主論文の要約

TTF-1/NKX2-1 binds to DDB1 and confers replication stress resistance to lung adenocarcinomas

（TTF-1/NKX2-1 は DDB1 に結合し、肺腺癌に複製ストレス耐性を付与する）

名古屋大学大学院医学系研究科　細胞情報医学専攻
腫瘍病態学講座　分子腫瘍学分野
（指導：髙橋　隆　教授）
刘　卓然

Introduction

Lung cancer is the leading cause of cancer death throughout the world, with adenocarcinomas arising from peripheral lung tissue the most frequent manifestation. Activated oncogenes induce stalling and collapse of DNA replication forks, imposing replication stress (RS) in cancer cells. DDR activation triggered by genomic insults including DNA RS leads to cell death or senescence, thereby functioning as an intrinsic barrier to malignant transformation.

TTF-1/NKX2-1, is a homeodomain transcription factor necessary for peripheral lung morphogenesis. This transcription factor functions as a lineage-survival oncogene with double-edged sword-like characteristics. Nevertheless, *TTF-1* remains an enigmatic oncogene and elucidation of the full picture of TTF-1-mediated regulation in lung adenocarcinoma development is keenly awaited for better understanding of its molecular pathogenesis.

Methods

The immortalized human bronchial epithelial cell line BEAS-2B as well as lung adenocarcinoma cell lines NCI-H441, NCI-H2087, PC-9 and SK-LU1 were used in this study. Western blot co-immunoprecipitation analyses were performed according to the standard procedures. SWATH-MS proteomic analysis was carried out to search for binding partners of TTF-1. Chemo-sensitivities of various cell lines were measured using colorimetric assay. TTF-1-inducible as well as TTF-1-knockout cell lines were established to analyze the biological functions of TTF-1. Protein and ubiquitylation levels were evaluated using western blotting.

Results

We first searched for the binding proteins of TTF-1 using NCI-H441 cells. Endogenous TTF-1 and associated proteins were immuno-precipitated in lysate, then examinations were subsequently performed using a proteomic approach that employed SWATH-MS analysis (Fig. 1a). Two independent experiments with and without DNase treatment identified 151 proteins in total with quantities at least 2-fold higher in the anti-TTF-1-immunoprecipitates as compared to those identified with control IgG. GO term annotations of the protein list derived from SWATH-MS analysis showed significant enrichment of those related to DNA replication and DDR, suggesting possible involvement of TTF-1 in these processes (Fig. 1b). IP-WB analysis validated the results of SWATH-MS analysis with regard to the interactions of TTF-1 with Ku80, PARP, DDB1, DNA-PKcs, PCNA, RPA32, and WRN in NCI-H441 cells (Fig. 1c, left panel). In addition, their specific bindings with TTF-1 were also confirmed using TTF-1-inducible BEAS2B-TTF-1 cells (Fig. 1c, right panel).

BEAS2B-TTF-1 and BEAS2B-CTRL cells were then exposed to 5-fluorouracil (5-FU), hydroxyurea (HU), carboplatin, or VP-16 in the presence or absence of doxycycline (DOX). We found that induction of TTF-1 overexpression by adding DOX in BEAS2B-TTF-1 cells conferred resistance to 5-FU or HU treatment, whereas BEAS2B-TTF-1 cells in a non-induced state were sensitive to both agents in manner similar to BEAS2B-CTRL cells with and without DOX treatment (Fig. 2a). In contrast to the findings

obtained with these agents, which induce DNA RS with distinct mechanisms, overexpression of TTF-1 did not confer resistance against carboplatin or VP-16, chemotherapeutic agents known to induce DNA damage. In addition, CRISPR/Cas9-mediated genome editing was applied to cells, followed by verification by sequencing for the presence of homozygous TTF-1 knockout. We consequently observed higher sensitivity to 5-FU in NCI-H2087 cells knocked out for TTF-1 (Fig. 2b). These findings suggested that TTF-1 confers resistance to DNA RS possibly through a functional interplay among the binding partners identified in the present proteomic search.

We then explored the mechanism related to how TTF-1-overexpression confers resistance to RS. Significant reductions of CHK1 levels in BEAS2B-TTF-1 and BEAS2B-CTRL cells without DOX treatment were observed in the presence of HU (Fig. 3a), a finding consistent with a previous report. Interestingly, TTF-1-induced BEAS2B-TTF-1 cells exhibited a sustained level of CHK1 expression even after exposure to HU, which was associated with a higher level of phosphorylated CHK1 (S317). In addition, TTF-1-induced BEAS2B-TTF-1 cells also showed reduced induction of γH2AX and phosphorylated CHK2, which are known to be induced by the presence of prolonged replication stress, reflecting the presence of DSBs and consequential DDR, respectively. BEAS2B-TTF-1 as well as TTF-1-inducible PC9-T lung adenocarcinoma cells were also treated with HU at a higher dose (5 mM) for 4 hours with and without TTF-1 induction, and those results indicated that overexpression of TTF-1 alleviated the reduction of CHK1 induced by HU treatment (Fig. 3b). These findings demonstrated that TTF-1 overexpression attenuates DNA RS-induced CHK1 reduction, thus maintaining CHK1 expression and consequential occurrence of DSBs. In addition, the half-life of CHK1 was found to be significantly prolonged by TTF-1 overexpression, which consequently led to an increased level of phospho-CHK1 (S317) (Fig. 3c).

When treated with siTTF-1, NCI-H2087, SK-LU-1 and NCI-H441, representative TTF-1-positive lung adenocarcinoma cell lines, exhibited significantly decreased CHK1 levels in WB analysis (Fig. 4a), We also examined CHK1 levels in NCI-H2087 cells knocked out for TTF-1, which clearly showed that endogenously overexpressed TTF-1 is required for maintaining the level of CHK1 in cells under replication stress (Figure 4b). In addition, we examined the half-life of CHK1 protein in 5-FU-treated NCI-H2087 cells and observed a significantly shortened half-life (Figure 4c), a finding that was opposite of the result obtained with TTF-1-overexpressing BEAS2B cells. Together our findings demonstrate that TTF-1 overexpression plays a role to sustain CHK1 under RS.

CHK1 is known to be targeted by DDB1 to the Cul4A E3 ligase complex, which positively regulates CHK1 ubiquitylation and proteasome-dependent degradation. Along this line, we noted in our proteomic search that DDB1 was repeatedly identified as a potential binding partner of TTF-1, while experimental results in this study confirmed it to be a TTF-1 binding partner. While HU treatment readily decreased CHK1 expression in BEAS2B-TTF-1 cells, DDB1 knockdown maintained that expression in the presence of HU regardless of induction of TTF-1 (Fig. 5a). It is also interesting to note that induction of TTF-1 did not further enhance CHK1 expression. We also showed that CHK1 expression was maintained by co-knockdown of DDB1 and TTF-1 in NCI-H2087 cells (Fig. 5b), in contrast to reduced CHK1 expression

induced by TTF-1 knockdown alone. These findings support the notion that TTF-1 binds with DDB1 and mitigates DDB1-directed degradation of CHK1.

Various expression constructs carrying full-length (TTF-1-myc) or truncated TTF-1 (TTF-1-ΔHD-myc, TTF-1-ΔC1-myc, TTF-1-ΔC2-myc) were transiently transfected into BEAS-2B cells in order to determine which domain of TTF-1 is involved in the interaction with DDB1. IP-WB analysis consequently revealed that the homeodomain of TTF-1 is required for their interaction (Fig. 5c). Consistently, BEAS-2B cells transfected with TTF-1 lacking the homeodomain failed to maintain CHK1 expression when treated with HU (Fig. 5d).

The association between CHK1 and DDB1 was significantly enhanced in the presence of HU in BEAS2B-TTF-1 cells, in accordance with a previous report. It was also notable that TTF-1 overexpression clearly attenuated the interaction between CHK1 and DDB1 under the same conditions with persistent RS (Fig. 6a). Furthermore, immunoprecipitation of CHK1 in HA-ubiquitin-expressing BEAS2B-TTF-1 cells revealed that overexpression of TTF-1 repressed CHK1 ubiquitylation induced by HU treatment (Fig. 6b).

Discussion and conclusion

CHK1 is activated by DNA RS in an ATR-dependent manner to trigger S-phase checkpoints, then its activation suppresses inappropriate firing of late or cryptic DNA replication origins and maintains replication fork integrity. ATR-mediated phosphorylation activates CHK1, consequently promoting its ubiquitylation and proteasome-dependent degradation. Our findings demonstrate that TTF-1 interacts with DDB1 and interferes with binding between DDB1 and CHK1, which in turn attenuates ubiquitylation and subsequent degradation of CHK1 (Fig. 6c), providing novel insight into how this lineage-survival oncogene contributes to lung adenocarcinoma development by conferring tolerance to DNA RS.