RESEARCH ARTICLE

B3GNT2, a Polylactosamine Synthase, Regulates Glycosylation of EGFR in H7721 Human Hepatocellular Carcinoma Cells

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Abstract

The epidermal growth factor receptor (EGFR) is an important surface receptor with *N*-glycans in its extracellular domain, whose glycosylation is essential for its function, especially in tumor cells. Here, we demonstrated that polylactosamine is markedly increased in H7721 hepatocellular carcinoma cells after treatment with EGF, while it apparently declined after exposure to all-trans retinoic acid (ATRA). In the study of the enzymatic mechanism of this phenomenon, we explored changes in the expression of poly-*N*-acetyllactosamine (PLN) branching glycosyltransferases using RT-PCR. Among the four glycosyltransferases with altered expression, GnT-V was most elevated by EGF, while GnT-V and B3GNT2 were most declined by ATRA. Next, we conducted co-immunoprecipitation experiments to test whether B3GNT2 and EGFR associate with each other. We observed that EGFR is a B3GNT2-targeting protein in H7721 cells. Taken together, these findings indicated that the altered expression of B3GNT2 will remodel the PLN stucture of EGFR in H7721 cells, which may modify downstream signal transduction.

Keywords: B3GNT2 - EGFR - poly-*N*-acetyllactosamine - glycosylation - H7721 cells

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Introduction

β-1, 3-*N*-acetylglucosaminyltransferase (B3GNT2, β3GnT-2) is a polylactosamine synthase that synthesizes a backbone structure of carbohydrate structures onto glycoproteins (Akira et al., 2007). Poly-*N*-acetyllactosamine (PolyLacNAc, PLN) is a linear carbohydrate polymer composed of alternating galactose (Gal) and *N*-acetylglucosamine (GlcNAc) residues. This polysaccharide can be incorporated into either *N*-linked or mucin-type *O*-linked glycans and can act as a marker for development, apoptosis, and metastasis (Kasai and Hirabayashi, 1996; Elola et al., 2005). PolyLacNAc polymers can be further modified by various glycosyltransferases to create branched structures and display terminal epitopes such as the sialyl Lewis X modification, an important adhension marker (Hakomori, 1999; Dall'Olio, 2000; Zhou, 2003).

PolyLacNAc is biosynthesized by the alternating addition of GlcNAc by a UDP-GlcNAc: βGal β-1, 3-*N*-acetylglucosaminyltransferase (B3GNT) and Gal by a UDP-Gal: βGlcNAc β-1, 4-galactosyltransferase (B4GALT). The primary glycosyltransferases within the B3GNT families is B3GNT2 (Togayachi et al., 2010).

N-glycosylation has been reported to regulate several properties of EGFR, including conformation, transportation to cell surfaces, ligand binding, dimerization, endocytosis, and degradation (Takahashi et al., 2008). Epidermal growth factor receptor (EGFR) is a member of ErbB family (ErbB1-4) of receptor tyrosine kinases (RTKs) that mediates cellular responses to EGF and transforming growth factorα (TGFα) and plays a crucial role in promoting tumor cell motility and invasion (Sebastian et al., 2006). Upon EGF binding, EGFR dimerizes and becomes autophosphorylated at multiple tyrosine sites within its cytoplasmic tail. The human epidermal growth factor receptor contains 12 putative *N*-glycosylation sites located in extracellular domain I-IV (Ullrich et al., 1984), and *N*-linked glycosylation of EGFR appears to be essential for its functions, especially the glycosylation in domain III, the major binding site for EGF and TGFα (Greenfield et al., 1989; Lemmon et al., 1997; Tsuda et al., 2000). Studies have shown that EGFR function can be modulated by changes in GnT-Va-related *N*-glycan expression. The overexpression of GnT-Va in human hepatocarcinoma cells, for example, caused aberrant *N*-glycosylation of EGFR and increased MAPK signaling mediated by EGF (Guo et al., 2004; Liu et al., 2013).

Elevated expression and activity of β-1, 3-*N*-acetylglucosaminyltransferase (B3GNT2) in

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hepatocellular carcinoma (HCC) is a common early event involved in tumor invasion during hepatocarcinogenesis. A better understanding of the functional role and the molecular mechanism for B3GNT2-targeted protein and downstream signaling pathway behind hepatoma invasion and metastasis is urgently needed. Our results suggested that B3GNT2 catalyzed the glycosylation of EGFR, resulting in reduced invasiveness-related behaviors mediated by EGF.

Materials and Methods

Materials

The H7721 human hepatocarcinoma cell line was obtained from the Institute of Cell Biology. RPMI 1640 medium was purchased from GIBCO/BRL. Rabbit monoclonal antibodie against EGFR was from Abcam. Goat monoclonal antibody against B3GNT2, HRP-labeled anti-rabbit, and anti-goat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). The Proteo-Extract Transmemberan Protein Extraction kit (TM-PEK) was from Novagen. Polyvinylidene difluoride (PVDF) membrane was purchased from Bio-Rad. FITC-conjugated LEL was product of Vector Laboratories (Burlingame, CA).

Cell culture and treatment

Cells were cultured in RPMI-1640 complete medium at a 37°C humidified atmosphere containing 5% CO_2. The mediums are renewed every two and three days for the cells cultured. The cells were harvested when they were in logarithmic growth phase, and adjusted to $2\sim3\times10^6$ cells/ml, then subjected to RNA extraction and RT-PCR. In the experiments using EGF or ATRA, the cultured cells were adjusted to 5×10^5/ml. EGF or ATRA was added to the culture medium in a final concentration of 20ng /ml or 10-5M respectively for 18h, or 72 h before the cells were harvested.

Immunofluorescence analysis

The H7721 cells grown overnight on coverslips were fixed in 4% paraformaldehyde/PBS at room temperature for 30min. After treatment with FITC-labeled tomato lectin (20μg/ml) at room temperature for 1h, the cells were stained with DAPI. After washing with PBS, cells were imaged by confocal fluorescence microscopy.

RNA extraction and RT-PCR

Total RNA was extracted from cells using TRIzol/ Chloroform / isopropanol method according to the protocol provided by Promega. Complementary DNAs (cDNAs) were synthesized from 4 μg of the total RNA with 150 ng random primer, 25mmol/L dNTP Mix 1ml, 40U RNase inhibitor, and 200U Superscript II RNase H reverse transcriptase in 20 μl reaction mixture. The mixture was incubated at 37°C for 60 min and 95°C for 5 min. After the addition of 2U RNase H, the PCR was performed in a volume of 25ml containing 1ml cDNA, 10pmol/L primer, 0.2 mmol/L of each dNTP, 5 IU Taq E polymerase and PCR buffer. The cDNA was subjected to denaturation at 94°C for 5 min, followed by 25 cycles of PCR. Each cycle included denaturation at 94°C for 30s, annealing at 55°C for 45s and elongation at 72°C for 75s. Finally, the samples were further incubated for elongation at 72°C for 7 min. After completion of the RT-PCR, 10 μl products or pUC Mix DNA marker were applied to 1.5% agarose gel for electrophoresis and stained by ethidium bromide. The intensities of the amplified DNA bands were scanned by ImageMaster System (UVP), including the analysis of the screened photos with NIH Image software. The data were indicated as the relative expression of glycosyltransferases after normalized with the loading control (GAPDH) and set the value of untreated cells as 100%. Three independent and reproducible experiments were performed and the mean±SD was calculated.

Western blotting, and Co-immunoprecipitation

Subconfluent cells were harvested and lysed by TM-PEK. 20 μg protein extracts was electrophoresed on a 10% polyacrylamide mini gel, transferred onto a PVDF membrane, and then subjected to Western blotting. For immunoprecipitation, 500 μg protein were used and precipitates were then subjected to Western blotting.

Statistical analysis

All the data were processed with SPPS version 11.5 for Windows® software, and $p<0.05$ should be considered to be statistically significant by Student's test.

Results

Altered expression of PolyLacNAc in H7721 cells

By using FITC-labeled tomato lectin (*Lycopersicon esculentum*) as the probe, it was discovered that poly-*N*-acetyllactosamine (PolyLacNAc, [Galβ1, 4Gnβ1, 3]n) was moderately expressed on the cell surface of H7721 human hepatocarcinoma cell line.

After the treatment of 20 ng/ml EGF (epidermal growth factor) for 72 h, the expression of PolyLacNAc was highly increased. Oppositely, it was significantly decreased after the treatment of 10-5M of ATRA (all-trans retinoic acid) for 72 h.

The enzymatic mechanism of the altered expression of PolyLacNAc in H7721 cells

To elucidate the enzymatic mechanism of the altered

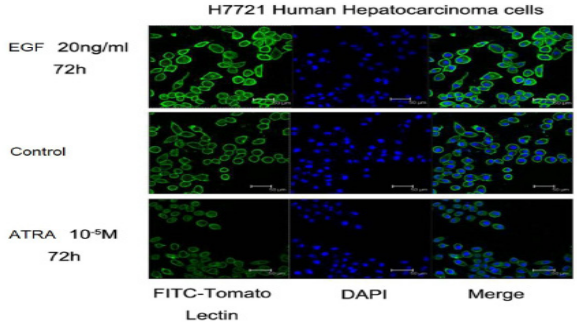

Figure 1. Confocal Immunofluorescence Images of H7721 Cells Labled with FITC-conjugated Tomato Lectin (LEL) after Treated with EGF or ATRA. The signals of LEL (reactive to polylactosamine) are shown.

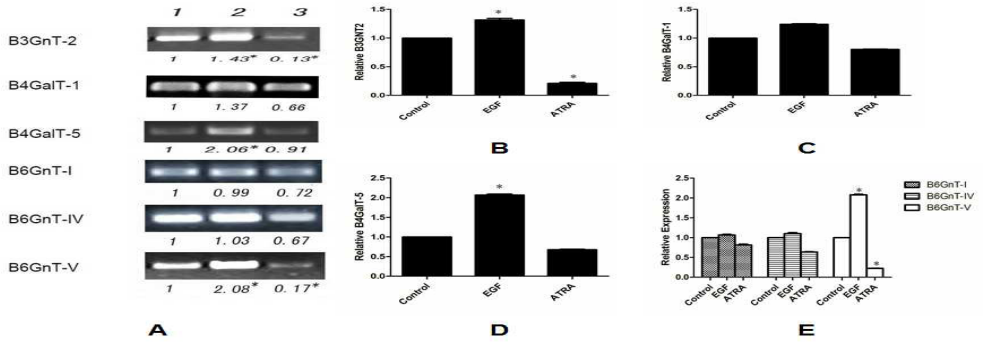

Figure 2. Effect of EGF or ATRA on the Expressions of Glycosyltransferases Related to the Synthesis of PolyLacNAc in H7721 Cells. A) RT-PCR profile of the expressions of glycosyltransferases in H7721 cells after EGF or ATRA treatment. The profile indicated is one of three independent and reproducible experiments; **B)** The relative expressions of β3GnT-2 during treatment with EGF or ATRA in H7721 cells; **C)** The relative expressions of β4GalT-1 during treatment with EGF or ATRA in H7721 cells; **D)** The relative expressions of β4GalT-5 during treatment with EGF or ATRA in H7721 cells; **E)** The relative expressions of GnTs during treatment with EGF or ATRA in H7721 cells; *: $p<0.05$. compared to "Control", n=3. The experimental procedure of RT-PCR was described in the "Method".

expression of PolyLacNAc, five glycosyltransferases related to the synthesis of PolyLacNAc were studied, including β6/β4-*N*-acetylglucosaminyltransferase-V/IVb (GnT-V/IVb), the branching enzymes in *N*-glycan synthesis; β4-galactosyltranferase-5 (β4GalT-5), the main enzyme transferring the galactose residue at the outside of the β6 branched *N*-acetylglucosamine; as well as β3-*N*-acetylglucosaminyltransferase-2 (β3GnT-2) and β4-galactosyltransferase-1 (β4GalT-1), two enzymes participated in the synthesis of [Galβ1, 4Gnβ1, 3]n in H7721 cells (this cell line does not express β3GnT-8, which is considered as the main enzyme for PolyLacNAc synthesis in other carcinoma cells such as column cancer). It was found by RT-PCR method that the expressions of GnT-V and GnT-IVb were elevated about 3 or 2 fold after the treatment of EGF, while they were declined apparently after treated with ATRA. β4GalT-5 was also up- or down-regulated by the treatment of EGF or ATRA respectively as GnT-IVb. The expression of β3GnT-2, the rate-limiting enzyme in PolyLacNAc synthesis, was approximately doubled after EGF and decreased to very low level after ATRA, while the expression of β4GalT-1 was changed slightly with no statistic significance after both EGF and ATRA treatment. Two other glycosyltransferases, β2-*N*-acetylglucosaminyltransferase-I (GnT-I) and β3-*N*-acetylglucosaminyltransferase-5 (β3GnT-5) selected as the negative controls, were almost unchanged after the treatment of EGF and ATRA.

EGFR is a B3GNT2-targeted protein in H7721 cells

Changes in the expression of *N*-glycan branching glycosyltransferases can alter cell surface receptor functions, involving their levels of cell surface retention, rates of internalization into the endosomal compartment, and subsequent intracellular signaling. To study in detail the regulation of the EGFR glycosylated by B3GNT2, we performed co-immunoprecipitation analysis to confirmed the interaction of B3GNT2 and the EGFR (Figure 3, lane 3).

We observed the expression of EGFR in H7721 cell membrane protein extracts quantity than those in cytoplasm (Figure 3, lane 1, 2). Moreover, after treated

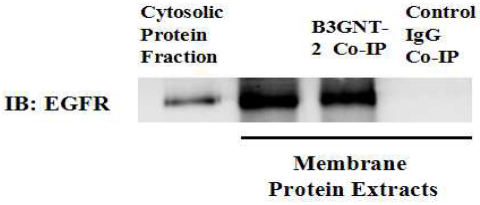

Figure 3. EGFR Western Blots Analysis of the B3GNT2 Immunoprecipitate from the Lysates of H7721 Cells

with EGF, the PolyLacNAc structure on the cell surface and the expression of B3GNT2 was highly increased in H7721 cells. Therefore, we hypothesized that B3GNT2 can catalyze the formation of PLN structure of EGFR on the surface of H7721 cells.

Discussion

Primary liver cancer (namely hepatocellular carcinoma, HCC) is worldwide the fifth most common cancer in men and the seventh one in women, and it represents the third most frequent cause of cancer death. HCC rates are particularly high in eastern/south-eastern Asia and in Africa (Bosetti et al., 2014). As a hallmark of tumor phenotype, altered glycosylation patterns during tumorigenesis include both the under- and overexpression of naturally occurring glycans, as well as neoexpression of glycans normally restricted to embryonic tissues (Dube et al., 2005). These tumor-associated glycans most often arise from changes in the expression levels of glycosyltransferases in the Golgi compartment of cancerous cells (Dube et al., 2005; Sun et al., 2013).

To investigate alterations of polylactosamine on memberane glycoproteins in H7721 cells, we performed immunofluorescence analysis with the *Lycopersicon esculentum* (tomato) lectin (LEL), which is known to bind to polylactosamines with at least three lactosamine unit repeats (Togayachi et al., 2010). Here we show that LEL-detectable polylactosamine on *N*-glycans is markedly increased in H7721 cells by EGF, but decreased from those cells by treated with ATRA.

To elucidate the enzymatic mechanism of the altered

expression of PolyLacNAc, five glycosyltransferases related to the synthesis of PolyLacNAc were studied by using RT-PCR. Among the four glycosyltransferases with altered expressions, GnT-V was most elevated by EGF, while GnT-V and β3GnT-2 were most declined by ATRA. However, which one is most important as a leading enzyme in the regulation of PolyLacNAc synthesis, will be further investigated.

Changes in the expression of *N*-glycan branching glycosyltransferases can alter cell surface receptor functions, involving their levels of cell surface retention, rates of internalization into the endosomal compartment, and subsequent intracellular signaling. The expression pattern of EGFR was membranous and cytoplasmic, more than half of the EGFR population is present in membrane rafts and smaller percentages are present in caveolae and clathrin-coated pits (Keating et al., 2008; Andreea et al., 2014). EGFR is an important surface receptor with *N*-glycans in its extracellular domain, and the glycosylation of EGFR is essential for its function (Soderquist et al., 1988; Bishayee, 2000). To study in detail the regulation of signaling of the EGF receptor (EGFR) by B3GNT2, we performed co-immunoprecipitation analysis to confirmed the interaction of B3GNT2 and EGFR. Therefore, we hypothesized that the glycosylation of EGFR catalyzed by B3GNT2 is closely related to the EGF signaling pathway. Although we have not determined the molecular basis of the regulatory mechanism of polylactosamine in H7721 cells yet, we did identify it on two important molecules, B3GNT2 and EGFR. We anticipate that these studies will lead to a better understanding of the regulatory mechanisms of EGFR and the biological functions of polylactosamine.

Acknowledgements

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