NEOPLASIA
www.neoplasia.com

Volume 20 Number 10 October 2018 pp. 1038–1044 1038

Critical Role of Estrogen Receptor Alpha O-Glycosylation by N-Acetylgalactosaminyltransferase 6 (GALNT6) in Its Nuclear Localization in Breast Cancer Cells[1,2]

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Abstract

Alteration of protein O-glycosylation in various human cancers including breast cancer is well known, but molecular roles of their aberrant glycosylations on cancer have not been fully understood. We previously reported critical roles of polypeptide N-acetylgalactosaminyltransferase 6 (GALNT6 or GalNAc-T6) that was upregulated in a great majority of breast cancer tissues. Here we further report O-glycosylation of estrogen receptor alpha (ER-α) by GALNT6 and the significant role of its nuclear localization in breast cancer cells. Knockdown of *GALNT6* expression in two breast cancer cell lines, T47D and MCF7, in which both ER-α and GALNT6 were highly expressed, by small interfering RNA could significantly attenuate expression of ER-α. Immunocytochemical analysis clearly demonstrated the drastic decrease of ER-α protein in the nucleus of these cancer cells. Accordingly, the downstream genes of the ER-α pathway such as *MYC*, *CCND1*, and *CTSD* were significantly downregulated. We confirmed GALNT6-dependent ER-α O-glycosylation and identified O-glycosylation of S573 in an F domain of ER-α by GALNT6 through LC-MS/MS analysis. We also obtained evidences showing that the glycosylation of ER-α at S573 by GALNT6 is essential for protein stability and nuclear localization of ER-α in breast cancer cells. Furthermore, we designed cell membrane–permeable peptides including the O-glycosylation site and found a significant decrease of the cell viability of breast cancer cells by treatment of these peptides in a GALNT6 expression–dependent manner. Our study suggests that targeting the GALNT6 enzymatic activity as well as the GALNT6/ER-α interaction could be a promising therapeutic approach to ER-α–positive breast cancer patients.

Neoplasia (2018) 20, 1038–1044

Introduction

Breast cancer is one of the major malignancies affecting women across the world. A total of 266,120 women are estimated to be diagnosed breast cancer and 40,920 women would die of breast cancer in the United States in 2018 [1]. Approximately 70% of breast cancers express/overexpress or have somatic mutations in an estrogen receptor-alpha (ER-α) gene, which plays critical roles in development and progression of breast cancer. Inhibitors of an estrogen/ER-α signaling pathway such as selective ER-α modulators (e.g., tamoxifen and raloxifene), ER-α downregulators (e.g., fulvestrant), and aromatase inhibitors (AIs) have been used for hormone receptor–positive breast cancer and significantly improved the prognosis breast cancer patients [2–4]. However, these treatment modalities often become ineffective because of the intrinsic and acquired endocrine

Abbreviations: GALNT6, N-acetylgalactosaminyltransferase 6; ER-α, estrogen receptor-alpha; GalNAz, tetraacetylated N-azidoacetylgalactosamine; *CCND*, Cyclin D1; *CTSD*, Cathepsin D; VVA, *Vicia villosa* agglutinin; GAPDH, glyceraldehyde phosphate dehydrogenase.

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[1] Statement of significance: Findings uncover the O-glycosylation of ER-α by GALNT6 and demonstrate effective strategy to ER-α–positive breast cancer treatment.

[2] Conflict of interest statement: Y. N. is a stock holder and a scientific advisor of OncoTherapy Science, Inc. J. P. is a scientific advisor of Cancer Precision Medicine Inc.

Received 4 May 2018; Revised 12 August 2018; Accepted 20 August 2018

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1476-5586
https://doi.org/10.1016/j.neo.2018.08.006

resistance [5,6]. Hence, development of novel molecular-targeted drugs for breast cancer to overcome endocrine resistance with higher efficacy and low risk of adverse reactions is crucial to further improve clinical outcome of breast cancer patients.

Polypeptide N-acetylgalactosaminyltransferase 6 (GALNT6) is an enzyme which mediates the mucin-type O-glycosylation and has been reported to be aberrantly expressed in many types of human cancer [7–9]. GALNT6 expression level was much higher in breast cancers compared to other cancer types [10], especially in the estrogen receptor (ER)–positive breast cancer tissues [11]. We previously reported upregulation of GALNT6 in a great majority of breast cancers and demonstrated its critical roles in breast cancer through decrease of cellular adhesion ability and disruption of mammary acinar morphogenesis [12,13]. We also found its upregulation in pancreatic cancer cells, in which GALNT6 could cause a cadherin switch (from E-cadherin to P-cadherin) affecting cellular adhesion to the underlying matrix [14]. High GALNT6 expression was also reported to be correlated with an increased risk of recurrence, lymph node metastasis, and chemoresistance in ovarian cancer [15]. It was also shown that GALNT6 was highly upregulated in colon adenocarcinomas compared with adjacent colon tissues, implying its important role in colon carcinogenesis [8]. GALNT6 was identified as an independent prognostic factor for the poor prognosis of gastric cancer patients; high GALNT6 was significantly associated with the low expression levels of E-cadherin as well as the high expression levels of MMP9 in gastric cancer tissues [16].

Here we demonstrate a possibility of ER-α as a novel substrate of GALNT6 and an essential role of GALNT6-mediated O-glycosylation for the nuclear localization of ER-α in breast cancer cells. We also show that cell membrane–permeable peptides including the O-glycosylation site of ER-α inhibit the interaction of ER-α/GALNT6, alter cellular phenotypes, and cause the cell death. Our data suggest that targeting the GALNT6 enzymatic activity as well as the GALNT6/ER-α interaction could be a promising therapeutic approach to ER-positive breast cancers.

Materials and Methods

Cell Culture

Human cancer cell lines T47D, MCF7, SKBR3, HCC1937, and HeLa were purchased from American Type Culture Collection and cultured according to provider's protocols. Three HeLa cell–derived cell lines stably expressing HA-tagged wild-type GALNT6 protein (HeLa-GALNT6-WT) or HA-tagged enzyme-dead H271D-substituted GALNT6 protein (HeLa-GALNT6-H271D) and the cells transfected with an empty vector (HeLa-Mock) were established as previously described [12], and these cells were cultured in the medium containing 0.8 mg/ml of G418 (Geneticin). Transfection of plasmids in the study was performed by using FuGENE 6 reagents (Roche) according to the manufacturer's protocols. Estradiol was purchased from Sigma-Aldrich, USA.

Screening of Novel O-Glycosylation Substrates Induced by GALNT6

For screening a candidate O-glycosylation substrate(s) of GALNT6, we performed *in vivo* metabolic labeling of O-glycosylated proteins using GalNAz (tetraacetylated N-azidoacetylgalactosamine), which is an azide-labeled sugar for O-glycans [17]. After 72 hours of treatment with 50 μM of GalNAz, total proteins were extracted from HeLa cells stably expressing mock, GALNT6-WT, or GALNT6-H271D by using CelLytic M reagent (Sigma-Aldrich) with 1% of Protease Inhibitor Cocktail Set III (Calbiochem). The O-glycosylated proteins containing GalNAz were co-conjugated with biotin by Click-iT reaction buffer kit (Invitrogen) and then were immunoprecipitated with NeutrAvidin beads (Thermo Fisher). After on-beads Lys-C/Trypsin digestion, eluted O-glycosylated proteins were analyzed by tandem mass spectrometry (MS/MS) analysis which performed beta-elimination reaction to remove O-glycans and simultaneously substitute originally-glycosylated Ser/Thr to [Ser-1Da] / [Thr-1Da] (-OH > -NH2).

GALNT6 Knockdown by Small Interfering RNA (siRNA)

We used siRNA (Sigma-Aldrich) for GALNT6 knockdown and SIC001 Mission siRNA Universal Negative Control (Sigma-Aldrich) as control. Briefly, GALNT6-siRNA or control siRNA was transfected into cells by using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's protocols. Seventy-two hours later, cells were collected for further analysis. The target sequences of siRNA are 5′-GAGAAAUCCUUCGGUGACA-3′ for si-GALNT6 as previously described [12,14].

Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA (1 μg) was reversely transcribed using SuperScript III First-Strand Synthesis System (Invitrogen) to generate cDNA. Aliquots of cDNA samples were quantified by the real-time RT-PCR method (qPCR). The qPCR was performed using primers listed below using the ViiA 7 system (Life Technologies). The expression levels of target genes were normalized with that of GAPDH. The PCR primers were as shown: *GALNT6* (Hs00926629_m1), *ESR1* (Hs00174860_m1), *MYC* (Hs00153408_m1), *CCND1* (Hs00765553_m1), *CTSD* (Hs00157205_m1), and *GAPDH* (Hs02758991_g1) TaqMan Gene Expression Assays (Thermo Fisher Scientific).

Western Blot

Western blot was performed as described previously [14]. Protein bands were visualized by ECL detection reagents (GE Healthcare). The primary antibodies used in this study were as follows: anti-human ER-α monoclonal antibody (1:400, Santa Cruz), anti-human GALNT6 polyclonal antibody (1:500, Sigma-Aldrich), anti-Flag M2 monoclonal antibody (1:1000, Sigma-Aldrich), anti-HA monoclonal antibody (1:1000, Roche), and anti–β-actin monoclonal antibody (1:10,000, Sigma-Aldrich). The secondary antibodies were goat anti-rabbit or anti-mouse IgG-HRP antibodies (1:10,000-1:30,000, Santa Cruz). For the detection of O-glycosylated proteins, we performed lectin blotting using biotinylated Vicia villosa agglutinin (VVA) lectin (1:1000, Vector Laboratories) and Streptavidin-HRP (1:10,000, Thermo Scientific).

Identification of an O-Glycosylation Site(s) of ER-α by MS Analysis

For identification of an O-glycosylation site(s) on ER-α protein, HeLa-GALNT6 stable cells (WT) were transfected with pCAGGSn3FC-ER-α expression vector [18,19] and collected after 48 hours of incubation. Cells were lysed with lysis buffer, and Flag-tagged ER-α protein was immunoprecipitated with anti-Flag monoclonal antibody and Protein A/G agarose (Invitrogen). After washing with the lysis buffer five times, immunocomplexes were

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