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Systematic proteome analysis identifies transcription factor YY1 as a direct target of miR-34a

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Abstract

MicroRNA 34a (miR-34a) is a potential tumor suppressor gene and has been identified as a miRNA component of the p53 network. To better understand the biological pathways involved in miR-34a action, a parallel global protein and mRNA expression profiling on miR-34a treated neuroblastoma cells (IMR32) was performed using isotope-coded affinity tags (ICAT) and Affymetrix U133plus2 microarray respectively. Global profiling showed that miR-34a causes much smaller mRNA expression changes compared to changes at the protein level. A total of 1495 proteins represented by 2 or more peptides were identified from the quantitative ICAT analysis, of which 143 and 192 proteins are significantly up- or down-regulated by miR-34a, respectively. Pathway analysis of these differentially expressed proteins showed the enrichment of apoptosis and cell death processes in up-regulated proteins but DNA replication and cell cycle processes in the down-regulated proteins. Ribosomal proteins are the most significant set down-regulated by miR-34a. Additionally, biological network analysis to identify direct interactions among the differentially expressed proteins demonstrated that the expression of the ubiquitous transcription factor YY1, as well as its downstream proteins, is significantly reduced by miR-34a. We further demonstrated that miR-34a directly targets YY1 through a miR-34a-binding site within the 3' UTR of YY1 using a luciferase reporter system. YY1 is a negative regulator of p53 and it plays an essential role in cancer biology. Therefore, YY1 is another important direct target of miR-34a which closely regulates TP53 activities.

Keywords

miR-34a; YY1; ICAT; proteomics; neuroblastoma

Introduction

miRNAs are 20 to 22 nucleotide RNAs that have been implicated in the regulation of proliferation, differentiation, and apoptosis¹. Many studies have provided evidence linking miRNAs to cancer formation. The miR-17-92 cluster, miR-372-373, miR-155 and miR-21

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have been implicated as proto-oncogenes while miR-15-16, let-7, miR-34, miR-29, miR-145, and miR221-222 have been suggested as potential tumor suppressor genes ¹⁻².

Neuroblastoma is the most common extracranial solid tumor in children and the high-risk neuroblastomas are associated with several genomic alterations including MYCN amplification, 17q gain, 1p36 deletion, and 11q loss. miR-34a is located in 1p36, a region frequently deleted in advanced stage tumors with MYCN amplification ³. miR-34a was first shown as a tumor suppressor in neuroblastoma ⁴ and the level of miR-34a expression is often lower in neuroblastomas with deletions at 1p36 ⁴⁻⁵. Low levels of miR-34a expression have also been shown in other cancers ^{1, 6}. Reintroduction of miR-34a in neuroblastoma cells with 1p36 deletion causes dramatic cell growth inhibition, cell cycle arrest, and apoptosis promotion ^{4-5, 7}. Several studies have found that miR-34a and its family members (miR-34b and c) are direct transcriptional targets of p53, and they mediate p53 tumor suppressor activity, including induction of cell-cycle arrest and promotion of apoptosis, while loss of miR-34a can impair p53-mediated cell death ^{1, 8-9}.

miRNA typically targets transcripts in the 3' untranslated regions and control their expression by mRNA degradation and translational repression ¹. Microarray analyses have been widely used to identify those miRNA targets with mRNA degradation ^{1, 10-11}. To discover targets with translational repression, several studies have used proteomic methods to evaluate the global changes in protein synthesis induced by miRNAs ¹²⁻¹⁵. In this study, we performed parallel proteomic and transcriptomic profiling on miR-34a treated neuroblastoma cells using isotope-coded affinity tags (ICAT) ¹⁶⁻¹⁹ and Affymetrix U133plus2 microarray respectively. Proteomic analysis revealed that miR-34a suppressed the level of YY1, a ubiquitous transcription factor that negatively regulates p53 ²⁰⁻²¹, as well as its downstream proteins. YY1 has also been associated with cell proliferation, anti-apoptosis, tumorigenesis and metastatic potential ²². Finally we showed that miR-34a directly targets YY1 through a miR-34a-binding site within the 3' UTR of YY1. The elucidation of the role of YY1 in miR-34a action may shed important light on tumor suppressive function of miRNA-34a.

Materials and Methods

Cell culture and preparation of protein

IMR32, a MYCN-amplified NB cell line, was cultured in EMEM media (Quality Biological, Gaithersburg, MD). SK-N-AS, a MYCN-not-amplified NB cell line, was maintained in RPMI 1640 media. All media were supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 1% glutamine and 1% P/S (Quality Biological, Gaithersburg, MD), and cells were cultured at 37°C. Synthetic microRNAs (160 fmol) were transfected into 1×10⁶ IMR32 cells using an Amaxa Nucleofector kit according to the manufacture's instruction (Amaxa Biosystems, Cologne, Germany). All synthetic meridian microRNAs were purchased from Dharmacon Technologies (Lafayette, CO). Protein preparation for ICAT experiment were done as previously described ¹⁹.

DNA microarray analysis

The affymetrix dataset from our previous study ⁵ was normalized in dChip to the median intensity using the PM-only model ¹⁹. In case of multiple probe sets representing the same gene (unique GeneID), the maximum of intensity values was taken.

ICAT labeling

Equal amounts of protein (300 µg each) from the IMR32 mimic control cells and the IMR32 cells transfected with miR-34 a were labeled with the light (cICAT-¹²C₉) and the heavy

(cICAT-¹³C₉) isotopic versions of the cICAT reagent (ABI, Framingham, MA), respectively, using the modified method described previously¹⁸. Briefly, each sample was dissolved in 240 μ L of 6 M Gdn-HCl in 50 mM Tris-HCl, pH 8.3, and reduced by adding 3 μ L of 100 mM TCEP-HCl and boiling in a water bath for 10 min. Each reduced sample was transferred to 3 vials containing either cICAT-¹²C₉ or cICAT-¹³C₉ dissolved in 60 μ L of CH₃CN in total and incubated at 37°C for 2 h. The two samples were combined, buffer-exchanged into 50 mM NH₄HCO₃, pH 8.3, using D-Salt Excellulose plastic desalting columns (Pierce, Rockford, IL) and digested with trypsin (Promega, Madison, WI) overnight at 37°C, using an enzyme to protein ratio of 1:30 (w/w). The digestion was quenched by boiling the sample in a water bath for 10 min and adding PMSF to a final concentration of 1 mM.

Affinity purification of cICAT-labeled peptides

UltraLink immobilized monomeric avidin columns with 0.6 mL bed volume were slurry-packed in glass Pasteur pipettes and equilibrated with 2 $\times$ PBS (0.2 M sodium phosphate, 0.3 M NaCl, pH 7.2). The stationary phase was blocked with 2 mM D-biotin in 2 $\times$ PBS, pH 7.2, and reversible biotin binding sites were stripped, using 30% CH₃CN/0.4% formic acid, and the columns were re-equilibrated with 2 $\times$ PBS, pH 7.2. The samples containing the cICAT-labeled peptides were boiled for 5 min, cooled to room temperature, loaded onto the avidin columns, and allowed to incubate for 15 min at ambient temperature. After washing the columns with 2 $\times$ PBS, pH 7.2, 1 $\times$ PBS, pH 7.2, and 50 mM NH₄HCO₃/20% CH₃CN, pH 8.3, the cICAT-labeled peptides were eluted with 30% CH₃CN/0.4% formic acid and lyophilized to dryness. The biotin moiety was cleaved from the cICAT-labeled peptides by treatment with the cleaving reagents provided by the manufacturer for 2 h at 37°C, and lyophilized to dryness.

μ RPLC-MS/MS of cICAT-labeled peptides

A 10 cm-long μ RPLC-electrospray ionization (ESI) column was coupled online with a 7-tesla hybrid linear ion trap-Fourier transform ion cyclotron resonance MS (LTQ-FT, Thermo Electron, San Jose, CA) to analyze each SCXLC fraction. To construct the μ RPLC-ESI columns, 75 μ m i.d. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) were flame-pulled to construct a 10 cm-fine i.d. (i.e., 5-7 μ m) tip against which 5 μ m, 300 Å pore size Jupiter C18 RP particles (Phenomenex, Torrance, CA) were slurry-packed using a slurry-packing pump (Model 1666, Alltech Associates, Deerfield, IL). The column was connected via a stainless steel union to an Agilent 1200 nanoflow LC system (Agilent Technologies, Palo Alto, CA), which was used to deliver mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in CH₃CN). Each SCXLC fraction was analyzed by μ RPLC-MS/MS. After 15-min loading of one-third content of each SCXLC fraction, the LTQ-FT MS began to acquire data while the column was maintained with 2% solvent B for another 15 min at a flow rate of ~200 nL/min, followed by a step gradient to elute the peptides: 2%-40% solvent B for 110 min and 40%-98% solvent B for 30 min. For LTQ-FT MS, the MS survey scan was performed in the FTICR part with a resolution of 5×10^4 , and the MS/MS scans were acquired in the LTQ part. The instrument was operated in a data-dependent MS/MS mode in which the seven most intense peptide molecular ions in the MS scan were sequentially and dynamically selected for subsequent collision-induced dissociation (CID) using a normalized collision energy of 35%. Dynamic exclusion was enabled with duration of 1 min to prevent repeated acquisition of MS/MS spectra of the same peptide for which the MS/MS spectrum had been acquired in the previous scan. The voltage and temperature for the capillary of the ion source were set at 10 V and 160°C, respectively.

Peptide identification and quantification

The raw MS/MS data were searched using TurboSEQUEST (ThermoElectron, San Jose, CA) against the human protein database (with total protein entries of 37542) from the European Bioinformatics Institute (EBI) (www.ebi.ac.uk) to identify light and heavy cICAT-labeled peptides. Static modification of Cys by mass addition of the cleaved light ICAT label (227.1270 Da) and dynamic modification of Cys by mass addition of 9.0302 Da were set in a single search to search for both light and heavy cICAT-labeled peptides. Peptide mass tolerance of 0.08 Da and fragment ion tolerance of 1.0 Da were allowed with trypsin specificity allowing two missed cleavages. SEQUEST criteria were $X_{corr} \geq 1.9$ for $[M+H]^+$ ions, ≥ 2.2 for $[M+2H]^{2+}$ ions and ≥ 2.9 for $[M+3H]^{3+}$ ions, and $\Delta Cn \geq 0.08$ for identification of fully tryptic peptides. The relative abundance of identified peptides was calculated using XPRESS (ThermoElectron, San Jose, CA), by which peak areas were integrated from their extracted ion chromatograms (XIC) using a minimum intensity threshold of 100 counts and smoothing point of 5. The cICAT dataset was further normalized by the mean ratio according to the method described previously¹⁹. We excluded proteins identified solely from a single peptide; Supplemental Table 5 shows the identification of proteins as well as the peptide sequences for the proteins with at least 2 peptide identifications. There are a total of 1495 unique proteins after removing the redundant identifications; the unique proteins and the number of peptides used for protein quantitation measurements are shown in Supplemental Table 4. The values of median ratio, mean ratio, median absolute deviation and standard deviation are available in Supplemental Table 4. In this study we use median ratio for all further analyses.

Western blotting

Total proteins were extracted from the IMR32 cells using radioimmuno precipitation assay buffer with 3% proteinase inhibitor cocktail (Sigma, St. Louis, MO). Twenty micrograms of protein was separated on a polyacrylamide gel under denaturing conditions and transferred to a nitrocellulose membrane (Invitrogen). The membranes were blocked for 1 h at room temperature, and then incubated overnight at 4 °C in Tris-buffered saline Tween-20 (TBST) containing 5% bovine serum albumin (BSA) and following antibodies: YY1 (H-10; Santa Cruz, CA); GAPDH (MAB374; Chemicon, Temecula, CA). Membranes were washed three times in TBST, and incubated with a secondary antibody conjugated with horseradish peroxidase (Rockland Immunochemicals, Gilbertsville, PA) in TBST and 0.5% BSA for 1 h at room temperature. After two washes with TBS, bands were detected by chemiluminescence using a SuperSignal Chemiluminescence kit (Pierce, Rockford, IL) on Biomax MR X-ray film (Kodak, Rochester, NY). Intensity of the bands was determined using ImageQuant software (GE Healthcare, Piscataway, NJ, USA) in the volume mode.

Plasmid constructs and luciferase assay

The partial YY1 3'-UTR containing miR-34a binding site (107bp, position 670-776 of YY1 3' UTR) was cloned into the pMIR-REPORT miRNA expression vector (Ambion, Austin, TX, USA) between SpeI and HindIII restriction sites to construct a wild-type plasmid Luc-YY1-WT (GeneArt, Toronto, Canada). A mutant plasmid Luc-YY1-mutant was constructed similarly but with 7 bp miR-34a binding site (ACTGCCA) being removed from the selected 107 bp YY1 3' UTR sequence (GeneArt, Toronto, Canada). Both constructs were sequenced to ensure sequence accuracy. For luciferase assays, we used Dharmafect 1 (Dharmacon Technologies) to co-transfect SK-N-AS cells with luciferase reporter construct plasmids, a b-galactosidase control plasmid and microRNAs (5 nM) per well in 48-well cell culture plates according to the manufacture's instruction. Luciferase activity was measured using a Dual light Luciferase and b-Galactosidase Reporter Gene Assay System (Applied Biosystems, Foster City, CA, USA) at 24h after transfection. Luciferase activity was then normalized by the b-galactosidase activity for transfection in each well.

Seed sequence enrichment analysis

We retrieved the 3' UTR sequences from Ensembl database (<http://www.ensembl.org>) for all transcripts whose proteins were detected by ICAT. When multiple 3' UTRs were annotated to the same gene, the longest 3' UTR was used. We searched the miR-34a seed sequence match for the sites of 6mer (match to position 2-7), 7mer (2-8), 7mer-A1 (2-7 with adenosine in position 1) and 8mer (1-8). The p-values for enrichment of miR-34a seed sequence match in down-regulated proteins were calculated using hypergeometric test.

Pathway and network analysis

To investigate the pathways and protein sets that are differentially regulated between the IMR32 mimic control cells and the IMR32 cells transfected with miR-34a, the gene set enrichment analysis (GSEA) method ²³ (<http://www.broad.mit.edu/gsea/>) was applied to the global protein expression profiling data. GSEA analysis was completed with a weighted enrichment statistics and proteins were ranked using log₂ ratio of protein expressions in miR-34a and mimic control. A minimal size of 15 overlapping genes in each set is required. A collection of curated gene sets in MSigDB (<http://www.broad.mit.edu/gsea/msigdb/>) was used in this analysis. Because of the limited number of samples, permutation tests were performed on gene sets with 1000 permutations for obtaining a FDR q-value. Gene sets with a $p < 0.001$ and FDR q-value < 0.1 were considered significant. Gene ontology (GO) analysis was performed using David bioinformatics resource (<http://david.abcc.ncifcrf.gov/>). The network analysis was performed using the network building tool MetaCore (GeneGo, St. Joseph, MI). MetaCore is an integrated software suite for functional analysis of experimental data and it contains curated protein interaction networks on the basis of manually curated database of human protein-protein, protein-DNA, protein-RNA and protein-compound interactions. Metacore uses a hypergeometric model to determine the significance of enrichment. The differentially expressed proteins from our experiment were used for generating network by a direct paths algorithm.

Results

miR-34a regulates systematic protein changes in neuroblastoma

To investigate the effect of miR-34a on global mRNA expression changes, we reanalyzed our previously published dataset which was obtained from IMR32 cells transfected with miR-34a or mimic control for 48 hours ⁵. After a quality filtering, a total of 16441 unique genes remained for analysis; miR-34a caused moderate mRNA expression changes with only 11 up-regulated genes and 9 down-regulated genes using a 2-fold cutoff (Supplemental Fig 1). miRNAs are well known to regulate target expression at the translational level, therefore the overall protein expression profile was examined using ICAT-based quantitative technology to identify global protein changes caused by miR-34a. Peptide identification was obtained at a confidence level greater than 95% as evaluated by searching a randomized sequence databases ¹⁹. After excluding proteins identified solely by a single peptide, a total of 1495 unique proteins remained for the further data processing and normalization procedure as described in the Materials and Methods section. The protein expression ratios (log₂ transformed) between miR-34a transfected cells and mimic control were normally distributed (Fig 1A) with a large number of proteins showing an abundance change in miR-34a treated cells compared to control cells. When using a minimum 2-fold cutoff, 143 and 192 proteins were up- and down-regulated, respectively. Comparing the mRNA expression ratios (Fig 1B) for the same set of genes identified by ICAT revealed that none of these genes exhibits greater than 2-fold mRNA expression change; however, we did observe a weak but significant correlation between mRNA and protein expression ($r = 0.1$, $p < 0.001$, spearman rank correlation). These data suggested that miR-34a exerts its biological effects mainly through translational rather than transcriptional regulation.

miR-34a seed sequence match in down-regulated proteins

miRNAs target protein coding genes for posttranscriptional repression primarily through binding sites in 3' UTRs through their seed sequence matches. We used miR-34a seed sequence matches for 6mer (match to position 2-7), 7mer (2-8), 7mer-A1 (2-7 with adenosine in position 1) and 8mer (1-8) respectively as defined in a previous reports¹⁴. The 3' UTR sequences were obtained from Ensembl database (<http://www.ensembl.org>) for all transcripts whose proteins were identified by ICAT and the enrichment of miR-34a binding sites in those 3' UTR sequences was determined. Many seed sequence matches have been found in the down-regulated proteins; however, there is no significant enrichment for any of those sites (Supplemental Table 1). The detailed match information for all down-regulated proteins is available in Supplemental Table 2.

Pathway analysis on proteins up- or down-regulated by miR-34a

We next examined the pathway enrichment among the differentially expressed proteins in miR-34a treated cells. Gene ontology (GO) analysis showed that in miR-34a treated cells the biological processes of apoptosis and cell death are significantly enriched in up-regulated proteins, while DNA replication and cell cycle related processes are significantly down-regulated (Table 1 and Supplemental Table 3; $p < 0.01$ and FDR < 0.1). Exploring the pathway maps using MetaCore returned similar results as GO analysis (Supplemental Fig 2). Cell cycle related map (Chromosome condensations in prometaphase, $p = 7.3e-09$) is the most significant map among the ten most notably down-regulated Genego pathway maps (Supplemental Fig 2A & 2B). Another cell cycle related map, sister chromatid cohesion, is also significantly down-regulated ($p = 5.7e-04$, Supplemental Fig 2A & 2C). In addition, an apoptosis map (Apoptosis and survival_HTR1A signaling, $p = 2/7e-05$) is one of the most significantly up-regulated Genego pathway maps (Supplemental Fig 2D). These protein pathway analysis results are consistent with previous miR-34a studies based on biological experiment or mRNA expression showing that miR-34a suppressed cancer cell growth through promotion of apoptosis and reduction of cell cycle and DNA synthesis^{4-5, 8-9, 24-25}.

Gene set enrichment analysis (GSEA) is another powerful pathway analysis tool focusing on groups of genes that share common biological function or mechanism²³. This method was applied to the quantitative proteomic data to investigate protein abundance changes induced by miR-34a at the pathway level. Surprisingly, only two protein sets involved in ribosome were shown to be significantly enriched in miR-34a down-regulated proteins, none was identified in up-regulated proteins ($p < 0.001$ and FDR q-value < 0.1 , Fig 2). These two sets are RIBOSOMAL_PROTEINS from GenMAPP and HSA0310_RIBOSOME from KEGG database; 25 and 20 proteins exist in the leading edge subsets respectively from a total of 37 and 28 overlapped proteins in database.

Protein networks in miR-34a regulation

We further investigated the protein interactions regulated by miR-34a for differentially expressed proteins using the MetaCore analysis tool and a direct interaction algorithm for which no additional objects are added to the network. Using a total of 335 proteins (143 up-regulated and 192 down-regulated), a network of 72 proteins with direct interactions was obtained (Fig 3). Four main sub-networks centered on YY1, Caspase-3, NF- κ B and STAT1 were observed. These proteins, as well as their networks, play important roles in cancer biology. Caspase-3 initiates degradation of DNA in the final stages of apoptosis; it is known to be induced by miR-34a⁴⁻⁵. Interestingly, almost all proteins in YY1 sub-network are down-regulated in the cells treated with miR-34a. Many of these proteins are ribosomal proteins, which may partially explain the enrichment of ribosomal proteins in miR-34a down-regulated proteins (Fig 2). In addition, other known YY1 target genes²⁶ including SMC2, CYP51A1, HMGB1, HMGB2, HMGB3, LMNB1, MTHFD2, MCM3, MCM4,

MCM5, MCM7, FDFT1, PRPS1, PLCB1, MCH2, MSH2 also have lower expression in miR-34a treated NB cells (Supplemental Table 4).

YY1 is a direct target of miR-34a

YY1, a ubiquitous transcription factor that negatively regulates p53, plays an important role in cancer biology²⁰⁻²¹. The previous protein network analysis revealed that YY1 sub-network is evidently regulated by miR-34a and YY1 3' UTR has conserved binding sites for miR-34a (Supplemental Table 2), therefore we hypothesized that miR-34a directly targets YY1. We used western blot analysis to validate the reduction of YY1 protein expression by miR-34a in 2 MYCN-amplified cell lines (IMR32 & SKNDZ) and 2 MYCN-single copy cell lines (SKNAS & SHSY5Y) transfected with miR-34a (Fig 4). Sequence analysis showed that the YY1 3' UTR contains a conserved binding site (position 720-726 of YY1 3' UTR) for miR-34a (Fig 5A). YY1 has also been predicted as miR-34a target by both TARGETSCAN and PICTAR-VERT (http://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MI0000268). To test the hypothesis that miR-34a directly targets YY1, a luciferase reporter using partial YY1 3' UTR (107bp, position 670-776 of YY1 3' UTR) with miR-34a binding site intact (Luc-YY1-WT) was constructed. We also constructed a luciferase reporter vector containing the same part of YY1 3' UTR but with miR-34a binding site removed (Luc-YY1-mutant). The resulting reporter constructs were transfected into SK-N-AS cells, a NB cell line that does not express miR-34a⁵, along with miR-34a or a mimic control microRNA. miR-34a decreases luciferase activity of the reporter vector containing YY1 3' UTR with a wild-type miR-34a binding site (Fig 5B, $p = 0.003$), but not for the reporter vector with mutated YY1 3' UTR. Taken together, these results demonstrated that miR-34a directly targets the YY1 gene through binding to YY1 3' UTR.

Discussion

Microarray analyses have been widely used to identify miRNA targets^{1, 10-11} and hundreds of putative, down-regulated miR-34 targets have been identified using this method^{8, 27-28}. Due to the translational regulatory mechanism of miRNAs, proteomic analysis becomes a powerful and direct tool for identifying miRNA targets and to quantify the contribution of translational repression to post-transcriptional gene silencing by miRNAs¹²⁻¹⁴. Two recent studies found that the changes in mRNA abundance are not only correlated with the repression of many targets, but also can account for most of the observed reduction in protein expression¹³⁻¹⁴. Using integrated genomics and proteomics approaches, we found that miR-34a over-expression caused moderate overall mRNA expression changes, but induced dramatic systematic protein level changes (Fig 1 and Supplemental Figure 1). A small but positive correlation ($r = 0.1$, $p < 0.001$) between transcript and protein fold changes was observed, suggesting that miR-34a regulates protein repression by both mRNA degradation and translational regulation. In the case where repression of protein expression is reflected by decreased mRNA level, microarray analysis might be sufficient for target identification without a need for sophisticated proteomics approaches^{13-14, 29}. However, in this study miR-34a over-expression in IMR32 cells caused very moderate changes in mRNA level and the correlation between mRNA and protein abundance level is low. Therefore proteomics is an invaluable technique for target identification. In this study, quantitative proteomics identified YY1 as a direct target of miR-34a; a result that would not be found using mRNA expression analysis alone. We found that the magnitude of mRNA expression changes induced by miR-34a in our study is much smaller than observed from a previous miR-34a study⁸. Several factors might explain these differences, such as the usage of different transfection methods (transient or stable transfection), different cell lines, the amount of miRNAs used, and the time points to collect data.

In this study, we identified and validated YY1 as a direct target of miR-34a. YY1 is a ubiquitous transcription factor that plays an essential role in development. This transcription factor has been associated with cell proliferation, anti-apoptosis, tumorigenesis and metastatic potential²². We showed that miR-34a inhibits YY1 expression as well as the expression of YY1 downstream genes. Many of the expressed YY1 downstream genes are ribosomal proteins. Pathway analysis of the global protein expression changes revealed ribosomal proteins as the only functional protein class significantly enriched in the group of miR-34a down-regulated proteins. It is known that YY1 binding sites exist in many ribosomal proteins³¹. Genes that express ribosomal proteins are reported to be most significant gene set up-regulated by YY1³², suggesting that the down-regulation of ribosomal proteins by miR-34a might be through YY1 pathway. There is also evidence showing MYCN enhances the transcription of a large set of ribosome biogenesis genes at the mRNA level³³. The enrichment of ribosomal proteins may also be related to MYCN function since this protein's expression is directly regulated by miR-34a⁵. We found that YY1 expression (both mRNA and protein level) was not reversely correlated with miR-34a expression. The correlation of YY1 with miR-34a expression is 0.039 for YY1 mRNA expression and 0.287 for YY1 protein level (Supplemental Figure 3). This was not surprising because the level of YY1 is likely to be controlled not only by miR-34a but also other mechanisms. The turnover of YY1 is probably also through ubiquitination and proteasomal degradation since it has been reported that the treatment of a proteasome inhibitor led to accumulated YY1 protein²⁰.

miR-34a has many potential target genes, with several of these, including MYCN, E2F3, CCND1, CCNE2, CDK4, CDK6, MET, BCL2, DLL1 and SIRT1, having been experimentally validated^{4-5, 8-9, 24-25, 30}. Among them, only CDK4 and CDK6 are shown in the list of proteins detected by ICAT. Both CDK4 and CDK6 are down-regulated (roughly 30-40% down) in miR-34a treated cells. ICAT has a limitation to detect many of those experimentally validated direct targets including MYCN¹⁹. While the reasons for this are not absolutely clear, it may be due to the unfavorable size of the cysteinyl residue containing peptides produced by tryptically digesting the protein, the resultant peptides having a low ionization efficiency, or the protein may be below the detection limit of the mass spectrometer¹⁹. In a recent study aimed at identifying differentially expressed proteins in miR-34a treated hepatocellular carcinoma HepG2 cells, YY1 was not identified¹⁵. This study was performed using MALDI-TOF/TOF mass spectrometry and only identified 19 up- and 15 down-regulated proteins (compared to 143 up- and 192 down-regulated proteins in this study). The difference of identification might be due to the usage of the different cellular context and proteomics methods. Obviously the greater coverage obtained using the method described in our study accounts for the ability to detect YY1 as being regulated by miR-34a in neuroblastoma cells.

Recent studies showed that the tumor suppressor protein p53 regulates the expression of a set of miRNAs including miR-34a which has been found to be a direct target of p53^{8-9, 24, 30}. p53 is a key regulator of cell cycle control, apoptosis, and genomic stability, and is commonly mutated in cancer. The levels and activity of p53 are tightly regulated by posttranslational modifications, including phosphorylation, ubiquitination, and acetylation. A p53-miR-34a feedback loop is proposed^{6, 25} in which p53 induces miR-34a expression, which in turn increases p53 acetylation by suppressing SIRT1 expression. The resultant increase of p53 activity prolongs miR-34a expression⁶. YY1 is an important negative regulator of p53, as it down-regulates this tumor suppressor's activity by stimulating p53 ubiquitination and degradation²⁰⁻²¹. Our study showed that miR-34a directly down-regulates YY1 expression, which may reversely regulate p53 activity. It is possible that YY1 might also be involved in the p53-miR-34a feedback loop; miR-34a up-regulated by p53 inhibits YY1 expression therefore decreasing p53 ubiquitination and degradation to form a

regulatory circuitry. Further extensive experimental studies are needed to prove this hypothesis.

In summary, we have demonstrated that quantitative proteomic methods are powerful for investigating the global protein expression changes regulated by miRNA and complement results obtained using microarray analysis. Using this proteomic approach we identified proteins regulated by miR-34a and experimentally validated that YY1 is a direct target of miR-34a. miR-34a is a transcriptional target of p53, miR-34a directly target YY1 and YY1 is a negative regulator of p53, therefore the elucidation of the role of YY1 in p53-miR-34a regulatory circuitry may shed important light on tumor suppressive function of miRNA-34a.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. miR-34a causes significantly higher protein level changes compared to mRNA changes
 Presented are 1441 genes which have both protein and mRNA detection by ICAT and Affymetrix U133plus2 microarray. **A.** Histogram of protein ratios ranged from -8.156 to 5.88. There are large changes in protein abundance. **B.** Histogram of mRNA expression change ranged from -0.964 to 0.416. There only exist moderate mRNA abundance changes.

A.

Gene set name	Number of overlapped genes	Number of genes in the leading edge	Sources	p-value	FDR
RIBOSOMAL_PROTEINS	37	25	GenMAPP	<0.0001	<0.0001
HSA03010_RIBOSOME	28	20	KEGG	<0.0001	<0.0001

B.

C.

Fig 2. GSEA analysis of protein expression changes induced by miR-34a

A. List of significantly enriched protein sets. Only 2 gene sets are significantly enriched in proteins down-regulated by miR-34a, none shown in up-regulated proteins. GSEA analysis was performed on the ranked proteins according to the $\log_2$ ratio of protein expression between miR-34a and mimic control miRNA treated NB cells. Gene sets with a FDR q-value of < 0.1 and $p < 0.001$ were considered significant. **B.** Enrichment plots of ribosomal_proteins and hsa03010_ribosome gene sets. The green curve shows the running sum of enrichment score (ES) for the ranked proteins. The blue vertical line specifies the maximum ES score. The proteins listed under the plot are the leading edge subset of proteins.

Fig 3. Protein networks associated with the proteins up- or down-regulated by miR-34a
 The network was generated by a direct interaction algorithm of MetaCore (GeneGo) using the list of proteins up- or down-regulated by miR-34a. Nodes represent proteins; lines between nodes indicate the interaction between proteins with green being activation, red inhibition and gray unspecified; the arrowheads indicate the direction of the interaction. Different shapes of the nodes represent the functional class of the proteins. ●: over-expressed; ●: under-expressed; ★: transcription factor.

A.

B.

Fig 4. miR-34a suppresses YY1 protein expression

4 neuroblastoma cell lines (IMR32, SKNDZ, SKNAS and SHSY5Y) were transfected with miR-34a or mimic miRNA control at doses of 20 and 40 μ M. The cell lysates were prepared for western blot analysis. **A.** Suppression of YY1 protein expression by miR-34a. **B.** The quantification of western blotting result. The protein level is normalized by GAPDH expression.

A.

B.

Fig 5. miR-34a directly targets YY1

A. miR-34a and its binding site within the YY1 3'UTR sequence. **B.** miR-34a suppresses YY1 by targeting the YY1 3'UTR. SK-N-AS cells were transfected with luciferase reporter constructs either containing a wild-type of YY1 3'UTR with miR-34a binding site (Luc-YY1-WT) or with miR-34a binding site removed (Luc-YY1-mutant). The cells were co-transfected with miR-34a or mimic miRNA control (5 nM). miR-34a reduces expression of luciferase containing a wild-type miR-34a binding site ($p = 0.003$) but not a mutant one.

Table 1

Significant GO processes of differential expressed proteins

	Term	Count	PValue	FDR
Up-regulated	GO:0009056~catabolic process	16	0.000	0.005
	GO:0006807~nitrogen compound metabolic process	12	0.001	0.015
	GO:0044248~cellular catabolic process	13	0.001	0.020
	GO:0006915~apoptosis	15	0.001	0.024
	GO:0012501~programmed cell death	15	0.001	0.026
	GO:0006519~amino acid and derivative metabolic process	10	0.001	0.027
	GO:0006520~amino acid metabolic process	9	0.002	0.028
	GO:0030163~protein catabolic process	8	0.002	0.040
	GO:0008219~cell death	15	0.002	0.043
	GO:0016265~death	15	0.002	0.043
	GO:0009057~macromolecule catabolic process	10	0.004	0.078
	GO:0006396~RNA processing	10	0.005	0.095
	GO:0009308~amine metabolic process	10	0.005	0.098
Down-regulated	GO:0006412~translation	28	0.000	0.000
	GO:0044249~cellular biosynthetic process	34	0.000	0.000
	GO:0009058~biosynthetic process	38	0.000	0.000
	GO:0009059~macromolecule biosynthetic process	28	0.000	0.000
	GO:0006261~DNA-dependent DNA replication	10	0.000	0.000
	GO:0043170~macromolecule metabolic process	101	0.000	0.000
	GO:0010467~gene expression	63	0.000	0.000
	GO:0044238~primary metabolic process	109	0.000	0.000
	GO:0044237~cellular metabolic process	108	0.000	0.000
	GO:0006974~response to DNA damage stimulus	13	0.000	0.002
	GO:0008152~metabolic process	114	0.000	0.002
	GO:0019538~protein metabolic process	58	0.000	0.003
	GO:0006260~DNA replication	11	0.000	0.004
	GO:0006281~DNA repair	11	0.000	0.006
	GO:0007076~mitotic chromosome condensation	4	0.000	0.009
	GO:0009987~cellular process	143	0.001	0.012
	GO:0009719~response to endogenous stimulus	13	0.001	0.013
	GO:0044267~cellular protein metabolic process	53	0.001	0.014
	GO:0016043~cellular component organization and biogenesis	44	0.001	0.016
	GO:0006259~DNA metabolic process	20	0.001	0.016
	GO:0044260~cellular macromolecule metabolic process	53	0.001	0.020
	GO:0030261~chromosome condensation	4	0.001	0.021
	GO:0022402~cell cycle process	18	0.001	0.023
	GO:0046483~heterocycle metabolic process	6	0.001	0.028
	GO:0006886~intracellular protein transport	12	0.002	0.044
	GO:0045005~maintenance of fidelity during DNA-dependent DNA replication	4	0.003	0.054

Term	Count	PValue	FDR
GO:0006284~base-excision repair	4	0.003	0.054
GO:0007049~cell cycle	19	0.003	0.060
GO:0000070~mitotic sister chromatid segregation	4	0.003	0.065
GO:0007059~chromosome segregation	5	0.003	0.065
GO:0065003~macromolecular complex assembly	14	0.004	0.070
GO:0000819~sister chromatid segregation	4	0.004	0.071
GO:0022613~ribonucleoprotein complex biogenesis and assembly	8	0.004	0.076
GO:0006397~mRNA processing	9	0.004	0.080
GO:0046907~intracellular transport	16	0.005	0.088