主論文の要旨

Bioinformatics and Functional Analyses Implicate Potential Roles for EOGT and *L*-fringe in Pancreatic Cancers

（バイオインフォマティクスと機能解析により示唆された
膵臓癌におけるEOGTと*L*-fringeの潜在的役割）

名古屋大学大学院医学系研究科　総合医学専攻
生物化学講座　分子細胞化学分野
（指導：岡島 徹也　教授）
Rashu Barua

【Abstract】

Notch signaling receptors, ligands, and their downstream target genes are dysregulated in pancreatic ductal adenocarcinoma (PDAC), suggesting a role of Notch signaling in pancreatic tumor development and progression. However, dysregulation of Notch signaling by post-translational modification of Notch receptors remains poorly understood. Here, we analyzed the Notch-modifying glycosyltransferase involved in the regulation of the ligand-dependent Notch signaling pathway. Bioinformatic analysis revealed that the expression of epidermal growth factor (EGF) domain-specific O-linked N-acetylglucosamine (*EOGT*) and Lunatic fringe (*LFNG*) positively correlates with a subset of Notch signaling genes in PDAC. The lack of *EOGT* or *LFNG* expression inhibited the proliferation and migration of Panc-1 cells, as observed by the inhibition of Notch activation. *EOGT* expression is significantly increased in the basal subtype, and low expression of both *EOGT* and *LFNG* predicts better overall survival in PDAC patients. These results imply potential roles for EOGT- and LFNG-dependent Notch signaling in PDAC.

【Methods and Materials】

EOGT and *LFNG* expression in pancreatic ductal adenocarcinoma (PDAC) were analyzed by using GEPIA2 database. The correlation between glycosyltransferase genes and Notch target genes was determined based on TCGA datasets.

Endogenous expression levels of EOGT in human PDAC cell lines were compared to a normal pancreatic ductal cell line (H6C7) by immunoblotting. Further, EOGT expression was verified by Immunostaining.

Cell growth inhibition by DAPT in Panc-1 cell line was determined by IncuCyte ZOOM Live-Cell Analysis System.

CRISPR/Cas9- mediated lentiviral gene editing technique was used to knockout *EOGT* in Panc-1 cell line. Evaluation of CRISPR/Cas9-mediated genome editing was confirmed by T7 endonuclease assay and immunoblotting.

Cell proliferation assay was performed in both wild type Panc-1 and *EOGT* lack Panc-1 cell line using IncuCyte ZOOM Live-Cell Analysis System.

Cell migration (Wound healing) was analyzed by using IncuCyte ZOOM Live-Cell Analysis system in in both wild type Panc-1 and *EOGT* knockout Panc-1 cell lines.

Knockout of *LFNG* was performed by CRISPR/Cas9-mediated lentivirus transduction. Genome editing was confirmed by T7 endonuclease assay and sequencing.

Cell proliferation in Panc-1 cells lacking *LFNG* were analyzed by IncuCyte Zoom system.

Wound healing migration assay to compare the motility of wild-type and LFNG-Ko Panc-1 cells was analyzed by IncuCyte Zoom system.

Kaplan-Meier survival curve for overall survival in pancreatic ductal adenocarcinoma patients was analyzed by EZR software (version 1.52), which is a graphical user interface for

R software (version 4.0.2).

【Results】

Among Notch target genes, *HES1* and *HEY1* expressions were higher in both basal and classical subtypes of PDAC. In contrast, the expression of *EOGT* expression is significantly increased in basal subtype of PDAC, which represent a more aggressive phenotype. *LFNG* expression was not significantly altered (Figure 1A). However, these data did not exclude the possibility of multiple, rather than a single, glycosyltransferases contributing to dysregulated Notch signaling in PDAC. Correlation analysis between glycosyltransferase and upregulated Notch target genes revealed a significant positive correlation between *EOGT* and *HEY1* ($r = 0.47$, $p = 4.2x\ 10^{-11}$). In contrast, a positive correlation between *LFNG* and *HES1* ($r = 0.48$, $p = 8.4 \times 10^{-12}$) was found (Figure 1B).

We found that four PDAC cell lines (Panc-1, BxPC3, Panc03.27, and CAPAN-2) out of ten showed higher EOGT expression compared to H6C7 cells (Figure 2A). We also verified the expression of EOGT by Immunostaining in the four PDAC cell lines (Figure 2B). Based on the immunoblotting and Immunostaining assays, we selected Panc-1, which showed prominent EOGT expression for functional analysis.

We performed gene editing with the CRISPR/Cas9-mediated lentivirus technique in Panc-1 cell. After lentiviral transduction and Blasticidin S selection, the lack of EOGT was confirmed by the anti-EOGT (AER61) antibody (Figure 3A & 3B).

To investigate whether the decreased Notch activity inhibits Panc-1 cell proliferation, Panc-1 cells were treated with DAPT, a γ-secretase inhibitor that blocks Notch signaling. The inhibitory effect of DAPT on the proliferation of Panc-1 cells was observed in a time-dependent manner (Figure 4). This result suggested that decreased Notch activity can substantially reduce the cell proliferation of Panc-1.

To examine the effect of knockout of *EOGT* in the Panc-1 cells, we observed the proliferation between wild-type and *EOGT*-KO cells at an interval of 6h over 66h. *EOGT*-KO cells exhibited slow growth relative to wild-type parental control cells. This result showed that *EOGT* promotes cell proliferation in Panc-1 cells (Figure 5A).

Analysis of the relative wound healing density revealed that *EOGT*-KO Panc-1 showed significant decreases in re-capturing the wound portion compared to wild-type control cells (**Figure 5B**). Compared to the control cells, *EOGT*-KO clones showed a slower migration rate, presenting a depletion of wound density highest 20%, within 18h. The differences in the migration rate among the KO clones appear to be due to the cancer cells' clonal variation.

To compare the effect of EOGT and LFNG in the growth and motility of Panc-1 cells, we generated *LFNG*-KO cells by CRISPR/Cas9-mediated lentivirus transduction. The cutting efficacy was analyzed by T7 endonuclease assay. Then, by limiting dilution, single-cell clones were isolated. GFP-positive clones were selected for sequencing to confirm LFNG knockout

in Panc-1 cells (Figure 6).

To evaluate the roles of *LFNG* in cell proliferation, Panc-1 cells lacking *LFNG* were analyzed. The result showed that the cell proliferation of *LFNG*-KO cells was lower than that of parental control cells (Figure 7A). These data suggested that *LFNG* promotes cell proliferation of Panc-1 cells. (Figure 7B).

Kaplan-Meier survival curves revealed no association between *EOGT* expression and overall survival in PDAC (Figure 8A). Additionally, no association was observed between *LFNG* expression and overall survival (Figure 8B). In contrast, lower of both *EOGT* and *LFNG* is associated with a better prognosis of PDAC (p= 0.0195) (Figure 8C).

【Discussion】

Bioinformatic analysis data suggested that the contribution of *EOGT* and *LFNG* to Notch signaling is qualitatively different, possibly through the differential impact on multiple Notch-ligand pairs, which leads to the transactivation of distinct sets of Notch signaling target genes, including *HES1* and *HEY1*. Further in-depth bioinformatics analyses included Biclustering methods, will help elucidate the pathological relevance of the observed correlations in tumor progression.

We showed the roles of *EOGT* in cell proliferation and migration assay in cancer cells. In our study, loss of *EOGT* or *LFNG* resulted in a similar cellular phenotype, suggesting that both genes cooperate in the tumor properties of PDAC cells. Furthermore, the combination of lower *LFNG* and *EOGT* expression in PDAC serves as an excellent prognostic marker, implying that both Notch modifiers modulates disease progression. Given that *EOGT* and *LFNG* modulate ligand-induced Notch signaling, these results suggested that changes in the expression of these glycosyltransferases result in altered Notch activity, as indicated by the correlation between *LFNG* and *HES1*, or *EOGT* and *HEY1* expression in the PDAC database. Therefore, dysregulated Notch signaling mediated by changes in the expression of *EOGT* and *LFNG* would affect the prognosis of PDAC. Although this study implicates potential roles for *EOGT*- and *LFNG*-dependent Notch signaling in PDAC, further studies will be necessary to clarify the roles of Notch-modifying enzymes in dysregulated Notch activity and the development and progression of PDAC at the molecular level.

【Conclusion】

EOGT- and *LFNG*- dependent Notch signaling plays critical roles in PDAC. Both *EOGT* and *LFNG* impact cell proliferation and migration in cancer cells. The dysregulated Notch signaling due to higher expression of EOGT or LFNG leads to poor prognosis.