Lewis(y) antigen stimulates the growth of ovarian cancer cells via regulation of the epidermal growth factor receptor pathway

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Abstract. Lewis(y) antigen is an oligosaccharide containing two fucoses, and is expressed variously in 75% of ovarian tumors, where its high expression level predicts poor prognosis. The effect and the possible mechanism of Lewis(y) on the proliferation of human ovarian cancer cells are still largely unkown. We report here that transfecting α1,2-FT gene into RMG-I cells increased the expression of Lewis(y) and promoted cell proliferation. In α1,2-FT-transfected cells, the Lewis(y) content of EGFR was increased dramatically. Tyrosine phosphorylation of EGFR was elevated. Concomitantly, tyrosine phosphorylation of Akt, ERK1/2 was also upregulated. Moreover, the expression of HER2/neu mRNA and protein, the tyrosine phosphorylation of HER2/ neu were also elveated, while the expression of p27 was significantly reduced. However, the expression of EGFR and the relative content of Lewis(y) on HER2/neu were unchanged. The above-mentioned alterations were correlated with the Lewis(y) content of EGFR and α1,2-FT expression in cells. In addition, the phosphorylation intensity and difference in phosphorylation intensity between cells with different expression of α1,2-FT were attenuated significantly by the inhibitor of EGFR tyrosine kinase and by the mono-antibody to Lewis(y). Meanwhile, the reduction in p27 and the difference in its expression among the two cell lines were also blocked by the Lewis(y) antibody. The PI3K signaling pathway was more important than the MAPK pathway in the regulation of p27 expression. These findings provide strong evidence that increased expression of Lewis(y) promotes cell proliferation through regulating the phosphorylation and expression of some molecules involved in the EGFR/ PI3K-signaling pathway.

Introduction

The cell membrane of mammalian cells is composed of glycolipids, glycoproteins and proteoglycans, and these carbohydrate structures will undergo conformational change during cellular differentiation and transformation (1). One distinct characteristic of malignant tumor cells is the abnormality in the glycosylation of the cell membrane and one of the most commonly seen changes is observed in the ABH and Lewis related histo-blood group antigens (2,3). Fucose participates in the composition of various glycosidic chains. It is generally thought that the synthesis of a carbohydrate chain will terminate after the binding of fucosyl residue to its terminal, and thus, the bound fucosyl residue will participate in the composition of the carbohydrate structure of some essential growth factor receptors, to play an important role in carcinogenesis. Lewis(y) antigen is an oligosaccharide with two fucoses, and its chemical structure is Fucα1→2Galß1→4[Fucα1→3] GlcNAcß1→R, belonging to the Lewis related histo-blood group antigens family with specific fucosylation of the terminal end of carbohydrate structure catalyzed by the α1,2-fucosyltransferase (4,5). The expression of Lewis(y) antigen primarily occurs during the embryogenesis period, but its expression in adults is limited on the surface of granulocytes and epithelium (6). However, elevated expression of Lewis(y) has been found in the majority of carcinomas, including breast, ovary, and colon cancers. Lewis(y) expression is related to clinical degree and progression (7-9). The tumor marker CA_{125} contains the Lewis(y) structure (10), suggesting a correlation between the ovarian cancer and Lewis(y).

In our previous studies, the stable ovarian cancer cell line with high expression of Lewis(y), RMG-I-H, was established by gene transfection technique to introduce the gene of human α1,2-fucosyltransferase (α1,2-FT) into the ovarian cancer cell line RMG-I. Through the study of these cell line models, it was discovered that the levels of α1,2-FT gene and Lewis(y) antigen increased significantly after transfection. Also, the RMG-I-H cells become highly tolerant

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to the anti-tumor drugs, 5-FU, and carboplatin (11,12). It suggested that the Lewis(y) antigen possessed the function of boosting the survival ability of ovarian cancer cells.

The molecular mechanisms by which Lewis(y) causes the malignancy of cancer cells have not been completely understood. Studies found that treatment of the cells with an antibody directed against Lewis(y) blocks the activation of epidermal growth factor receptor (EGFR) and EGFR mediated mitogen-activated protein kinase (MAPK) signaling (13). Anti-Lewis(y) antibody binds to EGFR and inhibits cell proliferation (14). Studies suggests that glycosylation of EGFR is important for the binding of EGFR with its ligand (15,16). Glycosylation is important and the most common form of post-translational modification that regulates many aspects of protein function (17,18). In recent years, increased attention has been paid to the relationship between structural changes in surface glycans and surface receptor signaling. It has been reported (19) that overexpression of *N*-acetylglucosaminyltransferase(GnT)-III introducing a bisecting *N*-acetylglucosamine(GlcNAc) into the *N*-glycans of EGFR in U373 MG glioma cells led to decreased epidermal growth factor (EGF) binding and autophosphorylation of EGFR, as well as reduced cell proliferation upon EGF stimulation. However, the above-mentioned structural changes in receptor glycans are located in the core portion of *N*-glycan, and whether alteration of the terminal residue on the outer chain of either *N*- or *O*-glycan can also modify surface receptor signaling remains unclear.

In this report, the cell growth rate and the cell cycle were studied in the ovarian cancer cell line with or without transfection of α1,2-FT cDNA. Furthermore, the expression of some cell proliferation-related proteins, such as growth factor receptors, signaling pathways as well as cell cycle-related proteins was determined, to elucidate the molecular mechanism of Lewis(y) effects on cell growth. The results suggest that α1,2-FT up-regulates the expression of Lewis(y) on EGFR, then the overexpression of Lewis(y) increases the tyrosine phosphorylation of EGFR and promotes the signal transduction of growth factor into the cells mainly via PI3K/Akt signaling pathway, resulting in the accelerated gene transcription in nucleus and the inhibition of p27 expression, stimulating the synthesis of DNA, finally leading to the passage of the cells through G1 check-point to the S stage of cell cycle. This leads to inreased cell proliferation.

Materials and methods

Materials. The human ovarian cancer cell line, RMG-I, which was established from the tissues of human ovarian clear cell carcinoma, donated by Professor Iwamori Masao of Tokyo University of Japan. The following reagents were purchased from commercial sources: PI3K inhibitor LY294002 from Promega; MEK inhibitor PD98059 from Cell Signaling; EGFR tyrosine kinase inhibitor ZD1839 from AstraZeneca; DMEM and fetal bovine serum (FBS) from Hyclone; trypsin and ethylenediamine tetraacetic acid (EDTA) from Amresco; propidium iodide (PI) from Sigma; TRIzol, primers and reverse transcription polymerase chain reaction (RT-PCR) reagents from Takara; mouse anti-human Lewis(y) monoclonal antibody from Abcam; HRP-labeled

second antibodies and protein G plus-agarose from Santa Cruz. For immunoblot analysis, the following antibodies were used: HER2/neu, EGFR, p-EGFR, p-HER2/neu, p27 and ß-actin from Santa Cruz; Akt, p-Akt, ERK1/2 and p-ERK1/2 from Cell Signaling. Protein content in cell lysates was measured by the BCA method.

α1,2-FT transfected RMG-I cell line was established as previously reported (11), named as RMG-I-H.

Cell culture and treatment. Cells were cultured in DMEM supplemented with 10% FBS at 37˚C under 5% CO_2 in humidified air. For the treatment with EGFR tyrosine kinase inhibitor, specific inhibitors of cell signaling and anti-Lewis(y) antibody, the final concentration of ZD1839, LY294002, PD98059 and anti-Lewis(y) antibody were 5, 25, 50 μM and 20 μg/ml, respectively. The duration of treatment was 24 h.

Determination of cell growth. Cells growing at the logarithmic stage ($4x10^4$ cells/well) were plated to 24-well plates. The next day, three wells of each group were selected randomly on each day, to be counted and averaged for consecutive 7 days. The growth curve would then be plotted.

Analysis of the cell cycle. Cells were synchronized using the serum starvation (2% FBS in DMEM) method for 48 h, collected, washed and treated with EDTA and 75% ethanol at -20˚C for 2 h, then subjected to FACScan according to the previously reported method (20). The percentage of cells in different phases of the cell cycle was sorted using the ModFit program.

Determination of the expression of EGFR, HER2/neu mRNAs with semi-quantitative RT-PCR. Total RNA was extracted from the transfected and control cells using TRIzol reagent. The cDNA was synthesized using Takara RNA PCR Kit and was used as a template for PCR analysis. The following primers were used: EGFR forward, 5'-TGTGAGGTGGTCCTTGGGAATTTGG-3'; EGFR reverse, 5'-TGCTGACTATGTCCCGCCACTGGA-3' (fragment size, 339 bp); HER2/neu forward, 5'-CCTGCTGAACTGGTGTATGCA-3'; HER2/neu reverse, 5'-TCAGAGTCAATCATCCAACATTTG-3' (fragment size, 420 bp); ß-actin forward, 5'-GGACTTCGAGCAAGAGATGG-3'; ß-actin reverse, 5'-ACATCTGCTGGAAGGTGGAC-3 (fragment size, 404 bp). The PCR protocol for EGFR consisted of denaturation at 95˚C for 5 min, 30 cycles of denaturation at 94˚C for 1 min, annealing at 66˚C for 30 sec and extension at 72˚C for 30 sec, final extension at 72˚C for 10 min. The PCR protocol for HER2/neu consisted of denaturation at 94˚C for 5 min, 30 cycles of denaturation at 94˚C for 45 sec, annealing at 51˚C for 45 sec and extension at 72˚C for 45 sec, final extension at 72˚C for 10 min. After amplification, 10 μl of each reaction mixture was detected by 2% agarose gel electrophoresis.

Analysis of the proteins or phosphorylated proteins of EGFR, HER2/neu, Akt, ERK1/2 and p27 with Western immunoblotting. Cells were washed twice with ice-cold PBS, scraped in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP40, 100 mM NaF, 200 μM Na_3VO_4, and 10 μg/ml each aprotinin, leupeptin, PMSF, and pepstatin], and incubated
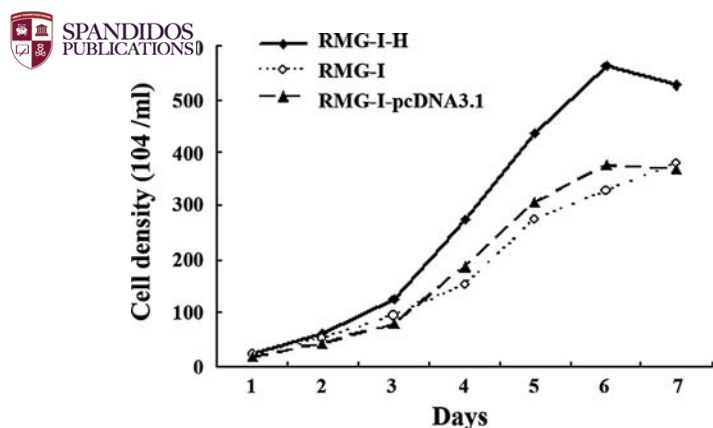
Figure 1. The growth curves of each group of cells before and after the transfection.

for 20 min at 4˚C while rocking. Lysates were cleared by centrifugation (15 min at 13,000 rpm, 4˚C). For immunoblot analysis, 75 μg of total protein were resolved by SDS-PAGE and transferred to poly(vinylidene difluoride) membranes. Membranes were blocked with TTBS [25 mM Tris-HCl, 150 mM NaCl (pH 7.5), and 0.1% Tween-20] containing 5% non-fat milk and incubated overnight at 4˚C with primary antibody in TBST/1% non-fat milk. Blots were washed in TTBS and incubated with the appropriate horseradish peroxidase-linked IgG, and immunoreactive proteins were visualized with ECL detection system.

Immunoprecipitation of EGFR and HER2/neu. Washed mono-layer cells were lyzed with 200 μl lysis buffer as described above. After protein determination, cell lysate containing 500 μg protein was incubated with 5 μg of one of the following antibodies (antibody to EGFR or HER2/neu), and incubated at 4˚C overnight. Protein G plus-agarose was added and the samples were incubated at 4˚C for 3 h for immunoprecipitation.

Analysis of Lewis(y) expression on EGFR and HER2/neu using Western immunoblotting. In brief, immunoprecipitated EGFR and HER2/neu were subjected to SDS-PAGE, then transferred to a poly(vinylidene difluoride) membrane and treated with 1:500 diluted anti-Lewis(y) and anti-EGFR or anti-HER2/neu sera in Tris-buffered saline with 5% fat-free dry milk, followed by 1:1000 HRP-labeled secondary

antibody. Finally, the color was developed with enhanced chemiluminescence reagents, and followed by densitometric scanning.

Statistical analysis. The SPSS 12.0 statistical analysis soft-ware was used, while the analysis of variance was employed. $p < 0.05$ was regarded as with statistical significance.

Results

Lewis(y) overexpression promotes cell proliferation. The cell growth curves plotted by the cell count method showed that the growth rate of the post-transfection cells, RMG-I-H, was much higher than the non-transfected group and the group of transfected vector alone ($p < 0.05$). Also, there was no significance difference between the RMG-I and RMG-I-pcDNA3.1 ($p > 0.05$) (Fig. 1).

The impact of Lewis(y) overexpression on cell proli-feration was further determined by cell cycle progression analysis. Flow cytometry with PI-stained cells showed that RMG-I and RMG-I-pcDNA3.1 were presented in G_0/G_1 (74.14±2.31%, 74.31±2.21%), S (23.95±2.66%, 23.96±1.52%), and G_2/M (2.05±0.166%, 2.91±0.77%) phases, respectively. While in RMG-I-H cells, S phase fraction was increased (33.27±3.59%, $p < 0.05$) and G_0/G_1 fraction was decreased (59.46±3.45%, $p < 0.05$) (Table I).

Expression of the mRNAs of EGFR, HER2/neu in α1,2-FT transfected cells. A semi-quantitative RT-PCR was adopted to analyze the expression of the mRNAs of EGFR, HER2/neu. Results showed the mRNA of HER2/neu was increased to 2.55-fold in RMG-I-H cells as compared with RMG-I cells ($p < 0.01$), while the mRNA of EGFR was unchanged in α1,2-FT transfected cells (Fig. 2). This finding suggests that the expression of EGFR, HER2/neu is mainly regulated at the transcription level.

Expression of cell EGFR, HER2/neu and their Lewis(y) in α1,2-FT transfected cells. Results from Western immuno-blotting indicated that the protein expression of HER2/neu in transfected cells was increased to 2.52-fold of the non-transfection level ($p < 0.01$), while the protein of EGFR was unchanged ($p > 0.05$) (Fig. 3A). This result was compatible with the increased mRNA of HER2/neu and the unaltered mRNA of EGFR as shown in Fig. 2. The expressions of Lewis(y) on EGFR and HER2/neu were observed after

Table I. Cell cycle of α1,2-FT transfectant and control cells (%, $\bar{x} \pm s$).

Group	Times	G_0/G_1	S	G_2/M
RMG-I	3	74.14±2.31	23.95±2.66	2.05±0.166
RMG-I-pcDNA3.1	3	74.31±2.21	23.96±1.52	2.91±0.77
RMG-I-H	3	59.46±3.45[a]	33.27±3.59[a]	7.32±0.44

[a]Comparison with the control group, RMG-I and RMG-I-pcDNA3.1, $p < 0.05$.

A

B
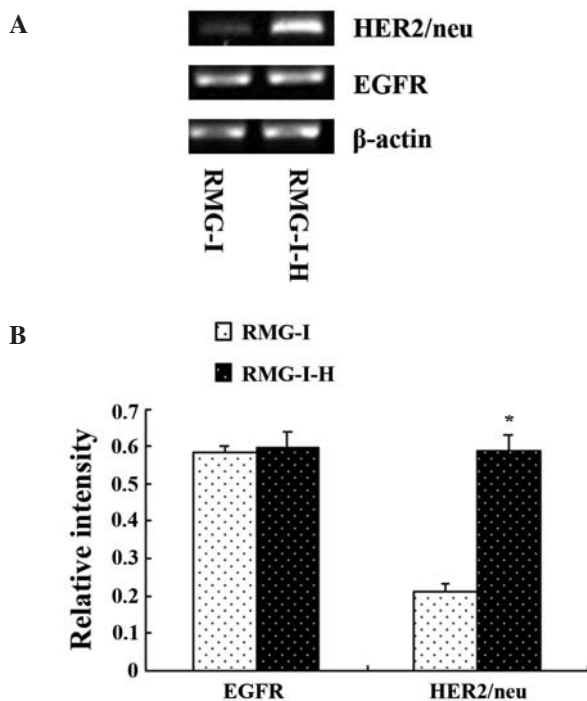

Figure 2. RT-PCR analysis of HER2/neu and EGFR after cells transfected with α1,2-FT gene. (A) RT-PCR profiles of HER2/neu and EGFR. (B) Densitometric quantification of RT-PCR profiles. The data were expressed as the intensity ratio of HER2/neu or EGFR to ß-actin (mean ± SD). *p<0.01 compared to RMG-I. 'A' is representative of three independent and reproducible experiments.

immunoprecipitation of these two proteins and Western immunoblotting with the monoclonal antibody against Lewis(y). It is of interest to find that the total amount of Lewis(y) on HER2/neu was increased in α1,2-FT transfected cells, up to 2.98-fold of the parental cells (p<0.01). However, their relative amount, calculated from the ratio of total Lewis(y) on HER2/neu to HER2/neu protein was unaltered, because the protein of HER2/neu was elevated in the same magnitude as total Lewis(y). However, both the total and relative amount of Lewis(y) on EGFR were all increased in α1,2-FT transfected cells (p<0.01) (Fig. 3B and C).

Tyrosine phosphorylation of EGFR, HER2/neu in α1,2-FT transfected cells. The relative intensity of tyrosine phosphorylation in total protein of EGFR or HER2/neu was calculated from the intensity ratio of the phosphorylated band to the unphosphorylated band. As shown in Fig. 4, the amount of total protein of EGFR was also unchanged following α1,2-FT transfection, and the tyrosine phosphorylation of EGFR was increased to 4.98-fold of the non-transfection value in α1,2-FT transfected cells (p<0.01). Meanwhile, the results in Fig. 4 also show that the HER2/neu protein was also increased in α1,2-FT transfected cells, when relative tyrosine phosphorylation was calculated as above, it was found that the level of phosphorylated HER2/neu was increased to 3.57-fold of the non-transfection value in α1,2-FT trans-fected cells (p<0.01).

Expression and phosphorylation of Akt, ERK1/2 in α1,2-FT transfected cells. The major pathways activated by EGFR
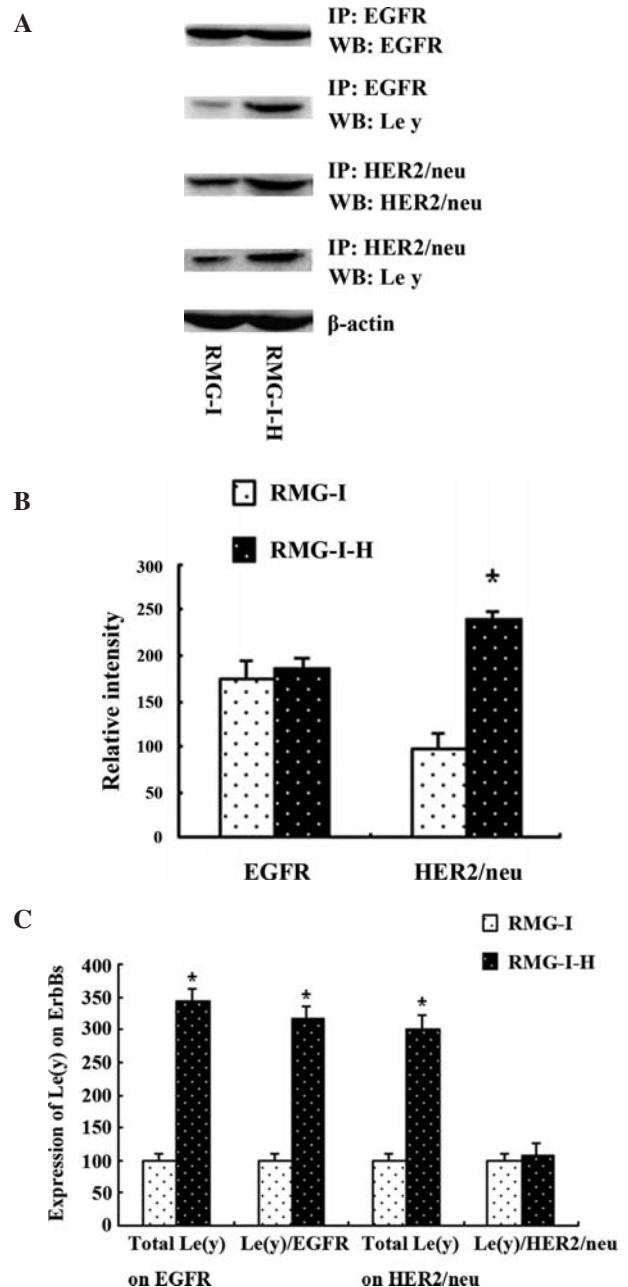
A

B

C

Figure 3. Effects of α1,2-FT transfection on expression of EGFR and HER2/neu and the Lewis(y) content of the glycans of EGFR and HER2/neu. (A) Western blot profiles of immunoprecipitated EGFR and HER2/neu proteins using corresponding antibodies and Lewis(y) antibody and HRP-labeled second antibodies. (B) Densitometric quantification of protein expression of EGFR and HER2/neu in (A). (C) Densitometric quantification of Lewis(y) in (A) and calculation of Lewis(y) expression/ErbBs (set the RMG-I cells as 100%) (n=3). *p<0.01 compared to RMG-I. Le y, Le(y), Lewis(y); IP, Immunoprecipitation by the antibody to EGFR and HER2/neu; WB, Western immunoblot with the antibodies to EGFR and HER2/neu or Lewis(y). 'A' is representative of three independent and reproducible experiments.

include Ras/MEK/MAPK; PI3K/Akt, both of which lead to the transcription of target genes that may contribute to ovarian cancer progression (21-23). As shown in Fig. 5, expression of Akt and ERK1/2 proteins was not obviously altered in α1,2-FT transfected cells, but the relative phos-phorylation of Akt (calculated from the ratio of the staining intensity of phosphorylated protein to unphosphorylated protein after normalization with ß-actin) was apparently
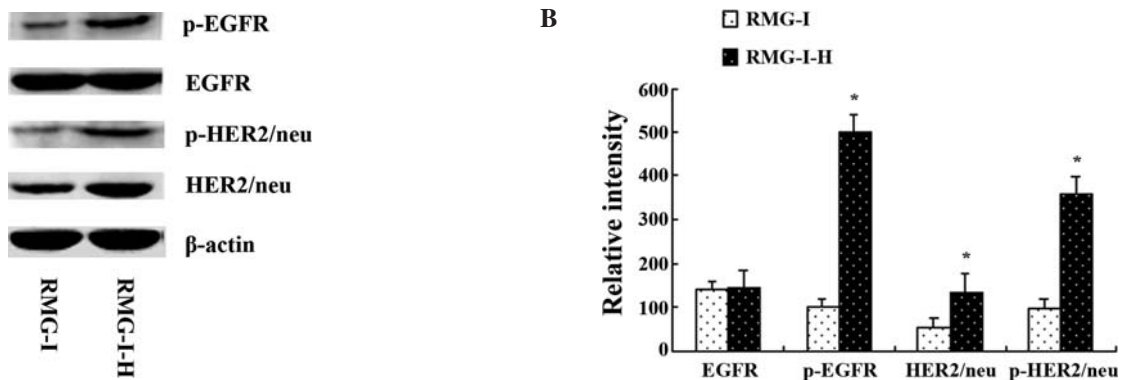
Figure 4. Effects of α1,2-FT transfection on the tyrosine phosphorylation of EGFR and HER2/neu. (A) Western blot profiles of p-EGFR, p-HER2/neu, EGFR and HER2/neu after staining with specific antibodies and HRP-labeled secondary antibody. (B) Densitometric quantification of protein expression of A (n=3). *p<0.01 compared to RMG-I. 'A' is representative of three independent and reproducible experiments.
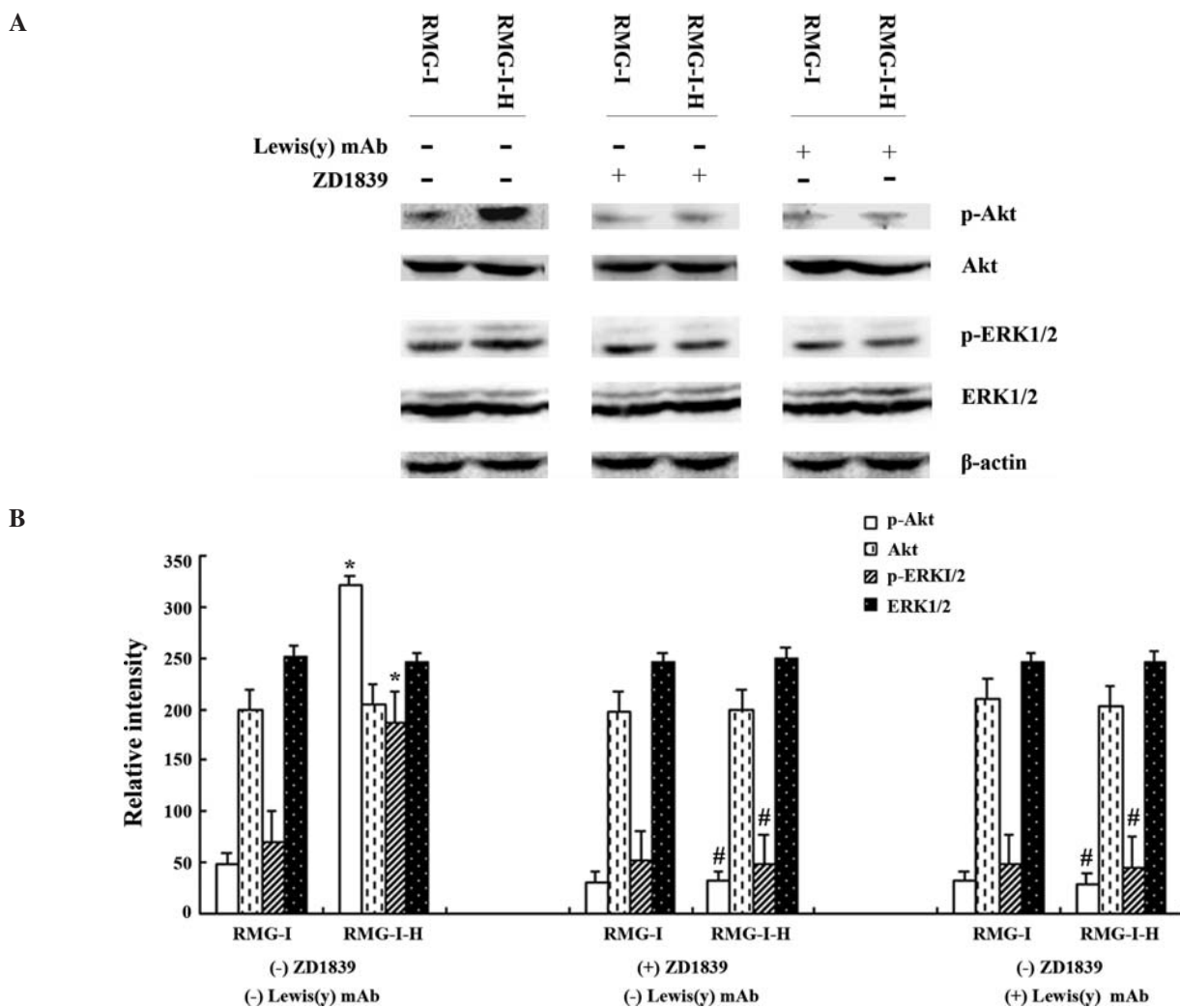

Figure 5. Effects of α1,2-FT transfection on the protein expression and tyrosine phosphorylation of Akt and ERK1/2, and the effect of ZD1839 and anti-Lewis(y) antibody on the phosphorylation of Akt and ERK1/2. (A) Western blot profiles of Akt, p-Akt, ERK1/2 and p-ERK1/2 in non- and α1,2-FT transfected cells, as well as in the absence and presence of ZD1839 and anti-Lewis(y) antibody. (B) Densitometric quantification of protein expression of A (n=3). *p<0.01 compared to RMG-I. #p<0.01 compared to RMG-I-H cells without ZD1839 or anti-Lewis(y) antibody treatment. Lewis(y) mAb, anti-Lewis(y) monoclonal antibody. 'A' is representative of three independent and reproducible experiments.

upregulated to 6.51-fold of the non-transfection value in α1,2-FT transfected cells, and the relative phosphorylation of ERK1/2 (the ratio of p-ERK1/2 to ERK1/2) was also increased in RMG-I-H cells, being 3.92-fold the non-trans-

fection value (both p<0.01). However, some differences in the phosphorylation intensities of Akt and ERK1/2 were observed in non- and α1,2-FT-transfected cells, but the differences were not statistically significant.
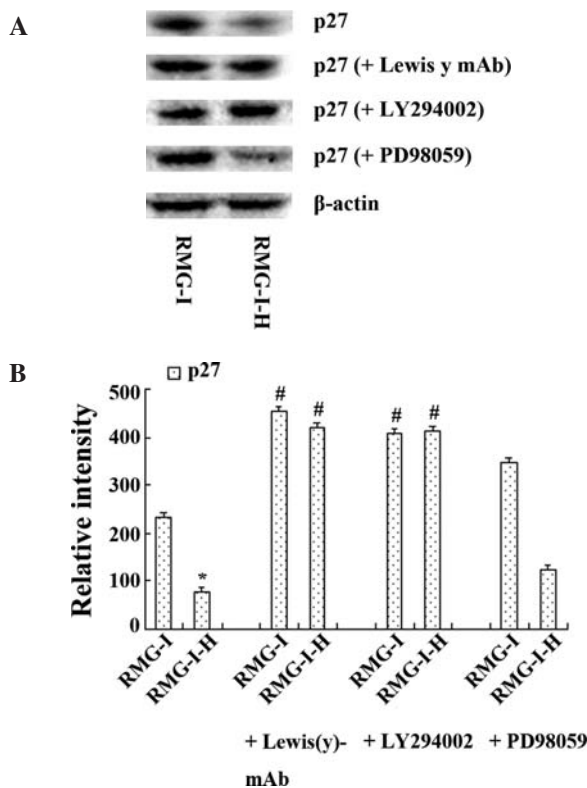
A

B

Figure 6. Effect of $\alpha 1,2$-FT transfection on the expression of p27, and the effect of anti-Lewis(y) antibody, LY294002 and PD98059 on the expression of p27. (A) Western blot profiles of p27 in non- and $\alpha 1,2$-FT-transfected cells, as well as in the absence and presence of anti-Lewis(y) antibody, LY294002 and PD98059. (B) Densitometric quantification of protein expression of A (n=3). *p<0.01 compared to RMG-I. #p<0.01 compared to RMG-I or RMG-I-H cells without anti-Lewis(y) antibody or LY294002 treatment. Lewis(y) mAb, anti-Lewis(y) monoclonal antibody. 'A' is representative of three independent and reproducible experiments.

Effect of ZD1839 and anti-Lewis(y) antibody on the phosphorylation of Akt, ERK1/2. In order to study whether the alteration in the phosphorylation of Akt and ERK1/2 was mediated by EGFR kinase and surface Lewis(y), phosphorylation of these two signaling molecules was treated with 5 μM ZD1839 (a specific inhibitor of EGFR tyrosine kinase) or 20 μg/ml anti-Lewis(y) antibody for 24 h; corresponding untreated cells were used as the control. As shown in Fig. 5, when ovarian cancer cells were treated by ZD1839, phosphorylation of both Akt and ERK1/2 were apparently decreased in non- and $\alpha 1,2$-FT-transfected cells. The decrease in phosphorylation of Akt and ERK1/2 was 87.3 and 75.7% of the corresponding untreated control value in $\alpha 1,2$-FT-transfected cells (p<0.01). By contrast, differences in phosphorylation intensity for Akt and ERK1/2 among non- and $\alpha 1,2$-FT-transfected cell groups were attenuated in ZD1839-treated cells. Meanwhile, in the presence of anti-Lewis(y) antibody, phosphorylation of both Akt and ERK1/2, and the differences in their phosphorylation intensities among the two cell lines were also decreased significantly (Fig. 5). The reduction in phosphorylation of Akt and ERK1/2 in $\alpha 1,2$-FT-transfected cells was 88.5 and 76.2% of the corresponding untreated control value, respectively (both p<0.01). When the cells were treated by ZD1839 or anti-Lewis(y) antibody, the rate of inhibition of

phosphorylation was correlated with expression of $\alpha 1,2$-FT, which was $\alpha 1,2$-FT > non-transfected cells.

Expression of p27 in $\alpha 1,2$-FT-transfected cells. Cell cycle progression is positively regulated by multiple cyclins and cyclin-dependent kinases (Cdks) and cyclin/Cdk complexes are negatively regulated by a number of Cdk inhibitors including p27 (24-26). Degradation of p27 is a critical event for the G_1/S transition. Loss of p27 is strongly associated with aggressive tumor behavior and poor clinical outcome in ovarian cancer (27,28). Our findings obtained from Western immunoblotting revealed that the expression of p27 was significantly decreased in the $\alpha 1,2$-FT-transfected cells, and the levels in $\alpha 1,2$-FT-transfected cells was 33.4% of the non-transfection value (p<0.01) (Fig. 6).

Effect of anti-Lewis(y) antibody on the expression of p27 protein. To further study the relationship between the expression of p27 and that of cell surface Lewis(y) (the product of $\alpha 1,2$-FT), the expression of p27 was determined after cell surface Lewis(y) had been blocked by the monoclonal antibody of Lewis(y). p27 was apparently increased in all the anti-Lewis(y) antibody-treated cell lines, including the parental and the transfected cells (p<0.01) (Fig. 6), when compared with the findings from the experiments without anti-Lewis(y) antibody treatment. Furthermore, the intensity difference of p27 among the two different cell lines almost disappeared.

Effect of LY294002, PD98059 on the expression of p27 protein. PI3K/Akt and Ras/MEK/MAPK signaling pathways have been reported to be involved in the regulation of p27 protein expression (29,30). The role of these two signaling pathways in reduction of p27 in $\alpha 1,2$-FT-transfected cells was also detected by use of the specific inhibitor of PI3K (LY294002) and MEK (PD98059). The results in Fig. 6 show that the expression of p27 protein was increased in both LY294002- and PD98059-treated cell lines, including non- and $\alpha 1,2$-FT-transfected cells. However, the intensity difference of p27 among different cell lines was still obvious in PD98059-treated cells, but almost abolished in LY294002-treated cells. These findings indicated that the PI3K/Akt signaling pathway might contribute more in the downregulation of p27 in $\alpha 1,2$-FT-transfected ovarian cancer cells.

Discussion

Glycans are important components of cell membrane, which play essential roles in cell-cell interaction, cell-molecule recognition, as well as involve in signal transduction and molecule adhesion, therefore closely relate to many important life processes such as cell growth, apoptosis, mobility and differentiation (31-33). Upon cancerous transformation, cell membrane glycans, especially their carbohydrate chains, undergo structural and quantitative changes. The major presentation of ovarian cancer is alteration in type II carbohydrate chains, such as Lewis(y) antigen. A previous study showed that 75% of ovarian cancers have varying degree of Lewis(y) overexpression, and increased expression is associated with poor prognosis of patients (34). In our

ry study, we introduced α1,2-FT gene into human ancer cell line RMG-I through gene transfection and established cell model overexpressing α1,2-FT gene and Lewis(y) (11). By comparing cell proliferation status before and after transfection, we found that cell proliferation after gene transfection was accelerated. Meanwhile, cell cycle test results showed that Lewis(y) overexpression enhanced DNA synthesis and promoted cells in G_0-G_1 phase to enter S and G2-M phase, leading to shortened cell cycle. Our preliminary study proved that Lewis(y) was the most dramatically changed fucosylated antigen after α1,2-FT gene transfection, which increased by 20-fold more than that of before transfection. Lewis(x) was slightly decreased, while Lewis(a) and Lewis(b) could barely be detected after transfection. Although H-1 antigen was predominant among all cells after transfection, its content was only 1/4 of the Lewis(y) level (11). Lewis(y) blocking experiments also provided further evidence for its function (35).

The molecular mechanism by which Lewis(y) antigen enhances the malignant behavior of ovarian cancer cells is still not clear. In previous studies, we tested the differences in oncogene expression before and after α1,2-FT gene transfection using gene chips technology (36). Results showed that: there were 88 differentially expressed genes after cell transfection, 60 of which were upregulated including c-erbB-2 gene. Altered genes mainly involved the genes regulating cell proliferation, signal transduction, protein amino acid phosphorylation, and transcription. Most cell surface receptors are glycoproteins, studies showed that changes in glycosyltransferase expression might affect the structure of carbohydrate chains on cell surface receptors and therefore impacted the expression and function of those glycoprotein receptors (15,16). Thus, it is possible that Lewis(y) may be an important component in signaling transduction pathway participating in signal transduction in the cells and further promoting proliferation of ovarian cancer cells.

Epidermal growth factor receptors (EGFR) are membrane receptors with tyrosine protein kinase activity, which can be activated by epidermal growth factor (EGF) or transforming growth factor-α (TGF-α) and transmit signals into nucleus via many downstream signaling pathways to further act on target genes and involve in regulating the pathogenesis and development of multiple tumors. Studies showed that after activation by growth factors, EGFR could regulate processes such as cell proliferation through downstream PI3K/Akt or MAPK signaling pathways (37-39). However, downstream transduction pathways for EGFR signaling used by tumor cells with different origins also vary (40,41). Research (42) showed that Lewis(y) antigen was a structural component of EGFR and our experimental results further verified this conclusion. Our study found that the expression of EGFR in the transfected cells at both protein and mRNA level had no significant change, but the relative content of Lewis(y) on EGFR after α1,2-FT gene transfection increased compared to that before transfection. Our study also found that the tyrosine phosphorylation level of EGFR significantly increased after gene transfection. Meanwhile, PI3K/Akt and Ras/MEK/ MAPK signaling transduction pathways downstream to EGFR were stimulated concomitantly. The elevated phosphorylation of downstream signaling molecules was presumed to be mediated by increased tyrosine phosphorylation of EGFR; the latter resulting from the increased Lewis(y) content of EGFR. It was verified by the following findings: i) the intensity of phosphorylation in downstream signaling molecules was positively correlated with the intensity of tyrosine phosphorylation in EGFR, and tyrosine phosphorylation was proportional to the Lewis(y) content of EGFR; ii) inhibition of EGFR tyrosine phosphorylation by EGFR tyrosine kinase inhibitor (ZD1839), led to an obvious reduction in the phosphorylation of Akt and ERK1/2, and obvious attenuation of the difference in phosphorylation intensity among two cell lines with different α1,2-FT expression; iii) blockage of cell surface Lewis(y) by anti-Lewis(y) antibody also resulted in significant attenuation of the phosphorylation of Akt and ERK1/2, as well as the difference in phosphorylation intensity among the two cell lines.

In addition, we proved for the first time that Lewis(y) antigen was not only an integral part of EGFR, but also a component of HER2/neu. HER2/neu is a transmembrane glycoprotein encoded by proto-oncogene c-erbB-2 with tyrosine protein kinase activity. It has high homology with EGFR and is highly expressed in most tumors, such as breast and ovarian cancer. The amplification of HER2/ neu gene and overexpression of its protein product closely relate to the poor prognosis in cancer patients (43,44). Studies showed that when HER2/neu expression increased to a certain level, it would be self-polymerized to acquire sustained activity, causing deregulation of downstream signaling molecules (45). In opposite to EGFR, it was found that although the expression of HER2/neu at both protein and mRNA level significantly increased after α1,2-FT gene transfection, but the relative content of Lewis(y) on HER2/ neu had no significant change before and after gene transfection. However, the tyrosine phosphorylation of HER2/neu still significantly increased after gene transfection. After α1,2-FT gene transfection, increase of HER2/neu both at protein level and at transcription level may also relate to changes in carbohydrate chains on cell membrane receptors after gene transfection, i.e., α1,2-FT gene transfection causes increase in Lewis(y) content on EGFR so that downstream signaling transduction pathways are activated and growth signals are delivered to the nucleus, leading to accelerated gene transcription of HER2/neu in nucleus, and finally promoting the expression of HER2/neu.

Increase in Lewis(y) content on cell membrane EGFR caused increase in its phosphorylation level. The possible mechanism may be that: i) as the exposed carbohydrate chain of EGFR on cell surface, increase in Lewis(y) content affects the 3-dimensinal structure of EGFR and expose more EGFR ligand binding sites, leading to overactivation of EGFR and downstream signaling molecules; ii) α1,2-FT gene transfection causes increased expression of EGFR ligand and enhances autocrine circle, leading to sustained activation of EGFR. Research show that tumor cells secreted EGF and or TGF-α in autocrine manner to act on its own membrane receptor EGFR, forming a circuit to stimulate self proliferation. This mechanism may play important roles in the processes of tumor pathogenesis and development, and is closely related to tumor cell proliferation (46). Our study

found that α1,2-FT gene transfection caused significant increase in EGF and TGF-α expression in nude mice grafted with ovarian cancer cell line RMG-I. Thus we speculate that α1,2-FT gene transfection might also cause ovarian cancer cell line RMG-I to secrete excessive EGF and TGF-α in autocrine manner, so that the latter continuously acts on its own membrane receptor EGFR to form an autocrine loop, and ultimately causes sustained activation of EGFR and the downstream signal transduction pathways.

Deregulation of cell cycle is a major factor to cause uncontrolled cell proliferation leading to cancers, while abnormal expression of signal transduction pathway proteins and mutation in cell cycle regulatory proteins may lead to malfunction of cell cycle checkpoints. In the present investigation, it was found that the expression of p27 protein significantly decreased in α1,2-FT transfected cells. We speculate that the influence of α1,2-FT on p27 expression was mediated by Lewis(y), because the increase in Lewis(y) content on the sugar chains of cell surface receptor might alter the conformation of the receptor, resulting in the enhancement of the signaling of cell proliferation-related pathway. Finally, some factors regulating the synthesis and/or degradation of p27 protein were decreased and/or increased, respectively, leading to a reduction in p27 protein. This result further elucidate the molecular mechanism of Lewis(y) enhancing the cell proliferation once again.

In summary, α1,2-FT gene transfection could increase the content of Lewis(y) of cell surface EGFR in ovarian cancer cell line RMG-I and eventually promote cell cycle progress and proliferation by influencing the carbohydrate chain structure of EGFR to regulate downstream signal transduction pathways. Although the specific mechanisms still need to be further studied, our results should provide novel ideas for understanding the mechanisms of pathogenesis and development, as well as the treatment of ovarian cancer.

Acknowledgements

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