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学位論文題目：Molecular Mechanisms of Anti-proliferative Effect of Lectin ZG16p on Colorectal Cancer Cells

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[Background]

In Japan, the number of deaths due to colorectal cancer has been increasing and more than 50,000 patients died in 2016. Recently, it is reported that the expression of ZG16p (zymogen granule protein 16) is downregulated in colorectal tumor tissue compared with normal tissue and low expression of ZG16p is related with shorter survival of colorectal cancer patients. However, the implication of downregulation of ZG16p in colorectal cancer remains unclear. ZG16p is a soluble 16 kDa protein and is highly expressed in human pancreas, liver and intestine. ZG16p has a β-prism fold structure similar to Jacalin-related lectins (JRLs) and binds to mannose via GG and GXXXD loops which are conserved among JRLs. ZG16p also interacts with negative-charged heparin/heparan sulfate via basic amino acids. In this study, to elucidate whether or not ZG16p is involved in the anti-cancer mechanism in the intestinal epithelia, the effect of ZG16p on the proliferation of colorectal cancer cells and patient-derived colorectal tumor organoids was investigated.

[Experiments and Results]

Human colorectal cancer cell line Caco-2 was transfected with ZG16p vector and growth assay was performed. The growth of Caco-2 cells overexpressing ZG16p was slower than mock cells. Cell proliferation of four colorectal cancer cell lines, Caco-2, LS174T, HCT116 and HCT15, was inhibited by incubating with a recombinant ZG16p. Caco-2 cells was the most sensitive against ZG16p and the sensitivity to ZG16p was the lowest in HCT15 cells. Furthermore, the proliferation assay using patient-derived colorectal tumor organoids was performed. The growth of an organoid line of which EGF is necessary for the growth was inhibited by ZG16p. ZG16p did not inhibit the proliferation of an organoid line which has *BRAF* mutation and is able to growth without any niche factors. Therefore, it is suggested that ZG16p suppresses the colorectal cancer cell proliferation by inhibiting RAS/MAPK signaling pathway.

Caco-2 cell growth assay using two ZG16p mutants, ZG16p-D151A lacking mannose-binding activity and ZG16p-M5 (K36A, R37A, R53A, R55A and R79A) lacking heparin-binding activity, revealed that both mutants did not inhibit cell proliferation. Immunofluorescent cell staining elucidated that ZG16p and ZG16p-D151A bound to Caco-2 cell surface, whereas ZG16p-M5 failed to bind. These results suggest that ZG16p binds to Caco-2 cell surface via heparin-binding site and that mannose-binding site, Asp151, plays a key role to inhibit cell proliferation.

Binding of ZG16p to four colorectal cancer cell lines was assessed and ZG16p strongly bound to Caco-2 and HCT15 cells. Binding of ZG16p to Caco-2 and HCT15 cells was decreased by inhibiting the formation of the sulfate donor using sodium chlorate, suggesting that ZG16p interacts with the cell surface via sulfate

groups. Although CEA (carcinoembryonic antigen), which is highly expressed in colorectal cancer and is used as a tumor marker, is abundantly localized on Caco-2 and LS174T cell surfaces, CEA and a ligand of ZG16p were not co-localized on the cell surfaces. Therefore, ZG16p does not bind to a GPI (glycosylphosphatidylinositol)-anchored glycoprotein including three mannoses.

The mechanism underlying cell growth suppression by ZG16p was investigated by using Caco-2 cells. Apoptotic cells were not detected in Caco-2 cells incubated with ZG16p by Annexin-V staining. There was no difference of senescence associated β-galactosidase activity between cells treated with or without ZG16p. The number of Ki67-positive cells did not differ between the control and ZG16p treated cells, indicating that ZG16p does not induce G0-phase transition. EdU incorporation assay showed the remarkable reduction of S-phase cell ratio in the cells incubated with ZG16p. PCR array analysis was performed to elucidate the change of cell cycle-related gene expression by culturing with ZG16p. It revealed that the expression of *WEE1*, *CDK6*, *TP53*, *RAD1*, *CCND1* and *ATR* was upregulated and that of *STMN1*, *MCM4* and *CDKN1A* was downregulated. Although the expression of tumor suppression gene *TP53* was upregulated in ZG16p treated cells, *TP53* is mutated in Caco-2 cells. In addition, the results that the upregulation of *CCND1* and *CDK6* and the downregulation of *CDKN1A* suggest that G1/S transition might be activated in Caco-2 cells treated with ZG16p. Moreover, the downregulation of *STMN1* and *MCM4* and the upregulation of *WEE1*, *RAD1* and *ATR* would represent cell cycle arrest during S or G2/M phase by ZG16p.

[Conclusion]

In this study, it is elucidated that ZG16p suppresses the proliferation of colorectal cancer cell lines and patient-derived colorectal tumor organoids. The heparin-binding site of ZG16p binds to the colorectal cancer cell surface via sulfate groups and the mannose-binding site is essential to inhibit cell proliferation. It is also suggested that ZG16p inhibits RAS/MAPK signaling pathway, inducing the growth suppression. These results suggest a novel pathway mediated by cell-surface carbohydrate in the growth regulation of colorectal cancer cells.