UBL3 overexpression enhances EV-mediated Achilles protein secretion in conditioned media of MDA-MB-231 cells

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論文題目

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(UBL3 の過剰発現は、MDA-MB-231 細胞の条件培地における EV を介したアキレスタンパク質の分泌を促進する)

論文の内容の要旨

[Introduction]

Cancer cells communicate within the tumor microenvironment through extracellular vesicles (EVs), which transport biomolecules to facilitate cancer progression. Ubiquitin-like 3 (UBL3) is known to facilitate protein sorting into small EVs. However, its role in EV-mediated protein secretion remains unexplored. This study investigates the role of UBL3 overexpression on EV-mediated Achilles protein secretion in MDA-MB-231 (MM) cells. Achilles is a fluorescent reporter and a faster maturing yellow fluorescent protein than another yellow fluorescent protein (Venus). A dual-reporter system integrating Akaluc and Achilles, tagged with Ubiquitin and connected by a self-cleaving P2A linker, was utilized to study this effect.

[Materials and Methods]

All experimental protocols and animal procedures were followed the guidelines set by the Ethics Committee of Hamamatsu University School of Medicine (Hamamatsu, Japan) (ethical approval number: 2020062). MM cells stably expressing Ubiquitin-Akaluc-P2A-Achilles (Ubi-Aka/Achi) were generated, and the expression of Ubiquitin-Akaluc (Ubi-Aka) and Achilles in conditioned media (CM) was detected by fluorescent and bioluminescent assay. EVs were derived from CM by ultracentrifugation and characterized by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). EV-mediated Ubi-Aka and Achilles protein secretion in CM of MM-Ubi-Aka/Achi was observed by EV uptake luciferase assay and fluorescence imaging (FLI). The effect of UBL3 overexpression was investigated by fluorescent and bioluminescent assay. A xenograft mouse model was generated by inoculating MM-Ubi-Aka/Achi cells orthotopically in severe combined immunodeficiency disease (SCID) mice. The expression of Ubi-Aka and Achilles was found *in vivo* by bioluminescence imaging and FLI, respectively.

[Results]

MM cells stably expressing Ubi-Aka/Achi were generated. It is known that most Ubiquitin-bound proteins usually undergo a proteasome pathway, while most of UBL3-bound proteins are sorted into EVs. In our study, both the bioluminescence of

Ubi-Aka and the fluorescence of Achilles secretion were observed. EVs were derived from CM of them. NTA and TEM results indicated that the size and shape of particles were allowable to be identified as EVs. Recipient cells showed significantly higher bioluminescence when added with EVs from MM-Ubi-Aka/Achi cells, indicating EV-mediated secretion of Ubi-Aka. The co-localization of Achilles and CD63 validated EV-mediated secretion of Achilles in the CM of MM-Ubi-Aka/Achi cells, and the fluorescence signals were highly specific for EVs.The intensity of Ubi-Aka was thirty times lower, while the Achilles was four times lower than the intensity of corresponding cells.This indicates that the Ubi-Aka signal is 7.5 times lower than the Achilles. They were also detected within EVs using an EV uptake luciferase assay and FLI. To investigate the effect of the UBL3 overexpression in CM, Ubi-Aka/Achi was transiently transfected into MM-UBL3-KO, MM, and MM-Flag-UBL3 cells. We found that the relative fluorescence expression of Achilles in CM of MM-UBL3-KO, MM, and MM-Flag-UBL3 cells were 30%, 28%, and 45%, respectively, indicating that EV-mediated secretion of Achilles proteins is enhanced by UBL3 overexpression. These findings demonstrated that UBL3 overexpression enhances EV-mediated Achilles protein secretion in CM of MM cells. As the Akaluc system is optimal for deep-tissue imaging, the next Ubi-Aka/Achi expression in MM-Ubi-Aka/Achi cells was observed *in vivo.*

[Discussion]

This study found that UBL3 overexpression enhances EV-mediated Achilles protein secretion in MM cells, investigated via a dual-reporter system integrating Akaluc and Achilles tagged with Ubiquitin. Despite the conventionally undetectable bioluminescence of Akaluc in CM from CD63-Akaluc expressing PC3 cells and serum samples from Akaluc-labeled HEK-293T injected mice without ATP and $MgSO_4$ supplementation, our study detected Ubi-Aka bioluminescence in CM of stably expressing MM cells. This is due to the delayed sorting into the ubiquitin-proteasome pathway. EVs were isolated using ultracentrifugation and characterized by NTA and TEM, providing insights into their size, concentration, and morphology. The concentration discrepancy with previous studies might result from sample origin variations, NTA settings, or PBS dilution. The bioluminescence and fluorescence of Ubi-Aka within EVs were confirmed by luciferase assay and FLI, respectively. The dual-reporter system showed UBL3 overexpression enhancing EV-mediated Achilles secretion, suggesting a UBL3-EVs pathway. This method is antibody-free, reducing detection costs. Ubi-Aka was undetected in CM of MM-Flag-UBL3, MM-UBL3-KO, and transiently expressing Ubi-Aka/Achi cells, likely due to lower expression levels. The Akaluc system proved reliable for deep tissue imaging and tracking tumor

progression *in vivo*, presenting new opportunities to study tumor progression and treatment efficacy.

[Conclusion]

These findings demonstrated that UBL3 overexpression enhances EV-mediated Achilles protein secretion in CM of MM cells. Targeting UBL3 could lead to novel therapies for cancer metastasis and neurodegenerative diseases by reducing the secretion of pro-metastatic and neurotoxic proteins, thereby inhibiting disease progression.