miR-375 Is Activated by ASH1 and Inhibits YAP1 in a Lineage-Dependent Manner in Lung Cancer

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Abstract

Lung cancers with neuroendocrine (NE) features are often very aggressive but the underlying molecular mechanisms remain elusive. The transcription factor ASH1/ASCL1 is a master regulator of pulmonary NE cell development that is involved in the pathogenesis of lung cancers with NE features (NE-lung cancers). Here we report the definition of the microRNA miR-375 as a key downstream effector of ASH1 function in NE-lung cancer cells. miR-375 was markedly induced by ASH1 in lung cancer cells where it was sufficient to induce NE differentiation. miR-375 upregulation was a prerequisite for ASH1-mediated induction of NE features. The transcriptional coactivator YAP1 was determined to be a direct target of miR-375. YAP1 showed a negative correlation with miR-375 in a panel of lung cancer cell lines and growth inhibitory activities in NE-lung cancer cells. Our results elucidate an ASH1 effector axis in NE-lung cancers that is functionally pivotal in controlling NE features and the alleviation from YAP1-mediated growth inhibition. Cancer Res; 71(19); 6165–73. ©2011 AACR.

Introduction

Lung cancer has long been the leading cause of cancer-related death in economically developed countries, and a better understanding of the molecular pathogenesis of this fatal disease is greatly anticipated for preventive and/or therapeutic breakthroughs (1). Accumulated evidence strongly suggests that alterations of microRNA (miRNA) expressions are involved in the development of human cancers (2–5). Our previous studies identified let-7 as a miRNA family with growth inhibitory activities, which were also found to be frequently downregulated in lung cancers in association with poor prognosis (6). In marked contrast to the tumor suppressor-like let-7 miRNA family, the miR-17-92

miRNA cluster plays roles as oncogene-type miRNAs in the development of lung cancers (7, 8).

Lung cancer is classified into 2 major classes, small cell lung cancer (SCLC) and non-SCLC (NSCLC), of which SCLC characteristically exhibits neuroendocrine (NE) features and an aggressive clinical course. In addition, a small proportion of NSCLCs such as large cell NE carcinoma also share such characteristics. Therefore, it is conceivable that elucidation of the underlying mechanisms involved in the acquisition of those characteristics in lung cancers with NE features may provide important clues for a better understanding of carcinogenic processes. Along this line, we previously reported that A549 lung adenocarcinoma cells exhibited NE properties when introduced with achaete-scute homologue 1 (ASH1/ASCL1), a proneural basic helix-loop-helix (bHLH) transcription factor (9), whereas ASH1 knockdown elicited prominent apoptosis in SCLC lung cancer cell lines (10). We also found that ASH1 mediates lineage-survival signal in SCLC at least in part through its transcriptional repressor activity toward putative tumor suppressor including DKK1 and E-cadherin (9). However, to date, virtually nothing is known about the possible involvement of miRNAs downstream of this dual function transcription factor, which is crucially involved in the biology of SCLC.

In this study, we investigated whether miRNAs are also governed by ASH1 and have roles downstream of ASH1 downstream in the development of lung cancers with NE features. Consequently, miR-375 was identified as a miRNA directly and highly transactivated by ASH1. The involvement of miR-375 in acquisition of NE phenotypes and growth regulation in lung cancers with NE features is also discussed.
Materials and Methods

Cells and expression constructs

An A549 lung adenocarcinoma cell line without NE differentiation and a typical SCLC cell line, ACC-LC-172, as well as A549 cells stably transduced with ASH1-expressing (A549-ASH1) or empty (A549-VC) lentiviruses (9) were maintained in RPMI-1640 with 5% FBS. ASH1-expressing lentiviral and plasmid vectors were constructed with CSII-CMV-MCS-IRES2-Blasticidin and pcDNA3 (Invitrogen), respectively, as previously described (9). Yes-associated protein 1 (YAP1) cDNA was purchased from OriGene and inserted into CSII-EF-MCS-IRES2-Venus. The lentivirus vectors were kindly provided by Dr. H. Miyoshi (RIKEN BioResource Center). Venus (improved YFP) was provided by Dr. A. Miyawaki (RIKEN Brain Science Institute).

Reporter assay

A 1,028-bp-long putative promoter fragment spanning from 992 bp upstream of the pre-miR-375 sequence to 3 bp upstream of the mature miR-375 sequence was amplified from human genomic DNA and cloned into a pGL4.10 basic reporter (pGL4-375P in Fig. 1B). pGL4-D1, -D2, and -D3 truncated reporter plasmids were constructed by reamplification of the pGL4-375P plasmid, with each containing 103, 324, and 681 bp regions 5’ to the pre-miR-375 sequence (Fig. 1B; Supplementary Fig. S1B). E-box deletion mutant reporters were also constructed by PCR-mediated in vitro mutagenesis of pGL4-Δ3. Each of these miR-375 promoter reporters was transfected into A549 cells using Lipofectamine 2000 (Invitrogen) together with an ASH1 expression vector, pcDNA3-ASH1, or control empty vector, pcDNA3, with the renilla luciferase reporter pRL-TK used as an internal control.

microRNA microarray and gene expression microarray analysis

Microarray analysis was conducted to examine miRNA expression profiles using a Human miRNA Microarray, pre-commercial version 6.0 (Agilent) with 470 miRNA probes, according to the manufacturer’s instructions. A549 cells were infected with an ASH1-expressing or empty lentivirus and harvested 4 days later. miRNA microarray data were log2 transformed and normalized to the 75th percentile. Microarray analysis by a Whole Human Genome 4 x 44K Microarray G4112F (Agilent) was also conducted to examine changes in expression of potential target genes of miR-375 by transfection of Pre-miR-375 or Pre-miR-NC#2 (Ambion) in A549 cells, which were then harvested at 12, 24, 48, and 96 hours after transfection. RNA samples were prepared by using an RNeasy kit (Qiagen) as previously described. All the microarray data used for this study are available at Gene Expression Omnibus accession numbers GSE31565 and GSE31566).

Quantitative reverse transcriptase PCR

Quantitative reverse transcriptase PCR (RT-PCR) analysis was carried out by using primers for chromogranin A (CHGA), chromogranin B (CHGB), secretogranin II (SCG2), secretogranin III (SCG3), ASH1, YAP1, and β-actin (Supplementary Table S1), along with Power SYBR Green PCR Master Mix (Applied Biosystems), and an ABI Prism7500 (Applied Biosystems), as previously described. Expression levels were calculated by using the standard curve method and normalized with that of the noncoding RNA RNU48.

Quantitative ChIP assay

ChIP assays were carried out as described previously, using ChIP E1/2 and ChIP E4/5 primers, which were designed for amplification of genomic fragments containing E1 and E2 E-boxes and E4 and E5 E-boxes, respectively (Fig. 1B). ChIP analysis using "distant" primers for amplification of a genomic
region approximately 5.3 kb downstream of pre-miR-375 (Supplementary Fig. S1) as well as CHGB primers were used as negative and positive controls, respectively. The primer sequences are shown in Supplementary Table S1. The amounts of chromatin-immunoprecipitated genomic DNA were measured by the ΔΔCt method to compare various ChIP primers and β-actin primers and the results of quantification were obtained as fold changes of A549-ASH1 against A549-VC. Antibodies against acetylated histone H3 (H3Ac), trimethylated H3 lysine 4 (H3K4me3), and trimethylated H3 lysine 27 (H3K27me3) were purchased from Upstate.

**In situ hybridization**

We employed a Fluorescein isothiocyanate–labeled locked nucleic acid (LNA) probe for mmu-miR-375 and a scrambled sequence (Exiqon). Probes were diluted to 40 nmol/L in hybridization buffer (Ambion). In situ hybridization was conducted according to the manufacturer’s protocol, as previously described (11). In brief, after deparaffinization, neutral formalin-fixed specimens on slides were incubated in proteinase K solution (20 μg/mL) at 37°C. After fixing the specimens with 4% paraformaldehyde, endogenous peroxidase activities were quenched in methanol containing H2O2 [0.3% (v/v)], then the probes were hybridized overnight at 37°C. After washing with SSC with 50% formamide, a CSA II biotin-free catalyzed signal amplification system (Dako) was used to visualize miRNA expression as brown precipitates. Nuclear staining was done with hematoxylin.

**Immunohistochemical analysis**

Slides were subjected to an antigen retrieval procedure by using Imunosaver (Nisshin EM) and then endogenous peroxidase activities were quenched. Next, the slides were incubated with rabbit polyclonal antisynaptophysin antibody (Dako) followed by goat anti-rabbit immunoglobulins/horseradish peroxidase (Dako), and then visualized with liquid 3,3′-diaminobenzidine (Dako). Nuclear counterstaining was done with hematoxylin.

**Transfection of Pre-miR-375 and LNA**

Both Pre-miR-375 and Pre-miR-NC#2 were purchased from Ambion. Antisense and scramble oligonucleotides against mature miR-375 were synthesized by using LNAs (Greiner). Each of oligonucleotides was introduced into A549 cells at 10 to 15 nmol/L, using 2.5 μL/mL of Lipofectamine RNAiMax (Invitrogen) according to the instructions of supplier.

**Results**

This study was initiated to investigate the potential involvement of miRNAs downstream of ASH1 in acquisition of characteristics of lung cancers with NE features. To this end, we first carried out genome-wide expression profiling of miRNAs to search for those significantly affected by ASH1 transduction in a lung cancer cell line without NE features. As a result, we identified 12 upregulated (>5-fold) and 8 downregulated (>5-fold) miRNAs in ASH1-transduced A549 cells (Table 1), of which miR-375 was found to be the most highly regulated.

**Table 1. Up- and downregulated miRNAs in ASH1-transduced A549 cells**

<table>
<thead>
<tr>
<th>miRNA</th>
<th>A549-ASH1a</th>
<th>A549-VCa</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulated miRNAs (&gt;5-fold)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-375</td>
<td>62,806</td>
<td>0.038</td>
<td>1,659.802</td>
</tr>
<tr>
<td>hsa-miR-193a</td>
<td>8,071</td>
<td>0.726</td>
<td>11.120</td>
</tr>
<tr>
<td>hsa-miR-489</td>
<td>0.332</td>
<td>0.030</td>
<td>11.105</td>
</tr>
<tr>
<td>hsa-miR-10a</td>
<td>13,254</td>
<td>1.333</td>
<td>9.940</td>
</tr>
<tr>
<td>hsa-miR-196b</td>
<td>0.231</td>
<td>0.030</td>
<td>7.731</td>
</tr>
<tr>
<td>hsa-miR-181a</td>
<td>4,906</td>
<td>0.683</td>
<td>7.188</td>
</tr>
<tr>
<td>hsa-miR-181a*</td>
<td>0.185</td>
<td>0.026</td>
<td>7.108</td>
</tr>
<tr>
<td>hsa-miR-95</td>
<td>0.178</td>
<td>0.026</td>
<td>6.830</td>
</tr>
<tr>
<td>hsa-miR-326</td>
<td>0.353</td>
<td>0.052</td>
<td>6.785</td>
</tr>
<tr>
<td>hsa-miR-9*</td>
<td>2.559</td>
<td>0.397</td>
<td>6.441</td>
</tr>
<tr>
<td>hsa-miR-628</td>
<td>0.149</td>
<td>0.026</td>
<td>5.720</td>
</tr>
<tr>
<td>hsa-miR-181b</td>
<td>8.344</td>
<td>1.512</td>
<td>5.517</td>
</tr>
<tr>
<td><strong>Downregulated miRNAs (&gt;5-fold)</strong></td>
<td>0.018</td>
<td>1.008</td>
<td>0.018</td>
</tr>
<tr>
<td>hsa-miR-30a-3p</td>
<td>0.018</td>
<td>0.608</td>
<td>0.029</td>
</tr>
<tr>
<td>hsa-miR-137</td>
<td>0.018</td>
<td>0.455</td>
<td>0.039</td>
</tr>
<tr>
<td>hsa-miR-30a-5p</td>
<td>1.092</td>
<td>13.985</td>
<td>0.078</td>
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<tr>
<td>hsa-miR-149</td>
<td>0.116</td>
<td>1.363</td>
<td>0.085</td>
</tr>
<tr>
<td>hsa-miR-618</td>
<td>0.041</td>
<td>0.255</td>
<td>0.161</td>
</tr>
<tr>
<td>hsa-miR-422b</td>
<td>0.463</td>
<td>2.485</td>
<td>0.186</td>
</tr>
</tbody>
</table>

*aNormalized signal intensity.*
upregulated, which was also verified by quantitative RT-PCR, using an miR-375–specific TaqMan probe (Fig. 1A). An ASH1 lentivirus was also transduced into 4 other NSCLC cell lines, 3 of which showed marked miR-375 induction (Supplementary Fig. S2A). A survey of the genomic region harboring miR-375 indicated that this miRNA resides in an intergenic region between the CCDC108 and CRVBA2 genes at chromosome 2q35 (Supplementary Fig. S1A), whereas a region approximately 1 kb in length was found to be evolutionarily highly conserved (shown in red). Of the 5 conserved E-boxes, 4 were CACCTG whereas the other (E2) was CATCTG. To verify the promoter activity and responsiveness to ASH1, luciferase reporter constructs of the putative miR-375 promoter and its various mutants were cotransfected with an ASH1 expression vector, pcDNA3-ASH1, or an empty vector into A549 cells. The pGL4-375P showed marked transactivation by ASH1 (Fig. 1B). pGL4-D containing 3 E-boxes (E1 to E3) showed robust responsiveness to ASH1, whereas pGL4-D2 with a further deletion failed to respond. Reporters, each of which contained a single E-box deletion mutation (pGL4-ΔE1, -ΔE2, and -ΔE3), showed moderate reductions in ASH1 responsiveness, whereas pGL4-ΔE123, carrying a deletion of all 3 E-boxes (E1 to E3), lost responsiveness to a level similar to that of pGL4-D2, indicating their crucial involvement in ASH1 responsiveness. To further confirm the promoter activity of this region, ChIP assays with antibodies against various histone H3 modifications were carried out by A549 cells infected with either ASH1-carrying or empty viruses (Fig. 1C). Consequently, specific induction of activating histone modifications (H3Ac and H3K4me3) in the genomic regions encompassing these 3 E-boxes were clearly shown in ASH1-expressing A549 cells. In addition, a ChIP assay with an anti–myc-tag antibody against the myc-tagged ASH1 protein indicated a direct interaction of ASH1 with the E1/2 region (Supplementary Fig. S3).

The association between ASH1 and miR-375 expression was then analyzed in fetal mouse lung (Fig. 2A). Although neuroepithelial bodies (NEB) known to consist of ASH1-expressing pulmonary NE cells and epithelial progenitor cells (12), showed positive immunohistochemical staining for the NE marker, synaptophysin, and H3K4me3 in the genomic regions encompassing these 3 E-boxes were clearly shown in ASH1-expressing A549 cells. This was further confirmed by quantitative RT-PCR analysis results (Fig. 3B). As this finding strongly suggests that miR-375 alone is capable of inducing NE
markers in the absence of ASH1, we then investigated whether miR-375 is required for NE marker induction by ASH1. A549 cells were first infected with an ASH1-expressing lentivirus and subsequently transfected with miR-375 antisense or scramble LNAs, which resulted in marked inhibition of various NE markers in the presence of miR-375 antisense, but not negative control, miR-375 scramble (Fig. 3C). These findings clearly showed that ASH1-inducible miR-375 is required for NE marker induction by ASH1 in lung cancer cells. To verify the specificity of NE marker induction by miR-375, we also transfected unrelated miRNAs into A549 cells. As shown in Supplementary Fig. S2B, various unrelated miRNAs scarcely induced CHGB expression, suggesting the specificity of miR-375-mediated NE marker induction. In addition, miR-375 was transfected into 2 other NSCLC cell lines and 2 immortalized normal lung epithelial cell lines, HPL1D and BEAS2B, which confirmed CHGB induction at varying degrees in all 4 cell lines (Supplementary Fig. S2C).

To study the direct effects of miR-375, we analyzed changes in the expression profiles of target genes for miR-375 predicted with TargetScan4.1 (http://www.targetscan.org/) and observed leftward shifts of the expression profile histograms, especially at 24 and 48 hours after miR-375 transfection (Fig. 4A), which indicated moderate but significant down-regulation of the predicted target genes of miR-375. In contrast, histograms of genes residing in chromosome 1 did not show any shifts, confirming specificity. Among the potential target genes affected by miR-375, transcriptional coactivator YAP1 was the most significantly repressed after miR-375 transfection (Fig. 4B), which was also confirmed by Western blotting analysis (Fig. 4C). Two potential miR-375 binding sites were also noted within the 3’-UTR of YAP1 mRNA, thus we carried out a luciferase assay by using YAP1 3’-UTR reporters (miR-375 × 1 and miR-375 × 2) containing either 1 or 2 potential miR-375 binding sites (Fig. 4D). A549 cells transfected with these reporter constructs along with either Pre-miR-375 or negative control Pre-miR-NC#2 showed significant suppression of luciferase activity in a target site-dependent manner. The YAP1 3’-UTR reporter with deletion of the potential miR-375 binding site abrogated miR-375-mediated suppression of luciferase activity (Supplementary Fig. S5A). The specificity of the miR-375 target sites was also supported by our findings of lack of suppression of the wild-type YAP1 3’-UTR reporter activity by various unrelated miRNAs (Supplementary Fig. S5B). The relationship of miR-375 with YAP1 was also substantiated by the significant negative correlation (R = 0.793, P < 0.0001) between miR-375 and YAP1 in a panel of 29 lung cancer cell lines and 2 immortalized normal airway epithelial cell lines (Fig. 4E, left). In addition, we observed a histologic type-dependent expression pattern with low YAP1 expression in SCLC and abundant expression in NSCLC cell lines, indicating an expression pattern opposite to that of miR-375, which has abundant expression in SCLC (Fig. 4E, right). These relationships among ASH1, miR-375, and YAP1 were also observed in primary lung cancer specimens (Fig. 4F), suggesting the existence of robust regulatory relationships in the ASH1–miR-375–YAP1 pathway in lung cancers with NE features.

The negative correlation between miR-375 and YAP1 found in a histologic type-related manner prompted us to investigate YAP1 functions in lung cancer cells of both histologic types. A549 adenocarcinoma and ACC-LC-172 SCLC cell lines were infected with YAP1-expressing or an empty lentivirus expressing the fluorescent protein Venus from an internal ribosomal entry site. Fluorescent microscopic examination revealed marked reduction of the fluorescence-positive population, which was indicative of successful infection by the YAP1-expressing virus, in contrast to robust growth in fluorescence-negative uninfected ACC-LC-172 cells (Fig. 5A), which were also confirmed by fluorescence-activated cell sorting analysis (Fig. 5B). In contrast to ACC-LC-172, A549 cells seemed to be tolerant to the introduction of YAP1, because the fluorescence-positive YAP1-infected population gradually increased (Fig. 5B). Time courses of fluorescent signals in lentivirus-infected A549 and ACC-LC172 cells are shown in Supplementary Figure S6A. YAP1-virus infection significantly inhibited the increase of fluorescent signals in ACC-LC-172 cells, but not in A549, suggesting a lineage-dependent growth-suppressive effect of YAP1. These findings were also confirmed by Western blotting analysis.
Figure 4. YAP1 inhibition by miR-375. A, histogram of gene expressions at 4 time points after Pre-miR-375 transfection. Gene expressions at 12 to 96 hours after transfection are shown as log2 ratio values between A549 cells transfected with Pre-miR-375 and Pre-miR-NC#2. Top, miR-375 predicted target genes; bottom, genes residing in chromosome 1, which was used as a control. B, time course of expression of each miR-375 predicted target gene after Pre-miR-375 transfection. C, Western blotting analysis of YAP1 protein. A549 cells transfected with Pre-miR-RNAs (15 nmol/L) were analyzed by Western blotting with the antibody against endogenous YAP1 protein. The intensity of YAP1 bands was determined with a densitometer and normalized with lamin B bands. Pre-miR-375 transfection downregulated the level of YAP1 protein by 58%. This downregulation is shown with a bar graph, in which the extent of downregulation is also numerically indicated. D, YAP1 3'UTR reporter assay. YAP1 mRNA contains 2 miR-375 target sites at the 3'UTR. Two reporter constructs, miR-375 x1 and miR-375 x2, carry 1 and 2 miR-375 sites, respectively. A549 cells were transfected with reporter constructs and Pre-miR-RNAs (10 nmol/L). The ratio of luciferase activity of Pre-miR-375 transfectants to that of Pre-miR-NC#2 transfectants is shown. miR-375 significantly suppressed luciferase activity in a target site-dependent manner. The alignment of 2 miR-375 target sites with mature miR-375 is also shown. The nucleotide positions in 3'UTR are indicated. E, negative correlations of miR-375 and YAP1 expressions in lung cancer cell lines. Left, YAP1 and miR-375 expressions showed significant negative correlations among the tested lung cancer cell lines. Right, YAP1 was frequently overexpressed in AD and SQ cells, whereas its expression was strongly suppressed in SCLC. In contrast, most SCLC showed overexpression of miR-375, whereas miR-375 was scarcely expressed in the AD and SQ cell lines. F, correlations of ASH1, miR-375, and YAP1 expression levels in primary lung cancer specimens. ASH1 and miR-375 expressions were positively correlated, whereas inverse correlations were present between miR-375 and YAP1, as well as between ASH1 and YAP1. NL, normal lung; AD, adenocarcinoma; SQ, squamous cell carcinoma; LA, large cell carcinoma; AS, adenosquamous carcinoma.
dead cells were frequently observed in YAP1-introduced exogenous YAP1 expression in A549 cells (Fig. 5C). Cleavage of caspase-7 and caspase-3 was detected in YAP1-introduced ACC-LC-172 as opposed to the abundant endogenous YAP1 expression in ACC-LC-172 cells infected with the YAP1-virus. In contrast, ACC-LC-172 cells did not express endogenous YAP1. In addition, exogenous YAP1 was scarcely detected. D, Western blotting analysis of caspases. Five days after infection, activated cleavage of caspase-7 and caspase-3 was detected in YAP1 virus-infected ACC-LC-172 cells. Normalized intensities of cleaved caspases are numerically indicated.

Discussion

The present results clearly show that ASH1 directly transactivates miR-375, resulting in an NE lineage-specific upregulation of miR-375 in lung cancers. Although NeuroD1 and Pdx1 potentially bind to the E-box in the miR-375 promoter (13), their expression was rarely detected and showed no correlations with miR-375 expression (data not shown). Therefore, we believe that ASH1 plays a major role as a transcriptional activator of miR-375. Our finding of NEB-specific expression of miR-375 also supports the existence of an ASH1–miR-375 signaling axis in the lung. It is important to note that miR-375 was recently reported to be detectable in pancreatic islet cells under the regulation of NeuroD1 and Pdx1, as well as in pituitary and adrenal glands (13–16). Herein, we clearly showed the functional importance of 3 E-boxes (E1, E2, and E3) in ASH1-mediated induction of the promoter activity of miR-375. Furthermore, a recent report described the promoter activity of a similar genomic region 5′ to murine miR-375 in a β-cell line, though its responsiveness to potential activators such as NeuroD1 and ASH1 was not examined (17).

Downregulation of miR-375 has been reported in a few other types of cancer (18–20). Interestingly, miR-375 was suggested to play tumor suppressor roles in those cancer types, whereas target genes for miR-375 thus far reported include PDK1, 14-3-3ζ (19), HuD (21), and JAK2 (22). Also, hepatocellular carcinoma was recently added to the list of cancers with miR-375 downregulation and YAP1 has been suggested to be a target gene relieved by that downregulation. Consistent with those findings, we observed moderate downregulation of PDK1, 14-3-3ζ, HuD, and JAK2 when miR-375 was introduced to A549 cells, though YAP1 showed the most significant downregulation in our experimental settings (Supplementary Fig. S8B). In addition, we did not observe clear growth inhibition of A549 cells stably introduced with a lentivirus expressing miR-375 (Supplementary Fig. S8A), even though YAP1 was effectively downregulated by miR-375 (Fig. 4B–D). Along this line, it is notable that miR-375 knockout mice were shown to be hyperglycemic in association with decreased β-cell mass as a result of impaired proliferation of β-cells (15) and that miR-375 was shown to regulate a number of genes other than YAP1, which potentially control cellular growth and proliferation of pancreatic islets (15). It is also interesting that estrogen receptor-α (ERα)-expressing breast cancers showed ERα-signal dependency and a high expression of miR-375 (23). ERα binds the miR-375 promoter and induces its expression. miR-375 in turn represses the RAS, dexamethasone-induced 1 (RASD1) gene, which negatively regulates ERα expression, suggesting the existence of a positive feedback loop between ERα and miR-375, as well as a growth-promoting role of miR-375 in ERα-positive breast cancers (23). Taken together, it is conceivable that miR-375 plays distinct roles in various can-
cers, depending on the cellular context and transcriptomes including its potential target genes, and that downregulation of YAP1 by ASH1-transactivated miR-375 promotes rather than inhibits growth of SCLC cells.

YAP1 has several domains including a TEAD binding region and 2 WW domains, whereas it lacks a DNA binding domain and functions as a transcriptional coactivator through interactions with DNA binding transcription factors (24–26). YAP1 interacts with the TEAD family through a TEAD binding domain, and transactivates growth-promoting genes, whereas it also binds to PPXY motif-containing molecules including p73 through WW domains, thus enhancing p73-dependent apoptosis in response to DNA damage (27–29). It has been reported that phosphorylation by AKT or repression by ΔNp63 downregulates the proapoptotic activity of YAP1 (30), and that PML is also involved in regulation of p73-YAP apoptotic signaling through sumoylation and stabilization of YAP (31). Therefore, accumulating evidence enhances the notion that YAP1 exerts both oncogenic and tumor-suppressive activities in a context-dependent manner (32,33). The present findings show that YAP1 moderately promotes NSCLC proliferation when overexpressed, whereas it significantly suppresses SCLC growth, suggesting its lineage-dependent dual roles in lung cancers.

In conclusion, we identified miR-375 as a direct transcriptional target for ASH1 and showed that it has a crucial role for mediating signals required for ASH1-mediated inhibition of NE features in lung cancers. In addition, the present findings indicate that miR-375 directly downregulates YAP1, whereas we also found that it shows NE lineage-specific growth inhibitory activities in lung cancers. A future study of the downstream genes of the ASH1–miR-375 axis will be of great interest to fully elucidate the underlying signaling networks involved in NE differentiation and highly malignant behaviors of NE lung cancers including SCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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