主論文の要旨

Inhibition of heat shock protein 90 destabilizes receptor tyrosine kinase ROR1 in lung adenocarcinoma

（肺腺癌において heat shock protein 90 阻害は
受容体型チロシンキナーゼ ROR1 を不安定化する）

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【Introduction】

Lung cancer is the leading cause of cancer death in both male and female. Adenocarcinoma is the most common histological subtype, arising in the distal lung and accounting for ~ 40 % and more than 50 % of all lung cancer cases in the United States and in Japan, respectively.

We have previously identified receptor tyrosine kinase-like orphan receptor 1 (ROR1) as a direct transcriptional target of TTF-1/NKX2-1, a lineage-survival oncogene in lung adenocarcinoma. ROR1 sustains pro-survival signaling from multiple receptor tyrosine kinases (RTKs) including EGFR, MET, and IGF1R in kinase-dependent and independent manners, in part by maintaining the caveolae structure as a scaffold protein of cavin-1 and caveolin-1. While expression levels of ROR1 in normal tissues are attenuated after birth, ROR1 is significantly upregulated in a variety of cancers. The oncofetal expression pattern, as well as multiple roles crucial to sustain survival signaling in cancer, point to a notion that ROR1 may be an effective drug target for cancer therapies development.

In the present study, we aimed at identifying compounds which potentially target ROR1-cavin-1 interaction to inhibit the scaffold function of ROR1.

【Materials and methods】

A FluoPPI assay was conducted by co-introducing expression constructs of the ROR1 intracellular domain fused with an N-terminal fluorescent protein tag (hAG-ROR1-ICD) and cavin-1 with a C-terminal assembly helper tag (cavin-1-Ash) in HeLa cells. For high throughput screening of a natural product library, HeLa clones stably expressing hAG-ROR1-ICD and cavin-1-Ash were seeded into 384-well plates at a density of $5x10^3$ cells/well. Formation of foci was quantified by calculating the mean fluorescent intensity in foci divided by that intensity in cells.

【Results】

To identify compounds that potentially inhibit ROR1-cavin-1 interaction, we employed the cell-based FluoPPI assay, in which protein-protein interaction can be evaluated as the formation of fluorescent foci in living cells (Figure 1A). A high throughput screening of an isolated natural product library containing 2,560 compounds identified geldanamycin, an HSP90 inhibitor, as a potential inhibitor of ROR1-cavin-1 interaction (Figure 1B). Immunoprecipitation-Western blot analysis using ROR1 and cavin-1 antibodies revealed that geldanamycin decreased ROR1 protein expression, but not cavin-1 protein expression or physical interaction between ROR1 and cavin-1 (Figure 1C and D), suggesting ROR1 as a potential client of HSP90. Immunoprecipitation-Western blot analysis and knockdown using siRNA demonstrated that, among four major HSP90 paralogs, only HSP90α can bind to ROR1 and is involved in ROR1 protein expression.

As some other receptor tyrosine kinases (RTKs) including EGFR, MET, and IGF1R, have been known as HSP90 clients, we examined whether geldanamycin would reduce expression levels of these proteins in lung adenocarcinoma cell lines as well. While the sensitivity of EGFR, MET,

and IGF1R to geldanamycin was widely different among lung adenocarcinoma cell lines, ROR1 was highly sensitive to geldanamycin in all six lung adenocarcinoma cell lines tested in this study (Figure 2A). Interestingly, geldanamycin significantly decreased cell proliferation in these six cell lines, suggesting the potential of HSP90 inhibitors in ROR1-positive lung adenocarcinoma (Figure 2B). To determine whether geldanamycin can suppress activation of bypass signaling that potentially drives resistance to tyrosine kinase inhibitors, we treated gefitinib-sensitive PC-9 cells with HGF to activate MET signaling, which can compensate the inhibitory effect of gefitinib on EGFR. Treatment with HGF restored cell proliferation reduced by gefitinib, and geldanamycin negated the effect of HGF (Figure 2C), suggesting that geldanamycin overcame HGF-mediated resistance to gefitinib in PC-9 cells.

We determined molecular mechanisms for geldanamycin-mediated regulation of ROR1 protein. While treatment with cycloheximide, an inhibitor of protein biosynthesis, did not largely affect expression levels of ROR1 protein, treatment with geldanamycin combined with cycloheximide more rapidly decreased ROR1 protein than treatment with geldanamycin alone, suggesting that geldanamycin reduced the stability of ROR1 protein. We then examined whether ROR1 protein is degraded through the ubiquitin/proteasome pathway or lysosomal degradation pathway. A proteasomal inhibitor MG132, but not a lysosomal degradation inhibitor Chloroquine, partially restored geldanamycin-mediated reduction of ROR1 protein levels (Figure 3A and B). In addition, ROR1 protein was polyubiquitinated upon geldanamycin treatment (Figure 3C), suggesting that ROR1 was degraded by geldanamycin through the ubiquitin/proteasome pathway.

We next determined the region of ROR1 protein responsible for geldanamycin-mediated proteasome degradation. ROR1 wild type and a deletion mutant of the C-terminal region (ΔS/T1+P+S/T2), but not a deletion mutant of the kinase domain (△TK), were degraded by geldanamycin (Figure 4A). In addition, immunoprecipitation of ROR1 revealed that HSP90α did not bind to △TK (Figure 4B). Our findings indicate that the kinase domain of ROR1 is responsible for binding with HSP90α and geldanamycin-mediated proteasome degradation, which are consistent with a recent study that identified the amino acid sequence ELHHPNIV in the kinase domain of ROR1 as a binding motif of HSP90. Kinase activity or N-linked glycosylation was not involved in interaction of ROR1 with HSP90α or proteasome degradation.

【Discussion】

We and others have reported that cell survival of a subset of lung adenocarcinoma depends on TTF-1/NKX2-1 signaling, but TTF-1/NKX2-1 cannot be considered as a molecular target because of its crucial role for maintaining physiological functions of normal lung. In this connection, we previously found that ROR1 is a transcriptional target for TTF-1/NKX2-1 and sustains EGFR-mediated pro-survival signaling in TTF-1/NKX2-1-positive lung adenocarcinoma cell lines. Further, ROR1 was shown to act as a scaffold protein of cavin-1 and caveolin-1, two essential structural components of caveolae, sustaining caveolae formation and pro-survival

signaling through multiple additional RTKs such as MET and IGF1R. Since bypass signaling through diverse RTKs confers EGFR-TKI resistance, the scaffold function of ROR1, therefore, appears to be an attractive target for overcoming EGFR-TKI resistance due to bypass signaling. It is also of note that given its broad expression in common solid tumors and hematologic malignancies with minimal expression in healthy adult tissues, ROR1-targeted therapies using CAR-T cells, monoclonal antibodies, and small molecule inhibitors are also being developed in a variety of cancer.

EGFR, MET, and IGF1R are known clients of HSP90, and geldanamycin indeed reduced those RTK proteins in the six lung adenocarcinoma cell lines examined in the present study. Furthermore, it is interesting to note that the concentrations of geldanamycin required to decrease each RTK protein were noticeably different among the cell lines. In contrast, ROR1 protein was consistently decreased in all of the lung adenocarcinoma cell lines treated with geldanamycin at a concentration lower than that required for significant reduction of other RTK proteins, which was accompanied by significant cell growth inhibition. ROR1 itself has been shown to sustain signaling of multiple RTKs in both kinase-dependent and independent manners; thus it is difficult to determine the contributions of ROR1 or other RTKs to the inhibitory effects of geldanamycin on cell growth. Nevertheless, these findings suggest a critical role for ROR1 in lung adenocarcinoma cell growth.

In conclusion, we have shown that ROR1 is a novel client protein of HSP90 with specific binding to HSP90α isoform. Our present findings indicate that HSP90 is required to sustain the expression of ROR1 crucial for lung adenosarcoma survival, suggesting promising potential of HSP90 inhibitors in ROR1-positive lung adenocarcinoma.