

EFEMP2 Mediates GALNT14-Dependent Breast Cancer Cell Invasion



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Abstract

N-Acetylgalactosaminyltransferase-14 (GALNT14) is a member of acetylgalactosaminyltransferases family. We have shown that GALNT14 could promote breast cancer cell invasion. However, the underlying molecular mechanism is unclear. Here, using yeast two hybrid, we find that EGF-containing fibulin-like extracellular matrix protein 2 (EFEMP2) interacts with GALNT14. Both *in vitro* and *in vivo* binding assays show that EFEMP2 is associated with GALNT14. Moreover, we find that GALNT14 mediates glycosylation of EFEMP2. EFEMP2 significantly increased the invasion ability of breast cancer cells including MCF-7 and MBA-MD-231 cells, and this phenomenon is suppressed by knockdown expression of GALNT14. In addition, the GALNT14-dependent O-glycosylation of EFEMP-2 regulates the stability of EFEMP-2 protein in breast cancer cells. Taken together, our results demonstrate a novel molecular mechanism underlying breast cancer invasion.

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Introduction

Alterations in protein glycosylation facilitate tumor development including tumor cell growth, differentiation, transformation, adhesion, invasion, and immune surveillance [1]. The major changes of protein glycosylation are in both O-glycans (GalNAc-Ser/Thr) and N-glycans, which may occur in cancer progression and metastasis. Alterations in glycosyltransferase expression, defined as production of transcripts and enzyme activity, may not only significantly impact the generation of glycans but also govern the degree of glycan branching, which in turn can alter overall glycan structure and function [2]. Moreover, other complicated mechanism such as the ratio of enzyme versus substrate as well as the localization of the enzymes may also regulate protein glycosylation. Thus, understanding the molecular mechanism of aberrant glycosylation is important for cancer research.

N-acetylglucosamine galactosyltransferase (GALNT) family consists of at least 24 different members residing in the Golgi apparatus. These enzymes initiate protein O-glycosylation by transferring the GalNAc residue from UDP-GalNAc to the hydroxyl group of Ser or Thr [3,4]. Several GALNTs are overexpressed during tumor development. For example, overexpression of GALNT6 has been found in breast cancer, which induces aberrant glycosylation and contributes to mammary carcinogenesis [8]. Thus, the expression of GALNT6 has been considered as a novel biomarker for breast cancer diagnosis [5]. Moreover, mutations of GALNT3 and GALNT13

have been identified in both of bone cortex hyperplasia [6,7] and bone marrow neuroblastoma [8]. The overexpression of GALNT2 induces hepatocellular carcinoma by modifying epidermal growth factor receptor (EGFR) [9]. GALNT14 was originally characterized by Wang et al. in 2003 [10]. GALNT14 is overexpressed in colorectal carcinoma and pancreatic cancer. It is associated with altered sensitivity to TRAIL-induced apoptosis through modulation of the O-glycosylation of death receptors in these tumors [11]. The expression of GALNT14 correlates with poor prognosis of serous epithelial ovarian cancer patients. It has been found that the potential use of GALNT14 is able to be served as a novel prognostic epithelial ovarian cancer biomarker [12]. Mutations of GALNT14 have been identified in resected cholangiocarcinoma and are associated with perineural invasion, lymph node metastasis, and its overall survival

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[13]. However, the underlying mechanism of how these GALNTs are upregulated in human tumors is not fully understood.

Our previous studies have shown that GALNT14 is highly expressed in breast cancer and regulates malignant progression of breast cancer [14]. GALNT14 contributed to breast cancer invasion and migration by altering the cell proliferation, motility, and expression levels of epithelial-mesenchymal transition genes and stimulating MMP2 activity, suggesting that GALNT14 could be not only an important biomarker of breast cancer diagnostic but also a potential target for breast cancer treatment [15]. However, the underlying mechanism by which GALNT14 mediates breast cancer invasion remains elusive.

In this study, using yeast two-hybrid (Y2H), we found EFEMP2 as a novel partner of GALNT14. We demonstrate that GALNT14 mediates the O-glycosylation of EFEMP2, which facilitates breast cancer invasion.

Materials and Methods

Cell Culture

MCF-7 and MBA-MD-231 cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (GIBCO, Carlsbad, CA) with 10% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin at 37°C and 5% CO₂ in a humidified incubator.

Plasmid Construction

The complete 1656-bp cDNA fragment of human GALNT14 (GenBank accession number: AB078144) was subcloned in frame into the *EcoRI-SalI* site of the DNA-Binding Domain (BD) vector pGBKT7 (CLONTECH) to generate an expression plasmid pGBKT7-GALNT14. The expression of bait GALNT14/GAL4 BD fusion protein was determined by Western blot of lysates from AH109 yeast cells transfected with this construct.

Yeast Two-Hybrid Screen

pGBKT7-GALNT14 and pGADT7 were transfected into yeast AH109; cells were plated on SD-Leu-Trp medium and cultured at 30°C until colonies appeared. Then, colonies were plated on SD-Leu-Trp-His-Ade medium and cultured at 30°C for 2 to 3 days to test the autoactivation of the bait. The pGBKT7-GALNT14 bait vector and the human mammary gland Matchmaker cDNA library (CLONTECH, Cat. No. 638811) in the pACT2 vector were co-transfected into AH109 that were grown on SD-Leu-Trp-His-Ade medium at 30°C for 3 to 5 days to test potential interactions. Approximately 5×10⁶ independent transfectants were pooled and replated on selection media (Ade⁻, His⁻, Leu⁻ and Trp⁻) containing 2% galactose (Gal) to induce the expression of cDNAs. In total, four positive colonies were identified. Plasmids from these transformants were extracted, isolated, and sequenced. To eliminate false-positive results, candidate plasmids isolated from the primary screen were transfected into AH109 strain containing pGBKT7-GALNT14 plasmid to assay the activation of reporter genes and reconfirm the positive candidates.

GST Pull-Down Assay

To reconfirm the interaction between GALNT14 and the cDNA clone identified by yeast two-hybrid system, GST pull-down assay with purified GST-GALNT14 fusion protein and His-EFEMP2

fusion protein was performed according to the method described in our previous work [16]. Briefly, the full-length cDNA of EFEMP2 was amplified from human 293T cDNA library. EFEMP2 and GALNT14 were subcloned into pET-28c and pGEX-4T-1 to construct pET-28c-EFEMP2 and pGEX-4T-1-GALNT14, respectively. The correct recombinant constructs were confirmed by sequencing. Expressions of EFEMP2 and GALNT14 were induced by IPTG. Target proteins were purified from the induced supernatants by using Glutathione-Sepharose 4B beads (GE Healthcare) and Ni-NTA agarose (GE Healthcare) according to the manufacturer's instructions. Mix 1 μg GST-GALNT14 or GST protein with 1 μg His-EFEMP2 fusion protein, after incubation and washing, and the bound proteins were analyzed by 12 % SDS-PAGE followed by Western blotting using anti-His antibody. The experiments were repeated three times.

Co-Immunoprecipitation

Co-immunoprecipitation (co-IP) analysis of EFEMP2 and GALNT14 followed the method that was described in detail [16]. Briefly, cells were harvested and lysed, and the protein contents were quantified using BCA protein quantitative kit (Sigma Aldrich). Fifty-microliter cell lysates were analyzed by Western blotting to quantify the expression level of the protein studied. EFEMP2 was precipitated by anti-EFEMP2 antibody (Santa Cruz), and using anti-GALNT14 antibody (Abcam) for Western blotting. The result was visualized by an enhanced chemiluminescence kit (Thermo Scientific).

Western Blot Analysis

For extraction of total protein, cells from two breast cancer cell lines, MCF7 and MBA-MD-231, were harvested and lysed in NP-40 lysis buffer. Extracted protein samples were measured by BCA protein assay (Pierce Chemical Co., Rockford, IL). Proteins were separated using SDS-PAGE and transferred onto nitrocellulose membrane for antibody detection. Antibodies against EFEMP-2 and GAPDH were obtained from Santa Cruz (USA).

Lectin Blot

Total protein was extracted from MCF-7 or MBA-MD-231 cells or MCF-7 or MBA-MD-231 cells transfected with shRNA targeting GALNT14 (shRNA-T14) or control shRNA (shNC). EFEMP-2 protein also was immunoprecipitated from MCF-7 or MBA-MD-231 cells and subjected to Vicia Villosa Lectin (VVL) blot. After being separated by 10% SDS-PAGE, proteins were transferred to NC membrane, and NC membrane was blocked in blocking solution (5% BSA in TBST). Membrane was incubated with a 0.2-μg/mL biotinylated VVL solution (B-1235, VECTOR Laboratories) and then membrane was incubated in secondary antibody: Streptavidin HRP conjugate (S911, Thermo Scientific). The result was visualized by an enhanced chemiluminescence kit (Thermo Scientific).

Cell Invasion Assay

The membrane inner surface of the Transwell chamber was coated with Matrigel, 1 × 10⁵ MCF-7 or MBA-MD-231 cells transfected with pCMV-Myc-EFEMP2 or pCMV-Myc empty plasmid, or co-transfected with shRNA-GALNT14 or shRNA-NC respectively. The cells were resuspended in 200 μl serum-free medium and added to the upper chamber, while 600 μl DMEM containing 20% fetal bovine serum was added to the lower chamber. After culturing for 24 hours, the cells were fixed with 4% paraformaldehyde in PBS buffer and

stained with 0.1% crystal violet to observe tumor cell infiltration across the membrane in order to determine the changes in the *in vitro* invasion of tumor cells. The migrated cells were photographed and counted at 200× magnification in 5 different fields per filter. The experiments were performed in triplicates.

Statistical Analysis

Data of cell invasion assay were analyzed using SPSS 19.0 software. $P < 0.05$ was considered as statistically significant. All experiments were independently repeated at least 3 times.

Results

Yeast Two-Hybrid Screening of GALNT14

To identify novel partners of GALNT14, we performed yeast two-hybrid screening. Using a human mammary gland cDNA library, total of $\sim 5 \times 10^6$ transfectants were screened. Four positive colonies were selected. The plasmids were extracted from these colonies, amplified in *E. coli*, and reintroduced into yeast cells to confirm the interaction. All of four colonies were shown to be positive clones. Subsequently, these positive clones were sequenced (Table 1). The strongest positive clone encodes partial EFEMP2 (161-443a.a.), and we further characterized it.

GALNT14 Interacts with EFEMP2 In Vitro and In Vivo

To further confirm the direct interaction between GALNT14 and EFEMP2, we generated the recombinant GST-GALNT14 and His-EFEMP-2, incubated these two proteins together, and performed a GST pull-down assay. We have also included GST as a negative control and found that His-EFEMP2 protein was bound to GST-GALNT14 but not GST (Figure 1A), which confirms the interaction results obtained from the yeast two-hybrid screening.

To verify the interaction of GALNT14 with EFEMP2 *in vivo*, we next performed co-IP assay in MCF-7 cells. The protein extracts were immunoprecipitated with protein A/G beads coupled to an anti-GALNT14 antibody. The immunoprecipitated complex was analyzed by Western blotting using an anti-EFEMP2 antibody. Western blot analyses show that EFEMP2 is weakly associated with GALNT14 (Figure 1B).

Since GALNT14 is a galactosyltransferase, the weak but direct interaction between GALNT14 and EFEMP2 indicates that EFEMP2 may be a substrate of GALNT14. To test the hypothesis, we first performed Lectin blot to examine the level of overall GalNAc conjugate proteins in MCF-7 and MBA-MD-231 cells. We found that there was an obvious decrease of GalNAc conjugating proteins in the GALNT14 knockdown MBA-MD-231 cells detected by VVL blot (Figure 2A). In addition, some specific protein bands detected by VVL blot were also decreased in the cell lysates of GALNT14 knockdown MCF-7. We further examined the glycosylation status of EFEMP-2 in MCF-7 and MBA-MD-231 cells when GALNT14 was

upregulated or downregulated. Interestingly, in the presence of equal amount of EFEMP2, O-glycosylation of EFEMP-2 was reduced when GALNT14 was knockdown, while O-glycosylation of EFEMP-2 was increased when GALNT14 was overexpressed (Figure 2B). Thus, the results indicate that GALNT14 mediates the O-glycosylation of EFEMP-2.

GALNT14 Regulates the Stability of EFEMP2

When the protein loading was normalized by GAPDH, we found that the protein level of EFEMP-2 was reduced when GALNT14 was knocked down. In contrast, EFEMP-2 was upregulated when GALNT14 was overexpressed (Figure 3A). It indicates that GALNT14-dependent O-glycosylation may regulate the stability of EFEMP-2. It has been shown that O-glycosylation plays an important role in maintaining protein stability [8]. Thus, we investigated the effect of O-glycosylation on the stabilization of EFEMP-2 protein. After treatment with a protein synthesis inhibitor, cycloheximide (CHX), we examined the stability of EFEMP-2 protein at different time points. As shown in Figure 3B, we found that EFEMP-2 protein was more stable in GALNT14 overexpressing MCF-7 cells than the control cells. However, Western blot analysis after CHX treatment of MCF-7 cell lines with GALNT14 knockdown showed rapid decrease of EFEMP-2 protein. Taken together, these results have suggested that O-glycosylation by GALNT14 is likely to be important for stabilization of the EFEMP-2 protein.

GALNT14 Knockdown Reduces EFEMP2-Induced Cell Invasion

Protein O-glycosylation is associated with cancer cell invasion. To evaluate the biological function of GALNT14-dependent regulation of EFEMP2 in breast cancer cells invasion, we performed Matrigel-coated Transwell invasion assay in MCF-7 and MBA-MD-231 cells. Representative micrographs were taken from the lower surface of the Transwell filter, and the migrated cells were counted. As shown in Figure 4, cells with ectopic-expressing EFEMP2 exhibited a higher invasive ability than mock-transfected control cells, indicating that EFEMP2 mediates breast cancer cell invasion. Moreover, when we knocked down endogenous GALNT14 by shRNA, EFEMP2-mediated breast cancer cell invasion was clearly suppressed, suggesting that GALNT14 regulates EFEMP2-mediated breast cancer cell invasion.

Discussion

Tumor cells display a wide range of glycosylation alterations compared with their nontransformed counterparts. Protein glycosylation increases molecular heterogeneity as well as the functional diversity within cell populations. This heterogeneity occurs because aberrant glycan modifications are protein specific, site specific (different sites on a given protein can be differentially glycosylated), and cell specific. In general, a shift from the normal glycosylation pathway occurs in cancer cells, leading to altered glycans expression due to under- or overexpression of glycosyltransferases (owing to dysregulation at the transcriptional level) [17,18].

In our previous work, we identified GALNT14 as a potential biomarker for breast cancer by immunohistochemistry [14]. We also found that GALNT14 played a critical role in cell migration, invasion, and proliferation of breast cancer by promoting the epithelial-mesenchymal transition of breast cancer cells. Since

Table 1. Yeast Two-Hybrid Screening from Human Mammary Gland cDNA Library Using GALNT14 as a Bait

Positive Clones	Residues (a.a.)	β -gal Assay
1. EFEMP-2	161-443	+++
2. MT2A	1-61	++
3. ALDH2	346-517	+
4. C1QC	27-245	+

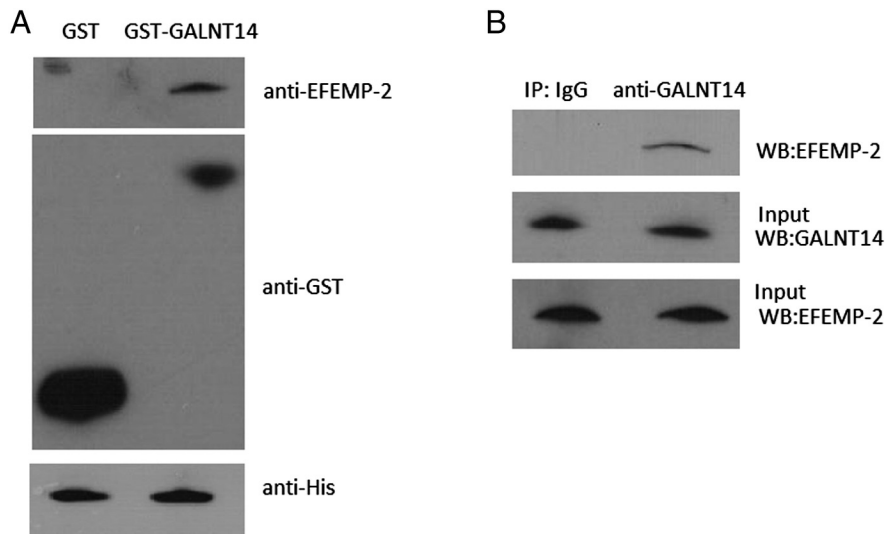


Figure 1. GALNT14 interacts with EFEMP2 both *in vitro* and *in vivo*. (A) GST pull-down assay demonstrating the binding between GALNT14 and EFEMP2 *in vitro*. GST-GALNT14 fusion protein (1.0 μ g) was mixed with purified His-EFEMP2 (1.0 μ g) and precipitated with glutathione-agarose followed by Western blot analysis. (B) GALNT14 co-IP with EFEMP2 in breast cancer cells MCF-7. Cell extracts were IPed with anti-EFEMP2 antibodies. Western blots were performed with indicating antibodies.

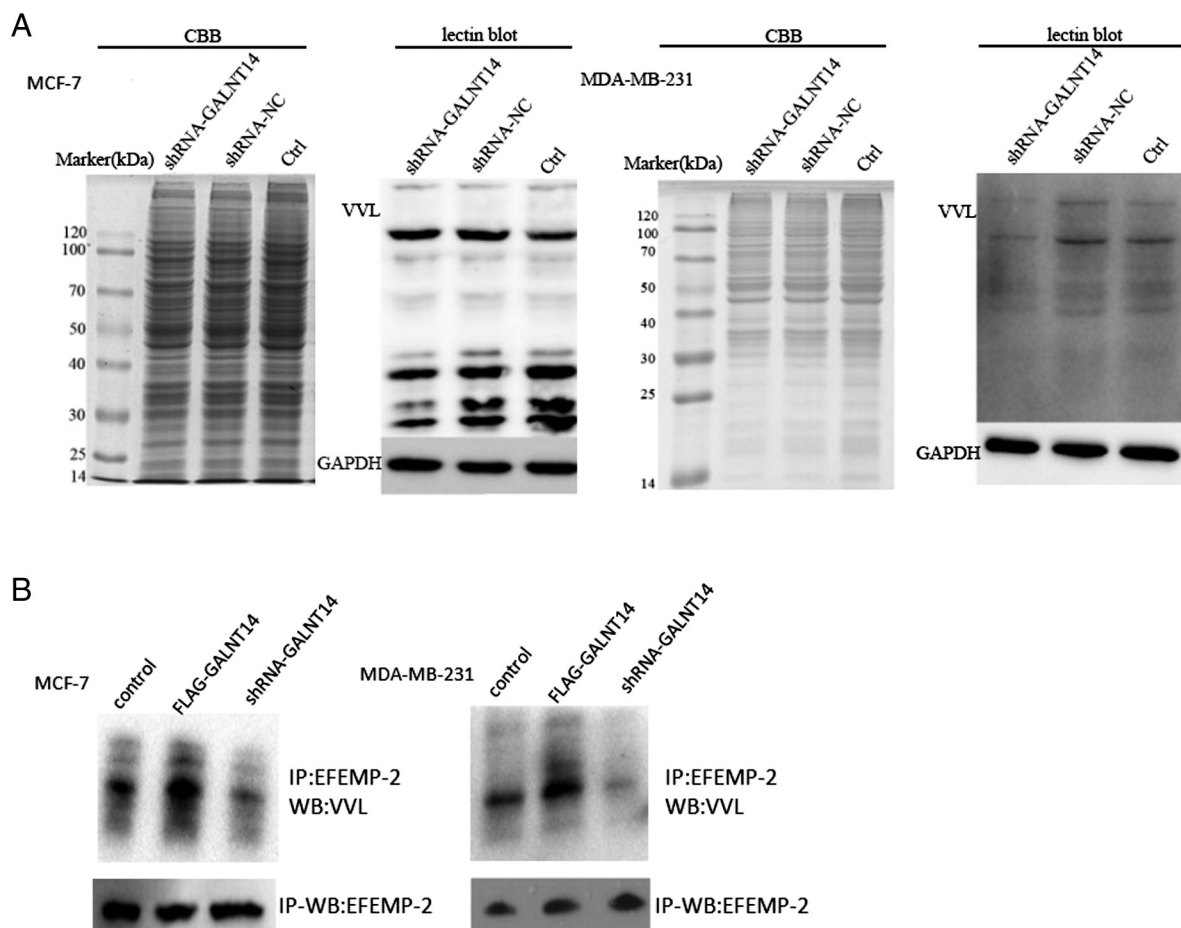


Figure 2. GALNT14 regulates the O-glycosylation of EFEMP-2. (A) The total O-glycosylation in GALNT14 knockdown (shRNA-GALNT14) MCF-7 and MBA-MD-231 cells was examined by VVL blotting. The cell lysate protein was stained using Coomassie blue. (B) The O-glycosylation of EFEMP-2 protein was examined in GALNT14 overexpression (FLAG-GALNT14) or knockdown (shRNA-GALNT14) MCF-7 and MBA-MD-231 cells by IP with anti-EFEMP2 and lectin blot.

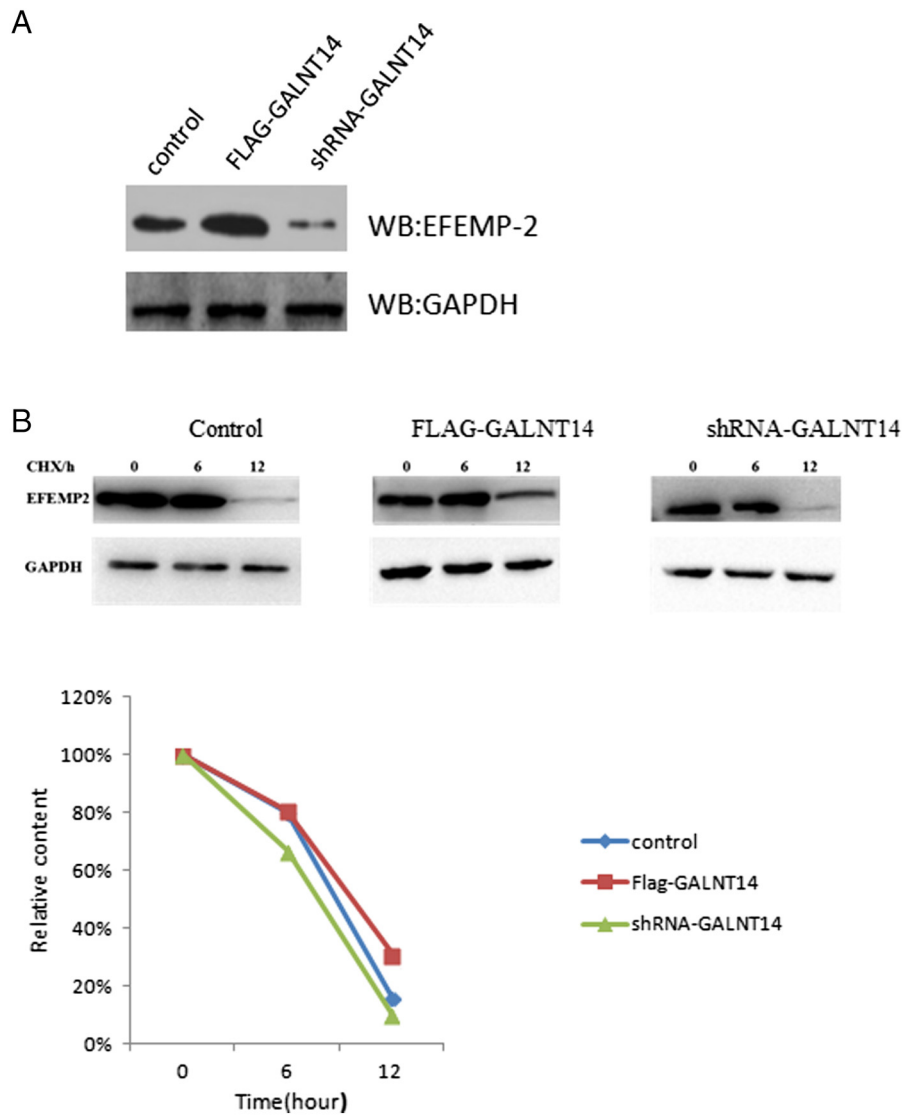


Figure 3. GALNT14 regulates the stability of EFEMP-2. (A) MCF7 cells were transfected with vectors encoding FLAG-GALNT14 or shRNA-GALNT14. The protein level of EFEMP-2 was examined by Western blot. GAPDH was used as the protein loading controls. (B) MCF7 cells were treated with 100 $\mu\text{g}/\text{ml}$ CHX for indicated hours. The level of EFEMP-2 was examined and illustrated in the graph (the lower panel).

overexpression of GALNT14 might contribute to mammary carcinogenesis, it would be valuable for development of novel therapeutic target against breast cancer [15]. Here, with unbiased approach, we found that GALNT14 mediated the O-glycosylation of EFEMP-2. EFEMP2 is a member of Fibulin family. Fibulin protein family is a family of glycoproteins consisting of a group of homologous proteins and facilitates cell motility [19,20]. Similar to other members in this family, EFEMP-2 facilitates breast cancer invasion. This activity is enhanced by GALNT14-dependent glycosylation. As the glycosylation stabilizes EFEMP-2, it prolongs the oncogenic functions in cancer cells. A previous study suggested that EFEMP-2 has two consensus sites for N-glycosylation, with Asn198 and Asn394, and a putative O-glycosylation site within the N-terminal end of human EFEMP-2 [16]. However, due to the limitation of the detection of O-glycosylation, we currently cannot map the O-glycosylation site. Future study may reveal the functional significance of each glycosylation event on EFEMP-2. Nevertheless, to the best of our knowledge, we demonstrate for the first time that GALNT14 O-glycosylates EFEMP-2, which may add

a new insight for the function of EFEMP-2 protein in breast cancer cell invasion.

Conclusions

In summary, we find EFEMP2 as a novel partner of GALNT14. We demonstrate that GALNT14 mediates the O-glycosylation of EFEMP2, which facilitates breast cancer invasion. Thus, our study might reveal GALNT14 as a novel target for clinical treatment of breast cancer.

Acknowledgments

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Author Contributions

Wu C. and Yu X. conceived and designed the study. Zuo T., Shan J. S., Liu Y., Xie R., Wang X., and Xiao B. H. performed the

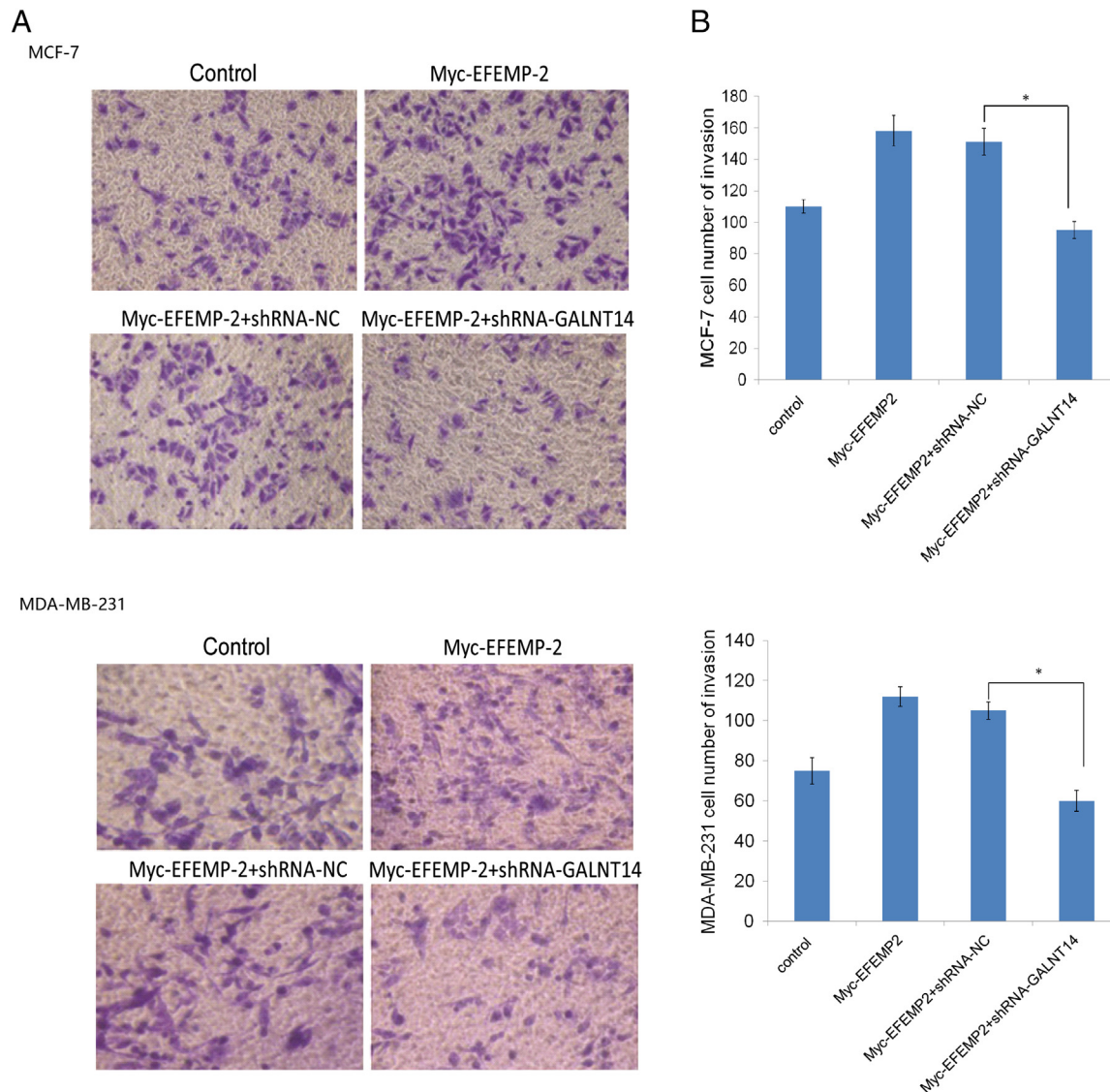


Figure 4. EFEMP-2 regulates breast cancer cell invasion via GALNT14. A total of 1×10^5 cells were plated onto the upper part of the Transwell chamber. After incubation for 24 hours, invasive cells were stained and counted at the lower part of transwell filter. (A) Photomicrographs obtained at 200 \times magnification. (B) Transmigration cells were counted for each of the indicated cells. The graph showed the mean and standard deviation of three separate experiments. *: $P < 0.05$.

experiments and collected the data. Liu Y., Liu C., and Wu C. C. analyzed the data. Zuo T., Yu X., and Wu C. wrote the manuscript. All authors read and accepted the final version of the manuscript submitted for publication.

Competing Interests

The authors declare that they have no competing interests.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tranon.2018.01.021>.

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