Suppression of FUT1/FUT4 expression by siRNA inhibits tumor growth

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Received 17 June 2007; received in revised form 10 October 2007; accepted 11 October 2007
Available online 24 October 2007

Abstract

Lewis Y (LeY) antigen is highly expressed in a variety of human carcinomas of epithelial cell origin. Recent studies suggest functional blockade of LeY may provide a novel therapeutic approach for the treatment of cancers. However, suppressing LeY expression by genetic manipulation and its impact on neoplastic cell proliferation has not been investigated. We report here that different fucosyltransferases (FUTs) were expressed with the greatest expression of fucosyltransferase I or IV (FUT1/4), the two key enzymes for the synthesis of LeY in human epidermoid carcinoma A431 cells. Knocking down FUT1/4 expression by short interfering RNA technique dramatically reduced the expression of FUT1/4 and LeY and inhibited cell proliferation through decreasing epidermal growth factor receptor (EGFR) signaling pathway. Treatment of A431 cells that were inoculated into the nude mice with FUT1 siRNA or FUT4 siRNA greatly impeded tumor growth. Suppressing FUT1/4 expression also blocked EGF-induced tyrosine phosphorylation of EGFR and mitogen-activated protein kinases. In conclusion, suppressing the expression of FUT1/4 by RNAi technology reduces the synthesis of LeY and inhibits cancer growth. It may serve as a potential methodology for the treatment of cancers that express LeY glycoconjugates.

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Keywords: Fucosyltransferase; siRNA; Tumor growth; Lewis antigen

1. Introduction

Lewis Y is carried by glycoconjugates (glycoproteins and glycolipids) on cell surface. It participates in a variety of physiological and pathological processes in euakaryotic organisms, including embryo implantation, infection, inflammation and cancer metastasis [1–4]. Elevated expression of LeY antigen has been found in 60–90% of the human carcinomas of epithelial cell origin, such as breast, pancreas, endometrium, ovary, colon, stomach, urothelium, lung and liver cancers[4–12], and correlates with the enhanced neoplastic cell proliferation, invasion, metastasis and poor prognosis [4,6,13,14]. Accumulated evidence suggests that LeY may not only be regarded as a crucial cancer-associated marker, but also as a potential target for the treatment of LeY-positive cancers.

LeY is a difucosylated oligosaccharide [Fucα1→2Galβ1→4(Fucα1→3)GlcNAcβ1→R] [15,16]. The two terminal fucosylation reactions of it are catalyzed by the specific fucosyltransferases (FUTs). FUTs are a group of fucosylation syntheses. By catalyzing the transfer of fucose (Fuc) residue from the donor substrate, GDP-Fuc, to the oligosaccharide acceptor in α1, 2-, α1, 3/4-, and α1, 6-linkage, FUTs promote the synthesis of difucosylated oligosaccharide chain of glycoconjugates [17–19]. The synthesis of LeY is mainly controlled by an α1, 2-fucosyltransferase I (FUT1), and an α1, 3-fucosyltransferase IV (FUT4) [20–23]. Based on type 2 [Galβ1→4GlcNAcβ1→R] epitope, LeY is synthesized by the addition of a Fuc residue to galactose (Gal) terminal with the α1, 2 linkage (H type 2 epitope) and a Fuc residue to N-acetylgalactosamine (N-GlcNAc) with the α1, 3 linkage [24–27].
It is well established that the alteration of cancer-associated carbohydrate antigens is greatly impacted by the aberrant control of different glycosyltransferases. FUT1 and FUT4 are overexpressed in some cancers, e.g., FUT1 in human and rat colon cancer [28–30], and FUT4 in human colon cancer [31], pancreatic cancer [32], gastric cancer [33] and lung adenocarcinoma [34]. The augmented LeY synthesis in cancers may be caused by the abnormal expression of FUT1/4. Transfection of mouse or human FUT1 gene to ovarian carcinoma-derived RMG-1 cells significantly elevates the expression level of LeY glycolipids, and stimulates the viability of the transfectants even in the presence of an anticancer drug [35]. The regulatory role of FUT4 gene expression has not been well elucidated. By developing a stable FUT4 gene overexpressing A431 cells, we have shown that FUT4 enhanced synthesis of LeY oligosaccharide which correlates with the increased cell proliferation [36].

The molecular mechanisms by which LeY causes the malignancy of cancer cells have not been completely understood. Glycosylation of epidermal growth factor receptor (EGFR) is important for the binding of EGFR with its ligand (EGFR) is important for the binding of EGFR with its ligand, and stimulated the viability of the transfectants even in the presence of an anticancer drug [35]. The regulatory role of FUT4 gene expression has not been well elucidated. By developing a stable FUT4 gene overexpressing A431 cells, we have shown that FUT4 enhanced synthesis of LeY oligosaccharide which correlates with the increased cell proliferation [36].

In this report, we developed RNA interference-mediated suppression of FUT1 (EC 2.4.1.69) and FUT4 (EC 2.4.1.152) expression, and evaluated their impact on LeY expression and tumor growth. We have also observed the effect of FUT1/4 siRNAs on EGFR/MAPK phosphorylation. These studies indicate that knocking down FUT1/4 expression in A431 cells decreases LeY expression, inhibits EGF-induced tyrosine phosphorylation of EGFR and MAPK, which leads to reduced cell proliferation and tumor growth. To our knowledge, it is the first time to study the role of genetic manipulation of LeY expression on tumor growth using both in vivo and in vitro models. It may contribute to the development of a novel therapeutic methodology to treat LeY-positive cancers.

2. Materials and methods

2.1. Cell culture

A431 cells obtained from the American Tissue Culture Collection (Manassas, VA) were maintained in DMEM/F12 (1:1, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 50 μg/ml streptomycin at 37 °C under 5% CO2 in humidified air.

2.2. Generation of FUT1/4 RNA interfering plasmids

siRNA oligomers were designed according to the sequence of human FUT1 gene (GenBank accession number: M35531) and human FUT4 gene (GenBank accession number: M55896). Using siRNA-designing program (Ambion Inc.), six potential siRNA oligomers of FUT1/4 with BamHII and HindIII restriction sites at the 5′ and 3′ ends were developed and commercially generated (Sangon Company, China). After being analyzed by BLAST search to ensure the accuracy of each oligomer, the efficiency of these siRNAs in knocking down the expression of FUT1 or FUT4 was tested by transfection of these siRNAs into A431 cells. Two siRNAs from each gene group provided the greatest decrease in FUT1 or FUT4 expression. The siRNA sequences were: FUT1-1 siRNA: 5′-GATCTTGGCGGTGTGGTTAATCAAGATCTGATTCAACCCGCCATTA-3′, 5′-AGCTTTAAGGCCTGTGCTCACTATCCTTCGAATTGAGCGAGGCCGTTTTCTTA-5′, 5′-AGCTTTAAGCTTGGCTCTCACTATCCTTCGAATTGAGCGAGGCCGTTTTCTTA-5′; FUT1-2 siRNA: 5′-GATCCAGATTGCGGTGTGGTTAATCAAGATCTGATTCAACCCGCCATTA-3′, 5′-AGCTTTAAGCTTGGCTCTCACTATCCTTCGAATTGAGCGAGGCCGTTTTCTTA-5′, 5′-AGCTTTAAGCTTGGCTCTCACTATCCTTCGAATTGAGCGAGGCCGTTTTCTTA-5′.

2.3. Transient transfection

A431 cells were trypsinized and seeded onto 6- or 96-well plates. When cells reached 80% confluence, FUT1-1 siRNA, FUT1-2 siRNA, FUT4-1 siRNA or FUT4-2 siRNA was transiently transfected into A431 cells using 400 μg of plasmid and 2 μl of Lipofectamine™ Reagent and Plus™ Reagent following the manufacturer’s instructions (Invitrogen). The transfection reagent was removed after 12 h and the cells were harvested after 48 h.

2.4. Semi-quantitative RT-PCR

Total RNA was extracted and purified from untransfected and transfected cells using Trizol reagent (Invitrogen). The cDNA was synthesized using RNA PCR Kit (AMV) Ver 3.0 (Takara). Primers used for detecting the expression profile of FUT genes in A431 cells were indicated in Table 1 [32]. PCR reactions were carried as follows: initial denaturation at 94 °C for 4 min, 30 cycles of 94 °C for 30 s, 55–57 °C for 50 s, 72 °C for 30 s, and a final extension for 10 min at 72 °C in a 50 μl reaction mixture containing 2 μl each cDNA, 0.2 μM each primer, 0.2 mM dNTP, and 2 U of Taq DNA polymerase.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Size of fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUT1</td>
<td>442 F: 5′-TGGCTTCTCCTGCTAGTCTGT-3′</td>
</tr>
<tr>
<td>FUT2</td>
<td>342 F: 5′-CTAGCGAAGATTTCAAGGCTGTAAGG-3′</td>
</tr>
<tr>
<td>FUT3</td>
<td>1086 F: 5′-ATGGATCCCCTGGGTGCAGCCAAG-3′</td>
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<tr>
<td>FUT4</td>
<td>456 F: 5′-CGGACGTCTTTGTGCCATCT-3′</td>
</tr>
<tr>
<td>FUT5</td>
<td>408 F: 5′-CTATGGTAGAAACCCGGCTATGCT-3′</td>
</tr>
<tr>
<td>FUT6</td>
<td>963 F: 5′-CTCGAGGAGGAGGAGG-3′</td>
</tr>
<tr>
<td>FUT7</td>
<td>497 F: 5′-CCACCTGGAGCCACACTCTCAAC-3′</td>
</tr>
<tr>
<td>FUT9</td>
<td>460 F: 5′-CTGATTTGGCCAGCTTTCTTGCTGCT-3′</td>
</tr>
<tr>
<td>PCNA</td>
<td>838 F: 5′-GCTCCATCCTCAAAAGAAGTGT-3′</td>
</tr>
</tbody>
</table>

Table 1

Primers used for RT-PCR in this study

F: forward primer; R: reverse primer.
After amplification, 5 μl of each reaction mixture was detected by 1% agarose gel electrophoresis, and the bands were then visualized by ethidium bromide staining, followed by analysis with Labworks 4.6 (UVP, Inc). Beta-actin expression was detected as the internal control, and the primers of proliferation cell nuclear antigen (PCNA) were used to detect the PCNA gene expression (Table 1).

2.5. Immunofluorescence staining

Forty-eight hours after transfection, the cells grown on the coverslips were fixed in 4% paraformaldehyde for 15 min after washing with PBS. In FUT1/4 assay, cells were permeabilized with 0.1% Triton-PBS for 10 min at room temperature. After blocking with complete serum for 2 h at 4 °C, goat anti-FUT1 antibody (1:100), goat anti-FUT4 antibody (1:100, Santa Cruz) or mouse anti-LeY antibody (BG-8, 1:100, Signet Laboratories) was applied to incubate with the slide overnight at 4 °C. Image was captured after incubation of cells with FITC-conjugated mouse anti-goat IgG (1:100) for FUT1/4 and PE-conjugated rat anti-mouse IgM (1:100, Santa Cruz) for LeY by 1 h using Olympus BX51 microscope (Japan).

2.6. Flow cytometry assay

Cells prepared as indicated above were gently trypsinized, and the single cell suspension was collected. After washing with PBS, 0.1% Triton-PBS was added and incubated for 10 min at 4 °C in FUT1/4 assay. The cells were then incubated with goat anti-FUT1 antibody (1:100), goat anti-FUT4 antibody (1:100, Santa Cruz) or mouse anti-LeY antibody (1:100, Santa Cruz) or mouse anti-FUT1 antibody (1:100, GlycoTech), anti-LeX antibody (1:100, GlycoTech), anti-sLeX antibody (1:100, GlycoTech), respectively, for 1 h at room temperature. Unbound antibodies were removed by washing with PBS for three times at room temperature. FITC-conjugated mouse anti-goat IgG (1:100) for FUT1/4 or PE-conjugated rat anti-mouse IgM (1:100) for LeY, H type 2, LeX and sLeX was then applied to incubate with the cells for 45 min at room temperature. The unbound secondary antibody was discarded and the cell mixture was adjusted to 500 μl with PBS for measurement in a FACSscan flow cytometer.

2.7. Cell proliferation assay

Cells (2 × 10^4 cells/well) were seeded onto each well of the 96-well plates in 140 μl complete medium for 4 h before starvation of serum. Twenty-four hours after starvation (cells reached to 80% confluence), transfection was performed as described above. After transfection was terminated, cells were washed with or without either mouse anti-LeY antibody (0.2 μg/well) or LeY-PAA–biotin (0.1 μg/well, GlycoTech) for 3 or 24 h at 37 °C. Twenty μl of 5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma) was added and incubated for 10 min at 4 °C. The cell suspension was collected. After washing with PBS, 0.1% Triton-PBS was applied to incubate with the cells for 45 min at room temperature. The slide overnight at 4 °C. Image was captured after incubation of cells with FITC-conjugated mouse anti-goat IgG (1:100) for FUT1/4 and PE-conjugated rat anti-mouse IgM (1:100) for LeY, H type 2, LeX and sLeX was then applied to incubate with the cells for 45 min at room temperature. The unbound secondary antibody was discarded and the cell mixture was adjusted to 500 μl with PBS for measurement in a FACSscan flow cytometer.

2.8. Western blotting

Total protein from the whole cell lysate was prepared and applied for Western blotting. In brief, cells prepared as indicated were washed, with PBS (pH 7.4) and incubated with lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM Na3VO4, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40) for 30 min on ice. Cell lysates were clarified by centrifugation at 9000 × g for 10 min, and the supernatant was collected. Protein concentration was determined with Coomassie Protein Assay Reagent (Bio-Rad) using bovine serum albumin as a standard. Total protein (8–40 μg) from the whole cell lysate was separated by 8–12% SDS-PAGE mini-gel. Proteins separated in the gel were transferred electrophoretically onto nitrocellulose membrane. After blocking with TTBS (50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween-20) containing 5% fat-free dry milk for 2 h, the membrane was incubated with specific monoclonal antibodies directed against PCNA (1:1000), EGFR (1:1000), phosphorylated EGFR (pEGFR) (1:500), phosphorylated ERK 1/2 (pERK1/2) (1:1000), or phosphorylated tyrosine (pTyr) (1:1000, Transduction Laboratories) overnight at 4 °C. Anti-β-actin antibody (Santa Cruz) was used to confirm the equal loading. After washing with TTBS three times, 5 min each, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse antibody (1:1000, Santa Cruz) for 1 h at 37 °C. The membrane was then developed using an enhanced chemiluminescence (ECL) detection system (Amersham).

2.9. Xenograft cancer model

Female nude mice (Balb/c nu/nu) were from Lab Animal Center in Dalian Medical University (PR China). All experimental procedures involving animals were conformed to the Experimental Animal Management Regulations of
The animals (35±2 days) were maintained under sterile conditions during the entire experimental period. On the 1st experimental day, A431 cells (2×10⁶) suspended in 0.2 ml PBS were injected into the right flank subcutaneously. On the next day, FUT1-1 siRNA, FUT4-1 siRNA or empty vector (150 μg DNA/mouse), and PBS were injected at the tumor cell inoculation sites. Treatment was repeated every 48 h. Tumor volume was measured by Vernier calipers every five days from the 5th day till the 25th day after tumor inoculation. The tumor volume was computed according to the formula (volume=length×width²×0.5). At the end of the experiment (the 25th day), the tumor mass was weighed.

2.10. Immunohistochemistry staining of LeY in xenograft tumors

Each excised tumor tissue was fixed in 10% formalin for 24 h, embedded in paraffin, and processed into 5 μm sections. For immunohistochemical analysis of LeY expression, slides were incubated overnight at 60 °C, deparaffinized, and rehydrated using standard techniques. Tissue slides were blocked for 1 h at 37 °C with complete serum, then incubated overnight at 4 °C with mouse anti-LeY antibody (1:100). LeY immunostaining was performed using avidin–biotin peroxidase complex kit (Santa Cruz). Image was captured using Olympus BX51 microscope.

2.11. Statistical analysis

All data were analyzed statistically by Student’s t test, with p<0.05 considered to be significant. Data were expressed as means±standard deviation.

3. Results

3.1. Expression profile of FUT genes in A431 cells and inhibition of FUT1/4 expression by their siRNAs

The expression profile of α1, 2- and α1, 3/4-FUT genes in A431 cells was detected by semi-quantitative RT-PCR. Results revealed that FUT1, FUT4, FUT5 and FUT9 were all strongly expressed with FUT1 and FUT4 exhibiting greatest expression (Fig. 1A, lanes 1, 4, 5 and 8), whereas the expression of FUT2, FUT3 and FUT6 was low (Fig. 1A, lanes 2, 3 and 6). FUT7 expression was not detectable (Fig. 1A, lane 7).

The acceptor of FUT5 is a sialyl α2, 3-GalNAc-R, and FUT9. Considering that the synthesis of LeY is mainly regulated by FUT1 and FUT4, siRNA plasmids designed to target FUT1 (FUT1-1 siRNA and FUT1-2 siRNA) and FUT4 (FUT4-1 siRNA and FUT4-2 siRNA) were developed. Semi-quantitative RT-PCR detection found that the expression of FUT1 (Fig. 1B,
lanes 2 and 3 of top row) and FUT4 (Fig. 1D, lanes 2 and 3 of top row) genes was dramatically decreased by their relative siRNAs in comparison to untransfected controls (top rows, lane 1 of B and D) \( (p<0.01) \). Relative density analysis of FUT1 (Fig. 1C) or FUT4 (Fig. 1E) gene expression vs. their internal control \( \beta\)-actin expression indicated FUT1 gene expression was decreased 4.46-fold with FUT1-1 siRNA and 6.06-fold with FUT1-2 siRNA; and that of FUT4 was reduced 4.31-fold with FUT4-1 siRNA and 5.88-fold with FUT4-2 siRNA.

3.2. Transient transfection of siRNAs of FUT1/4 decreases the expression of both enzymes and LeY

To detect the protein expression level of FUT1/4 and LeY in the cells, immunofluorescence staining and flow cytometry were employed. Immunofluorescence staining revealed that the protein expression level of FUT1/4 (Fig. 2A) was much lower in siRNA-transfected A431 cells (b, c, e, and f) in comparison to untransfected controls (a and d). Results indicate that these siRNAs effectively interfere with the enzyme expression of both FUT1 and FUT4 in vitro. The inhibitory effect on LeY expression level was also seen as detected by immunofluorescence analysis. The fluorescence intensity of LeY in untransfected control cells (Fig. 2B, a) was much stronger than that in FUT1 (b and c) and FUT4 (e and f) knocking down cells.

Flow cytometry studies showed the protein level of FUT1 (Fig. 2C, a), FUT4 (Fig. 2D, a) and LeY (b of C and D) was significantly decreased in siRNA transfected A431 cells in comparison to untransfected controls (Fig. 2C, D). No significant changes of H type 2 (Fig. 2C and D, c) and LeX (not shown) was seen in cells with FUT1 and FUT4 knockdown. No alterations in the sLeX expression were detected in cells with FUT1 and FUT4 knockdown. No alterations in the sLeX expression were detected in FUT1 or FUT4 siRNAs transfected cells (data not shown). These results suggest that siRNAs of FUT1 and FUT4 strongly silence the expression of their target enzymes and significantly prevent the synthesis of LeY antigen.

3.3. Knocking down FUT1/4 expression inhibits cell proliferation

To study the impact of down-regulating FUT1/4 expression on cell proliferation, MTT assay was employed. The number of proliferated cells was determined by measuring the absorbance at a wavelength of 490 nm. Cell proliferation in FUT1 knocking down cells was reduced 1.95–2.02-fold (Fig. 3A, lanes 4 and 6), and 1.55–1.58-fold in FUT4 knocking down cells (Fig. 3A, lanes 8 and 10) in comparison to the untransfected control cells (Fig. 3A, lane 1). No significant difference in the proliferation rate was seen between the empty vector-transfected and untransfected cells (Fig. 3A, lane 2 vs. 1). The addition of anti-LeY antibody exerted the inhibitory effect on cell proliferation.
(Fig. 3A, lane 3), and LeY oligosaccharide added to the FUT1 or FUT4 knocking down cells partially reversed the inhibitory effect of these siRNAs on cell proliferation (Fig. 3A, lane 5 vs. 4, 7 vs. 6, 9 vs. 8 and 11 vs. 10). This indicates that silencing of FUT1/4 may prevent cell proliferation by downregulating LeY expression.

PCNA is a commonly used marker to detect cell proliferation. The difference in PCNA expression among these cells prepared as indicated above was measured by semi-quantitative RT-PCR (Fig. 3B) and Western blotting (Fig. 3C). Consistent with the results obtained from MTT assay, expression of PCNA gene (B) and protein (C) was significantly decreased in the cells transfected with siRNAs of FUT1 or FUT4. The expression of PCNA gene was decreased 3.07–3.08-fold (Fig. 3B, lanes 2 and 3 vs. 1 in a, lanes 2 and 3 vs. 1 in b) in FUT1 knockdown cells, and 3.63–3.75-fold in cells when FUT4 was suppressed (Fig. 3B, lanes 5 and 6 vs. 4 in a, lanes 2 and 3 vs. 1 in c). The protein expression level of PCNA was decreased correspondingly in the cells with reduced FUT1 (Fig. 3C, lanes 2 and 3 vs. 1) or FUT4 (Fig. 3C, lanes 5 and 6 vs. 4).

3.4. Knocking down FUT1/4 decreases the growth of A431 xenograft tumor in nude mice

siRNA plasmids directed against FUT1 or FUT4 down-regulated the synthesis of LeY and inhibited cell proliferation in

Fig. 4. Knocking down the expression of FUT1-1/FUT4-1 decreases xenograft A431 tumor growth in nude mice. A431 cells xenograft nude mice were injected with DNAs of FUT1-1 siRNA, FUT4-1 siRNA, empty vector, or PBS (control). The animal assay was carried out for 25 days. (A) Display of sacrificed nude mice with xenograft tumor on day 25. Arrows indicate the location of tumors; Tumor volume (B) and tumor mass curves (C) were generated with data from 6–8 mice in different groups; (D) Immunohistochemistry staining of LeY expression in excised xenograft tumors. Arrows indicate the expression of LeY in different groups. Magnification, ×200. Control, Vector, FUT1-1 siRNA or FUT4-1 siRNA represents tumor sample from PBS, empty vector, FUT1-1 siRNA or FUT4-1 siRNA treated mice, respectively.
vitro. To further investigate the inhibitory effect of these siRNAs on tumor growth in vivo and the impact of them on LeY expression in tumors, A431 cells were inoculated into the nude mice. After local injection of siRNA (FUT1-1 siRNA, FUT4-1 siRNA), empty vector or PBS (control) every other day for 25 days at the A431 cells-inoculated site, tumor volume and tumor weight were measured and recorded. Both FUT1-1 siRNA and FUT4-1 siRNA greatly impeded tumor growth (Fig. 4A–C). At the end point, compared to the PBS or empty vector-treated group, FUT1-1 siRNA reduced tumor volume and tumor mass 1.95-fold (B) and 1.82-fold (C), respectively; FUT4-1 siRNA reduced tumor volume and tumor mass by 1.72-fold (B) and 1.89-fold (C), respectively (p < 0.01).

The expression of LeY in the excised tumor tissues was detected by immunohistochemistry staining. Four tumor samples were randomly selected from 4 experimental groups and applied for immunohistochemical analysis using anti-LeY antibody. Results showed that LeY expression level was decreased in tumors treated with siRNA plasmids directed against either FUT1 (Fig. 4D, c) or FUT4 (d) in comparison to that in PBS-treated- or empty vector-treated ones (a and b).

3.5. Knocking down FUT1/4 expression decreases the tyrosine phosphorylation of EGF-mediated EGFR/MAPK

To study whether the decreased FUT1/4 expression affects EGFR/MAPK signaling pathway, tyrosine phosphorylation of EGFR and ERK1/2 was assessed by Western blotting. After stimulation with EGF (100 ng/ml) for 10 min, antibodies directed against EGFR, pEGFR and pERK1/2 were used to probe the cell lysate prepared as described above. As expected, the significant reduction in the phosphorylation of EGFR (Fig. 5, second row) and ERK1/2 (third row) was found in FUT1 (lanes 2 and 3 vs. 1) and FUT4 (lanes 5 and 6 vs. 4) knocking down cells. In addition, anti-pTyr antibody was selected to further assess the changes of phosphorylated tyrosine kinase (fourth row).

4. Discussion

Abnormal elevation in the expression of LeY are seen in many cancers and correlates with the malignancy of cancers. To develop the potential therapeutic approaches in the treatment of LeY-positive cancers, much attention has focused on blocking LeY function with anti-LeY antibodies [39–41], but little attention has been paid to generate a method by inhibiting the synthesis of LeY to prevent cancer growth.

LeY synthesis can be regulated at the transcriptional level by FUT1/4 genes [23,42]. The overexpression of FUT1/4 elevates the synthesis of LeY [35,22]. Among the eight α1, 2- and α1 3/4-FUT genes, FUT1 and FUT4 are predominant FUTs expressed in A431 cells. Although the strong expression of FUT5 and FUT9 was also seen in A431 cells, the acceptor of FUT5 is sialyl α2, 3-GalNAc-R which does not belong to type 2 epitope, and FUT9 exhibits more efficient activity for the synthesis of LeX [43]. We hypothesized that knocking down the expression of FUT1 and FUT4 genes would more specifically decrease LeY synthesis in A431 cells and inhibit tumor growth. In this study, we have developed siRNAs to specifically knock down the expression of FUT1/4 genes. Their impact on the synthesis of LeY and tumor growth was also studied. We found that the gene and protein expression levels of FUT1/4 were significantly reduced by the siRNA transfection. These siRNAs also showed significantly inhibitory effects on tumor growth both in vitro and in vivo models. These results indicate that inhibition of either FUT1 or FUT4 down-regulates the synthesis of LeY and decreases neoplastic cell proliferation and tumor growth.

The α1, 2-fucosylation and α1, 3-fucosylation are crucial for the synthesis of LeY which correlates to cancer malignancy. The expression of Fucα1→2Galβ1 motif is related to the cancer progression in colon [28,44]. Goupille et al. [45] found that transfection of rat colon carcinoma cells with the antisense oligonucleotides of FUT1 gene reduced the cancer malignancy in syngeneic animals. Our results showed that treatment with FUT1 RNAi plasmids decreased the synthesis of LeY and reduced neoplastic cell proliferation. Alpha 1, 3-fucosylation of LeY is mainly regulated by FUT4 [31]. Increased FUT4 has been found in two ovarian cell lines which highly express LeY and LeX [22]. The overexpression of FUT4 is associated with the progression of ovarian cancer. Both FUT4 and LeY are also increased during the early human embryo development [1,46], and the elevated LeY, a fetotumor, probably correlates with the overexpressed FUT4. Our data showed that FUT4 RNAi plasmids significantly decreased the synthesis of LeY. FUT4 has two different transcription initiation sites that produce long- and short-form mRNAs of FUT4, respectively. The expression level of long-form mRNA is higher than the short-form FUT4 genes. Our data showed that FUT4 RNAi inhibits the synthesis of LeY and decreases neoplastic cell proliferation and tumor growth.
expression of FUT1 or FUT4 was reduced. It is possible that FUT9 plays a more important role on LeX though FUT4 also affects the synthesis of LeX. FUT7 is responsible for synthesis of sLeX [18]. In our study, no expression of FUT7 was detected in A431 cells. In order to understand detailed mechanisms of regulation of fucosylated sugar chains by specific fucosyltransferases, further study needs to be explored.

LeY expression is related to the cancer growth and metastatic potential. The higher expression level of LeY is linked to the more progressive clinical grade of endometrial carcinoma [6]. Suppression of the specific fucosyltransferases by RNAi technique will not only help to elucidate the function of fucosylated antigens in carcinogenesis, but also provide a new approach for the treatment of cancers through inhibiting fucosylated antigen synthesis. We found that knockdown of FUT1/4 expression reduced the synthesis of LeY and inhibited cancer growth in both in vitro and in vivo models. Functional blockade of LeY antigen and injection of anti-LeY antibody into A431 xenograft nude mice induced the significant reduction of cancer weight and volume [39,40]. In this study, we found that directly interfering LeY synthesis by disrupting FUT1/4 gene expression with RNAi technique inhibited neoplastic cell proliferation and tumor growth. siRNA is a small molecule which is easier to access to its specific target cells in comparison with the antibody. It may also have fewer side effects than the treatment with an antibody. siRNA-mediated inhibition of LeY synthesis would be an effective novel approach for anticancer treatment.

LeY is mainly distributed on the plasma membrane of cancer cells [42], and carried by different glycolipids [47] and glycoproteins, such as CD44v6 (30), EGFR [48] and MUC6 [9,49], which are related to carcinogenesis. Using ABL364 and IGN311 (antibodies directed against LeY antigen), EGFR activation is prevented and neoplastic cell growth is inhibited [39,40]. The activation was also decreased by FUT1 siRNAs or FUT4 siRNAs, indicating that down-regulation of LeY may inhibit EGFR activation, consequently influencing EGFR/MAPK signaling pathway mediated cancer cell proliferation. The other LeY-containing glycoconjugates mentioned above may also be changed in FUT1/4 knocking down cells, and this issue deserves further investigation.

In addition to the stimulatory effect of LeY on EGFR/MAPK signaling and tumor growth, LeY may also function as an adhesive molecule in cancer cell adhesion and migration. We have previously shown that LeY was stage-specifically expressed during embryo development. Injection of anti-LeY antibody into the uterus of pregnant mouse or preincubation of the embryo with anti-LeY antibody impaired the implantation of embryo to the uterus. These treatments also reduced the secretion and expression of matrix metalloproteinase (MMPs) [1,24,50]. LeY expression level is increased in the angiogenesis-rich rheumatoid arthritis compared with that in nonangiogenic osteoarthritis. It suggests that LeY may serve as an angiogenic mediator [51]. Accumulated evidence suggests that LeY not only plays a role in the inflammatory angiogenesis, but also regulates the cancer angiogenesis and promotes tumor growth and metastasis. Therefore, it is important to inhibit LeY synthesis in anti-inflammation and anticancer treatment.

In summary, our current study demonstrates that FUT1/4 siRNA plasmids down-regulate LeY synthesis level, inhibit EGFR activation, and effectively reduce tumor growth. To our knowledge, this is the first report to study the suppression of the expression of LeY prevention of the tumor cell proliferation by knockdown of FUT1/4 expression with RNAi technique. Our results also implicate that the siRNAs of FUT1/4 could be a potential therapeutic agent for treatment of human LeY-expressing cancers.

Acknowledgments

Grant support: National Natural Science Foundation of China Research grants (30270329, 30670465 and 30672753) and Liaoning Provincial Core Lab of Glycobiology and Glycoengineering grant.

We thank Dr. Zhengmei Zhu for her skillful technical supports.

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