Gene 578 (2016) 232–241

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

# Increased fucosylation has a pivotal role in multidrug resistance of breast cancer cells through miR-224-3p targeting FUT4

Xiaobin Feng [a,1], Lifen Zhao [a,1], Shuhang Gao [b], Xiaobo Song [c], Weijie Dong [d], Yongfu Zhao [e], Huimin Zhou [f], Lei Cheng [g], Xiaolong Miao [e], Li Jia [a,*]

[a] College of Laboratory Medicine, Dalian Medical University, Dalian 116044, Liaoning Province, China
[b] Department of Oncology, The First Affiliated Hospital of Dalian Medical University, Dalian 116011, Liaoning Province, China
[c] Department of Medical Biology, Faculty of Health Sciences, University of Tromsø, Tromsø, Norway
[d] Department of Biochemistry, Dalian Medical University, Dalian 116044, Liaoning Province, China
[e] Department of General Surgery, The Second Affiliated Hospital of Dalian Medical University, Dalian 116027, Liaoning Province, China
[f] Department of Microbiology, Dalian Medical University, Dalian 116044, Liaoning Province, China
[g] Department of Laparoscopic Surgery, The First Affiliated Hospital of Dalian Medical University, Dalian 116011, Liaoning Province, China

ARTICLE INFO

*Article history:*
Received 23 October 2015
Received in revised form 8 December 2015
Accepted 12 December 2015
Available online 14 December 2015

*Keywords:*
Fucosylated N-glycan
Fucosyltransferase
Multidrug resistance
Human breast cancer cell lines
miR-224-3p

ABSTRACT

Fucosylation is the final step in the glycosylation machinery, which produces glycans involved in tumor multidrug resistance development. MicroRNAs (miRNAs) are endogenous negative regulators of gene expression and have been implicated in most cellular processes of tumors, including drug resistance. This study was undertaken to determine the roles of fucosylation and miR-224-3p in multidrug resistance of human breast cancer cell lines. Comparative analysis revealed differential modification patterns of fucosylation of the fucosylated N-glycans in drug-resistant T47D/ADR cells and sensitive line T47D cells. The expressional profiles of fucosyltransferase genes in two pairs of parental and chemoresistant human breast cancer cell lines showed that FUT4 was up-regulated highly in MDR cell lines. Altered level of FUT4 affected the drug-resistant phenotype of T47D and T47D/ADR cells both in vitro and in vivo. By bioinformatics analysis, we identified FUT4 as one of the miR-224-3p-targeted genes. Further studies showed an inverse relationship between of FUT4 and miR-224-3p in parental and ADR-resistant breast cancer cells, wherein miR-224-3p was downregulated in resistant cells. 3′-UTR dual-luciferase reporter assay confirmed that miR-224-3p directly targeted 3′-untranslation region (3′-UTR) of FUT4 mRNA. In addition, miR-224-3p overexpression sensitized T47D/ADR cells to chemotherapeutics and reduced the growth rate of breast cancer xenografts in vivo. Our results indicate that FUT4 and miR-224-3p are crucial regulators of cancer response to chemotherapy, and may serve as therapeutic targets to reverse chemotherapy resistance in breast cancer.



## 1. Introduction

Breast cancer is the most common cancer in women and the second leading cause of cancer death after lung cancer (Bugano et al., 2008). Although the efficacy of initial treatment may be high, the multidrug resistance (MDR) can be developed during disease progression in 90% of breast cancer patients (Gottesman & Ling, 2006; Sims et al., 2013). Several mechanisms have been reported to activate MDR in such cancers, including reduced apoptosis, advanced DNA damage and repair, altered drug metabolism, mutation expression of drug targets, drug inactivation, or efflux of the drug from the cell (Szakacs et al., 2006). Since glycan alterations are associated with resistance to chemotherapy of cancer cells, researchers have focused on studying the role of glycosyltransferases as the new molecular targets for treating MDR cancer cells.

Cell-surface glycans involve in numerous physiological and pathological processes (Becker & Lowe, 2003; de Vries et al., 2001). Fucosyltransferases (FUTs) are the biosynthetic enzymes to catalyze the L-

*Abbreviations:* MDR, multidrug resistance; FUTs, fucosyltransferases; miRNAs, microRNAs; 3′-UTRs, 3′-untranslated regions; GDP-Fuc, guanosine diphosphate β-L-fucose; 5-Fu, fluorouracil; PNGaseF, enzyme peptide:N-glycosidase F enzyme; HLB, hydrophilic-lipophilic balance; PBS, phosphate buffered saline; Tween 20, polyoxyethylene sorbitan monolaurate; PBST, phosphate buffered, saline with Tween-20; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MS, mass spectrometric; ADR, adriamycin; VCR, vincristine; TAXEL, paclitaxel; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 2,5-DHB, 2,5-dihydroxybenzoic acid; CDS, coding sequence; GFP, green fluorescent protein; DMSO, dimethylsulfoxide; IHC, immunohistochemical; Puro, puromycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC50, half maximal inhibitory concentration; SD, standard deviation; shRNA, short hairpin RNA; qRT-PCR, real-time PCR; VP, etoposide phosphate; MRP-1, multidrug resistance associated protein; ER, estrogen receptor.

* Corresponding author at: College of Laboratory Medicine, Dalian Medical University, 9 Lvshunnan Road Xiduan, Dalian 116044, Liaoning Province, China.
*E-mail address:* jiali0386@sina.com (L. Jia).

[1] The two authors contributed equally to this work.

http://dx.doi.org/10.1016/j.gene.2015.12.028
0378-1119/

fucose transfer from the donor substrate guanosine diphosphate β-L-fucose (GDP-Fuc) to various sugar acceptor substrates, including oligosaccharides, glycoproteins and glycolipids (Oriol et al., 1999). According to the fucosylation site, FUTs are classified into α-1,2 (FUT1 and FUT2), α-1,3/4 (FUT3, FUT4, FUT5, FUT6, FUT7 and FUT9), α-1,6 (FUT8) and protein O-(POFUT1 and POFUT2) fucosyltransferases (Merino et al., 2012). Increased FUT1 and FUT2 expression in human ovarian carcinoma-derived RMG-I cells promoted cell proliferation and resistance against anticancer drugs, such as 5-FU and carboplatin (Iwamori et al., 2005). Recently, we reported that FUT4, FUT6 and FUT8 were useful markers for observing MDR in human hepatocellular carcinoma cell lines (Cheng et al., 2013). The altered levels of FUT4, FUT6 and FUT8 were responsible for changed drug-resistant phenotype of BEL7402 and BEL/FU cells both in vitro and in vivo (Cheng et al., 2013). Therefore, it appears that fucosylation is a frequent post-translational modification that can be found in MDR. The biologic functions of oligosaccharides, however, differ in various cancer types.

MicroRNAs (miRNAs) are small non-coding RNAs that repress the expression of target genes by inhibiting translation and/or stability of mRNAs (Bartel, 2004). Numerous miRNAs function as either tumor suppressors or oncogenes, and the aberrant miRNA expression is directly associated with cancer progression, metastasis and multidrug resistance, and observed in a wide variety of human malignancies (Di Leva & Croce, 2010). Recently, miRNAs have emerged as crucial mediators in regulating the cellular responses of breast cancer cells to chemotherapy. MiR-139-5p not only attenuated the development of breast cancer cells but also mediated drug-resistance by regulating the expression of the downstream target gene Notch (Zhang et al., 2015a). MiR-217 overexpression induced drug resistance and invasion of breast cancer cells by targeting PTEN signaling (Zhang et al., 2015b). MicroRNA-7 inhibited multiple oncogenic pathways and reversed trastuzumab resistance in breast cancer cells (Huynh & Jones, 2014). MiR-34a expression was downregulated in breast cancer MCF7/ADR cells compared with MCF7 cells. Ectopic miR34a expression reduced breast cancer stem cell properties and increased sensitivity to doxorubicin treatment by directly targeting NOTCH1 (Park et al., 2014). However, there is no report regarding the miR-224-3p on FUT-mediated breast cancer MDR.

To date, the regulatory role of FUTs on drug resistance of breast cancer remains complex and unclear. In this study, we compared human breast cells with their chemoresistant cell counterpart in terms of fucosylated glycans and FUT gene expression. In addition, the present study was to determine the association of miR-224-3p with FUT4 in human breast cancer so as to provide a better understanding of a mechanism underlying breast cancer MDR.

## 2. Materials and methods

### 2.1. Membrane protein extract and release of N-glycans from cell membrane proteins

Membrane protein extract was determined by using a CelLytic MEM Protein Extraction kit according to the manufacturer's protocol (Sigma, St. Louis, MO, USA). The membrane protein concentration was measured with a Micro BCA Protein Assay kit (PIERCE, Rockford, IL). For releasing of N-glycans, three 100 μg aliquots of lyophilized cell membrane proteins were first digested with trypsin (10 μg) and chymotrypsin (10 μg) dissolved in 25 mM ammonium bicarbonate (25 μl) at 37 °C for 18 h. The digest was left in a water bath (85 °C, 5 min) and after cooling N-linked oligosaccharides were released from peptides by treatment with PNGase F enzyme (2 μl; 6 U) at 37 °C (18 h) followed by Pronase digestion (10 μg) at 37 °C (8 h). During the incubation time, the reaction sample was mixed occasionally. The released N-glycans were purified using an Oasis HLB cartridge (60 mg/3 ml; Waters) and then were lyophilized.

### 2.2. Mass spectrometric analysis

Glycomic profiles were acquired on a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (Bruker Corp., Billerica, MA, USA). To increase sensitivity and provide more informative fragmentation, the released glycans were permethylated. 0.5 μl of permethylated glycans was spotted on a MALDI plate and allowed to dry in air. 0.5 μl of sodiated 2,5-dihydroxybenzoic acid (2,5-DHB) (10 mg/ml DHB in 50% acetonitrile with 100 mM sodium chloride) was added on top. MALDI-TOF experiments were performed with a 4800 Proteomics Analyzer (Applied Biosystems). All MS (mass spectrometric) spectra were obtained from $Na^+$ adductions.

### 2.3. Cell culture

Cell lines MCF-7 and T47D were obtained from the KeyGEN Company (Nanjing, China) and cultured in DMEM, supplied with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin–streptomycin (Gibco, Grand Island, NY, USA) at 37 °C in a humidified and 5% $CO_2$ incubator. Adriamycin (Sigma, St. Louis, MO, USA) was added to parental cell cultures in stepwise increasing concentrations from 1 mg/l to 5 mg/l for 2 months to develop an adriamycin-resistant (ADR) subline, named MCF-7/ADR and T47D/ADR, correspondingly. To maintain the resistant phenotype, MCF-7/ADR and T47D/ADR cells were kept in the medium containing 1 mg/l adriamycin (ADR) and were cultured in drug-free medium for 48 h before the experiments.

### 2.4. Real time PCR analysis

Total RNA was isolated from cell lines with the RNeasy Mini Kit (QIAGEN, Valencia, CA), and cDNA was synthesized using QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. FUT mRNAs were quantified by SYBR-Green-quantitative real-time PCR (Takara, Otsu, Shiga, Japan) and normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The sequences of the upstream and downstream primers are shown in Table 1. The expression level of the target genes was determined relatively to GAPDH and calculated as $2^{-(Ct_{\text{Target gene}} - Ct)_{\text{GAPDH}}}$.

The expression of miR-224-3p was determined by using mirVanaTM qRT-PCR microRNA Detection Kit according to the manufacturer's protocol (Ambion Inc., Austin, TX, USA) and was normalized with the $2^{-\Delta\Delta CT}$ method relative to U6-small nuclear RNA. All the PCR reactions were done in triplicates.

### 2.5. Western blot analysis

Whole cell proteins were electrophoresed under reducing conditions in 10% polyacrylamide gels. The separated proteins were transferred to a polyvinylidene difluoride membrane. After blocked with 5% skimmed milk in PBS containing 0.1% Tween 20 (PBST), the membrane was incubated with FUT4 (1/1000 diluted; Abcam, Cambridge, UK) and then with peroxidase-conjugated anti-rabbit IgG (1/10,000 diluted; GE Healthcare UK Ltd., Little Chalf-ont, UK). A GAPDH antibody (1/200 diluted; Santa Cruz Biotech) was used as a control. All bands were detected using ECL Western blot kit (Amersham Biosciences, UK), according to the manufacturer's instruction. The bands were analyzed with LabWorks (TM ver4.6, UVP, BioImaging systems).

### 2.6. Lentivirus production and infection

The FUT4 coding sequence (CDS) was obtained and inserted into the NotI and BamHI sites of the pGLV5/H1/GFP + Puro lentiviral plasmid, respectively. The FUT4 shRNA sequences were inserted into the BamHI and EcoRI sites of the pGLV3/H1/GFP + Puro lentiviral plasmid. Lentivirus plasmids were co-transfected with PG-P1-VSVG, PG-P2-REV and PG-P3-RRE plasmids into 293T cells (Invitrogen), and virus-