主論文の要旨

miR-342-3p regulates MYC transcriptional activity via direct repression of E2F1 in human lung cancer

（ヒト肺癌において、miR-342-3p は E2F1 の発現を抑制し、MYC の転写活性を制御する）

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Introduction

Lung cancer has long been the leading cause of cancer death. Multiple oncogenes and tumor suppressor genes are involved in the molecular pathogenesis of lung adenocarcinoma, while accumulating evidence also indicates that altered expression of miRNAs is crucially involved. MYC is one of the most frequently amplified and overexpressed oncogene in cancer. The MYC gene encodes a transcription factor that regulates a wide variety of genes involved in control of cell growth, proliferation, and apoptotic cell death. The transcriptional activity of MYC is tightly controlled for proper transcriptional regulation through various mechanisms, which include MYC expression itself at both transcriptional and posttranscriptional levels, as well as its interaction with cofactors that functionally cooperate with MYC. Unfortunately, very little is known thus far about how MYC is regulated by miRNAs in lung cancer cells, especially via the latter mechanism involving MYC cofactors. In the present study, we attempted to identify miRNAs involved in the regulation of MYC transcriptional activity in lung cancer.

Materials and methods

We determined that the MYC module was a surrogate of MYC transcriptional activity, based on changes in mRNA expression in response to doxycycline (Dox)-inducible MYC in were transduced BEAS-2B and SK-LC-3 cells. We then conducted microarray analysis of mRNA expression using RNAs obtained up to 48 hours after MYC induction. We also performed microarray analysis of mRNA and miRNA expression profiles of 76 surgically resected lung adenocarcinoma tumors. MYC module activity was calculated for each of the 76 lung adenocarcinoma cases. Correlation co-efficient values and statistical significance were then calculated to analyze the association of the expression of each miRNA with MYC module activity. miRNAs with a significant association were selected for further analyses by flow cytometric, cell viability and colony formation assays (Figure 1).

Results

A total of 146 genes with early and continued response to MYC induction were selected as comprising the MYC module (Figure 2). Based on the expression profiles of 76 lung adenocarcinoma specimens, 77 candidate miRNAs with significant associations with MYC module activity were identified. Of these, 39 with readily detectable expression in either MYC-induced or -non-induced BEAS-2B cells were selected for further analysis (Table 1). We found that seven and five miRNAs were increased and repressed, respectively, in response to MYC induction. These miRNAs were previously reported MYC-regulated miRNAs including miR-17-92 cluster, miR-22, miR-26a, miR-30a-3p, and miR-30e-3p, supporting the robustness of our integrative strategy. Among the remaining miRNAs not affected by MYC induction, 20 with significant negative correlations with MYC module activity attracted our attention, as we

considered that these miRNAs may directly target MYC or indirectly modulate MYC module activity by targeting genes interacting/cooperating with MYC, thereby affecting MYC-mediated transcriptional regulation. Among the top 5 miRNAs in terms of their negative correlations with MYC module activity, cell cycle profiles were found to be affected by introduction of miR-342-3p (Figure 3A). We therefore decided to focus on miR-342-3p to elucidate how this miRNA may affect MYC-mediated transcriptional activity. In the present study, MYC module activity clearly showed a significant inverse correlation to miR-342-3p expression levels ($r=-0.490$, $P=3.37\times10^{-6}$) and miR-342-3p module activity ($r=-0.806$, $P=1.17\times10^{-19}$) (Figure 3B). In addition to that, a significant inverse correlation was observed with the dataset from the NCI Director's Challenge Consortium composed of 442 lung adenocarcinoma cases ($r=-0.726$, $P=1.94\times10^{-73}$) , as well as with the dataset consisting of 226 lung adenocarcinomas ($r=-0.694$, $P=9.05\times10^{-34}$) reported by the National Cancer Center of Japan (Figure 3C and 3D).

Target prediction programs did not predict MYC as a direct target of miR-342-3p, suggesting the possibility of indirect regulation. We nominated genes that were previously reported to cooperate with MYC and as the potential direct targets of miR-342-3p using (TargetScan, Human Release 6.2). The qRT-PCR analysis showed that E2F1 was markedly reduced in response to miR-342-3p introduction into NCI-H2009 (Figure 4A). We also validated this finding using western blotting analysis (Figure 4B) as well as with two additional lung adenocarcinoma cell lines, NCI-H23 and NCI-H441 (Figure 4C). Conversely, treatment with miR-342-3p antisense LNA resulted in a readily noticeable increase in E2F1 expression in the NCI-H1975 and ACC-LC-94 cell lines (Figure 4D). Next, a luciferase assay using a reporter construct carrying wild-type or mutant 3' UTR of E2F1 was performed with NCI-H1975 cells treated with either miR-342-3p antisense LNA or a scrambled negative control demonstrated that miR-342-3p directly targets E2F1 3' UTR. For biologic effects, we observed marked reduction in numbers of colonies and significant reduction of cell proliferation by miR-342-3p introduction, as well as siE2F1 and siMYC (Figure 5A and 5B). FACS analysis with aphidicolin thymidine double block indicated retarded cell cycle progression through the G1 and S phases in NCI-H2009 cells transfected with miR-342-3p mimics (Figure 5C). Consistent with these biologic effects, GO term analysis (Figure 5D) and GSEA (Figure 5E) also support that miR-342-3p plays important roles to inhibit cell cycle progression and proliferation.

Discussion

The present study was initiated to identify miRNAs that play roles in regulation of the functional activity of MYC in the molecular pathogenesis of lung cancers. To this end, we utilized an integrative approach with combinatorial usage of miRNA and mRNA expression profiling datasets of patient tumor tissues, as well as those of MYC-inducible cell lines. Our

results allowed us to identify multiple miRNAs reported as either directly downstream or upstream of MYC, supporting the robustness of our strategy. The former examples included the miR-17-92 cluster, miR-22, miR-26a, miR-30a-3p, and miR-30e-3p, all of which were previously shown to be under MYC-mediated transcriptional regulation, while the latter instances were comprised of let-7, miR-34a and miR-24, which have been reported to directly repress MYC expression via binding to a target site at the 3'UTR of MYC. Intriguingly, our integrative approach also led us to identify miR-342-3p, which we found to be a miRNA indirectly regulating MYC activity via direct inhibition of E2F1.

Previous studies have clearly shown that MYC exerts its transcriptional functions by collaborating with its obligated binding partner MAX and other interacting proteins. In addition, other molecules are thought to cooperate with MYC via binding to a genomic region in the proximity of the MYC binding site. Along this line, the E2F1 motif has been shown to be significantly enriched near MYC-bound genomic regions, especially those containing the canonical E-box motif, suggesting that this functional cooperation between MYC and E2F1 is crucially involved in cancer development. In the present study, we found that direct repression of E2F1 by miR-342-3p is indirectly involved, at least in part, in the mechanism underlying regulation of MYC activity.

In conclusion, we employed an integrative approach and identified miR-342-3p as a miRNA indirectly regulating MYC activity. Our study also revealed that direct repression of the MYC-cooperating transcription factor E2F1 is responsible, at least in part, for the MYC activity-regulating effect of miR-342-3p in lung adenocarcinomas. A future study is warranted to fully elucidate the functional roles of miR-342-3p, considering that miRNAs can inhibit multiple targets. Our results also clearly demonstrate the usefulness of our approach to utilize expression profiling datasets of both patient tumor tissues and experimental findings in vitro in an integrated manner.