学 位 論 文 の 要 旨

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学　位　論　文　名　Protective Role of Cytoplasmic $p21^{Cip1/Waf1}$ in Apoptosis of CDK4/6 Inhibitor-induced Senescent Breast Cancer Cells

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論 文 内 容 の 要 旨

INTRODUCTION

Several molecular-targeting drugs have been developed for breast cancer. These include CDK 4/6 inhibitors, and these drugs can induce growth arrest and increase expression of the cell-cycle inhibitors, including $p16^{Ink4a}$ and $p21^{Cip1/Waf1}$. This state is called senescence and induced via DNA damage. Senescent cells secrete several growth factors and inflammatory cytokines, known as the senescence-associated secretory phenotype (SASP). However, as well as activating and promoting accumulation of immune cells and tumor clearance, SASP promotes cancer recurrence and metastasis.

Anti-apoptotic Bcl-2 family proteins protect cancer cells from therapy-induced apoptosis. To overcome this resistance, several inhibitors targeting these Bcl-2 family proteins have been developed. ABT-263 (navitoclax) is a small-molecule inhibitor targeting Bcl-2, Bcl-xL, and Bcl-w. Alternatively, several drugs, called as senolytics, have been reported to preferentially target senescent cells for death, and ABT-263 is a representative.

We recently reported that a CDK4/6 inhibitor abemaciclib can induce senescence in human breast cancer cells. Therefore, in this study, we examined the antitumor effects of abemaciclib and ABT-263 using two human breast cancer cell lines and investigated the underlying mechanisms.

MATERIALS AND METHODS

Two human breast cancer cell lines (MDA-MB-231 and MCF-7) were used. Cell viability

was measured by Cell Counting Kit-8. Cell death was assessed using the annexin V-FITC Apoptosis Detection Kit and PI. For immunoblot assay, the following antibodies were used: anti-$p21^{Cip1/Waf1}$, anti-$p16^{Ink4a}$, anti-γH2AX(Ser^{139}), anti-Bcl-2, anti-Bcl-xL, anti-Mcl-1, anti-survivin, anti-cFLIP, anti-TATA- binding protein, anti-PARP, anti-caspase 3, anti-c-Myc, anti-GAPDH, and anti-β-actin. Nuclear and cytoplasmic proteins were prepared using the LysoPure™ Nuclear and Cytoplasmic Extraction Kit. Protein bands were visualized using an Amersham ImageQuant™ 800 Biomolecular Imager. To examine confocal images, cells were stained with anti-p21 rabbit IgG and anti-caspase-3 mouse IgG, followed by Alexa 488-conjugated anti-rabbit antibody and Cy5-conjugated anti-mouse IgG. Observation was done by confocal laser scanning microscopy FV1000-D. siRNA transfection was performed using Lipofectamine RNAiMAX. In *in vivo* xenograft model, female BALB nude mice were injected with MDA-MB-231 cells and Matrigel into the mammary pad. When the tumor diameter was approximately 5–6 mm, abemaciclib (50 mg/kg) was orally administered on day 0 - 7 after grouping. On days 2, 4, and 6 after grouping, breast cancer-bearing mice were administered intraperitoneally ABT-737 (50 mg/kg). All experiments with animals in this study were approved by the Animal Care and Use Committee of Shimane University (IZ3-74). To utilize clinical database, the Kaplan–Meier plotter was used for univariate analysis of survival time according to CDKN1A gene expression in breast cancer. TRGAted was used to analyze survival according to tumor p21 protein level in patients with breast invasive carcinoma. Student's *t*-test (two groups) and analysis of variance (ANOVA) with the Tukey–Kramer test (more than two groups) were used. P value < 0.05 was judged statistically significant.

RESULTS AND DISCUSSION

Abemaciclib and ABT-263 in combination additively decreased the viability of MDA-MB-231 cells, but not MCF-7 cells. ABT-263 alone increased the proportions of annexin V^+ apoptotic MDA-MB-231 cells, whereas the combination drastically increased the them. In contrast, their combination increased the proportions of annexin V^+ MCF-7 cells, albeit only slightly. The abemaciclib and ABT-263 combination induced cleavage of caspase-3 and PARP, as well as the induction of γH2AX expression, in MDA-MB-231 cells.

Given that abemaciclib and ABT-263 are orally administered, we used ABT-737 because its specificity is identical to ABT-263 but it can be administered systemically. As a result, the abemaciclib and ABT-737 combination suppressed the tumor volume of MDA-MB-231 in nude mice on days 7, 10, and 14.

The abemaciclib and ABT-263 combination decreased cytoplasmic p21 expression in MDA-MB-231 cells but increased it in MCF-7 cells. However, combination treatment decreased and increased cytoplasmic p21 expression in MDA-MB-231 and MCF-7 cells, respectively,

compared with the untreated group. Confocal imaging revealed that p21 and caspase-3 were colocalized in the cytoplasm of MDA-MB-231 cells, and p21-overexpressing MDA-MB-231 cells exhibited increased resistance to apoptosis. On the other hand, siRNA-mediated knockdown of p21 or pharmacological inhibition of p21 increased the sensitivity of MCF-7 cells to agents or TRAIL.

The clinical database was used to elucidate roles of p21 in breast cancer patients. Patients were split into low or high expression groups using the best cutoff. Untreated breast cancer patients with $p21^{high}$ showed a poorer prognosis compared to those with $p21^{low}$. Interestingly, chemotherapy-administered patients with $p21^{high}$ showed a poor prognosis but endocrine therapy-administered patients with $p21^{high}$ showed a better prognosis.

Given that p16 and p21 play crucial roles in senescence and that both cell lines used in this study lack p16, we focused on p21 in the death of treated breast cancer cells because p21 exerts multiple activities in cancer cells. Abemaciclib monotherapy increased p21 expression in both cell lines, a hallmark of senescence, whereas its combination with ABT-263 decreased and increased p21 expression in MDA-MB-231 cells and MCF-7 cells, respectively. p21 is a target of p53, and MDA-MB-231 and MCF-7 cells carry mutant and wild-type p53, respectively. In addition, CDK4/6, a target of abemaciclib, is a downstream molecule of p21. These findings raised the question of why CDK4/6 inhibitors increase the upstream molecule p21. Given that c-Myc represses p21 expression via binding to the *p21* promoter, and that cellular senescence has been reported to be responsible for tumor regression upon c-Myc inactivation, we examined the expression of c-Myc and found that abemaciclib treatment decreased their expression of c-Myc. Presumably, abemaciclib suppressed c-Myc, resulting in an increase in the p21 level.

We determined whether the p21 expression on the prognosis of breast cancer patients by utilizing clinical database and found that breast cancer patients with $p21^{high}$ showed a poorer prognosis than those with $p21^{low}$. As a result, endocrine therapy-administered patients with $p21^{high}$ showed a better prognosis but chemotherapy-administered patients with $p21^{high}$ showed a poor prognosis. However, the endocrine-treated/chemotherapy-naive group might represent patients with estrogen receptor $(ER)^{+}$ luminal breast cancer, while the endocrine-naïve/ chemotherapy-treated group might be patients with ER^{-} or triple-negative breast cancer (TNBC). The roles of p21 seems to be different between ER^{-} and ER^{+} breast cancer patients.

CONCLUSION

In conclusion, cytoplasmic p21, which was increased in CDK4/6 inhibitor-induced senescent cancer cells, can protect human breast cancer cells from therapy-induced apoptosis. These lines of evidence suggest that increased p21 in therapy-induced senescent cancer cells has potential as a target for preventing tumor invasion and metastasis after anticancer therapy.