Profound Regulation of EGFR Activation and Degradation Through O-glycosylation — Lessons learned from the study of colorectal cancer cells

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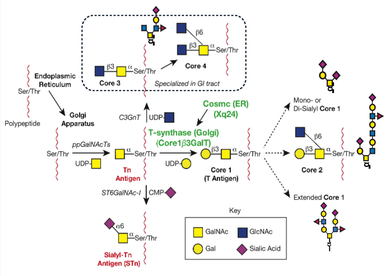
Abstract

Protein O-glycosylation is one major type of posttranslational modifications. The O-glycans on glycoproteins play important roles in many biological processes. Core-1 based O-glycans are the most predominant ones found in all animal cells, while Core-3 O-glycans appear to be restricted to proteins from epithelial cells of intestines. Notably, the Core-3 O-glycans were reportedly to play significant suppressive roles in colorectal tumor biology, while truncated O-glycans, Tn and SialylTn antigens, play promoting roles in carcinomas, such as colon cancer. The mechanisms underlying Core-3 O-glycans' tumor suppression, and Tn/STn's tumor promotion, however, are not well understood. Membrane glycoprotein, EGFR plays essential roles in cell proliferation, migration, differentiation, and survival, and it is often mutated or over-expressed in malignancy such as carcinomas. EGFR is shown to be highly N-glycosylated; its O-glycosylation remains controversial. Herein, we established isogenic colorectal carcinoma cell sub-lines with the expression of Tn/STn antigens, and Core 3 O-glycans respectively by manipulating corresponding glycogenes in parental HT-29, HCT116 and SW620 cell lines, and investigated the possible role of O-glycosylation in regulation of EGFR pathway. While Core 3 O-glycans caused inhibition of cell proliferation, migration and colony formation, expression of Tn/STn antigens promoted these malignancy phenotypes. Mechanistically, the altered cell proliferation and migration was due to the down- and up-regulation of EGFR-AKT-GSK3β-Cyclin D1 axis through accelerated and attenuated degradation of EGFR and Cyclin D1 in Core 3 O-glycan and Tn/STn expressing cells, respectively. Furthermore, isogenic cells with Tn expression were significantly less sensitive to the Cetuximab-induced ADCC due to the lower binding of cetuximab to the EGFR in Tn expressing-cells than the parental cells even though the EGFR was expressed at similar level between those isogenic cells. These data demonstrated that the Core 3 O-glycosylation of EGFR attenuates, while tumor antigens, Tn/STn on EGFR elevate the EGFR pathway in human colon cancer cells, highlighting the profound role of O-glycosylation in regulation of EGFR pathway. Overall, this study aids in our better understanding of the regulation mechanisms of EGFR pathway and the mechanism of action by anti-EGFR mAb drugs, and facilitate the development of better therapeutics targeting EGFR in solid tumors.

Background



The common biosynthetic pathways for mucin-type O-glycan core structures 1, 2, 3 and 4 in human cells: The O-glycosylation is initiated by a family of ppGalNAcTs mainly in Golgi apparatus which transfer the GalNAc from UDP-GalNAc to the Serine(Ser)/Threonine(Thr) residues of proteins transported from the endoplasmic reticulum (ER) to form GalNAcα1-Ser/Thr, known as the Tn antigen. Then, the Tn antigen will be further modified/elongated by other glycosyltransferases which are indicated in each step to form core 1-based complex O-glycans in all cell types and core 3-based complex O-glycans mainly in the gastrointestinal tract epithelial cells. Among those glycosyltransferases, T-synthase (Core 1 β3GalT) is the key enzyme in the O-glycan biosynthesis by converting the Tn antigen to the Core 1 structure (Galβ1,3GalNAcα1-Ser/Thr) also known as T antigen; and ER chaperone Cosmc which is encoded by a gene *Cosmc* on human Xq24 assists T-synthase's folding. Some Tn antigen could be also converted to Sialyl-Tn (STn) antigen by the ST6GalNAc-I. Importantly, O-glycans on glycoproteins play critical roles in the molecular interactions that govern many important biological systems.

Summary

1. Three CRC cell (isogenic) lines with three different O-glycosylations: SialylCore 1-based, Tn antigen, and sialylCore 3-based O-glycans were established.

2. *Cosmc*-KO CRC cells with expression of Tn antigens (CKO cells) poses significantly higher proliferation, colony formation and migration, while CRC cells with expression of SialylCore 3 (C3GnT cells) appear the opposite: lower proliferation, colony formation and migration.

3. CKO cells have significantly higher Cyclin D1, while C3GnT cells appear to have much less cyclin D1. Treatment of cells with a proteasomal inhibitor restored the Cyclin D1; suggesting that accelerated degradation of cyclin D1 occurs in cells expressing sialylCore 3 O-glycans.

4. CKO cells have significantly more EGFR, and pEGFR, while C3GnT cells appear to have much less EGFR, and pEGFR. Treatment of cells with a proteasomal inhibitor restore the EGFR; suggesting accelerated degradation of EGFR occurs in cells expressing sialylCore 3 O-glycans.

5. O-glycosylation in colorectal cancer cells regulates EGFR/AKT/cyclin D1 pathway through regulation of EGFR activation and degradation.

6. CKO cells are significantly less sensitive to Cetuximab induced ADCC although they appear to have similar expression of EGFR to the control cells expressing the sialylCore 1 o-glycans.

7. C3GnT is not expressed, and pEGFR and Tn are correlatedly expressed in human CRC tumor tissue.

Results

Establishment of CRC cell lines with expression of Tn antigens and Core 3 O-glycans respectively

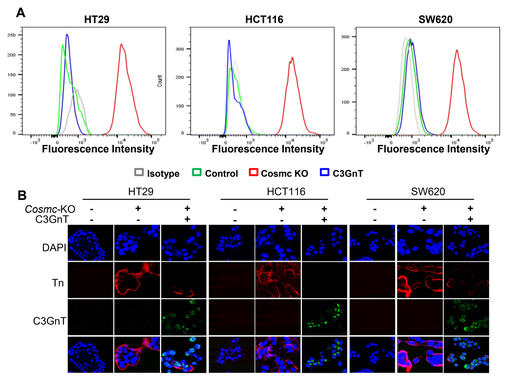


Figure 1. Establishment and characterization of *Cosmc*-KO and C3GnT-expressing CRC cell lines resulted in significantly reduced expression of Tn antigens. A: FACs analysis with anti-Tn mAb; B: IF staining

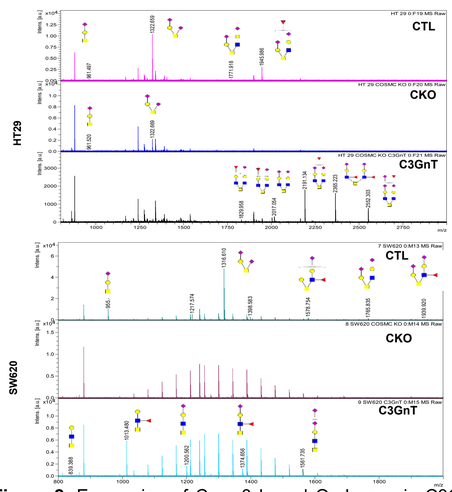


Figure 2. Expression of Core 3 based O-glycans in C3GnT-exprtessing CRC cell lines: in *Cosmc*-KO (CKO) cells resulted in production of Core 3-based O-Glycans. O-glycans were analyzed by CORA. O-glycans of cell lines derived from HT29 and SW620 were shown.

Characterization of CRC cell lines with expression of Tn and Core 3 O-glycans

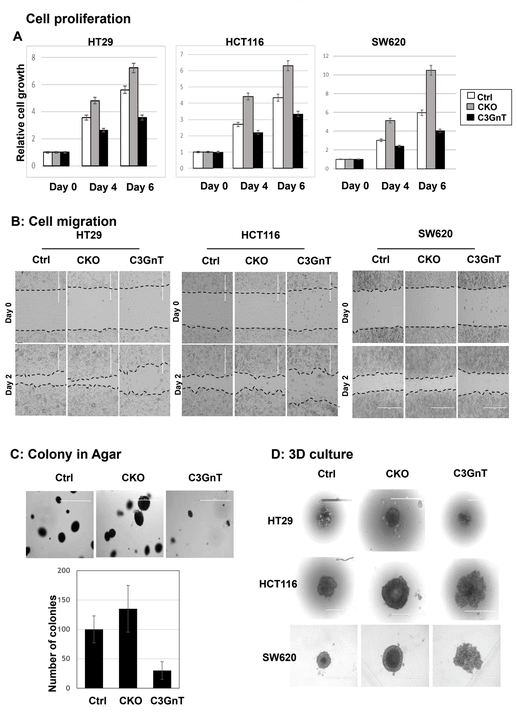


Figure 3. Expression of core 3 O-glycans in CRC cell lines attenuates, while expression of Tn antigens promotes the malignancy of CRC cells: A: cell proliferation: B: Cell migration, C: cell growth in Agar, D: Spheroids in 3D culture

Cells with different O-glycosylation proliferate differently— cell cycle analysis

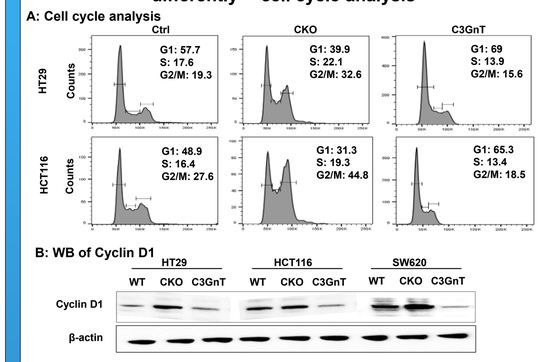


Figure 4. Cell cycle facilitation by Tn antigen expression and inhibition by core 3 O-glycan expression on cells are mediated by regulation of Cyclin D1. A: Cell cycle analysis; B: WB analysis of Cyclin D1.

O-glycosylation affects EGFR activation and degradation, consequently, regulates Cyclin D1 degradation through EGFR/AKT/GSK3/Cyclin D1 Axis

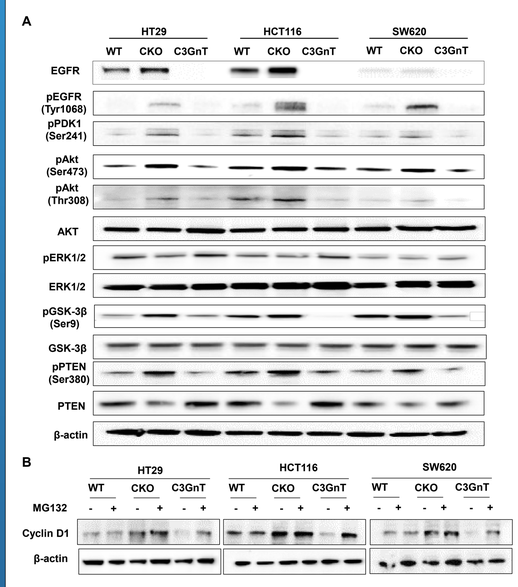


Figure 5. Analysis of EGFR/AKT/GSK3β/Cyclin D1 Pathways. A: EGFR signaling was analyzed by Western blot analyses. B: Cells were treated with MG132 for 6hrs and Cyclin D1 were analyzed by WB analysis.

O-glycosylation regulates EGFR degradation

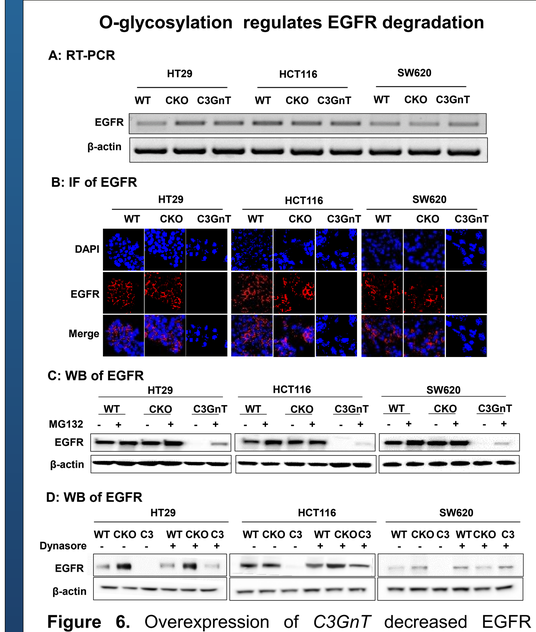


Figure 6. Overexpression of *C3GnT* decreased EGFR protein stability through the proteasome dependent degradation. **A**: RT-PCR of EGFR. **B**: IF of EGFR. **C** and **D**: Cells were treated with MG132 and Dynasore for 6hrs and EGFR was analyzed by WB.

O-glycans on EGFR affect NK cell–mediated cytotoxicity induced by cetuximab

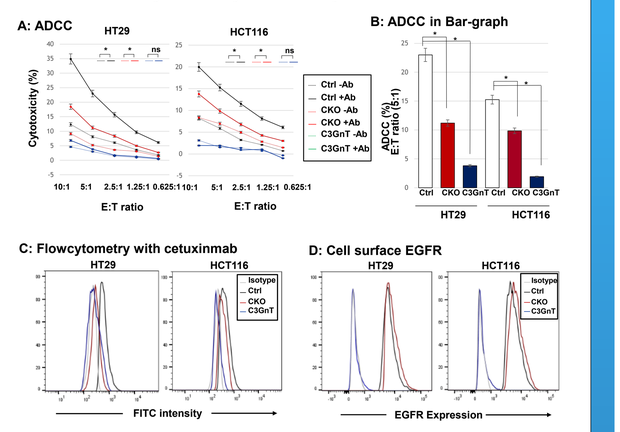


Figure 8. Cetuximab-mediated cytotoxicity against CRC cells with different O-glycosylation. A. The effect of cetuximab on the survival of CRC cells was determined. The percentage of ADCC (5:1 target ratio) showed in B. C. CRC cells were incubated with 20 μg/ml cetuximab or isotype control, subsequently followed by incubation with FITC-F(ab')2 anti-human IgG and then analyzed by flow cytometry. D) Representative histograms of surface EGFR staining of the different O-glycoengineered CRC cells using flow cytometry.

C3GnT was not expressed, and pEGFR and Tn were correlatedly expressed in CRC tumor cells

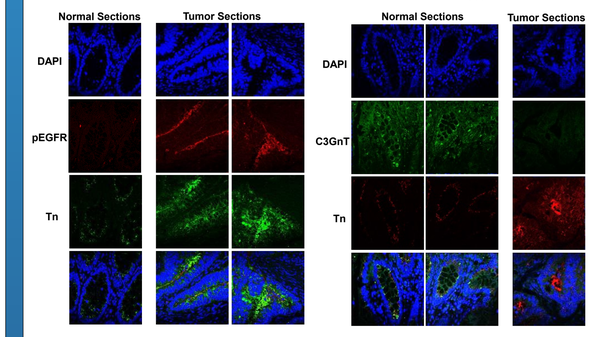
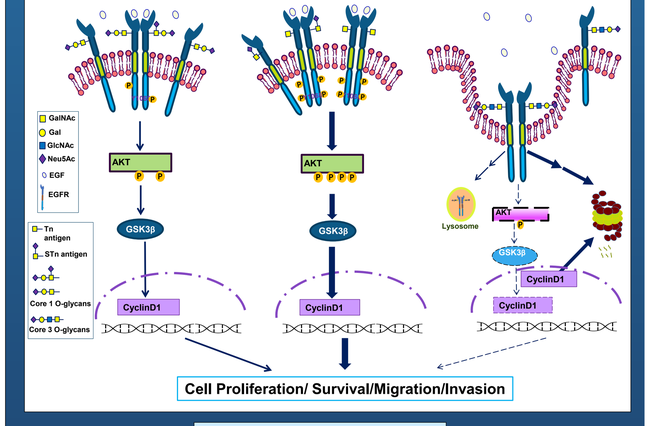


Figure 9. Immunofluorescent (IF) staining of human colon tissue arrays: representative images of paired normal and tumor tissues sections stained with anti-Tn, p-EGFR were shown.

Working Model: Expression of different O-glycans on CRC cells attenuates CRC cell proliferation through regulation of activation and degradation of EGFR and CyclinD1 via EGFR/AKT/GSK3β/Cyclin D1 pathway.



FDA Mission Relevance

Our research advances better understanding of important role of O-glycans in colon cancer that can assist in development of potential therapeutics in prediction of patient response to the targeted therapies. The acquired information also has implications in helping the FDA reviewers in making more informed regulatory decisions regarding specific drug combination strategies and interpretation of clinical safety and efficacy data.

Acknowledgements

This work was supported by the FDA/CDER intramural research funding.