Negative Regulation of the Tumor Suppressor p53 Gene by MicroRNAs

Munish Kumar¹,#, Zhongxin Lu¹,2,#, Apana Agha L. Takwi¹,#, Weiqun Chen¹,2, Natalie S. Callander³, Kenneth S. Ramos¹, Ken H. Young³, and Yong Li¹,*

¹Department of Biochemistry and Molecular Biology, Center for Genetics and Molecular Medicine, School of Medicine, University of Louisville, 319 Abraham Flexner Way, Louisville, KY, 40202 USA
³Department of Pathology and Laboratory Medicine/Oncology, Paul P. Carbone Comprehensive Cancer Center, University of Wisconsin School of Medicine and Public Health, 600 Highland Avenue, Madison, Wisconsin 53792 USA

Abstract

The tumor suppressor p53, encoded by the TP53 gene, is recognized as the guardian of the human genome because it regulates many downstream genes to exercise its function in cell cycle and cell death. Recent reports have revealed that several microRNAs (miRNAs) are important components of the p53 tumor suppressor network with miR-125b and miR-504 directly targeting TP53. In this report, we use a screening method to identify that two miRNAs (miR-25 and miR-30d) directly target the 3'UTR of TP53 to down-regulate p53 protein levels and reduce the expression of genes that are transcriptionally activated by p53. Correspondingly, both miR-25 and miR-30d adversely affect apoptotic cell death, cell cycle arrest, and cellular senescence. Inhibition of either miR-25 or miR-30d expression increases endogenous p53 expression and elevates cellular apoptosis in several cell lines, including one from multiple myeloma that has little TP53 mutations. Thus, beyond miR-125b and miR-504, the human TP53 gene is negatively regulated by two more miRNAs: miR-25 and miR-30d.

Keywords

microRNA; miR-25; miR-30d; p53; senescence; apoptosis; cell cycle arrest; multiple myeloma

---

Users may view, print, copy and download text and data-mine the content in such documents for the purposes of academic research, subject always to the full Conditions of use: http://www.nature.com/authors/editorial_policies/license.html#terms

*Correspondence should be addressed to Yong Li, yong.li@louisville.edu, Tel: 502-852-7551; Fax: 502-852-6222.
²Present Address: Department of Medical Laboratory and Central Laboratory, The Central Hospital of Wuhan, Wuhan, Hubei, 430014 China
#These authors contributed equally.

Authorship Contributions
MK, ZL, AK, and WC performed research; NC and KY contributed human specimens; ZL, MK, KR, KY, and YL designed research, analyzed data, and wrote the paper; and all authors reviewed the manuscript.

Disclosure of Conflicts of Interest
The authors declare no conflicts of interest.
Tumorigenesis is widely accepted as a multi-step process that requires activation of oncogenes and inactivation of tumor suppressor genes (Hanahan and Weinberg 2000). Tumor protein p53 (p53) is recognized as the guardian of the human genome. It responds to diverse stressors to transactivate many downstream genes, such as p21, Bax, and Puma, which are critical to apoptotic cell death, cell cycle arrest, and cellular senescence (Kruse and Gu 2009, Riley et al 2008, Vogelstein et al 2000, Vousden and Prives 2009). Restoring p53 function alone is sufficient to cause regression of several types of tumors in mice (Ventura et al 2007, Xue et al 2007), supporting the notion that p53 function has to be disrupted for tumor development. Indeed, somatic mutations of TP53, the gene that encodes p53, are estimated to occur in ~50% of all human cancers. In many other cancers, TP53 is inactivated indirectly through amplification or overexpression of the MDM2 gene encoding the murine double minute 2 (Mdm2) protein or its homolog MDM4. This mechanism is reported to occur in ~10% to 20% of all cancers (Toledo and Wahl 2006). Other mechanisms of p53 inactivation occur at much lower frequencies and include allelic deletion of CDKN2A encoding the p14ARF protein, viral protein inactivation of p53, or mislocation of p53 to the cytoplasm (Vogelstein et al 2000). We reasoned that if p53 malfunction is required for all cancers, there are a significant number of cancers with unknown p53 inactivation mechanisms and that a newly discovered class of noncoding regulatory RNA molecules called microRNAs (miRNAs) might be potential inhibitors of p53 gene expression.

miRNAs are short 20–25 nucleotide RNA molecules that negatively regulate gene expression by targeting the 3'UTRs of mRNAs (Bartel 2004). Several reports shed light on the involvement of miRNAs in the p53 pathway. The Agami laboratory has shown that miR-372 and miR-373 permit proliferation and tumorigenesis of primary human cells that harbor both oncogenic RAS and active wild-type p53. They further demonstrated that these two miRNAs neutralize p53-mediated CDK inhibition through negatively regulating the tumor suppressor LATS2 gene (Voorhoeve et al 2006). The Hannon laboratory profiled miRNA gene expression in wild-type and p53-deficient cells and found that the miR-34 gene family (including miR-34a, b, and c) was among the most upregulated in wild-type p53 cells. They further found that miR-34s were transcriptionally activated by p53 and that ectopic expression of miR-34 induced cell cycle arrest through down-regulating the expression of CDK4 and other genes (He et al 2007). The finding that miR-34s are critical components of the p53 network has been independently corroborated by several other laboratories (Bommer et al 2007, Chang et al 2007, He et al 2007, Raver-Shapira et al 2007, Tarasov et al 2007). Taken together, these results support a pivotal downstream role of miRNAs in the regulation of the p53 pathway.

The initial dissection of human miRNA function, mostly by identifying target genes and characterizing related phenotypes has been approached using three distinct methods. The first approach, as exemplified by let-7 (Johnson et al 2005) targeting RAS, is to explore the human homolog of a miRNA with known function in nematodes or other organisms. The second, as exemplified by miR-34a (He et al 2007) targeting genes in the p53 network, is to profile miRNA expression in disease end points, tissues or cells treated with various agents.
before selecting a single miRNA for examination. The last approach as exemplified by miR-372 and miR-373 (Voorhoeve et al 2006) targeting LATS2, is to screen for a desired phenotype using a pooled miRNA library. The method of let-7 & RAS is not applicable for human TP53 as the 3'UTR of human p53 mRNA is not homologous to that of mouse or other lower eukaryotes. The second approach, i.e. general expression profiling of most human tumor tissues might miss the miRNA:TP53 interaction as p53 is mutated in ~50% of cancers. Park et al., (2008) used a reporter assay to screen 91 miRNAs and found that the miR-29 family can upregulate p53 by targeting p85α and CDC42 though it is vague about how p85α and CDC42 activate p53 (Park et al 2009). miR-125b, a brain-enriched microRNA, was identified as a bona fide negative regulator of p53 in both zebrafish and humans (Le et al 2009). More recently, Hu et al., (2010) demonstrated that miR-504 directly represses p53 expression and function in human cell lines (Hu et al 2010). Here we report the development of a two-tier screening assay to identify miRNA that negatively regulate p53 signaling through direct interaction with TP53 3'UTR. We screened over 400 miRNAs and found that miR-25 and miR-30d down-regulate p53 expression and interfere its function in cell cycle arrest, apoptosis, and senescence. These findings demonstrated that multiple miRNAs directly inhibit p53 expression and function by interacting with its 3'UTR, adding a new layer of complexity of p53.

MATERIALS AND METHODS

Plasmid constructs

The construction of the genetic library for miRNA minigenes is described elsewhere and all constructs are available at Genecpoeia, Inc. (Germantown, MD). The 3'UTR of TP53 was PCR amplified from genomic DNA and cloned into pRL-TK (Promega) downstream of the Renilla luciferase gene. Mutations in pRL-p53UTRmut were cloned by PCR to contain the following sequences: ACGTTAT (position 92–98, mutated from TGCAATA) for miR-25, CAAATGT (position 287–293, mutated from GTTTACA) for miR-30d, and GTCACAA (position 960–966, mutated from CAGTGTT) for miR-200a. For constructs expressing TP53 and its 3'UTR variants, the wt or mutant 3'UTR of TP53 were cut from pRL-p53UTR or its mutant and cloned downstream of the p53 expression cassette of pCMV-p53 (Clontech, Mountain View, CA). All constructs were confirmed by DNA sequencing.

Statistical Analyses

All values in Fig. 1C, 1D, 1E, 1G, 1H, 2C, 3C, 4B, 4C, 5B, 5D, and 6C were represented as means with SEM from three to six independent experiments. Statistical analyses were performed using Student’s t-test with results considered statistically significant at P ≤0.05. Pearson Correlation analyses in Fig. 6A were performed using SPSS 11.5 (SPSS, Inc., Chicago, IL). Cell culture, Western blotting, qRT-PCR, cell cycle analysis, apoptosis, and senescence assays were performed using routine procedures and details were provided in Supplemental Text 1.
Results

Dual-reporter screening to identify miRNAs targeting TP53 in 293T cells

We performed a screen using the tier-1 assay (Fig. 1A) to determine whether any of the 366 miRNA constructs carrying 419 mature miRNAs modulate the basal p53 activity in 293T cells (Supplemental Data Set 1). We found a large number of miRNAs which activate or suppress p53 signaling as judged by alteration of the luc/Rluc readout. Overall, there were 105 miRNAs upregulating and 65 down-regulating the basal luc expression by more than 25%. The 25% cut off was used as it is the minimum threshold observed in two previously published articles on miRNA: 3'UTR interactions (Lewis et al 2005, Miranda et al 2006).

Next, we performed computational analyses to predict which miRNAs might target the human TP53 gene with four commonly used miRNA target prediction methods: Miranda (John et al 2004), TargetScan (Lewis et al 2005), PicTar (Krek et al 2005), or RNA22 (Miranda et al 2006). By using 4 prediction methods with relaxed stringencies, we maximized the number of miRNAs chosen to undergo the tier-2 assay to avoid missing TP53-targeting miRNAs. These analyses identified 67 miRNAs predicted to target the human p53 3'UTR by at least one method. Only 8 out of these 67 miRNAs down-regulated reporter expression with a cutoff of 25% in the tier-1 assay (Fig. 1C; Supplemental Fig. S1), indicating a number of predicted miRNA:mRNA interactions are probably false positives and can be excluded by experimental validation such as the tier-1 assay. The tier-2 assay (Fig. 1B) was then performed to determine which of the miRNAs predicted to target TP53 down-regulate the expression of Rluc located upstream of the TP53 3'UTR. Only 3 miRNAs (miR-25, miR-30d, and miR-200a) down-regulated both Rluc in the tier-2 assay and luc expression in the tier-1 assay by more than 25% and emerged as putative regulators of p53 (Fig. 1C & Fig. 1D).

We next examined the protein levels of p53 when miR-25, miR-30d, or miR-200a was overexpressed in 293T cells (Supplemental Table S1). The protein levels of p53 were down-regulated significantly by miR-25 and miR-30d, but not by miR-200a (Fig. 1E). To determine whether these miRNAs target the TP53 3'UTR specifically, we performed a mutational analysis, in which the nucleotides corresponding to the seed sequence of each miRNA were mutated in pRL-p53UTR (Fig. 1F). A tier-2 assay performed using mutant TP53 3'UTRs instead of pRL-p53UTR demonstrated that a mutation in the corresponding miR-25 region abolished reporter regulation by miR-25, but not by miR-30d. Similar results were observed with mutations for miR-30d (Fig. 1G), while a control mutant in which a mock miR-200a binding site was mutated did not affect reporter expression by either miR-25 or miR-30d (Fig. 1G). These results suggest that the down-regulation of the reporter gene by these miRNAs is dependent on specific segments of the TP53 3'UTR, which are partially complementary to the respective miRNAs. Finally, we examined the TP53 mRNA levels after introduction of the miRNAs. TP53 mRNA was down-regulated about 60% or 85% with the introduction of miR-30d or miR-25 (Fig. 1H), indicating that TP53 mRNA was degraded and that corresponding changes in protein levels were likely mediated by mRNA turnover. Taken together, these results suggest that TP53 is an authentic target of miR-25 and miR-30d in 293T cells.
miRNAs inhibit the effect of p53 on apoptotic cell death and G2 arrest in 293T cells

One of the major endpoints of p53 activation is apoptosis, which partially depends on the induction of Bax. Apoptosis can be enhanced by genotoxic agents such as etoposide. We examined the protein levels of p53, p21, and Bax in 293T cells overexpressing miR-25 or miR-30d with or without etoposide treatment. We found that p53, p21, and Bax expression decreased with miRNA overexpression in the presence or absence of the DNA damaging agent (Fig. 2A, left panel). As p53 & Bax-dependent intrinsic apoptosis pathway is known to be mediated by caspase 9 (initiator caspase) and caspase 3,6,7 (effector caspase) (Mayer and Oberbauer 2003), we measured both caspase 9 and caspase 3,7 activities in 293T cells expressing miRNAs in the presence or in the absence of etoposide. As shown in Fig. 2B and 2C, there was a significant decrease in caspase activities when either miRNAs was overexpressed in 293T cells with or without etoposide treatment, coinciding with p53 and Bax down-regulation (Fig. 2A). Gadd45α (growth arrest and DNA-damage-inducible), a protein regulated by p53, has been reported to induce cell cycle arrest at the G2-M phase (G2 arrest) (Wang et al 1999). In our experiment, Gadd45α was down-regulated along with p53 in 293T cells transfected with both miR-25 and miR-30d (Fig. 2A, right panel) and a cell cycle analysis showed that fewer cells were arrested at the G2-M phase than in the control (Fig. 2D). These data suggest that miR-25 and miR-30d down-regulate the expression of p53, Bax, and Gadd45α and subsequently reduce both apoptosis and G2 arrest in 293T cells. Similar G2 arrest was observed in p53 wild-type, non-immortalized, primary human lung fibroblast WI-38 cells (Supplemental Figure S2). Since miR-25 is reported to target Bim in gastric cancer cells (Petrocca et al 2008) and Barrett's esophagus (Kan et al 2009) and PCAF in multiple myeloma cancer cells (Pichiorri et al 2008), we determined the expression of these two proteins in 293T cells. In contrast to previous reports, the expression levels of both Bim and PCAF were not altered significantly by miR-25 or miR-30d in 293T cells (Fig. 2A, right panel). This result suggests that down-regulation of p53 by miR-25 is unlikely due to indirect effects of miR-25 overexpression on other genes such as Bim or PCAF.

miRNAs inhibit p53 function on apoptosis and G1 arrest in p53-null H1299 cells with ectopic p53 expression

We next constructed three distinct p53 expression cassettes based on pCMV-p53 carrying a wt p53 gene instead of pRL-TK with Rluc, all with wt TP53 coding sequences: one with a wt 3’UTR (p53/3UTRwt) and the other two with mutations that disrupt a respective miRNA: 3’UTR interaction (p53/3UTRmut25 and p53/3UTRmut30d). We introduced these p53 constructs and miRNAs into human lung adenocarcinoma p53-null H1299 cells and found that both miR-25 and miR-30d down-regulated the expression of the ectopic p53 gene with a wt 3’UTR (Fig. 3A). However, p53 expression levels were not changed in cells co-transfected with a miRNA and a p53 gene with a respective mutant 3’UTR (Fig. 3A). A similar expression pattern was observed for the p53-regulated genes p21, Puma, and Bax (Fig. 3A).

We next determined cell apoptosis as both Puma and Bax were down-regulated in cells having p53 with a wt 3’UTR but not in those with a mutant 3’UTR (Fig. 3A). As demonstrated in Fig. 3B and Fig. 3C, when Puma and Bax were down-regulated, apoptotic

Oncogene. Author manuscript; available in PMC 2011 August 17.
cell death was reduced in cells with each miRNA and an ectopic p53 gene with a wt 3’UTR. In contrast, there was no significant change in apoptosis in cells carrying a p53 gene with a mutant 3’UTR, which can no longer be targeted by the respective miRNA (Fig. 3C). This coincided with the unaltered expression levels of both p53 and Puma/Bax in these cells (Fig. 3A). We then performed a cell cycle analysis and found that fewer cells were arrested at the G1-S phase (G1 arrest) when miR-25 or miR-30d was co-overexpressed with the p53 gene with a wt 3’UTR but not with the one with a respective mutant (Fig. 3D). This is presumably due to the functional output of the p53-regulated, CDK inhibitor p21, which is significantly down-regulated in cells overexpressing a miRNA targeted TP53 gene with a wt 3’UTR but not in those with mutant 3’UTRs (Fig. 3A). These results support the view that miRNAs target p53 to down-regulate Puma/Bax and p21 expression and adversely affect apoptosis and G1 arrest in H1299 cells carrying an ectopic p53 expression construct.

**miRNAs inhibit the effect of p53 on cellular senescence in HCT116 cells**

To further determine p53 regulation by miRNAs, we utilized a pair of cell lines that have been widely used in studies of p53: HCT116 with a wt TP53 gene and HCT116 p53−/− in which both TP53 alleles are deleted (Bunz et al 1998). We introduced miR-25 or miR-30d into these two cell lines and monitored the expression levels of Bax, p21, and p53. We found that miRNAs down-regulated the expression of p53 and two p53-transactivated genes in HCT116, the cell line with a wt TP53 gene but not in HCT116 p53−/− cells which do not have TP53 (Fig. 4A, left), indicating that down-regulation of p21 and Bax by miRNAs was p53-dependent.

We transfected p53 constructs with 3’UTR variants and miRNAs into HCT116 p53−/− cells and determined the expression levels of p53, p21, and Bax. We found that both miR-25 and miR-30d down-regulated the expression of the ectopic p53 gene carrying a wt 3’UTR but not the one with a mutant (Fig. 4A). The expression of both p21 and Bax was either reduced or minimally changed, following p53 expression. We then determined cellular senescence using β-galactosidase staining as p21 is thought to be responsible for irreversible cellular arrest, i.e. senescence. As shown in Fig. 4B, senescence was reduced in HCT116 cells with miRNA overexpression and lower levels of p53 and p21 (Fig. 4A). Conversely, cellular senescence was reduced in HCT116 p53−/− cells with either miRNA and an ectopic p53 gene with a wt 3’UTR (and lower levels of p21, Fig. 4A), but the reduction of senescence was abolished in HCT116 p53−/− cells co-expressing either miRNA and a p53 gene carrying a respective mutant 3’UTR (Fig. 4C and Supplemental Figure S3). These results suggest that miR-25 and miR-30d regulate cellular senescence through down-regulating p53 expression and subsequently the expression of p21 and that such regulation depends on miRNA binding sites of TP53 3’UTR.

**Inhibition of miRNA expression increases p53 expression and cellular apoptosis in HCT116 and A549 cells**

To further illuminate whether endogenous p53 expression is regulated by miRNAs, we next determined whether inhibition of miRNA expression increases p53 expression. We transfected miRNA inhibitors into two cell lines, HCT116 and A549, both possessing a wild-type p53 gene. We observed that the endogenous level of p53 protein was elevated...
when any of the inhibitors to miR-25 or miR-30d was introduced (Fig. 5A). p21 and Puma were also up-regulated in both cell lines (Fig. 5A and 5C). Furthermore, an increase in apoptosis was observed, coinciding with the elevated levels of Puma in both HCT116 and A549 cells (Fig. 5B and 5D).

Inhibition of miRNA expression increases p53 expression and cellular apoptosis in multiple myeloma cells

Most of the miRNA expression profiling in cancers conducted to date does not distinguish tumors with wild-type TP53 from those with TP53 mutations, so the association of miRNA dysregulation and p53 inactivation may not be revealed if overexpression of miRNAs only inactivates the wild-type TP53 gene. We reasoned that if these miRNAs were involved in p53 inactivation, they could be overexpressed in cancers with an extremely low p53 mutation rate. Multiple myeloma is a neoplasm characterized by excessive infiltration of abnormal plasma cells in the bone marrow. Various studies of p53 mutations in multiple myeloma with clinical heterogeneity in the study cohorts and small sample sizes reported a prevalence of p53 mutation ranging from 0 to 20% (Fonseca et al 2004). The latest comprehensive study with the largest number of newly diagnosed patients reported TP53 mutations are rare as only 9 of 268 samples (3%) tested were positive for monoallelic mutations (Chng et al 2007). Biallelic deletion of TP53 is observed in only 2% of patients (Xiong et al 2008). Moreover, other known mechanisms of p53 inactivation also have relatively low incidence in multiple myeloma (Supplemental Table S2). It has been reported that miR-25 is overexpressed in primary plasma cells from multiple myeloma patients (n=16) ~71 fold (P=3E-07) and miR-25 is indeed the most upregulated miRNA (Pichiorri et al 2008). In addition, miR-125b and miR-30d (but not miR-504) are also upregulated ~5.6 fold (P=0.05) and ~2.1 fold (P=0.04), respectively (Pichiorri et al 2008).

We found that miR-25 and miR-30d were overexpressed ~6.3- and ~11.2-fold (mean values; P = 0.0027 and 0.018) in plasma cells from a larger number of multiple myeloma patients (n=31) compared to that from healthy donors (n=6) (Fig. 6A). Additionally, we determined the TP53 mRNA levels in plasma cells and we found that TP53 mRNA is lower in plasma cells from multiple myeloma patients than in healthy donors (mean value is ~25% of that of the control; P = 0.0063; Fig. 6A). TP53 mRNAs were detectable in all myeloma specimens, indicating that there was no TP53 biallelic deletion in this cohort. We determined whether there was an inverse correlation of miRNAs expression and TP53 mRNA in multiple myeloma as measured by Pearson Correlation. miR-25 expression was moderately inversely correlated with TP53 mRNA levels (Coefficient = −0.22, P = 0.026) and so is miR-30d (Coefficient = −0.10, P = 0.037). Given that miR-25 and miR-30d down-regulate p53 expression through reduction in p53 mRNA levels (Fig. 1H), this result indicates the importance of miRNA overexpression in lowering p53 expression.

To further test whether miRNAs regulate endogenous p53 expression in multiple myeloma, we introduced miRNA inhibitors into an established multiple myeloma cell line H929 with a wild-type p53 gene. We found that the endogenous level of p53 protein was elevated when any of the inhibitors to miR-25 or miR-30d was introduced (Fig. 6B). Puma was also up-regulated (Fig. 6B). An increase in apoptosis was observed in H929 cells with inhibitors to
any of the two miRNAs (Fig. 6C). Taken together, these data suggest that dysregulation of miR-25 and miR-30d is pathologically important to p53 function in H929 cells. Thus, inhibition of miRNA to activate p53 could be a therapeutic tool for multiple myeloma intervention.

Discussion

The investigation of mechanisms of p53 inactivation in tumors has been the focus of intense interest as it may offer a clue as to where malignancy initiates (Vousden and Prives 2009). Several miRNAs, such as miR-372 and miR-373, miR-29a,b,c, and miR-34a,b,c, have been reported to be important components of the p53 transcriptional network. The identification of miRNAs that directly target TP53 is critical to understanding why approximately 50% of cells with wt TP53 have developed malignancies. In this report, we identify two miRNAs, miR-25 and miR-30d that interact with the 3'UTR of the human TP53 gene to negatively regulate p53 expression. Down-regulation of p53 protein and its transactivational genes such as p21, Bax, Puma, and Gadd45α by these miRNAs is sufficient to reduce cellular apoptosis, cell cycle arrest, and senescence. These findings suggest an important role for miR-25 and miR-30d in the regulation of the p53 tumor suppression function. As p53 is well-integrated in a complex molecular network and involved in a number of positive and negative feedback loops (Harris and Levine 2005), we were interested in determining whether p53 status affects the expression of miR-25 or miR-30d. We transfected pCMV-p53 carrying a functional p53 gene or pCMV-p53m153 with an inactive TP53 into 293T cells and H1299 cells and determined the expression of miRNAs by qPCR. The expression of miR-25 and miR-30d was not changed in either cell line. This result indicated that the expression of miR-25 and miR-30d is not regulated by p53. Similar results were reported by another group in a p53 temperature-sensitive cell line (Raver-Shapira et al 2007), indicating that p53 are unlikely to regulate the expression of TP53-targeting miR-25 or miR-30d. It is consistent with that there is no p53 TREs in the promoter of miR-25, which is in the same transcriptional unit with MCM7 (Suzuki et al 1998) or miR-30d.

It is noteworthy that other members of the miR-25 and miR-30 gene family were not identified to target p53 in our screen assay through they have the same seed sequence as that of miR-25 and miR-30d. These miRNAs include miR-92a, b and miR-30a, b, c, e (Supplemental Table S3). This may largely be due to the difference in the 3’ portion of these miRNAs. It has been proposed that 3–4 contiguous Watson-Crick pairs uninterrupted by bulges, mismatches or wobbles, involving the 3’ portion of miRNAs and the UTR of a target gene is important to miRNA:target interaction (Bartel 2009). These base pairs are called 3’ supplementary pairing. We noted that there is 3’ supplementary pairing between p53 3’UTR and miR-25/miR-30d but no supplementary paring between the 3’UTR and miR-92a,b/ miR-30a,b,c,e (Table S3). These minor differences were reflected by our tier-1 assay in which miR-92a and miR-30a did not down-regulate p53-dependent reporter activity (Fig. S1). We also recognize that miR-125b and miR-504, two known p53 regulators, were not scored in our method, which indicates the screening assay used in this study is by no means exhaustive and may have cell-line specificity limitations (Supplemental DataSet 1).
Interestingly, three TP53-targeting miRNAs, miR-25 and miR-30d in our study and miR-125b in a previous report (Le et al 2009) are evolutionally conserved in fly, zebra fish, mouse, and human and they are grouped into miR-125, miR-25, and miR-30 gene families (Griffiths-Jones et al 2006). The miR-504 gene located on the Chromosome X, on the other hand, is mammalian-specific. The mature sequences of these 4 miRNAs from mouse are identical to those of human, and the stem-loop pre-miRNAs of miR-25 from mouse are the same as those from human. However, the TP53 3’UTRs from mouse and human vary in size and sequence; and none of the four miRNAs are predicted to target mouse TP53 (Supplemental Table S4). The human TP53 3’UTR (Genbank No. NM_000546) is 1207 nts, while the mouse (NM_011640) is only 431 nts; and they share little sequence identity except a ~100 nt fragment (~77% identity). In contrast, both the coding DNA sequence and amino acid sequence of p53 from mouse (1173 nts & 390 aa) and human (1182 nts & 393 aa) are highly conserved with ~80% identity. This indicates that p53 regulation by miR-25, miR-30d, miR-125b, and miR-504 via the 3’UTR does not serve the same role in mice. This could contribute to differences in tumor development between human and mouse.

p53 function is known to be disrupted in the majority of human cancers. Yet in some tumors p53 mutation rates are extremely low. For instance, only a minute subset of multiple myeloma cases has TP53 genetic alterations (3% mutation and 2% biallelic deletion) at the time of diagnosis. Should disrupting p53 function be indispensable to tumorigenesis, there would be other mechanisms of p53 inactivation. We have demonstrated that miR-25 and miR-30d overexpression is inversely correlated with lower TP53 mRNA levels in plasma cells from multiple myeloma patients compared to that from healthy donors. Moreover, inhibition of miR-25 or miR-30d increased p53 and Puma expression levels and led to elevated cellular apoptosis. These results suggest that p53 inactivation by overexpressed miRNAs may be a crucial early step that primes plasma cells to malignancy as high levels of miRNAs could prevent p53 from guarding the genome. Beyond multiple myeloma, miR-25 and miR-30d are also upregulated in prostate, pancreatic, and stomach tumors compared to normal tissues (Yanaihara et al 2006). It has been reported that mutations of TP53 occur infrequently in early invasive prostate carcinoma (Prendergast et al 1996, Voeller et al 1994). Thus, categorizing cancers based on TP53 gene status may uncover more intriguing miRNA expression patterns and reveal the power of miRNAs as biomarkers and drug targets.

To summarize, the arsenal of p53 regulators includes not only proteins such as Mdm2 and Mdm4, but also miRNAs. Most known p53 regulation mechanisms by proteins are posttranslational, but miRNAs, including miR-25, miR-30d, miR-125b, and miR-405, regulate p53 expression posttranscriptionally by interacting with its 3’UTR. Their ability to act directly on p53 and adversely affect apoptosis, cell cycle arrest, and senescence of cancer cells makes them attractive candidates as drug targets. As the p53 gene is only mutated in about half of all human cancers, it could be inactivated by miRNA overexpression in malignancies such as multiple myeloma with little p53 mutations. Determination of TP53 gene status and miRNA expression and manipulation of miRNA levels in human tumors therefore may enable better cancer diagnosis, prognosis, and therapy.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

Y. L. is supported by the Director’s Biomarker Award from the Center for Environmental Genomics and Integrative Biology funded by NIEHS/NIH (P30ES014443), Kentucky Lung Cancer Research Program, NCI/NIH (R01CA138688), and NCRR/NIH (P20 RR024489). K. H. Y. is supported by University of Wisconsin Paul Carbone Comprehensive Cancer Center Award, Gundersen Medical Foundation Award, University of Wisconsin Pathology R & D Fund and NCI/NIH (P20CA103697 and 1RC1CA146299). The authors are grateful to the critical reviews from Drs. Korise E. Rasmusson and Nancy C. Martin.

References

Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004; 116:281–297. [PubMed: 14744438]
Bartel DP. MicroRNAs: Target Recognition and Regulatory Functions. Cell. 2009; 136:215–233. [PubMed: 19167326]
Bommer GT, Gerin I, Feng Y, Kaczorowski AJ, Kuick R, Love RE, et al. p53-Mediated Activation of miRNA34 Candidate Tumor-Suppressor Genes. Curr Biol. 2007; 17:1298–1307. [PubMed: 17656095]
Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, et al. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science. 1998; 282:1497–1501. [PubMed: 9822382]
Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, et al. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol Cell. 2007; 26:745–752. [PubMed: 17540599]
Chng WJ, Price-Troska T, Gonzalez-Paz N, Van Wier S, Jacobus S, Blood E, et al. Clinical significance of TP53 mutation in myeloma. Leukemia. 2007; 21:582–584. [PubMed: 17215851]
Fonseca R, Barlogie B, Bataille R, Bastard C, Bergsagel PL, Chesi M, et al. Genetics and cytogenetics of multiple myeloma: a workshop report. Cancer Res. 2004; 64:1546–1558. [PubMed: 14989251]
Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res. 2006; 34:D140–D144. [PubMed: 16381832]
Hanahan D, Weinberg RA. The Hallmarks of Cancer. Cell. 2000; 100:57–70. [PubMed: 10647931]
Harris SL, Levine AJ. The p53 pathway: positive and negative feedback loops. Oncogene. 2005; 24:2899–2908. [PubMed: 15838523]
He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, et al. A microRNA component of the p53 tumour suppressor network. Nature. 2007; 447:1130–1134. [PubMed: 17554337]
Hu W, Chan CS, Wu R, Zhang C, Sun Y, Song JS, et al. Negative Regulation of Tumor Suppressor p53 by MicroRNA miR-504. Mol Cell. 2010; 38:689–699. [PubMed: 20542001]
John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human MicroRNA targets. PLoS Biol. 2004; 2:e363. [PubMed: 15502875]
Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, et al. RAS is regulated by the let-7 microRNA family. Cell. 2005; 120:635–647. [PubMed: 15766527]
Kan T, Sato F, Ito T, Matsumura N, David S, Cheng Y, et al. The miR-106b-25 polycistron, activated by genomic amplification, functions as an oncogene by suppressing p21 and Bim. Gastroenterology. 2009; 136:1689–1700. [PubMed: 19422085]
Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, et al. Combinatorial microRNA target predictions. Nat Genet. 2005; 37:495. [PubMed: 15806104]
Kruse J-P, Gu W. Modes of p53 Regulation. Cell. 2009; 137:609–622. [PubMed: 19450511]
Le MTN, Teh C, Shyh-Chang N, Xie H, Zhou B, Korzh V, et al. MicroRNA-125b is a novel negative regulator of p53. Genes & Dev. 2009; 23:862–876. [PubMed: 19293287]
Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005; 120:15–20. [PubMed: 15652477]

Mayer B, Oberbauer R. Mitochondrial Regulation of Apoptosis. News Physiol Sci. 2003; 18:89–94. [PubMed: 12750442]

Miranda KC, Huynh T, Tay Y, Ang YS, Tam WL, Thomson AM, et al. A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. Cell. 2006; 126:1203–1217. [PubMed: 16990141]

Park SY, Lee JH, Ha M, Nam JW, Kim VN. miR-29 miRNAs activate p53 by targeting p85α and CDC42. Nat Struct Mol Biol. 2009; 16:23–29. [PubMed: 19079265]

Petrocca F, Visone R, Onelli MR, Shah MH, Nicoloso MS, de Martino I, et al. E2F1-Regulated MicroRNAs Impair TGFβ-Dependent Cell-Cycle Arrest and Apoptosis in Gastric Cancer. Cancer Cell. 2008; 13:272–286. [PubMed: 18328430]

Pichiorri F, Suh SS, Ladetto M, Kuehl M, Palumbo T, Drandi D, et al. MicroRNAs regulate critical genes associated with multiple myeloma pathogenesis. Proc Natl Acad Sci USA. 2008; 105:12885–12890. [PubMed: 18728182]

Prendergast NJ, Atkins MR, Schatte EC, Paulson DF, Walther PJ. p53 immunohistochemical and genetic alterations are associated at high incidence with post-irradiated locally persistent prostate carcinoma. J Urol. 1996; 155:1685–1692. [PubMed: 8627854]

Raver-Shapira N, Marciano E, Meiri E, Spector Y, Rosenfeld N, Moskovits N, et al. Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. Mol Cell. 2007; 26:731–743. [PubMed: 17540589]

Riley T, Sontag E, Chen P, Levine A. Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol. 2008; 9:402–412. [PubMed: 18431400]

Suzuki S, Adachi A, Hiraïwa A, Ohashi M, Ishibashi M, Kiyono T. Cloning and characterization of human MCM7 promoter. Gene. 1998; 151:1685–1692. [PubMed: 9714754]

Tarasov V, Jung P, Verdoott B, Lodygin D, Epanchintsev A, Menssen A, et al. Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. Cell Cycle. 2007; 6:1586–1593. [PubMed: 17554199]

Toledo F, Wahl GM. Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. Nat Rev Cancer. 2006; 6:909–929. [PubMed: 17128209]

Ventura A, Kirsch DG, McLaughlin ME, Tuveson DA, Grimm J, Lintault L, et al. Restoration of p53 function leads to tumour regression in vivo. Nature. 2007; 445:661–665. [PubMed: 17251932]

Voeller HI, Sugars LY, Pretlow T, Gelmann EP. p53 oncogene mutations in human prostate cancer specimens. J Urol. 1994; 151:492–495. [PubMed: 7904314]

Voigstein B, Lane D, Levine AJ. Surfing the p53 network. Nature. 2000; 408:307–310. [PubMed: 11099028]

Voorhoeve PM, le Sage C, Schrier M, Gillis AJ, Stoop H, Nagel R, et al. A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. Cell. 2006; 124:1169–1181. [PubMed: 16564011]

Vouwesen KH, Prives C. Blinded by the Light: The Growing Complexity of p53. Cell. 2009; 137:413–431. [PubMed: 19410540]

Wang XW, Zhan Q, Coursen JD, Khan MA, Kontny HU, Yu L, et al. GADD45 induction of a G2/M cell cycle checkpoint. Proc Natl Acad Sci USA. 1999; 96:3706–3711. [PubMed: 10097101]

Xiong W, Wu X, Sternees S, Johnson SK, Haessler J, Wang S, et al. An analysis of the clinical and biologic significance of TP53 loss and the identification of potential novel transcriptional targets of TP53 in multiple myeloma. Blood. 2008; 112:4235–4246. [PubMed: 18337559]

Xue W, Zender L, Miething C, Dickens RA, Hernandez E, Krizhanovsky V, et al. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature. 2007; 445:656–660. [PubMed: 17251933]

Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell. 2006; 9:189–198. [PubMed: 16530703]

Oncogene. Author manuscript; available in PMC 2011 August 17.
Figure 1.
A screening assay to identify miRNAs targeting TP53 in 293T cells. (A) Schematic presentation of the tier-1 assay measuring the basal p53 activity. (B) Schematic presentation of the tier-2 assay. (C) The tier-1 assay for 8 miRNAs that are predicted to target TP53. (D) The tier-2 assay for 8 miRNAs that are predicted to target TP53. (E) The protein levels of p53 were down-regulated by miR-25 and miR-30d. “p53 RQ” indicated the relative quantification of p53 normalized to β-actin. (F) TP53 3’UTR mutations. Three mutations were created in the TP53 3’UTR to disrupt the respective seed sequence: 3’UTR interaction...
for miR-25, miR-30d, and miR-200a. Red vertical bars denote mutant while black wt. (G) Rluc down-regulation by miRNAs is dependent on the 3'UTR of TP53. 293T cells were cotransfected with pRL-TK carrying a wt or mutant 3'UTR sequence. (H) p53 mRNA levels were decreased with miRNA overexpression. * P ≤0.05 with n=3–6.
Figure 2.
miRNAs down-regulate p53 expression and reduce apoptosis and cell cycle arrest in 293T cells. (A) p53, p21, Bax, and Gadd45α were down-regulated in 293T cells transfected with miRNAs with or without etoposide treatment. (B) Caspase 9 activities in 293T cells overexpressing miRNAs with or without etoposide treatment; the Y-axis denotes the relative luminescent units (RLU) from the Caspase-Glo® 9 Assay (n=4). (C) Caspase 3,7 activities in 293T cells overexpressing miRNAs with or without etoposide treatment; the Y-axis denotes the relative fluorescent units (RFU) from the Apo-ONE® Homogeneous.
Caspase-3/7 Assay (n=4). (D) miRNAs inhibit p53-mediated G2 arrest in cells treated with etoposide; the X axis denotes events (the number of cells) and Y denotes the emitted fluorescent light (DNA content) of the DNA dye (PI) (n=6). *, $P \leq 0.05$ and **, $P \leq 0.01$. 
miRNAs down-regulate p53 expression and reduce apoptosis and cell cycle arrest in H1299 cells. (A) p53, p21, Bax, and Puma were down-regulated in H1299 cells transfected with miRNAs and an ectopic p53 with a wt 3'UTR, but not those with mutant 3'UTRs. (B) A representative photo of flow-cytometry used to determine cellular apoptosis of H1299 cells. The Y-axis denotes the log values of signal density for Annexin V with X to that of PI. The percentage of cells in three quadrants was presented. (C) The bar graph for 6 independent runs of (B). Student’s t-tests were performed for samples with or without etoposide.
treatment, respectively. (D) miRNAs inhibit p53-mediated G1 arrest. “Control” was performed using parental vectors only (1:1 of miRNA empty vector and p53 empty vector). The X axe denotes events (the number of cells) and Y denotes the emitted fluorescent light of the DNA dye (PI), i.e., DNA content. * $P \leq 0.05$ with n=3–6.
Figure 4.
miRNAs inhibit p53 cellular senescence expression in HCT116 and HCT116 p53−/− cells. (A) p53, p21, and Bax expression in HCT116 and HCT116 p53−/− cells with miRNA overexpression. (B) miRNAs inhibit cellular senescence in HCT116 cells. (C) miRNAs inhibit cellular senescence in HCT116 p53−/− cells expressing an ectopic wt p53 with a wt 3’UTR, but not those with mutant 3’UTRs. The bar graphs in (B) and (C) represent 3 independent runs. “Control” in (C) was performed using parental vectors only (1:1 of miRNA empty vector and p53 empty vector). The Y axis in the graphs denotes the number of senescence-positive cells per microscope field. * P ≤0.05 with n=6.
Figure 5.
Down-regulation of miRNAs increases p53 expression and cellular apoptosis in HCT116 and A549 cells. (A) Inhibition of miRNA expression using miRNA inhibitors upregulates the expression of p53 and its target genes in HCT116 cells. (B) Inhibition of miRNA expression promotes cellular apoptosis in HCT116 cells. (C) and (D), similar experiments but in lung cancer A549 cells. “Neg contl”, the negative control; “Anti-miR-25/-30d”, the inhibitors to miR-25/-30d. * P ≤0.05 with n=6.
Figure 6.
Down-regulation of miRNAs increases p53 expression and cellular apoptosis in multiple myeloma cells. (A) miR-25, miR-30d, and TP53 mRNA expression in plasma cells from bone marrow aspirates of multiple myeloma patients (MM) and healthy donors (NC). Whisker-box plot where the boxes indicate the 25th and 75th percentile; thin lines in the boxes indicate the 50th percentile and thick lines denote the mean values; whisker caps indicate the 5th and the 95th percentile; filled circles indicate outliers. (B) Inhibition of miRNA expression using miRNA inhibitors increases the expression levels of p53 and its target genes in H929 cells. (C) Inhibition of miRNA expression promotes cellular apoptosis.
in H929 cells. “Neg contl”, the negative control; “Anti-miR-25/-30d”, the inhibitors to miR-25/-30d. * $P \leq 0.05$ with $n=6$. 

*Oncogene. Author manuscript; available in PMC 2011 August 17.*