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学位論文題目	Defect in chromosome alignment is a novel mechanism underlying ALK inhibitor-induced suppression of cell proliferation （染色体整列異常はALK阻害により誘導される細胞増殖阻害の新規メカニズムである）
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論文内容の要旨

Introduction

Receptor-type tyrosine kinases are the most prevalent therapeutic targets in anti-cancer drug development as they are aberrantly activated in a wide range of cancers. The receptor-type tyrosine kinase, anaplastic lymphoma kinase (ALK) is a member of the insulin receptor superfamily and is especially highly expressed in neonates and in the developing central and peripheral nervous system. Genetic alterations of ALK, such as gene amplification, fusion, and mutation, have been found in different cancers and ALK inhibitors have been approved for the treatment of non-small-cell lung carcinoma. Although these inhibitors suppress proliferation and induce apoptosis of cancer cells via the inhibition of the MEK/ERK and the PI3K/AKT pathways, resistance to these treatments is generally developed. Understanding the precise mechanism for suppressing the proliferation will enable an improved polytherapy to overcome the resistance. Therefore, novel mechanisms that suppress cell proliferation were explored. In this study, the effects of ALK inhibition by various inhibitors as well as ALK-targeted siRNAs (siALKs) on cell division were evaluated and the mechanism of action in neuroblastoma and lung cancer cells was investigated.

Chapter 1: ALK inhibition by inhibitors and knockdown causes delay in the early M phase

The ALK inhibitors, crizotinib, ceritinib, and TAE684 showed a concentration-dependent reduction in the proliferation of the neuroblastoma SH-SY5Y cells. To examine the effect on M-phase progression, cells were treated with inhibitors at around IC_{50} concentration for 1 h after release from arrest at the G2/M border caused by the CDK1 inhibitor RO-3306. The M phase is a process in which duplicated chromosomes are divided equally into two progeny cells and comprises 5 subphases: prophase (P), prometaphase (PM), metaphase (M), anaphase (A), and telophase (T), and followed by cytokinesis (Cyto) that splits cytoplasm. Based on microtubule and chromosome morphologies, the cells were classified into four groups: P/PM, M, A/T, and Cyto. Most control cells progressed to cytokinesis in an hour after release from the arrest at the G2/M border whereas the M-phase progression of ALK inhibitors-treated cells was largely delayed in P/PM. Interestingly, a significant percentage of

cells showed the misalignment of chromosomes upon inhibitor treatment, indicating the involvement of ALK in chromosome alignment. The western blotting analysis revealed that the autophosphorylation of ALK was almost completely abolished by inhibitors, indicating an inhibition of the kinase activity. Three different siALKs were used to knockdown ALK expression and caused delay in P/PM/M, excluding the off-target effects of inhibitors. Furthermore, the ALK knockdown-induced delay was suppressed via the re-expression of siALK-resistant ALK. Taken together, these results suggest that ALK is involved in the chromosome alignment in M phase and that ALK inhibition-induced delay in the early M phase may partly contribute to the suppression of cell proliferation.

Chapter 2: Delays in the early M phase and anaphase onset are accompanied by SAC activation

Proper chromosome segregation is achieved through several critical processes, such as the duplication of sister chromatids, spindle formation, kinetochore-microtubule attachment, and alignment of chromosomes at the mitotic equator. To find out the mechanism of ALK knockdown-induced delay in the early M phase, inhibitor- or siALK-treated cells were synchronized with RO-3306 and subsequently observed every 3 min for 5 h under time-lapse imaging. Hoechst 33342 was used to stain DNA for monitoring chromosome movement. The M-phase cells were classified into three stages based on chromosome morphology: P/PM, M, and A/T. In the control cells, the average duration of P/PM and M were 31 and 26 min, respectively. In sharp contrast, crizotinib treatment extended the duration of P/PM and M to 74 and 83 min, respectively. Likewise, it took siALK-treated cells 63 and 40 min to complete P/PM and M, respectively. 16% of siALK-treated cells failed to complete the M phase within 5 h. These results suggest that ALK downregulation hampers the chromosome alignment and thereby delays the onset of anaphase. Moreover, abnormal M-phase characteristics, such as spindle misorientation and chromosome misalignment were observed. Chromosome misalignment can be caused bt a defect in spindle formation and can activate the spindle assembly checkpoint (SAC). To confirm whether the delay in the early M phase was accompanied by SAC activation, the SAC components, the Mps1 kinase and the MAD2, were inhibited by an inhibitor and siRNA, respectively, and then cells were treated with crizotinib. In both conditions, crizotinib-induced delay in the anaphase onset was not observed and most of the cells progressed to cytokinesis within 1 h after release from the G2/M arrest. These findings indicate that SAC is responsible for the delay in the anaphase onset in ALK-inhibited cells.

Chapter 3: Inhibition of the EML4-ALK fusion protein causes delay in the M phase

The EML4-ALK fusion protein is one of the major oncoprotein identified in non-small-cell lung cancer. The non-small-cell lung cancer H2228 cells express the EML4-ALK fusion and have been used in preclinical cancer research. These cells are relatively resistant to crizotinib compared to EML4-ALK-expressing H3122 cells. In general, EML4-ALK triggers both the PI3K-AKT and MEK/ERK pathway and sustains this survival and proliferative signaling. However, the MEK/ERK pathway is activated independently of EML4-ALK in H2228 cells. Therefore, as a model of drug-resistant cancer cells, H2228 cells were used to examine whether ALK inhibitor affected the M-phase progression.

H2228 cells were treated with TAE684 at around IC_{50} and observed under time-lapse imaging for 12 h. The average duration of P/PM in control cells was 30 min, whereas this was prolonged to 130 min in TAE684-treated cells. Furthermore, the M phase was completed by only 40% of the cells, while the remaining cells either continued the M phase during the observation, prematurely exited, or died in the M phase. Western blotting analysis showed that the phosphorylation of EML4-ALK was suppressed. These results suggest that

inhibition of EML4-ALK causes the defect of the M-phase that may partly contribute to the suppression of the H2228 cell proliferation.

Conclusion:

The inhibition of ALK or EML4-ALK disrupts cell division through delays in the early M phase and anaphase onset. The latter is caused by SAC activation to which defects in chromosome alignment and spindle orientation lead. These results conclude that ALK is a novel regulator of the M phase and the delay in the M-phase progression is a novel mechanism that underlies the ALK inhibitor-caused suppression of cell proliferation. Understanding the mechanism of action of ALK inhibitors will undoubtedly support to the develop new therapeutic approaches.

審 査 の 結 果 の 要 旨

≪緒言≫

ALK はインスリン受容体スーパーファミリーに属する受容体型チロシンキナーゼであり、新生児や発生段階にある中枢及び末梢神経で発現している。種々のがん細胞において、ALK 遺伝子の変化、遺伝子増幅、他の遺伝子との融合、活性化型変異などが見出されて以来、治療標的として着目され、crizotinib などが開発され臨床で使用されてきた。しかしながら他の薬剤同様、耐性の獲得が問題となっている。細胞分裂進行に影響する低分子化合物のスクリーニングにおいて、ALK 阻害剤 crizotinib が細胞分裂に影響することを見出したことから、本研究では、ALK 阻害剤による細胞増殖抑制効果の新規メカニズムとして細胞分裂への影響を解析した。

≪審査結果の要旨≫

第１章 ALK 阻害による細胞分裂の遅延

第１世代の crizotinib、第２世代の ceritinib、特異性の高い TAE684 を用い細胞分裂への影響を調べた。神経芽細胞腫 SH-SY5Y 細胞の細胞周期を同調し、IC50 付近の濃度を用いて細胞分裂進行への影響を調べると、これらの ALK 阻害剤により自己リン酸化が阻害され、細胞分裂進行が遅延した。また、多くの細胞で染色体の整列異常が観察され、染色体整列への ALK の関与が示唆された。阻害剤のオフターゲット効果を排除するため、３種類の siRNA を用いて ALK をノックダウンすると、阻害剤と同様な結果が得られた。さらに、ALK をノックダウンした細胞で、siRNA に非感受性の ALK を再発現させると、siRNA により観察された細胞分裂遅延が抑制された。すなわち、ALK は細胞分裂に関与し、ALK の阻害は細胞分裂の遅延を引き起こすこと、さらにこの効果が ALK 阻害による細胞増殖阻害に部分的に寄与することが示唆された。

第２章 細胞分裂遅延に対する紡錘体チェックポイントの関与

細胞分裂遅延の機構を調べるため、阻害剤あるいはノックダウンにより ALK を阻害し、細胞周期を同調してタイムラプスイメージングを行なった。その結果、前中期、中期の延長と、染色体整列異常、紡錘体軸の配向性異常が観察された。染色体整列異常は紡錘体チェックポイントを活性化するため、チェックポイントキナーゼである Mps1 の阻害、さらには、チェックポイント関連分子 Mad2 の

ノックダウンを行うと、ALK 阻害により引き起こされる細胞分裂遅延が解除された。これらの結果から、ALK 阻害による中期の延長、すなわち後期開始の遅延に紡錘体チェックポイントの関与が示唆された。

第３章 EML4-ALK 融合タンパク質阻害による細胞分裂遅延

EML4-ALK 融合タンパク質は非小細胞肺癌の原因遺伝子の一つである。このタンパク質を発現する H2228 細胞は同じく EML4-ALK を発現する H3122 細胞より、crizotinib に対し耐性である。そこで、ALK 阻害剤耐性のモデルとして H2228 細胞に対する効果を調べると、H2228 細胞においても ALK 阻害による細胞分裂遅延が観察された。よって、EML4-ALK の阻害も細胞分裂異常を引き起こすこと、さらに、それが細胞増殖抑制に寄与することが示唆された。

≪審査の結論≫

本研究により、ALK 及び ALK 融合タンパク質の阻害は、染色体整列異常と紡錘体軸の配向性異常を引き起こし、その結果、紡錘体チェックポイントを活性化して後期開始を遅延させることを明らかにした。これらの細胞分裂への効果は ALK 阻害による細胞増殖抑制効果の新規機構であり、ALK 阻害剤に対する耐性克服を目的とした抗がん剤の併用療法のための重要な情報を与えると考えられる。

学位論文とその基礎となる報文の内容を審査した結果、本論文は博士（薬学）の学位論文としての価値を有するものと判断する。