Genetic Mutation of p53 and Suppression of the miR-17–92 Cluster Are Synthetic Lethal in Non–Small Cell Lung Cancer due to Upregulation of Vitamin D Signaling

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Abstract

Lung cancer is the leading cause of cancer-related fatalities. Recent success developing genotypically targeted therapies, with potency only in well-defined subpopulations of tumors, suggests a path to improving patient survival. We used a library of oligonucleotide inhibitors of microRNAs, a class of posttranscriptional gene regulators, to identify novel synthetic lethal interactions between miRNA inhibition and molecular mechanisms in non–small cell lung cancer (NSCLC). Two inhibitors, those for miR-92a and miR-1226, produced a toxicity distribution across a panel of 27 cell lines that correlated with loss of p53 protein expression. Notably, depletion of p53 was sufficient to confer sensitivity to otherwise resistant telomerase-immortalized bronchial epithelial cells. We found that both miR inhibitors cause sequence-specific downregulation of the miR-17–92 polycistron, and this downregulation was toxic only in the context of p53 loss. Mechanistic studies indicated that the selective toxicity of miR-17–92 polycistron inactivation was the consequence of derepression of vitamin D signaling via suppression of CYP24A1, a rate-limiting enzyme in the 1α,25-dihydroxyvitamin D, metabolic pathway. Of note, high CYP24A1 expression significantly correlated with poor patient outcome in multiple lung cancer cohorts. Our results indicate that the screening approach used in this study can identify clinically relevant synthetic lethal interactions and that vitamin D receptor agonists may show enhanced efficacy in p53-negative lung cancer patients. Cancer Res; 75(4); 666–75. © 2014 AACR.

Introduction

The existence of defined genetic abnormalities in non–small cell lung cancer (NSCLC) has enabled the development of targeted therapeutic approaches to NSCLC treatment. In particular, therapies targeting tumors carrying mutations in EGFR or a fusion of the EML4 and ALK genes have been clinically successful as first-line therapies (1–3). Targeted therapies, however, sacrifice breadth of treatable tumors for high efficacy in the presence of a specific biomarker: only 25% to 35% of NSCLC tumors will respond to the EGFR and EML4/ALK targeted therapies, and the current 5-year survival rate remains around 15%.

microRNAs (miRNA) are a class of posttranscriptional regulators of gene expression. In a sequence-driven process mediated by the RNA-induced silencing complex (RISC), the approximately 22 nucleotide long RNAs associate with 3′ untranslated regions (3′-UTR), leading to the downregulation of their targets (4, 5). miRNAs are found throughout the genome as either individual loci, within introns of host genes, or in polycistrons, single transcripts that produce multiple miRNAs. miRNAs have been implicated in developmental processes, drug response, and cancer initiation and progression (6–10), and can function as either tumor promoters (oncomiRs) or tumor suppressors, with some miRNAs able to play both roles, depending on the context (11). In a parallel to oncogene addiction, some cancer cells have been shown to be dependent on the expression of a single oncogenic miRNA. For example, whereas miR-21 has been shown to lead to a pre-B malignant lymphoid-like phenotype, inactivation of miR-21 leads to rapid and complete regression (12).
miRNAs are readily manipulated both in vitro and in vivo, and both gain and loss of miRNA function have been demonstrated to have substantial effects on tumor initiation and progression in in vivo models (6, 13, 14). Oligonucleotides complementary to a mature miRNA competitively bind the miRNA and prevent it from being loaded into the RISC (15). Such inhibitors have been demonstrated to have therapeutic efficacy in in vivo models due to their high target affinity and bioavailability, even without any packaging or carrier (14, 16, 17).

Our goal is to identify synthetic lethal inhibitor:genotype interactions in NSCLC. Here, we used a phased screening approach to identify miRNA inhibitors with selective toxicity across a genetically diverse collection of NSCLC cell lines. We were able to use the diversity of the cell lines in tandem with their mutational and transcriptional profiles to identify a dependency on the miR-17~92 cluster that arises after p53 loss in the lung epithelium.

Materials and Methods

Cell lines

Cell lines were obtained from the Hamon Center for Therapeutic Oncology Research at UT Southwestern Medical Center. All cell lines were grown in a humidified atmosphere with 5% CO2 at 37°C. HBECs and HCC4017 were grown in ALC-4 medium supplemented with 2% FBS (18, 19). All other cell lines were grown in the RPMI-1640 medium (Life Technologies) supplemented with 5% FBS (Atlanta Biologicals). Cell lines were DNA fingerprinted in October 2013 using the GenePrint PowerPlex 1.2 system (Promega) and confirmed against libraries maintained by ATCC.

Reagents

The miRCURY LNA microRNA Inhibitor Library - Human v14.0 was obtained from Exiqon. Inhibitors for miR-92a and miR-1226 were obtained from Exiqon and Dharmacon and mismatch and scrambled derivatives were synthesized by Exiqon. siRNA oligos were obtained from Dharmacon. p53 and β-tubulin antibodies were acquired from Santa Cruz Biotechnology and Sigma-Aldrich. 1α,25-dihydroxyvitamin D3 was acquired from Sigma-Aldrich.

miRNA inhibitor screen

Cells were plated in 96-well format, transfected with oligos, and incubated for 72 hours, after which medium was changed, and then incubated for an additional 72 hours. Cell viability was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Luminescence was quantified on an EnVision plate reader (PerkinElmer). Raw values were normalized using R (20) and cellHTS2 (21) to obtain cell viability ratios.

Cell viability assay

Cells were plated in 96-well format, transfected with oligos, and incubated for 72 hours, after which medium was changed and then incubated for an additional 72 hours. Cell viability was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Luminescence was quantified on an EnVision plate reader (PerkinElmer). Raw values were normalized using R (20) and cellHTS2 (21) to obtain cell viability ratios.

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Cell viability assay

Cells were plated in 96-well format, transfected with oligos, and incubated for 72 hours, after which medium was replaced and, as appropriate, supplemented with 1α,25-dihydroxyvitamin D3. Cells were then incubated for 72 hours. Cell viability was determined as above. Luminescence was quantified on a PheraStar FS plate reader (BMG LabTech).

1α,25-Dihydroxyvitamin D3 toxicity assay

Cells were plated in 96-well plates, allowed to recover for 48 hours, and then treated with an addition of growth media, growth media plus carrier (ethanol), or growth media plus 1α,25-dihydroxyvitamin D3 for 6 hours. Caspase-Glo 3/7 reagent (Promega) was added at equal volume to the growth media and incubated for 1 hour before luminescence was quantified as above.

Quantitative real-time PCR

Total RNA was prepared using the Ambion miRvana miRNA Isolation Kit (Life Technologies). miRNA and miRNA levels were assessed by qRT-PCR using an ABI PRISM 7900HT using pre-designed TaqMan primer and probe sets (Life Technologies). RN119 was used as a loading control for miRNA assays; ACTB and GAPDH were used as loading controls for pri-miRNA and gene expression assays. Threshold cycle times (Ct) were obtained and relative gene expression was calculated using the comparative Ct method.

Gene expression profiling

HBECC30KT and HBECC30KT-shTP53 cells were reverse transfected with 50 nmol/L miR-92a mismatch oligo or miR-92a inhibitor and plated in triplicate in 6-well plates. Forty-eight hours after transfection, total RNA was prepared as above. RNA quality was assessed by Bio-Rad Experion (Bio-Rad). RNA was then transcriptionally profiled on Illumina HumanHT-12 v4 Expression BeadChips (Illumina) as described previously. Data have been deposited in the GEO public repository under accession number GSE64007. Additional detail is provided in Supplementary Materials.

Protein expression profiling

Reverse-phase protein arrays (RPAs) were used to measure protein expression, as described above (22). Additional detail is provided in Supplementary Materials.

Survival analysis

Gene expression data from 182 lung adenocarcinoma tumor specimens were obtained from GEO dataset GSE41271. Samples were then ranked by CYP24A1 expression and the highest and lowest quintiles were then compared by the Mantel–Cox log-rank test. miRNA expression profiles and corresponding clinical data on 470 lung adenocarcinoma patients were obtained from The Cancer Genome Atlas (TCGA). Samples were then ranked by expression of miR-17-5p, miR-18a-5p, miR-19a-3p, miR-20a-5p, miR-19b-3p, and miR-92a-3p, with the highest and lowest quintiles compared for association with patient survival by the Mantel–Cox log-rank test.

Statistical analysis

Correlations were calculated by the Pearson product-moment correlation coefficient. P values were determined by two-tailed, unpaired Student t test with P < 0.05 used as a nominal threshold for significance.

Elastic net for predictive biomarker discovery

The elastic net is a penalized linear regression model that can identify multi-feature biomarker signatures whose additive patterns act to predict a response vector, in this case measurements of viability in response to treatment with the miR-92a inhibitor in a panel of cell lines. The elastic net also allows for weighting of identified features to enable predictions of sensitivities in untested cell lines. Candidate predictive features were selected from measures of gene expression (described above), with replicate measurements for the same gene averaged together. To determine the optimal parameter values to use in the model, we did 100 iterations of 10-fold cross-validation where, in each iteration, the cell lines were randomly resampled into different groups. Parameter values were chosen so as to give the minimum mean squared
error for each fold. The dataset was then subjected to 100 permutations of bootstrapping. Features were then ranked based on weights and bootstrapped frequency of occurrence. Additional detail is provided in Supplementary Materials.

**Results**

A miRNA inhibitor screen on two NSCLC cell lines identifies a potential genetic context-dependent vulnerability to miRNA inhibition

To identify miRNA inhibitors with genotype-driven selective toxicity in NSCLC, we implemented a tiered screen based on a comprehensive library of 918 single-stranded competitive inhibitors targeting 870 of the known human miRNAs (miRBase v.14). Two NSCLC cell lines derived from adenocarcinomas, H358 and H1993, which harbor distinct genetic alterations (Supplementary Table S1), were used for screening.

We observed several patterns of response to individual miRNA inhibitors (Fig. 1A), confirming that although histologically similar, the two cell lines can generate divergent responses to a specific perturbation. Specifically, 35 inhibitors (3.79%) decreased the viability of H358 cells more than 50%, and 11 (1.19%) decreased the viability of H1993 cells to the same extent. Among these, the inhibitors of miR-92a and miR-1226/C3 showed the most significant selective cytotoxicity to H358. Notably, the inhibitors are identical in sequence at 10 positions, including a CAGGCC motif at their 5' ends. Testing across a panel of cell lines with diverse genetic backgrounds (Supplementary Table S2) returned a significantly correlated toxicity profile, indicating that the two inhibitors may converge on a specific NSCLC vulnerability (Fig. 1B and C). Both inhibitors were benign in normal HBEC30-KT cells, indicating selectivity within tumorigenic contexts.

miR-92a is a member of the miR-25 family of miRNAs, which includes miR-25, miR-32, miR-92a, miR-92b, miR-363, and miR-367, all of which share a common seed sequence (Supplementary Fig. S1A). miR-92a is also a component of the miR-17~92 polycistron, a cluster of cotranscribed miRNAs, including miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a. However, none of the related inhibitors, whether by seed sequence or by cotranscription, exhibited cytotoxic effects similar to the miR-92a or miR-1226 inhibitors (Supplementary Fig. S1B and S1C).

p53 levels and activity are inversely correlated with miR-92a inhibitor toxicity, and loss of p53 sensitizes a resistant cell line to the miR-92a inhibitor

Therapeutic approaches based on genotype have shown promising returns in NSCLC (2, 23). We therefore sought a genetic predictor of response to the miR-92a/miR-1226 inhibitors. Interrogating the mutational status and gene expression of known oncogenes and tumor suppressors in NSCLC for correlations with inhibitor toxicity revealed an inverse correlation with TP53 mRNA accumulation (Fig. 2A). This was recapitulated by p53 protein abundance as quantified by RPPA (Fig. 2B).

To assess the relationship between miR-92a inhibitor response and p53 activity, we used a genetic model of p53 loss: an immortalized human bronchial epithelial cell line, HBEC30-KT, was

**Figure 1.** A miRNA inhibitor screen identifies distinct toxicity patterns across NSCLC cell lines. A, results from the 918 miRNA inhibitors used in the first phase of the screen. Each dot represents one inhibitor, with normalized cell viability in H358 on the x-axis and in H1993 on the y-axis. Red dots; miR-92a and miR-1226 inhibitors. B, cytotoxicity of miR-92a and miR-1226 inhibitors, shown as percent viability relative to negative control. Red bar, representative normal cell line. Error bars, standard deviations. C, viability of 27 NSCLC cell lines after miR-92a inhibitor or miR-1226 inhibitor treatment. Each red dot, one cell line; dashed line, line of best fit. Correlation and P value were determined by the Pearson product moment.
modiﬁed to constitutively express a short hairpin RNA (shRNA) to TP53 (24), creating a TP53-knockdown cell line HBE30KT-shTP53. Whole-genome transcript arrays from the cell lines on the tails of the viability distribution (the 6 most sensitive and the 6 most resistant lines shown in Fig. 1C), obtained from GEO dataset GSE32036, were used to identify the top 1% of differentially expressed genes by their signal-to-noise (S2N) values, calculated as (μsen − μres)/(σsen + σres), where μ and σ represent the mean and standard deviation of expression, respectively, for each class. We next deﬁned p53-dependent genes as those with at least 2-fold differential accumulation in HBE30KT versus HBE30KT-shTP53 cells. A hypergeometric distribution test indicated signiﬁcant overlap between genes that are decreased in HBE30KT-shTP53 relative to HBE30KT, and high in cell lines sensitive to the miR-92a inhibitor, as determined by hypergeometric test. D, genes identiﬁed as features that alone or in combination are predictive of response to the miR-92a inhibitor in the panel of 22 cell lines. Yellow and blue bars, positive and negative correlations with viability, respectively. E, p53 levels in H358 and H1993 cell lines 72 hours after a 50 nmol/L transfection of either a negative control oligo or the miR-92a inhibitor, as determined by Western blot. F, p53 levels in HBE30-KT and HBE30KT-shTP53 cells and viability of HBE30-KT and HBE30KT-shTP53 72 hours after transfection with 50 nmol/L of either the miR-92a inhibitor, the miR-1226 inhibitor or a negative control inhibitor. G, qRT-PCR quantiﬁcation of miR-92a in H358 and H1993 cells 72 hours following transfection with either a LNA- or RNA hairpin-based miR-92a inhibitor or negative control inhibitors. Fold change is relative to the negative control transfection in each panel. H, cell viability in H358 and H1993 cells 144 hours after transfection with 50 nmol/L of miR-92a inhibitor, miR-1226 inhibitor or negative control inhibitor. I and J, cell viability in H358 and HBEC30KT-shTP53 cells 144 hours after transfection with either unmodiﬁed or sequence-modiﬁed versions of the miR-92a and miR-1226 inhibitors, or a negative control inhibitor, as described in Supplementary Table S2. Results are shown as means and standard deviations of triplicate measurements, relative to the negative control. P values were determined by two-tailed, unpaired Student t test. ****, P < 0.0001.
These results show that there is a significant “p53-like” gene signature associated with response to the miR-92a inhibitor and that p53 network genes are sufficient to act as a multi-feature signature whose combinatorial expression can predict cellular response to the miR-92a inhibitor. H358, the sensitive cell line from the first phase of the screen, had the lowest observed TP53 and p53 expression of any of the cell lines in the panel (Fig. 2A). However, p53 abundance did not change in response to transfection with miR-92a inhibitor in either the sensitive or resistant cell line as measured by Western blot analysis 72 hours after treatment (Fig. 2E), indicating that the effect of miR-92a inhibitor on cell survival is not through regulation of TP53 expression.

To address the necessity and sufficiency of p53 loss with respect to cellular response to the miR-92a inhibitor, we returned to the HBEc30KT-shTP53 model. As expected, the unmodified HBEc30-KT did not respond to the miR-92a inhibitor, whereas the knockdown of p53 was sufficient to render the cell line sensitive to the miR-92a inhibitor (Fig. 2F).

Depletion of mature miR-92a is insufficient to induce toxicity in p53-depleted cell lines

One potential pitfall in any experiment involving short oligonucleotides is phenotypes caused by off-target effects (25) of the oligos. We therefore used a second design, a double-hairpin miRNA inhibitor (miridian miRNA inhibitors; Dharmacon). We observed successful knockdown of mature miR-92a with inhibitors based on either the LNA or RNA hairpin inhibitors in both cell lines (Fig. 2G). However, the hairpin miR-92a inhibitor did not reproduce the toxicity of the LNA inhibitor (Fig. 2H). Interestingly, the miR-1226 inhibitor replicated the phenotypes observed with the miR-92a inhibitors, which is consistent with their correlated toxicities.

To establish the sequence-dependence of the toxicity of the LNA-based miRNA inhibitors, we designed variants of the miR-92a and miR-1226 inhibitors (Supplementary Table S5). As shown in Fig. 2I and J, none of the variants displayed significant cytotoxicity in either H358 or HBEc30KT-shTP53 cells. The mature miR-92a inhibitor replicated the phenotypes observed with the miR-92a inhibitors, which is consistent with their correlated toxicities.

The miR-17–92 primary transcript is the likely mediator of the cytotoxicity of miR-92a inhibitor

Given the above observation, the sequence similarity between the inhibitors led us to consider sequence-dependent genomic targets other than mature miR-92a that they may both regulate. The transcript with the highest sequence complementarity to the miR-92a and miR-1226 inhibitors is the primary transcript of the miR-17–92 polycistron, a cluster of miRNAs in a single primary transcript (Fig. 3A). To address whether the levels of the primary transcript of miR-17–92 were altered in response to treatment with the miR-92a inhibitor, we measured its expression in both miR-92a–sensitive and -resistant cells 72 hours following transfection of the inhibitor. We observed a decrease in the miR-17–92 primary transcript in all four cell lines (Fig. 3B) following transfection of the LNA miR-92a inhibitor, although the extent of the decrease in HBEc30-KT cells was much less than in the other three cell lines. The RNA hairpin inhibitor to miR-92a, however, showed no effect on primary transcript levels in either H358 or H1993 cells (Fig. 3C). Treatment with the miR-1226 inhibitor also resulted in depletion of the miR-17–92 primary transcript (Fig. 3D), but did not deplete mature miR-92a in H358 cells (Fig. 3E), further confirming that inhibition of miR-92a is not required for toxicity. We also observed significantly higher endogenous levels of miR-17–92 primary transcript in tumor cells versus normal cells, and in resistant cell lines relative to sensitive cell lines (Fig. 3F), consistent with previous reports demonstrating upregulation of miR-17–92 in NSCLC (7). The low levels of the primary transcript in HBEc30-KT may explain the lack of toxicity of the inhibitors (Fig. 1C). Taken together, these results suggest that the cytotoxic effects of the LNA-based miR-92a and miR-1226 inhibitors are mediated by knockdown of the miR-17–92 primary transcript.

Given the depletion of the miR-17–92 primary transcript, we assayed the four cell lines for the abundance of the mature miRNAs from the miR-17–92 cluster following a 72-hour transfection with LNA miR-92a inhibitor. We observed depletion of mature mir-18a, mir-20, and miR-92a in H358 cells (Fig. 3G) and of mature mir-19a, mir-19b, and miR-92a in H1993 cells (Fig. 3H). The miR-92a locus demonstrated the most dramatic knockdown in all cases. All of the mature miRNAs from the locus were depleted in HBEc30KT-shTP53 cells, whereas only miR-92a was depleted in HBEc30KT cells (Fig. 3I). The downregulation of mature miRNAs in sensitive cell lines is consistent across both the NSCLC cell lines and HBECs, and is consistent with the synthetic lethal relationship between the miR-92a inhibitor, the miR-17–92 cluster, and p53 loss. Notably, in mouse cell models derived from a standard murine model for lung adenocarcinoma, we observed a decrease in levels of mature miR-92a in response to the miR-92a inhibitor, but no effect on the levels of the other mature miRNAs from the cluster or on levels of the primary transcript (Supplementary Fig. S2). In addition, treatment with miR-92a inhibitor had no effect on cell viability relative to the mismatch control inhibitor, further indicating the combinatorial participation of the miRNAs in the miR-17–92 locus in support of human p53 hypomorphs.

Expression profiling identifies a group of target genes of the miR-17–92 cluster that are derepressed by miR-92a inhibitor in p53-depleted cells, but not in p53-wild-type cells

To identify the candidate genes responsible for the response of p53-depleted cells to the miR-92a inhibitor, we profiled the two HBEc30KT cell lines for changes in miRNA expression following transfection of the miR-92a inhibitor or control. Consistent with the observation that the miR-92a inhibitor can deplete the miR-17–92 cluster, we observed a corresponding upregulation of genes in the miR-17–92 target space (Supplementary Fig. S3A). Notably, we observe that a substantially larger number of targets were upregulated in response to the miR-92a inhibitor in HBEc30KT-shTP53 cells (141, 64.6%) relative to HBEc30KT cells (29, 35.6%; Supplementary Fig. S3B). These results are consistent with the observation that HBEc30KT is resistant to miR-92a inhibitor-induced cytotoxicity.

The six miRNAs in the miR-17–92 primary transcript (miR-17 and miR-20; miR-19a and miR-19b; miR-92a) include three seed-sequence families and one miRNA with a unique seed sequence, which appear to share a combinatorial target space (Supplementary Fig. S3C).

miR-92a and p53 converge on CYP24A1, a key element of the vitamin D₃ response network, in regulating cell survival

Analysis of the 712 transcripts (representing 675 genes), modulated more than 2-fold by the miR-92a inhibitor in HBEc30KT-shTP53 cells, with the “Upstream Regulators” IPA module identified inflammation-related pathways as potential regulators of
the observed differential expression, among which the 1α,25-
dihydroxyvitamin D₃ pathway ranked second (Table 1). 1α,25-
Dihydroxyvitamin D₃ is the active form of vitamin D₃ (26–29),
and has shown efficacy in reducing proliferation and inducing
apoptosis in cancer cells (30–32). We found that 1α,25-
dihydroxyvitamin D₃ activated caspase 3/7 by approximately 4-fold
compared with control in the miR-92a inhibitor-sensitive H358 cells,
but not in the miR-92a inhibitor-resistant H1993 cells (Fig. 4B),
consistent with the observed activation of caspase 3/7 by miR-92a
inhibitor (Fig. 4C). Furthermore, analysis of the miR-17, miR-18,
miR-19, miR-20, and miR-92 target genes and the transcriptional
network of 1α,25-dihydroxyvitamin D₃ shows that there is an
overlap between the miR-17–92 targetome and the 1α,25-
dihydroxyvitamin D₃ response network (Supplementary Fig. S3C).
Altogether, these results suggest that the 1α,25-dihydroxyvitamin
D₃ response network is likely a key mediator of the miR-92a
inhibitor cytotoxicity.

Similar analysis of upstream regulators for the 3,842 transcripts
(representing 3,586 genes) that are modulated by
TP53 knockdown in HBEC30KT cells also returned an inflammation-like

Figure 3.
The miR-92a inhibitor downregulates the miR-17–92 primary transcript and dysregulates the expression of mature miRNAs from the miR-17–92 polycistron. A, the
miR-17–92 polycistron with predicted interactions between the primary transcript and the miR-92a and miR-1226 inhibitors. B, qRT-PCR for the primary transcript (pri-miRNA) of the miR-17–92 polycistron in H358, H1993, HBEC30KT-shTP53, and HBEC30-KT cells at two locations: one in the miR-18a locus (miR-18-pri) and one in the miR-92a-1 locus (miR-92a-1-pri). RNA was collected 72 hours after transfection with either the miR-92a inhibitor or a negative control inhibitor. C, qRT-PCR for miR-18-pri and miR-92a-1-pri in H358 and H1993 cells 72 hours after transfection with either the RNA hairpin miR-92a inhibitor or a negative control inhibitor. D, qRT-PCR for miR-18-pri and miR-92a-1-pri in H358 cells 72 hours after transfection with either the miR-1226 inhibitor or a negative control inhibitor. E, relative abundance of miR-92a following treatment with either miR-92a inhibitor or miR-1226 inhibitor. F, relative abundance of pri-miR-18 and pri-miR-92a in H358, H1993, HBEC30KT-shTP53, and HBEC30-KT cells. G–J, levels of mature miRNAs from the miR-17–92 polycistron 72 hours after transfection with either the LNA miR-92a inhibitor or the mismatched control inhibitor in H358, H1993, HBEC30KT-shTP53, or HBEC30KT cells, as measured by qRT-PCR. In all panels, results are shown as means and standard deviations of triplicate measurements, relative to the negative control. mm, mismatch. P values determined by the Student t test. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
response (Table 2). Of the top 20 upstream regulators identified, nine have known roles in inflammation and immune response, including six cytokines (TNF, IFNG, IL1B, OSM, IFNL1, and IFNA2, all of which are consistently activated after p53 loss), lipopolysaccharide, TLR3, and the NFκB complex. This observation suggests that inflammation-related pathways may confer a survival advantage to p53 hypomorphs but also confer collateral addiction to miR-17~92 expression.

Within the functional network described above, CYP24A1 was significantly upregulated in HBE30KT-shTP53 cells compared with HBE30KT cells. Notably, CYP24A1 is a rate-limiting regulator of 1α,25-dihydroxyvitamin D₃ degradation (32) and directly affects the intracellular half-life of 1α,25-dihydroxyvitamin D₃. CYP24A1 is decreased to basal levels by miR-92a inhibitor in HBE30KT-shTP53 cells (Fig. 4A), suggesting that it may participate in miR-92a inhibitor-induced cytotoxicity. Furthermore, the host gene for the miR-17~92 cluster (C13orf25) is overexpressed in human lung cancers, including both NSCLC and SCLC cell lines and patient specimens (7). To further examine the clinical relevance of the miR-17~92-mediated regulation of CYP24A1 expression, we analyzed primary lung adenocarcinoma tumors for associations between expression of miRNAs in the miR-17~92 cluster and patient survival as well as associations between CYP24A1 expression and patient survival and found that high expression of CYP24A1 correlates with poorer overall and recurrence-free survival. Based on stratifying the patients by expression and comparing the highest and lowest quartiles, lower levels of miR-18a, miR-20a, and miR-92a are significantly associated with longer overall survival (Fig. 4D). In all cases, median survival in patients with low expression is longer than in patients with high expression. In addition, high expression of CYP24A1 correlates with poorer overall and recurrence-free survival. Median recurrence-free survival was 3.1 years in the high CYP24A1 group and >9 years in the low CYP24A1 group (P = 0.004). Median overall survival was 5.2 years in the high CYP24A1 group and >9 years in the low CYP24A1 group (P = 0.01; Fig. 4E). This was confirmed in both the Director’s Challenge Lung Study, where the top and bottom decile by CYP24A1 expression (n = 45) showed median survival of 3.8 and 6.6 years, respectively, with P = 0.039 by log-rank test, and TCGA study, where the top and bottom deciles (n = 42 for adenocarcinoma; n = 40 for squamous cell carcinoma) showed median survival of 1.7 and 3.5 years, respectively, for adenocarcinoma, with P = 0.006, and 1.8 and 6.6 years, respectively, for squamous cell carcinoma, with P = 0.05 (33, 34).

Discussion

Here, we find that oncogenotype-selective vulnerability to a miR-92a LNA inhibitor is dependent upon downregulation of the miR-17~92 primary transcript. The oncogenic function of the miR-17~92 cluster has been well demonstrated in previous studies (7, 35–37), supporting the potential use of a miR-92 inhibitor as an anticancer therapeutic agent. Our study identifies p53 as a major mediator of resistance to miR-92a inhibitor-induced cytotoxicity in lung cancers, with low p53 levels a predictor of miR-92a inhibitor toxicity. The quantitative response of NSCLC cell lines to the miR-92a inhibitor in relation to p53 levels in lung cancer cells, coupled with the dramatic sensitization observed in HBE30-KT cells after p53 knockdown provide an experimental basis for selecting which patients are likely to benefit from the miR-92a inhibition and which are not. Because p53 mutation and loss are among the most common genetic events in tumor biology, identifying a compound with antineoplastic effects in a low p53 context represents a potential avenue to p53-directed therapeutic interventions.

The mechanism for the interaction between the miR-92a and miR-1226 inhibitors and the miR-17~92 primary transcript remains to be determined. LNA-based antisense oligos have demonstrated both in vivo and in vitro potency for silencing long mRNA transcripts, and our observation of miR-17~92 primary transcript depletion could certainly be through a RISC-independent, antisense-like mechanism (38–40). The observed differences in the relative abundance of mature miRNAs from the miR-17~92 cluster by following treatment with miR-92a inhibitor can have half-lives of hours to days in vivo, it has also been

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<td>IFNB1</td>
<td>Cytokine</td>
<td>~2.255</td>
<td>1.78 × 10⁻⁷</td>
</tr>
<tr>
<td>SMARCB1</td>
<td>Transcription regulator</td>
<td>3.000</td>
<td>3.14 × 10⁻⁷</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>Cytokine</td>
<td>3.14</td>
<td>1.57 × 10⁻⁶</td>
</tr>
<tr>
<td>E2F2</td>
<td>Transcription regulator</td>
<td>~2.200</td>
<td>1.79 × 10⁻⁶</td>
</tr>
<tr>
<td>IL4</td>
<td>Cytokine</td>
<td>2.647</td>
<td>1.94 × 10⁻⁶</td>
</tr>
<tr>
<td>IFNA2</td>
<td>Cytokine</td>
<td>~2.915</td>
<td>2.42 × 10⁻⁶</td>
</tr>
<tr>
<td>IRGM</td>
<td>Other</td>
<td>3.000</td>
<td>3.75 × 10⁻⁶</td>
</tr>
<tr>
<td>IFR3</td>
<td>Transcription regulator</td>
<td>~2.850</td>
<td>6.57 × 10⁻⁶</td>
</tr>
<tr>
<td>IL18RN</td>
<td>Cytokine</td>
<td>2.392</td>
<td>1.27 × 10⁻⁵</td>
</tr>
</tbody>
</table>

NOTE: Shown are upstream transcriptional regulators consistent with the observed changes in gene expression, activation z-score, and overlap P value. Activation z-score and overlap P value are derived by comparing the known targets of each regulator with observed differential expression, with respect to number of genes and magnitude and direction of change. Significance is generally attributed to P values < 0.01 and activation z-scores greater than 2 or smaller than −2.
demonstrated that mature miRNA species from miR-17-92 do not respond consistently to miR-17-92 depletion (41–43) and that the secondary structure of the miR-17-92 primary transcript affects miRNA processing (44). The lack of response to the miR-92a inhibitor in mouse cells derived from murine KRASmt and KRASmt/p53null lung tumors suggests that the interaction between the miR-92a inhibitor and the miR-17-92 primary transcript may differ between the two organisms. Although the mature miRNAs for miR-17-5p, miR-18a-5p, miR-19a-3p, miR-20a-5p, and miR-92a-3p are identical, there is only an 85.6% similarity in the region beginning with miR-17 and ending with miR-92a. More importantly, miR-92a differs slightly in sequence between the two organisms. Assessing the energy required to unfold the local region of the primary transcript around miR-92a also shows a difference, with a ΔG of −59.12 kcal/mol for the human transcript and −70.30 kcal/mol for the mouse transcript, reflecting differences in the tertiary structure of the miR-17-92 primary transcript that may affect the processing of the transcript into mature miRNAs (45) or accessibility of the transcript to the miR-92a inhibitor.

Finally, we observed upregulation of inflammation-related pathways after p53 loss, and activation of a 1α,25-dihydroxyvitamin D3 response in the high-confidence miR-17-92 targetome in HBECKT-shTP53 cells. Our results suggest that both an inhibitor of the miR-17-92 polycistron and 1α,25-dihydroxyvitamin D3 in NSCLC cellular growth and survival. G. working model for how repression of the miR-17-92 primary transcript by miR-92a and miR-1226 inhibitors can depress target genes that regulate CYP24A1.
Table 2. Twenty most significant upstream regulators in HBEC3KT-shTP53 versus HBEC3KT

<table>
<thead>
<tr>
<th>Upstream regulator</th>
<th>Molecule type</th>
<th>Activation z-score</th>
<th>P value of overlap</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF</td>
<td>Cytokine</td>
<td>7.189</td>
<td>6.18 × 10^-25</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Growth factor</td>
<td>-2.258</td>
<td>1.10 × 10^-50</td>
</tr>
<tr>
<td>IFNG</td>
<td>Cytokine</td>
<td>8.041</td>
<td>9.68 × 10^-25</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>Chemical drug</td>
<td>7.310</td>
<td>3.25 × 10^-42</td>
</tr>
<tr>
<td>NCK2-5</td>
<td>Transcription regulator</td>
<td>-4.911</td>
<td>1.86 × 10^-11</td>
</tr>
<tr>
<td>TP53</td>
<td>Transcription regulator</td>
<td>-3.673</td>
<td>2.93 × 10^-58</td>
</tr>
<tr>
<td>MAPK1</td>
<td>Kinase</td>
<td>-4.847</td>
<td>1.46 × 10^-21</td>
</tr>
<tr>
<td>OSM</td>
<td>Cytokine</td>
<td>4.194</td>
<td>3.00 × 10^-11</td>
</tr>
<tr>
<td>IL1β</td>
<td>Cytokine</td>
<td>5.596</td>
<td>7.98 × 10^-21</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Chemical drug</td>
<td>-6.822</td>
<td>1.35 × 10^-79</td>
</tr>
<tr>
<td>HRAS</td>
<td>Enzyme</td>
<td>2.353</td>
<td>1.05 × 10^-26</td>
</tr>
<tr>
<td>NFκB (complex)</td>
<td>Complex</td>
<td>6.191</td>
<td>5.39 × 10^-26</td>
</tr>
<tr>
<td>IFNL1</td>
<td>Cytokine</td>
<td>6.570</td>
<td>1.40 × 10^-23</td>
</tr>
<tr>
<td>IFNA2</td>
<td>Cytokine</td>
<td>6.380</td>
<td>2.42 × 10^-22</td>
</tr>
<tr>
<td>poly r(T)/r(C)-RNA</td>
<td>Chemical reagent</td>
<td>7.265</td>
<td>1.99 × 10^-22</td>
</tr>
<tr>
<td>TLR3</td>
<td>Transmembrane receptor</td>
<td>4.977</td>
<td>5.75 × 10^-22</td>
</tr>
<tr>
<td>TP63</td>
<td>Transcription regulator</td>
<td>3.700</td>
<td>2.09 × 10^-21</td>
</tr>
<tr>
<td>STAT3</td>
<td>Transcription regulator</td>
<td>2.076</td>
<td>3.20 × 10^-21</td>
</tr>
<tr>
<td>EZH2</td>
<td>Transcription regulator</td>
<td>3.200</td>
<td>8.20 × 10^-21</td>
</tr>
<tr>
<td>CHUK</td>
<td>Kinase</td>
<td>3.898</td>
<td>1.46 × 10^-10</td>
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</table>

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Borkowski, L. Du, E. McMillan, C.-R. Yang, J. D. Minna, M. A. White, A. Pertsemlidis

Writing, review, and/or revision of the manuscript: R. Borkowski, L. Du, E. McMillan, A. Kosti, A. F. Gazdar, J. D. Minna, M. A. White, A. Pertsemlidis

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Study supervision: M. A. White, A. Pertsemlidis

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**References**


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