nanoparticle-based inhibition of miR-155 decreases tumor growth in mouse models of Waldenstrom macroglobulinemia and lymphoma, respectively (61-63). In addition, a recent publication showed that knockdown of miR-155 in the doxorubicin-resistant cell line A549/dox reversed doxorubicin resistance and restored doxorubicin-induced apoptosis and cell cycle arrest, most likely through downregulation of multidrug resistance genes (MDR1 and MRP1) and the breast cancer resistance protein gene (BCRP) (64), further supporting that miR-155 might be a good target in chemosensitization of tumors.

Our in vivo toxicology studies did not uncover any adverse effects of anti-miR-155-DOPC in mice. These findings are important, especially in light of the recent early termination of the Phase I clinical study of MRX34, the first miRNA-based therapy to be evaluated in clinical trials for the treatment of human cancers, due to multiple immune-related severe adverse events observed in patients receiving MRX34 (ClinicalTrial.gov Identifier NCT01829971). Our approach is to combine chemotherapy with targeted anti-miR-155 therapy, which will significantly reduce the risks of adverse events, since lower doses of both drugs will need to be used to achieve clinical responses. In addition, the used carrier molecule, DOPC liposomal nanoparticles, is currently being tested in a Phase I clinical trial (ClinicalTrial.gov Identifier NCT01591356), and so far, no adverse events have been associated with the treatment. This suggests that treatment with anti-miR-155-DOPC will most likely be safe and well tolerated.
However, further systematic preclinical safety studies for anti-miR-155-DOPC in large animals are needed before its clinical value can be evaluated.

When we took the *TP53* mutational status into consideration for the survival analysis of the lung adenocarcinoma-TCGA cohort, we observed that miR-155 and the combination of miR-155 and *TP53* are significantly associated with shorter OS, only in cases with unmutated *TP53* or *TP53* mutations not affecting its function. Similar conclusions could be drawn from the NSCLC-Italy cohort, since all patients were selected for unmutated *TP53* status. In addition, we showed that overexpression of miR-155 in MEC1 and MEC2 cell lines (both carrying a deletion of the *TP53* locus) does not induce chemoresistance to fludarabine treatment (Supplementary Figure S1), suggesting that there is a difference in response in the context of wild-type and mutant *TP53* alleles. However, as the current data are very limited, further investigation is needed to assess the role of mutant *TP53* vs. wild-type *TP53* in the newly identified miR-155/*TP53* feedback loop.

In contrast with most of the literature (meta-analyses in (19, 20, 65)), we found that in most of the analyzed cancer datasets, miR-155 expression and *TP53* mRNA expression by itself were not sufficient to be associated with OS (Table 1). In fact, significant correlations between miR-155 and survival could only be found in the leukemia cohorts. In addition, a recent meta-analysis evaluating miR-155 as a prognostic factor for survival in 1,557 NSCLC patients from 6 different studies suggested that high expression levels of miR-155 alone may not be significantly related to lung cancer prognosis, except for Asian and American patients (66). Our data further support the importance to consider miRNA (miR-155) and target mRNA (*TP53*) to predict survival. Actually, when combined, we found that high miR-155 and low *TP53* expression significantly correlated with survival in 4 independent lung cancer datasets (Table 1), and that this combination remained independently associated with survival in the datasets analyzed in a multivariate analysis (Supplementary Table S5 and S6). We recently
demonstrated that a combination of miR-520d-3p and its target EphA2 is a better prognostic factor for ovarian cancer than each gene by itself, and that simultaneous targeting of miRNA/mRNA (miR-520d-3p/EphA2) results in a remarkable therapeutic synergy as compared to either monotherapy (31).

In conclusion, our study is innovative due to multiple reasons. We show for the first time that the most frequently altered human tumor suppressor TP53 is directly targeted by one of the most oncogenic miRNAs, miR-155, and that TP53 directly regulates the expression of this miRNA as a feedback loop. Second, a combination of TP53 and miR-155 expression seems to be a much better classifier for overall survival of lung cancer and possibly also leukemia, than miR-155 alone. Third, miR-155 and TP53 and their downstream targets are involved in resistance to multiple types of chemotherapeutic regimens in various histotypes. Finally, we propose to use anti-miR-155 as an additive to chemotherapy and not as a single agent, as was proposed by others (61-63). This means lower doses of drugs to be used and, consequently, less adverse reactions to occur in clinical trials. The identification of the miR-155/TP53 interaction will favor the advancement of new anti-miR-155 targeted therapies to overcome the development of drug resistance.

ACKNOWLEDGMENTS

The authors would like to thank Drs. Evan N Cohen and James M Ruben (The University of Texas MD Anderson Cancer Center) for their assistance with the cytokine assay, and A. Gordon Robertson (Canada’s Michael Smith Genome Sciences Center) for his assistance with the data collection of the TCGA datasets. We further acknowledge the support of the RNAi and non-coding RNA Center of the UT MD Anderson Cancer Center.
AUTHORSHIP CONTRIBUTIONS

Conceived and designed the experiments: KVR, FF, CVP, MJY, AF, RVD, IIW, AKS, GL, MF and GAC.

Performed the experiments: KVR, FF, TS, CR, EFM, IV, RSR, LD, XZ, VG, RR, FM, AKS.

Analyzed and interpreted the data: KVR, FF, TS, CI, CR, EFM, IV, RSR, LD, XZ, MSN, SR, VG, RR, MF, FM, AN, DA, SC, LX, IB, MN, HMK, AKS, GL, MJK, MF and GAC.

Contributed reagents/materials/analysis tools: PPR, VVR, LA, RO, WP and TML.

Wrote the first draft of the manuscript: KVR, FF, MF and GAC.

Contributed to the writing of the manuscript: KVR, FF, MF and GAC.

Statistical Analysis: KVR, CI, LX, MF, FM, AN, XW, MF and GAC.

Study Supervision: MF and GAC.

All authors critically reviewed the manuscript and approved the final version.
REFERENCES


### TABLES

#### Table 1 The analyzed patient datasets for survival analysis

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Reference</th>
<th>Total No. pts</th>
<th>TP53 (high)</th>
<th>TP53 (low)</th>
<th>miR-155 (high)</th>
<th>miR-155 (low)</th>
<th>Combined miR-155 and TP53 (high)</th>
<th>Combined miR-155 and TP53 (low)</th>
<th>Poor prognosis</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL – NEJM</td>
<td>(23)</td>
<td>94</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CLL – Clin Cancer Res</td>
<td>(24)</td>
<td>212</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>ALL – MDACC</td>
<td>MDACC</td>
<td>52</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>NSCLC - Italy</td>
<td>IRST</td>
<td>24</td>
<td>11</td>
<td>13</td>
<td>0.119</td>
<td>9</td>
<td>15</td>
<td>0.064</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>Lung adenocarcinoma - MDACC</td>
<td>MDACC</td>
<td>56</td>
<td>22</td>
<td>36</td>
<td>0.06</td>
<td>34</td>
<td>24</td>
<td>0.22</td>
<td>26</td>
<td>14</td>
</tr>
<tr>
<td>Lung adenocarcinoma - TCGA</td>
<td>TCGA</td>
<td>343</td>
<td>216</td>
<td>127</td>
<td>0.019</td>
<td>236</td>
<td>107</td>
<td>0.19</td>
<td>90</td>
<td>70</td>
</tr>
<tr>
<td>Lung squamous cell carcinoma - TCGA</td>
<td>TCGA</td>
<td>192</td>
<td>119</td>
<td>73</td>
<td>0.086</td>
<td>136</td>
<td>56</td>
<td>0.26</td>
<td>46</td>
<td>29</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>956</td>
<td>368</td>
<td>249</td>
<td>555</td>
<td>401</td>
<td>166</td>
<td>126</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CLL, chronic lymphocytic leukemia; NEJM, The New England Journal of Medicine; ALL, acute lymphoblastic leukemia; MDACC, The University of Texas MD Anderson Cancer Center; NSCLC, non-small cell lung cancer; TCGA, The Cancer Genome Atlas; pts, patients

*a* The TCGA data were downloaded from the data portal at https://tcga-data.nci.nih.gov/tcga (currently https://gdc.cancer.gov).

*b* High and low expression of mir-155 and TP53 were determined with the log-rank test as indicated in the Statistical Analysis section of the Materials and Methods.
**FIGURE LEGENDS**

**Figure 1** The effect of miR-155 modulation on drug resistance. (A) Cell viability and (B) dose-response curves for A549 cells treated with CDDP (left graph), REH cells (wt and shp53) treated with doxorubicin (middle graph) and JM1 cells treated with CDDP (right graph). (C) Clonogenic assay of A549 cells treated with CDDP. (D) Viability assay for H2009 cells treated with CDDP. CDDP, cisplatin; wt, wild-type; shp53, short hairpin for TP53; LVEV, lentivirus empty vector; LV, lentivirus. Error bars represent SEM, and each assay was performed at least three times.

**Figure 2** In vivo orthotopic lung cancer model for the role of miR-155 in chemoresistance. (A) Injection and treatment schedule for CDDP (green arrows) and anti-miR negative control (NC) or anti-miR-155 liposomal nanoparticles (red stars) for five different treatment groups: mice that were injected with A549-LVEV cells and untreated (group 1), injected with A549-LVEV cells and treated with anti-miR-NC and CDDP (group 2), injected with A549-155LV cells and treated with anti-miR-NC and CDDP (group 3), injected with A549-155LV cells and treated with anti-miR-155 alone (group 4), and injected with A549-155LV cells and treated with anti-miR-155 and CDDP (group 5). (B–C) Graphs of the primary tumor size (B) and the aggregate mass of nodules in the mediastinum (C) for each of the five treatment groups. (D) *In situ* hybridization for miR-155 for each of the five treatment groups. (E) Immunohistochemical analyses for Ki-67 (proliferation) and CD31 (angiogenesis), as well as the TUNEL assay (apoptosis) and TP53 immunostaining for each of the five treatment groups. Quantifications are presented in the histograms at the right side of the pictures. CDDP, cisplatin; LVEV, lentivirus empty vector; LV, lentivirus; NC, negative control. Error bars represent SEM. Scale bars in panels D and E represent 100 μm. The number of mice in each group is indicated.
**Figure 3** *in vivo* toxicology assessment of anti-miR-155-DOPC. (A) Blood chemistry analyses of blood urea nitrogen content (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine and lactic dehydrogenase (LDH) in mice (n=5/group) treated with anti-miR-NC-DOPC or anti-miR-155-DOPC. (B-C) Hematology analyses consisting of complete blood count (B) including white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin, hematocrit, average volume of RBC (mean corpuscular volume or MCV), average amount of hemoglobin in one RBC (mean corpuscular hemoglobin or MCH), average concentration of hemoglobin in one RBC (MCH concentration or MCHC), red cell distribution width (RDW), platelet count and mean platelet volume (MPV), as well as white blood cells differential count (C) including levels of segmented neutrophils, lymphocytes, monocytes, eosinophils, basophils and large unstained cells (LUC) in mice (n=10/group) treated with anti-miR-NC-DOPC or anti-miR-155-DOPC. (D) Cytokine assay detecting 25 pro-inflammatory cytokines in the serum of mice (n=10/group) injected with either anti-miR-NC-DOPC or anti-miR-155-DOPC. (E) Hematoxylin and eosin (H&E) staining in brain, heart, kidney, liver, lung and spleen of mice (n=5/group) treated with anti-miR-NC-DOPC or anti-miR-155-DOPC. BUN, blood urea nitrogen content; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; LDH, lactic dehydrogenase; WBC, white blood cell; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; MPV, mean platelet volume; Neutro, neutrophils; Lymph, lymphocytes; Mono, monocytes; Eos, eosinophils; Baso, basophils; LUC, large unstained cells. Error bars represent SD.

**Figure 4** *in vitro* validation of a miR-155/TP53 negative feedback loop. (A) Schematic representation of three predicted TP53 binding sites in the downstream region of miR-155. (B) Chromatin immunoprecipitation for TP53 binding to BS1/2 and BS3 in REH cells with normal TP53 expression (REH wt). (C) Luciferase reporter assay and mutagenesis for the TP53 binding
site BS3 downstream of miR-155 in A549 cells. (D) Western blot analysis of A549 and H460 cell lines with baseline miR-155 levels or overexpressing miR-155. (E) Western blot analysis of H2009 cells with relatively high basal miR-155 expression and after inhibiting miR-155. (F) Schematic representation of a predicted miR-155 binding site in the 3’ UTR of TP53 (BS-UTR). (G) Luciferase reporter assay and mutagenesis for BS-UTR in the TP53 null cell line H1299. (H) Luciferase reporter assay for the 3’ UTR of TP53 in the TP53 wild-type cell line H460. BS, binding site; UTR, untranslated region; SCR, scrambled. Error bars represent SD, and each assay was performed at least three times.
Figure 1
**Figure 2**

[Image: Diagram showing the experimental setup with A549-LVEV (n=20) and A549-155LV (n=30) cells.}

**A**
- No treatment (n=9)
- CDDP anti-miR-NC (n=10)
- CDDP anti-miR-155 (n=8)
- Anti-miR-155 (n=7)

**B**
- Primary tumor size (mm³)
- Aggregate mass of nodules from dissection (g)

**C**
- CDDP: -/+  
- Anti-miR: NC/155

**D**
- Images showing the histological analysis with 100 µm scale.
- A549-LVEV
- A549-miR-155

**E**
- Images showing expression levels of Ki-67, CD31, TUNEL, and TP53.
- KI-67
- CD31
- TUNEL
- TP53

**Graphs**
- Comparison of primary tumor size and aggregate mass of nodules between different treatment groups.

Downloaded from clincancerres.aacrjournals.org on January 10, 2021. © 2016 American Association for Cancer Research.
Figure 3
Figure 4
Combining anti-miR-155 with chemotherapy for the treatment of lung cancers
Katrien Van Roosbroeck, Francesca Fanini, Tetsuro Setoyama, et al.

Clin Cancer Res  Published OnlineFirst November 30, 2016.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-16-1025

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2016/12/21/1078-0432.CCR-16-1025.DC2

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/early/2016/11/30/1078-0432.CCR-16-1025. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.