主論文の要旨

TTF-1-regulated miR-532-5p targets KRAS and MKL2 oncogenes and induces apoptosis in lung adenocarcinoma cells

（TTF-1 により制御される miR-532-5p は KRAS および MKL2 を標的とし、肺腺癌の細胞死を誘導する）

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Introduction

We and others have previously identified TTF-1, also called NKX2-1, as a lineage survival oncogene in lung adenocarcinoma. Subsequent studies revealed double-edged sword characteristics of TTF-1 in the development of lung adenocarcinoma. TTF-1 elicits lineage-survival signaling by inducing transcriptional targets such as ROR1, while TTF-1 also inhibits tumor progression by decreasing cell motility through direct activation of genes such as MYBPH. It is thus clear that a more comprehensive picture of this still enigmatic lineage-survival oncogene needs to be elucidated. Whereas most of the previous studies on TTF-1 have focused on its regulation of protein-coding genes, little is known about regulation of microRNAs by TTF-1. In the present study, we combined in vivo patient data with in vitro data from lung adenocarcinoma cell lines, aiming at identifying miRNAs, which are transcriptionally regulated by TTF-1.

Methods

Cell lines used in this study were the immortalized human bronchial epithelial cell line BEAS-2B as well as lung adenocarcinoma cell lines NCI-H23, NCI-H1299, NCI-H441, NCI-H2009, PC-9, ACC-LC-94 and ACC-LC-319, all of which were confirmed to be absent of mycoplasma contamination. Four TTF-1-inducible cell lines were established to investigate global changes in mRNA- as well as microRNA expression upon TTF-1-induction using microarray analyses. Cells were transfected with siRNA, microRNA mimics or vector constructs according to the manufacturer's instructions.

qRT-PCR to analyze mRNA, as well as miRNA expression levels, and western blot analysis to check protein expression levels were performed according to the standard procedures. To investigate binding of TTF-1 to promoter regions, ChIP-qRT-PCR as well as dual-luciferase reporter assays were employed. Dual-luciferase reporter assays were also used to confirm binding of miR-532-5p to 3'UTR of potential target mRNAs.

The biological phenotype of miR-532-5p overexpression was evaluated by colony formation assay, a calorimetric assay to check cell proliferation, FACS analysis for subG1 cells as a measurement of apoptotic cells and western blot analysis. In addition, a xenograft assay was employed to investigate the biological effect *in vivo*.

Results

Our strategy to identify TTF-1-regulated miRNAs is described in Figure 1a. We first investigated changes in miRNA expression profile in TTF-1-inducible BEAS-2B cell line, which resulted in identification of 40 different miRNAs that showed greater than two-fold up-regulation by TTF-1. Next, we performed microarray analyses of four TTF-1-inducible cell lines to search for genes which were up- or down-regulated in at least two out of four cell lines upon TTF-1 induction; 81 genes were accordingly identified as TTF-1 module genes. By using this gene set, we then calculated TTF-1 module activity in 75 surgically resected lung adenocarcinoma tissues,

which were previously analyzed for mRNA as well as miRNA expression profiles. We next analyzed which miRNAs significantly correlated with TTF-1 module activity in these lung adenocarcinoma tissues, leading to the identification of 81 miRNAs. Finally, we combined this information with that from global miRNA expression profiling in TTF-1-induced BEAS-2B cells *in vitro*. Eventually, 11 miRNAs were identified as TTF-1-regulated miRNAs (Table 1). We selected miR-532-5p for further analysis, because of a high degree of TTF-1-mediated induction shown in a confirmatory experiment (Fig. 1b) and significant correlation with TTF-1 module activity (Fig. 1c).

MiR-532-5p was found to reside in intron 3 of the protein-coding gene CLCN5. ENCODE H3K4me3 and H3K27Ac ChIP-seq data of A549 cells strongly suggested the presence of two potential genomic regions harboring active promoters (Fig. 2a). TTF-1-knock-down significantly reduced miR-532-5p-, but not CLCN5 expression in NCI-H441 and NCI-H2009 cells (Fig. 2b). ChIP-qRT-PCR analysis showed binding of TTF-1 to the MIR532 promoter (Fig. 2c) and this binding was attenuated when TTF-1 binding site was deleted as revealed by dual luciferase assays (Fig. 2d, e). These data strongly suggest that TTF-1 regulates miR-532-5p independent of its host gene CLCN5.

We next were interested in potential target genes of miR-532-5p. Thus, we performed microarray analysis of miR-532-5p-introduced NCI-H1299 and NCI-H23 cells and identified potential target genes with the aid of TargetScan prediction algorithm. Among those target genes, KRAS attracted much of our subsequent attention. KRAS expression was reduced on mRNA- as well as protein level upon introduction of miR-532-5p into NCI-H23 and NCI-H1299 cells as shown by qRT-PCR and western blot analysis, respectively (Fig. 3a). Moreover, dual luciferase reporter assay revealed binding of miR-532-5p to the 3'UTR of KRAS (Fig. 3b), confirming KRAS as a genuine target of miR-532-5p. We next examined whether miR-532-5p introduction has an effect on lung adenocarcinoma proliferation, similar to KRAS knockdown, using three lung adenocarcinoma cell lines with mutant KRAS; NCI-H23, ACC-LC-94, and ACC-LC-319, as well as NCI-H1299 with wild-type KRAS (Fig. 3c). Whereas miR-532-5p introduction suppressed cell proliferation in all cell lines examined, knockdown of KRAS did not affect proliferation of ACC-LC-319 and NCI-H1299 cells, suggesting that miR-532-5p may also target another gene that plays a crucial role in lung adenocarcinoma development.

Among the list of potential miR-532-5p target genes, MKL2, a transcriptional co-activator of SRF, was shown to be an intriguing candidate accounting for significant growth inhibition of siKRAS-resistant ACC-LC-319 and NCI-H1299 cells. We confirmed MKL2 to be a genuine target of miR-532-5p by qRT-PCR, western blot analysis and dual luciferase assay (Fig. 4a, b). Next, we examined the effects of miR-532-5p, siKRAS, and siMKL2 treatment on colony formation and found significantly reduced numbers of colonies following miR-532-5p and siMKL2 treatment in all four cell lines (Fig. 4c). In contrast, siKRAS specifically inhibited colony formation in NCI-H23 and ACC-LC-94 cells, with a clear dependence on mutant KRAS-mediated

signaling, but not in ACC-LC-319 and NCI-H1299 cells. Consistent findings were obtained in flow cytometry measurements of sub-G1 cell populations (Fig. 4d).

Western blot analysis revealed that while miR-532-5p introduction inhibited MEK:ERK pathway similar to KRAS knockdown in NCI-H23 and ACC-LC-94 cells, this was not seen in ACC-LC-319 and NCI-H1299 cells (Fig. 5a). These results suggest that TTF-1-induced miR-532-5p plays a role as a negative regulator of at least two biologically pertinent and distinct pathways, the MAPK pathway via targeting KRAS and the MKL:SRF pathway by targeting MKL2. In order to investigate our speculation, we treated NCI-H23 and NCI-H1299 cells with two inhibitors of MKL:SRF-mediated gene transcription. Colorimetric assays revealed marked inhibition of cell proliferation in response to MKL:SRF pathway inhibition in both cell lines (Fig. 5b). Also, we observed significant induction of apoptosis by knockdown of SRF in both NCI-H23 and NCI-H1299 cells (Fig. 5c), suggesting that miR-532-5p-induced MKL2 repression elicits apoptosis, at least in part, due to consequential impairment of the transcriptional activating function of the MKL2:SRF complex. Finally, we investigated the effects of miR-532-5p introduction in a xenograft model using ACC-LC-319, NCI-H1299 and ACC-LC-94 cells and observed marked reduction of tumor weight in all cell lines analyzed (Fig. 5d)

Discussion and conclusion

By using an integrative approach that combined both *in vitro* and *in vivo* findings, we have found that TTF-1 transactivates miR-532-5p, which potently induces apoptosis in lung adenocarcinoma cells. MiR-532-5p was shown to inhibit two pathways crucial for survival of lung adenocarcinoma cells, which include the MEK-ERK axis and the MKL/SRF-mediated signaling by directly targeting KRAS and MKL2, respectively (Fig. 5e). These findings warrant future investigation to develop novel therapeutic strategies using miR-532-5p, which may ultimately prove effective for treating patients with this hard-to-cure cancer.