A combined array-based comparative genomic hybridization (aCGH) and functional library screening approach identifies mir-30d as an oncomir in cancer

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Abstract

Oncomirs are microRNAs (miRNAs) that acts as oncogenes or tumor suppressor genes. Efficient identification of oncomirs remains a challenge. Here we report a novel, clinically-guided genetic screening approach for the identification of oncomirs, identifying mir-30d through this strategy. mir-30d regulates tumor cell proliferation, apoptosis, senescence, and migration. The chromosomal locus harboring mir-30d was amplified in >30% of multiple types of human solid tumors (n=1,283). Importantly, levels of mir-30d expression were associated significantly with poor clinical outcomes in ovarian cancer patients (n=330, p=0.0016). Mechanistic investigations suggested that mir-30d regulates a large number of cancer-associated genes including the apoptotic caspase \textit{CASP3}. The guided genetic screening approach validated by this study offers a powerful tool to identify oncomirs that may have utility as biomarkers or targets for drug development.
Introduction

Cancer is a genetic disease involving multistep changes in the genome (1). Most studies to date have focused mainly on the role of protein-coding sequences in cancer. Although non-coding sequences constitute up to 98% of the human genome and have been implicated in tumorigenesis, our knowledge of the function of these genomic sequences in cancer is still limited (2-7). microRNAs (miRNAs) are non-coding RNAs about 22 nucleotides in length which regulate gene expression in a sequence-specific manner (2-4, 6, 8-13). Increasing evidence indicates that miRNAs may play a critical role in cancer (14-21). miRNAs act as key regulators of various fundamental biological processes, many of which share common pathways with the development of cancer. In addition, it has been widely reported that miRNA expression is remarkably deregulated in cancer. Several miRNAs have already been shown to play a critical role in cancer. Referred to as “oncomirs”, these miRNAs can act as either tumor suppressors or as oncogenes (4). Current rapid advances in oligonucleotide/nanoparticle therapies create a realistic optimism for the establishment of miRNAs as a new and potent therapeutic target for cancer treatment (22-25). However, the efficient identification of oncomirs remains a challenge. Although both microarray based high throughput genomic/genetic profiling (3, 18, 19, 26) and whole genome-wide genetic screening (16, 27-31) have been successfully applied to the identification of oncomirs in human cancer, both approaches have limitations. Genome-wide screening is time and labor intensive, and most importantly, can produce artifacts. The profiling strategies cannot distinguish “causal” from “bystander” genetic events. In this study, we report a novel, clinically guided genetic screening approach to identify oncomirs involved human cancer. Based on this approach, one promising oncomir candidate, mir-30d, was successfully identified.

Materials and Methods

Patients and specimens- All frozen ovarian cancer specimens were collected at the University of Turin. Detailed information is provided in Supporting Methods.
Cell lines and cell culture- Cancer cell lines 2008, OVCAR5, OVCAR10, OWA42, MDA-MB-231, T47D, HCT116, and BT549 were purchased from ATCC or DCTD Tumor/Cell Line Repository. Four independent immortalized human ovarian surface epithelial cell lines (IOSEs) were generously provided by Dr. Nelly Auersperg. All cancer cell lines were obtained directly from ATCC or DCTD that performs cell line characterizations and passaged in our laboratory for fewer than 6 months after receipt. The method of characterization used by ATCC or DCTD can be found in its website. Detailed information is provided in Supporting Methods.

Retroviral transductions- The retrovirus-based human miRNA expression vector (pmiRvec) was purchased from GeneService (27). Retroviral vectors containing human miRNAs were transfected into the packaging cell line PT67 (Clontech) using the FuGene6 Transfection Reagent (Roche). The growth media was changed 48 hrs post-transfection and the media containing retrovirus was collected 48 hrs later. Human tumor cells were infected with retrovirus in the presence of 8 μg/ml of polybrene and selected using the antibiotic blasticidin (Invitrogen).

Transfection of miRNA mimic oligos and of miRZip miRNA inhibitors- Pre-miR miRNA precursors and control oligos were purchased from Ambion. miRZips lentiviral-based miRNA inhibition vectors and control vectors were purchased from System Biosciences. The effects of transfection or infection on endogenous mir-30d expression were examined by real-time RT-PCR (Figure S1). Detailed information is provided in Supporting Methods.

RNA isolation and TaqMan miRNA assays- Total RNA was isolated from 100 to 500 mg of frozen tissue or 1 x 10⁶ cultured cells using TRIzol reagent (Invitrogen). The expression of mature miRNAs was analyzed using the TaqMan miRNA Assay (Applied Biosystems). Detailed information is provided in Supporting Methods.

MTT proliferation assays, cell cycle assays, caspase-3 activity assays, Annexin-V apoptosis assays, β-Gal senescence assays, and migration assays- Detailed information is provided in Supporting Methods.
Western blots- Anti-total caspase-3 and anti-cleaved caspase-3 primary antibodies were purchased from Cell Signaling Technology. Detailed information is provided in Supporting Methods.

Generation of the in vivo xenograft model and bioluminescence optical imaging- Six to eight week old female nude mice were used to generate xenograft tumor models. The IVIS Lumina II Bioluminescence and Fluorescence Imaging System (Caliper Life Sciences) was used for in vivo bioluminescent imaging of MDA-MB-231 tumors. Detailed information is provided in Supporting Methods.

Array-based comparative genomic hybridization (aCGH)- BAC clones included in the "1 Mb–array" platform have been previously described (32). Detailed information is provided in Supporting Methods.

Analysis of DNA copy number profiling data sets- We examined whether mir-30d exhibits a high frequency of genomic amplification in a variety of human cancers by analyzing a panel of high resolution DNA copy number profiling data sets. These data sets consisted of 1,282 primary tumors representing nine human cancer types. Detailed information is provided in Supporting Methods.

Single-nucleotide polymorphism (SNP) arrays- A total of 192 established tumor cell lines were analyzed using the Affymetrix 500K SNP chip (Affymetrix) (data were collected by Joel Greshock and Barbara L. Weber at GlaxoSmithKline). Detailed information is provided in Supporting Methods.

SNP data analysis- All SNP chip images (‘CEL files’), were extracted using Affymetrix Genotype software, and analyzed using the dChip software package. Detailed information is provided in Supporting Methods.

Tissue microarrays- Detailed information is provided in Supporting Methods.

miRNA in situ hybridization (ISH) and image analysis- In situ detection of miRNA expression was performed on formalin-fixed paraffin-embedded tissue microarray sections. Detailed information is provided in Supporting Methods.

cDNA microarrays and microarray analysis – A Human Gene 1.0 ST oligonucleotide array (Affymetrix) was used in this study. Detailed information is provided in Supporting Methods.
Bioinformatic analysis—mir-30d targets were predicted using TargetScan (33). miRNA effects to the expression levels of protein-coding transcripts were identified by DIANA-mirExTra (34).

Quantitative real time RT-PCR—Total RNA was reverse-transcribed using a High Capacity RNA-to-cDNA Kit (Applied Biosystems) under conditions provided by the supplier. cDNA was quantified by RT-PCR on an ABI Prism 7900 Sequence Detection System (Applied Biosystems).

Statistical analysis—Statistical analysis was performed using the SPSS or SAS statistics software package. All results were expressed as mean ± SD, and p<0.05 was used for significance.

Results

A clinically guided genetic screening approach to identify oncomirs

We hypothesized that the limitations of the approaches discussed above could be overcome by integrating these two approaches, i.e., using miRNA array profiling as a “clinical/biological” filter to generate a restricted/selected miRNA expression library, which could then be used for functional genetic screening. To test our hypothesis, we generated a restricted miRNA expression library as follows: 1) We used array-based comparative genomic hybridization (aCGH) profiles as the “clinical/biological” filter. miRNAs that are highly amplified in human cancer were selected based on our previous aCGH studies (32). In ovarian (n=109) and breast (n=73) cancer specimens, 55 and 166 miRNAs were identified, respectively, with DNA copy number amplification observed in >15% of cases (Figure 1A; Table S1). 2) These potential oncomirs were further concentrated by combining the miRNAs that were co-amplified in both ovarian and breast tumors (n=41) (Figure 1B; Table S1). 3) We used a whole genome human miRNA expression library (27) to derive this restricted library by selecting retrovirus miRNA expression vectors for these 41 miRNAs. This restricted library was used in an ovarian cancer cell line, 2008, to screen in vitro for miRNAs that are advantageous for cell growth in long-term culture. This was performed using a modification of a previously described whole genome-wide shRNA screening method (35, 36) (Figure 1C). Briefly, target cell populations were infected such that each cell contained, on...
average, a single integrated virus (27). After 72 hrs, the cells were antibiotic-selected, then propagated for five weeks to expand those cell clones with a growth advantage, and seeded again at low density in 150 cm culture dishes to isolate single cell colonies. Large and aggressively growing single colonies were collected and expanded in 6-well plates. DNA was extracted from >100 colonies and virus insertions were amplified by PCR and confirmed by DNA sequencing (Figure 1C).

**mir-30d regulates cancer cell proliferation, senescence, and apoptosis *in vitro***

Several potential candidate oncomirs, including mir-30d, were identified by the above approach. mir-30d represented 42% of the viral insertions sequenced. To validate and further characterize this finding, we first transfected a chemically synthesized mir-30d mimic into six cancer cell lines including 2008 and five independent cancer cell lines. Using an MTT assay, we found that forced mir-30d expression significantly increased cell growth in all six cell lines (Figure 2A). Meanwhile, a similar experiment was performed using a lentiviral mir-30d inhibitor. Consistent with our gain-of-function studies, blocking of endogenous mir-30d expression even more remarkably decreased the growth rates of cancer cell lines *in vitro* (Figure 2B). In 2008, MDA-MB-231 and HCT116, mir-30d inhibitor nearly completely blocked cell growth ability *in vitro* (Figure 2B). In both mimic and inhibitor experiments, total RNA was isolated from parallel transfection wells, and real-time RT-PCR was used to confirm the effect on mir-30d expression. Since blocking endogenous mir-30d significantly reduced tumor cell proliferation, we next examined the effect of mir-30d inhibitor treatment on the cell cycle, using DNA staining followed by flow cytometry. We found that mir-30d inhibitor transfection led to a significant cell cycle arrest at the G0/G1 phase (Figure 2C). Finally, we observed that transfection of a mir-30d inhibitor not only significantly blocked cancer cell proliferation (Figure 2B), but also remarkably increased the number of the cells with typical senescence morphology (Figure 2D). To confirm this observation, we examined β-galactosidase activity, a known characteristic of senescent cells that is not found in presenescent, quiescent, or immortal cells. 4
~ 6 days after transfection with a mir-30d inhibitor, the percentage of β-galactosidase positive cancer cells was significantly increased compared to controls (Figure 2E).

Most recent studies have suggested that the mir-30 family regulates apoptosis in cardiomyocytes (37) and cancer cells (30). It indicates that decreasing of cell death may be another mechanism to explain the advantage of mir-30d for cell growth in long-term culture (about two month selection in our genetic screening approach). Thus, we examined whether mir-30d regulated apoptosis in the cell lines used in this study. Based on a bioluminescent caspase assay, we found that transfection of a mir-30d mimic significantly reduced caspase-3 activity in camptothecin-induced apoptosis in cancer cells (Figure 3A). This result was further confirmed via Western blot. Figure 3B shows that the transfection of a mir-30d mimic remarkably reduced both total and cleaved caspase-3 protein in both control and camptothecin-induced apoptotic cells. To prove that mir-30d blocks chemotherapy drug induced apoptosis, the apoptotic status after camptothecin treatment was analyzed by flow cytometry using an Annexin-V assay and Western blots of PARP protein. Consistent with the above results, we found that the transfection of a mir-30d mimic significantly reduces the apoptotic cells induced by camptothecin treatment (Figure 3C and D). Taken together, above results demonstrate that mir-30d regulates proliferation, senescence, and apoptosis in cancer cells, suggesting that mir-30d may serve as an oncomir in human cancer.

**mir-30d blockade significantly inhibits tumor growth in vivo**

We next questioned whether mir-30d could regulate tumor growth in vivo. Cancer cells (MDA-MB-231) in which mir-30d had been knocked-down by a lentiviral inhibitor and control cells were injected subcutaneously (s.c.) into female nude mice (Figure 4). The cells in which mir-30d were knocked-down lost all in vivo growth ability, while the control cells grew well in vivo in the same animal (Figure 4A and B, n=5, p<0.05). To enhance tumorigenicity of mir-30d knocked-down tumors, we repeated the above experiments using tumor cells mixed with Matrigel, a mixture of basement membrane proteins, tissue plasminogen activators, and growth factors. To increase the sensitivity of tumor visualization, a luciferase...
reporter expression vector was transfected into the MDA-MB-231 cells. We found that the tumors produced by cells in which mir-30d had been knocked-down displayed slow growth \textit{in vivo} with the Matrigel mixture, consistent with the first experiment (without the Matrigel mixture). Thus, knockdown of mir-30d significantly decreases the growth rate of tumors \textit{in vivo} (Figure 4C to E, n=5, p<0.05). This \textit{in vivo} result provides further evidence indicating that mir-30d is an oncomir in human cancer.

\textbf{mir-30d exhibits a high frequency of genomic amplification in human solid tumors}

Our previous aCGH study indicated that the DNA copy number of mir-30d was amplified in about 50\% of the ovarian cancer (n=109) and 40\% of the breast cancer (n=73) specimens (32). First, we investigated whether there was a positive correlation between the DNA copy number amplification and mature mir-30d expression in primary tumors. We analyzed mature and pre mir-30d expression in 36 primary ovarian tumor specimens by real-time RT-PCR, and found that both mature and pre mir-30d expression was detectable in all specimens and significantly higher in the tumors with a high DNA copy number of mir-30d (n=21, mature mir-30d: p=0.014; pre-mir-30d: p<0.001) compared to tumors with no amplification of mir-30d DNA copy number (n=15). We also examined the 16 ovarian cancer cell lines. The average level of mir-30d expression was higher in the cell lines with amplified mir-30d DNA copy numbers (Figure 5A). In addition, the average level of mir-30d expression in these ovarian cancer cell lines was remarkably higher than in immortalized human ovarian surface epithelial cells (IOSEs, Figure 5A), which were used as control cells for epithelial ovarian cancer (38). These results demonstrate that genomic copy number amplification of mir-30d increases mature mir-30d expression in human cancer.

Next, we asked whether mir-30d also exhibits high levels of genomic amplification in other human cancers. We analyzed a panel of high resolution DNA copy number profiling data sets that consisted of 1,282 primary tumors representing nine human cancer types (detailed information provided in the methods section and Table S2). We found that the genomic locus harboring mir-30d was significantly amplified in 8 of these 9 human cancers (Figure 5B). For example, in breast cancer (n=327), 8.1\% of the
specimens revealed that the genomic locus harboring mir-30d was amplified to greater than five copies, and 30.4% of the specimens indicated that mir-30d was present in 3-5 copies (Figure 5B). Overall, mir-30d was amplified at a high frequency (> 30%) in human solid tumors (n=1,282). In contrast, 2.7% of tumors displayed a heterozygous deletion for mir-30d, and one specimen with a homozygous deletion of mir-30d was found (Figure 5B). These DNA copy number profiling results were further validated using a single nucleotide polymorphism (SNP) array on six matching human tumor types (detailed information provided in the methods section). One hundred and ninety-two established tumor cell lines were examined in this SNP array. The genomic locus containing mir-30d was examined in each cell line (Figure 5C). Consistent with the aCGH findings, mir-30d displayed remarkable amplification in tumor cell lines (Figure 5B). In breast cancer (n=29), in 6.9% of specimens the genomic locus harboring mir-30d was present in more than five copies, while 44.8% of the specimens had 3-5 copies (Figure 5B). Collectively, mir-30d was amplified in more than 30% of the cancer cell lines examined (Figure 5B, n=192).

**Higher levels of mir-30d expression were significantly correlated with poor clinical outcomes in ovarian cancer patients**

To study the expression of mature mir-30d in human cancer, *in situ* hybridization (ISH) was performed on ovarian cancer tissue arrays (n=330). We detected mature mir-30d in the majority of ovarian cancer specimens (81.2%, 268/330, Figure 5D). Strong cytoplasmic hybridization signal was mainly found in tumor islet but not stroma cells (Figure 5D). To further examine the clinical significance of mir-30d expression, the cytoplasmic ISH signals were scored as negative or weak positive (n=62), intermediate positive (n=161), and strong positive (n=107, Figure 5D). We found that higher expression levels of mature mir-30d in ovarian cancer were significantly correlated with poor clinical outcomes in these patients (Figure 5E, p=0.0016). The above clinical information (DNA copy number amplification and correlation with survival) provides robust evidence that mir-30d does indeed play a critical oncogenic role in the development and progression of human cancer.
mir-30d regulates a large number of transcripts/genes in cancer cells

Up to one-third of messenger RNAs (mRNAs) appear to be miRNA targets (33). Each miRNA can target hundreds of mRNA transcripts (39) affecting the production of proteins (40, 41), and more than one miRNA can converge on a single target transcript (42). Taken together, the potential regulatory circuitry afforded by specific miRNAs may be enormous, although typically this repression is relatively mild (39-41). Therefore, we believed that the oncomiric function of mir-30d would be mediated by repressing multiple signaling pathways rather than by targeting one or a few cancer-associated proteins. To test this hypothesis, we used microarray analyses to profile the transcriptional changes seen after the transfection of a mir-30d mimic into two cancer cell lines (Figure 6). Consistent with other reports in the literature (39-41), the repression of genome-wide transcription caused by mir-30d was typically relatively mild. We found that the mir-30d mimic down-regulated 462 and 376 transcripts in 2008 and MDA-MB-231 cells, respectively (Figure 6A to E; Tables S3 and S4). To confirm that the majority of the down-regulated transcripts were repressed by mir-30d directly, we undertook both bioinformatic and experimental validations. First, following a method described by Krutzfeld et al. (43), we calculated the number of nonoverlapping occurrences of each possible hexamer in every 3' UTR sequence of those down-regulated transcripts (377 and 313 annotated genes were included in 2008 and MDA-MB-231, respectively). After dividing this by the length of the 3' UTR, we applied a one-tailed Wilcoxon rank sum to each normalized hexamer count distribution in down-regulated versus unchanged 3' UTRs using the online program DIANA-mirExTra (34) (Figure 6 B and D). The three hexamers that were most overrepresented in the down-regulated genes were ACATTT, CATTGT and ATTTGT (p<10^{-19}) and corresponded to the seed positions of mir-30d (Figures 6B and D). We also predicted mir-30d targets using TargetScan (33), and found that 128 of 462 and 151 of 376 down-regulated transcripts in 2008 and MDA-MB-231, respectively, were mir-30d predicated targets (Figure 6E). This bioinformatic evidence strongly suggests that a large percentage of those down-regulated transcripts were indeed directly repressed by mir-30d.
That is, about 30-60% of the down-regulated transcripts were direct mir-30d targets. Additionally, about 1 of 3 (n=150) down-regulated transcripts were shared by both cell lines, and 79 of these were predicted to be mir-30d targets by TargetScan (Table S5). Finally, 11% (9 of 79) of these transcripts were selected and further validated by real-time RT-PCR in 2008 and MDA-MB-231 cells, as well as another two independent cell lines, HCT116 and A2780 (Figures 6F and Figure S2). Notably, three of known mir-30d targets identified by another group, \textit{GNAI2} (44) and \textit{GALNT1}, \textit{GALNT7} (45) were consistently found in our 79 transcript list (Figure 6E). Taken together, these results indicate that mir-30d represses a large number of transcripts, in cancer cells.

Most importantly, miRNA target prediction programs strongly indicated that \textit{CASP3}, a central protein in the execution-phase of cell apoptosis, was a potential target of mir-30d (Figure 7A). The mir-30d binding sites in the \textit{CASP3} 3'-UTR predicated by TargetScan were broadly conserved among different species (Figure 7A). Additionally, we found that both protein and mRNA levels of \textit{CASP3} were significantly repressed after transfection of mir-30d into cancer cells (protein: Figure 3B and mRNA: Figure 6E and 7B). Thus, we used a luciferase reporter assay to experimentally examine whether mir-30d directly regulated \textit{CASP3} expression via its 3'-UTR. The seeding sequences of mir-30d in the \textit{CASP3} 3'-UTR were mutated, and overexpression of mir-30d was found to significantly reduce luciferase activity in the wild type but not the binding site mutant \textit{CASP3} 3'-UTR reporters (Figure 7C). These results demonstrate that mir-30d directly regulates expression of \textit{CASP3}, a master regulator of apoptosis, in cancer cells. It is one of important molecular mechanisms to explain our observation on cellular function of mir-30d in apoptosis and long-term maintenance of tumor cell growth.

\textbf{Discussion}

In the present study, we report a novel, clinically guided, genetic screening approach to identify oncomirs in human cancer. This approach uses array-based profiling data as a clinical/biological filter to generate a selected functional genetic screening library. Our strategy can distinguish “causal” from
“bystander” genetic events to decrease noise and artificial results, and also requires less time and labor than whole-genome wide genetic screening approaches. With this novel strategy, one promising oncomir candidate, mir-30d, was successfully identified. This clinically guided genetic screening approach is a powerful tool to identify oncomirs as biomarkers for diagnosis or as targets for the development of cancer treatments. There are also limitations of our strategy to identify oncomirs. For example, only oncogenic miRNAs, especially miRNAs promoting tumor cell growth or survival, are able to be identified by our current approach. This limitation can be overcome by combining with other library screening approaches such as transwell assay (to identify miRNAs promoting migration and invasion), soft agar assay (to identify miRNAs inducing malignant transformation), and negative selection approaches (to identify tumor suppressor miRNAs). Finally, since our approach is based on an in vitro positive selection strategy, the oncomirs that give a growth advantage to tumor cells in vitro, such as promoting angiogenesis and regulating anti-tumor immune response, cannot be identified by our approach. Combination with an in vivo selection approach will more closely recapitulate the pathobiology of human cancers.

mir-30d was initially identified using this screening approach, where the cells harboring miRNAs producing a growth advantage could be expanded in vitro. Subsequently, our in vitro experiments demonstrated that mir-30d regulates cell proliferation, senescence, and apoptosis. In agreement with our observations, Kumar et al. also reported that mir-30d inhibits apoptosis in human cancer via regulation of the p53 pathway (30). A similar function of mir-30d was also observed in normal cells such as cardiomyocytes (37). Furthermore, using a xenograft model, we showed that inhibition of endogenous mir-30d expression remarkably blocked tumor growth in vivo. Two independent groups have provided strong evidence indicating that mir-30d promotes tumor invasion and metastasis in hepatocellular carcinoma via regulating GNAI2 (44) and in melanoma via repressing GALNT7 (45). Consistent with these studies, we also found that mir-30d repressed GNAI2 and GALNT7 expression in ovarian and breast cancer cell lines (Figure 6E), and using an in vitro migration assay, we observed that transfection of mir-30d significantly promoted the migration of BT549 and MDA-MB-231 cells in vitro (Figure S3).
study found that the DNA copy number of mir-30d was remarkably amplified (more than 30%) in 8 of 9 human cancer types that were examined. This suggests that mir-30d amplification in human cancer is a common oncogenic event. In agreement with our conclusion, two independent groups reported that mir-30d was amplified in brain tumors (46) and melanoma (45). Most importantly, we found that overexpression of mir-30d was significantly associated with a poor clinical outcome in human ovarian cancer patients. In addition, a recent genome-wide serum miRNA profiling study demonstrated that higher levels of serum mir-30d were significantly associated with shorter survival times in non-small cell lung cancer patients (47). Gaziel-Sovran et al. also reported that higher levels of mir-30d expression was correlated with shorter time to recurrence and lower overall survival in melanoma (45). Taken together, the above evidence strongly suggests that mir-30d serves as an oncomir in human cancer.

The results of this study indicate that mir-30d can down-regulate a large number of transcripts in human cancer cells, although as with most other miRNAs, this repression is relatively mild (39-41). We identified 79 protein coding genes which were predicted by bioinformatics to be potential direct targets of mir-30d. We then showed that the expression of these genes (involved in multiple cancer-associated pathways such as apoptosis and migration) were indeed down-regulated by the transfection of a mir-30d mimic into each of the two cell lines used in our microarray study as well as two independent cancer cell lines. We found that CASP3, a central protein in the execution-phase of cell apoptosis, is a direct target of mir-30d. Another mir-30d targets (GNAI2 which was identified in liver cancer (44); GALNT1 and GALNT7 which were identified in melanoma (45)) were also found in our target list. Notably, we also found that a relatively large number of predicted mir-30d target genes (49 of 462 in 2008 (ovarian cancer) and 72 of 376 in MDA-MB-231 (breast cancer) cells, respectively (Figure 6E) were only down-regulated in one cell line in our microarray study. This indicates that the function of mir-30d may be cell-context dependent or tumor-type specific. In agreement with our finding, Martines et al. showed that the mir-30 family regulates B-Myb expression in a cervical cancer cell line (48). Finally, because mir-30d regulates a large number of protein coding genes, it may regulate multiple cancer-associated pathways. For example,
beyond apoptosis and migration, the mir-30 family may also regulate autophagy (49), immune suppression (45) and cancer stem cell differentiation (50). Therefore, the biological function and clinical significance of mir-30d in human cancer may be complex and depended on the context of downstream targets and molecular networks in each specific cancer type.

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References


Figure Legends

Figure 1. A clinically guided genetic screening approach to identify oncomirs
A. aCGH frequency plots of ovarian (n=109) and breast (n=73) cancer specimens. Columns represent miRNAs which are ordered based on location in the genome (from chromosome 1 to 22). Green represents gain, and red represents loss. B. Venn diagrams of miRNA genes with copy number gain shared by ovarian and breast cancers. C. A clinically guided genetic screening approach to identify oncomirs in vitro.

Figure 2. mir-30d regulates cancer cell proliferation and senescence in vitro
A. Growth rates of the mir-30d mimic and control transfected tumor cells, measured using the MTT assay. B. Growth rates of the mir-30d miRZips inhibitor and control miRZips expressed in tumor cells, measured by cell counts. C. Cell cycle analysis of the mir-30d miRZips inhibitor and control miRZips expressed in tumor cells using flow cytometry. D. β-Gal staining in control and mir-30d inhibitor transfected cells. E. Percentage of β-Gal positive cells after control and mir-30d inhibitor lentiviral transduction. *p<0.05

Figure 3. mir-30d regulates cancer cell apoptosis in vitro
Tumor cells were transfected with mir-30d mimic or control mimic using Lipofectamine RNAiMAX transfection reagent. A. Caspase-3 activity after control or camptothecin treatment, measured using the caspase-3 assay. B. Total and cleaved caspase-3 after control and camptothecin treatment, detected by Western blots. C. Percentage of apoptotic cells measured by flow cytometry using an Annexin V assay. D. Total and cleaved PARP protein after control and camptothecin treatment, detected by Western blots. *p<0.05

Figure 4. Inhibition of mir-30d blocks xenograft tumor growth in vivo
**Figure 5. mir-30d genomic amplification and clinical significance in cancer patients**

A. Correlation analysis between mir-30d DNA copy number alteration and miRNA expression in cell lines. DNA copy number was analyzed by aCGH. Mature mir-30d expression was quantified by real-time RT-PCR.  
B. mir-30d exhibited genomic amplifications at high frequencies in multiple human solid tumors by aCGH (left) and SNP array (right).  
C. SNP array validation in human breast, colon and lung cancer cell lines.  
D. mir-30d expression in ovarian cancer was detected by *in situ* hybridization.  
E. Overall survival in patients with ovarian cancer (n=330) according to mir-30d staining scores.

**Figure 6. mir-30d regulates a large number of transcripts/genes in cancer cells**

A and C. The genome-wide transcriptional effect of mir-30d mimic transfection compared to control mimic transfection was analyzed using an Affymetrix Human Gene 1.0 ST oligonucleotide array in ovarian cancer cell line 2008 (A) and breast cancer cell line MDA-MB-231 (C). Red spots indicate down-regulated transcripts.  
B and D. Histograms of negative natural logarithms of P values derived from a one-tailed Wilcoxon rank sum test applied to the distributions of hexamers in the 3′ UTRs of all down-regulated versus unchanged transcripts.  
E. Venn diagrams of transcript numbers shared by down-regulated transcripts in mir-30d mimic transfections in 2008 and MDA-MB-231 cells and predicted targets of mir-30d by TargetScan.  
F. The microarray results were validated by real-time RT-PCR in four
tumor cells. Real-time RT-PCR validation of transcripts that were down-regulated in both 2008 and MDA-MB-231 cells after transfection with the mir-30d mimic, and that were also predicted mir-30d targets by TargetScan (E). Validations were performed in 2008 and MDA-MB-231 cells as well as in two independent cell lines, HCT116 and A2780. * P<0.05; ** P<0.01

**Figure 7. mir-30d regulates CASP3 in cancer cells**

**A.** The schematic diagram of the mir-30d binding site (marked in red) in the CASP3 3′-UTR, which was broadly conserved among different species. Hsa, Human; Ptr, chimpanzee; Mml, Rhesus; Mmu, mouse; Rno, Rat; Cpo, Pig; Sar, Shrew; Cfa, Dog; Fca: Cat; Eca, Horse; Bta, Cow; Dno, armadillo; Laf, elephant.

**B.** The mir-30d mimics and control mimic were transiently transfected into four cancer cell lines. At 48 hrs post-transfection, total RNA were collected, and the endogenous CASP3 expression was examined by real-time RT-PCR. **C.** Reporter assays using wild type and mir-30d binding site mutant CASP-3 3′ UTR. The mir-30d mimic transfection significantly reduced luciferase activity in the wild type but not the binding site mutant CASP3 3′ UTR reporters. * P<0.05; ** P<0.01
Figure 2. miR-30d regulates cancer cell proliferation and senescence in vitro
Figure 3. miR-30d regulates cancer cell apoptosis in vitro

A

B

C

D

HCT116

MDA-MB-231

A2780

2000

Caspase-3 (total)

Caspase-3 (cleaved)

beta-actin

Z-AMB

Z-AMB

Annexin-V

Apoptotic cells (%)

PARP (total)

PARP (cleaved)

beta-actin

Ctrl

Camp

Ctrl

Camp

Ctrl

Camp

Ctrl

Camp

Ctrl

Camp

Ctrl

Camp
Figure 4. Inhibition of mir-30d blocks xenograft tumor growth in vivo.

A. Without Matrigel

B. Right: control tumor; Left: inhibitor tumor

C. With Matrigel

D. Right: control tumor; Left: inhibitor tumor

E. Radiance (arbitrary units) X 10^6

Days post injection
Figure 5. miR-30d genomic amplification and clinical significance in cancer patients

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Number</th>
<th>Gain (3-5)</th>
<th>Loss (%)</th>
<th>Validation (SNP)</th>
<th>Number</th>
<th>Gain (3-5)</th>
<th>Loss (%)</th>
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<tbody>
<tr>
<td>Bladder</td>
<td>25</td>
<td>0</td>
<td>16.3</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>36.4</td>
</tr>
<tr>
<td>Breast</td>
<td>327</td>
<td>13.5</td>
<td>36.5</td>
<td>4.0</td>
<td>29</td>
<td>6.9</td>
<td>44.8</td>
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<tr>
<td>Colon</td>
<td>290</td>
<td>5.1</td>
<td>30.4</td>
<td>2.5</td>
<td>28</td>
<td>7.1</td>
<td>14.3</td>
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<tr>
<td>Lung</td>
<td>103</td>
<td>2.9</td>
<td>25.8</td>
<td>1.9</td>
<td>103</td>
<td>1.9</td>
<td>24.2</td>
</tr>
<tr>
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<td>220</td>
<td>3.2</td>
<td>24.1</td>
<td>2.3</td>
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<td>N/A</td>
<td>N/A</td>
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<tr>
<td>Neuroblastoma</td>
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<td>0</td>
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<td>2.4</td>
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<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Ovarian</td>
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<td>10.4</td>
<td>44.7</td>
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<td>12</td>
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<tr>
<td>Pancreatic</td>
<td>37</td>
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<td>30.0</td>
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<tr>
<td>Sarcoma</td>
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<td>27.2</td>
<td>1.2</td>
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<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Total 1282 7.9 25.2 2.7 0 192 2.6 32.6 4.2 1.0

C

D

E

p=0.0016

- Negative/weak positive (n=62)
- Intermediate positive (n=151)
- Strong positive (n=107)
Figure S. miR-30d regulates a large number of transcripts/genes in cancer cells

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z
Figure 7. miR-30d regulates CASP3 in cancer cells

(A) 5'...UUUUUUUGUGAGUGUUUAAC... hP53 3' UTR
3' GAAGCGUCACCCCGGUAAGAUUUCG

(B) Relative mRNA expression

(C) Normalized luciferase activity

Hoe  UGAAAUUUAUAUAUAUAUUAUAUUAU
Py  UGAAAUUUAUAUAUAUAUUAUAUUAU
Mas  UGAAAUUUAUAUAUAUAUUAUAUUAU
Mnsu  UGAAAUUUAUAUAUAUAUUAUAUUAU
Rho  UGAAAUUUAUAUAUAUAUUAUAUUAU
Glo  UGAAAUUUAUAUAUAUAUUAUAUUAU
Salt  UGAAAUUUAUAUAUAUAUUAUAUUAU
Cas  UGAAAUUUAUAUAUAUAUUAUAUUAU
Fca  UGAAAUUUAUAUAUAUAUUAUAUUAU
Fbn  UGAAAUUUAUAUAUAUAUUAUAUUAU
Dros  UGAAAUUUAUAUAUAUAUUAUAUUAU
Lat  UGAAAUUUAUAUAUAUAUUAUAUUAU
A combined array-based comparative genomic hybridization (aCGH) and functional library screening approach identifies mir-30d as an oncomir in cancer

Ning Li, Sippy Kaur, Joel Greshock, et al.

Cancer Res  Published OnlineFirst November 4, 2011.

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