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Lewis y Expressed in Oral Squamous Cell Carcinoma Attenuates Malignant Properties *via* Down-regulation of EGF Signaling

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Abstract. *Background/Aim: Lewis y is expressed in oral squamous cell carcinoma (OSCC) cells and tumors. Previously, we reported that Lewis y was not expressed in invasion areas, and attenuation of proliferation and invasion in OSCC cells was caused by over-expression of Lewis y. However, the roles of Lewis y in the attenuation of malignant properties have not been clarified. In this study, we investigated the roles of Lewis y in OSCC. Materials and Methods: The levels of Lewis y on EGFR and the phosphorylation levels of EGFR in OSCC cells were analyzed by immunoprecipitation and western blot. EGFR cross-linking and binding kinetics of EGF were performed. Results: Upon EGF stimulation, phosphorylation and dimer formation of EGFR were more prominent in Lewis y– cells. EGF binding kinetics showed reduced binding sites in Lewis y+ cells. Conclusion: Lewis y reduced EGF binding to EGFR, leading to suppression of malignant properties through suppression of EGF signaling.*

Glycoproteins and glycosphingolipids expressed on plasma membranes play important roles in maintaining the homeostasis of organisms and in the mechanisms of disease (1, 2). Glycosylation is also known to play a role in the malignant properties of cancer cells *via* the regulation of various receptors and effector molecules (3-5). For example, glycosphingolipids, ganglioside D3 (GD3) and ganglioside D2 (GD2), have been shown to promote proliferation and invasion through enhancing the phosphorylation of Src family kinases, p130Cas, and paxillin in melanoma, glioma, and osteosarcoma cells (5-8). As for glycoproteins, fucosylation of *N*-glycans on epidermal growth factor receptor (EGFR) has been reported to be involved in the signaling of cancer cells (9, 10). It has also been reported that truncation of *O*-glycans leads to the promotion of the malignant properties of gastric cancer cells (11).

Lewis y as well as A/B/O-type structures and Lewis a, b, x antigens are known as blood group antigens. Lewis y has core 2 (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) structures, which are modified by two fucosylations (12, 13); these fucosylations take place on *O*-glycans in mucin-type glycoproteins and glycosphingolipids (3). Lewis y is synthesized by α1,3 fucosyltransferase (α1,3-*Fut*) from type 2H, which is synthesized by α1,2 fucosyltransferase (α1,2-*Fut*) from type 2 chain that has core 2 (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) structures.

Previously, we reported that Lewis y is expressed in seven oral squamous cell carcinoma (OSCC) cell lines (number of positive/total: 7/7; positive rate: 100%) and OSCC tissues (number of positive/total: 21/23; positive rate: 91.3%) (14). On the other hand, in normal squamous epithelia, no expression was observed. Furthermore, Lewis y was not observed in the invasive areas, and over-expression of Lewis y in OSCC cell lines resulted in the attenuation of invasion and proliferation, and tumor reduction (14). Collectively, these results suggest that expression of Lewis y suppressed the malignant properties, although it was expressed in OSCC. However, the mechanisms of Lewis y function are not well-understood. To investigate these mechanisms, the levels of Lewis y on EGFR, as well as the tyrosine-phosphorylation levels of EGFR in OSCC cell lines were analyzed. To clarify the mechanism of the effects of Lewis y on EGFR signaling, we performed an EGFR cross-linking and binding study of

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^{125}I-EGF to measure the dimerization of EGFR following EGF treatment and the binding kinetics of EGF to EGFR, respectively. We also examined the invasion and cell adhesion activity of Lewis y+ and Lewis y– cells.

Materials and Methods

Cell lines and transfectant cells. HSC-2 (human OSCC from mouth), HSC-3 (human OSCC from tongue), HSC-4 (human OSCC from tongue), Sa3 (human OSCC from upper gingiva), SCCKN (human OSCC from tongue), HO-1-u-1 (human OSCC from mouth floor), and Ca9-22 (human OSCC from gingiva) were purchased from Riken Cell Bank (Tsukuba, Japan). Lewis y+ transfectant cells were established by transfecting with pcDNA3.1 vector carrying a neomycin resistant gene and the human *Fut1* cDNA, which encodes α1,2 fucosyltransferase, into HSC-3-21 (HSC-3 mutant cells; Lewis y negative cells), and selecting resistant cells using G418 (synonym; neomycin) as previously described (14). Then, Lewis y positive (Lewis y+) and Lewis y negative (Lewis y–) cells were isolated using FACS Vantage™ SE (BD Biosciences, Franklin Lakes, NJ, USA) (14). Lewis y knockdown cells (*Fut1*-knockdown (KD) cells) were established by transfecting HSC-3-34 cells (HSC-3 clone) with the *Fut1*-miRNA plasmid containing a blasticidin resistant gene, and selecting blasticidin resistant cells as previously described (14).

Cell culture. HSC-2, HSC-3, Sa3, SCCKN, and Ca9-22 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) containing 7.5% fetal bovine serum (FBS). HSC-4 and HO-1-u-1 cells were grown in Roswell Park Memorial Institute medium (RPMI-1640) (Sigma-Aldrich) containing 10% FBS. Lewis y+ transfectant cells were grown in DMEM containing G418 (400 μg/ml). Lewis y knockdown cells were grown in DMEM containing blasticidin (5 μg/ml). Cells were maintained at 37˚C in a humidified incubator containing 5% CO_2.

Antibodies. Anti-Lewis y antibody (H18A, mouse IgG3, catalog number: A2587, 1:100 dilution for flow cytometry, and 1:500 dilution for immunoblotting) was purchased from SEIKAGAKU CORPORATION (Tokyo, Japan). Anti-EGFR antibody (rabbit polyclonal IgG, catalog number: sc-03, 1:1,000 dilution) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine antibody (PY20, mouse IgG2b, catalog number: 610000, 1:1,000 dilution) and PY20 conjugated with HRP (catalog number: 610012, 1:1,000 dilution) were obtained from BD Transduction Laboratories (San Diego, CA, USA). Anti-mouse IgGs conjugated with HRP (catalog number: NXA931, 1:2,000 dilution) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Anti-rabbit IgGs conjugated with HRP (catalog number: #7074, 1:2,000) were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-mouse IgG3 conjugated with Alexa Fluor™ 488 (catalog number: A-11001, 1:100 dilution) was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Flow cytometry. Expression of Lewis y was analyzed by FACS Calibur™ (Becton Dickinson, Franklin Lakes, NJ, USA), as described previously (14). Control samples were prepared by using non-relevant normal mouse IgGs. For quantification of positive cells, the CELL Quest™ software for FACS Calibur (BECTON DICKINSON, San Jose, CA, USA) was used.

EGF stimulation. After serum starvation for 24 h, cells were treated with recombinant human epidermal growth factor (rhEGF) (R&D Systems, Minneapolis, MN, USA) for the indicated time periods. The cells were immediately washed with phosphate buffered saline (PBS) and lysed. The lysates were immunoprecipitated with an anti-EGFR antibody (Santa Cruz Biotechnology). The precipitated proteins were separated in SDS-PAGE and then immunoblotted with an anti-phosphotyrosine antibody (PY20) conjugated with HRP (BD Transduction Laboratories).

Preparation of cell lysates. Five $\times 10^5$ cells were seeded in a 6-cm dish. Cells were lysed with a lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 1 μg/ml of leupeptin) (7).

Immunoprecipitation. Immunoprecipitation was performed by incubating cell lysates with the anti-EGFR antibody bound to protein A-Sepharose at 4˚C overnight with rotation. After being washed with a washing buffer (50 mM Tris-HCl, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM EDTA), the precipitates were resuspended with 4× SDS sample buffer and analyzed by western immunoblotting.

Western immunoblotting. Proteins were fractionated in SDS-PAGE using 6 or 10% gels. The fractionated proteins were transferred to an Immobilon-P membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 3% bovine serum albumin (BSA) in PBS containing 0.05% Tween 20 for 1 h. The membrane was then incubated with primary antibodies followed by incubation with goat anti-mouse IgGs conjugated with HRP or goat anti-rabbit IgGs conjugated with HRP. After being washed, protein bands were visualized using an ECL™ detection system (PerkinElmer Life Sciences, Waltham, MA, USA) and detected using a film processing machine FPM100 (FUJI FILM, Tokyo, Japan).

Invasion assay. Invasion assays were conducted with a Boyden chamber, as described previously (6). In brief, Matrigel™ (BD Biosciences) was diluted with PBS, added to each filter (8-μm pore size, BD Falcon, Franklin Lakes, NJ, USA) and left to polymerize overnight. Culture medium containing serum was added in the lower chamber (6-well plate, BD Falcon) before setting the upper chamber. Cells (2×10^5 cells/well) were added to serum-free medium in the upper chamber and cultured for 24 h. After being stained with Giemsa (Wako, Osaka, Japan), the number of cells was counted under a microscope.

EGFR cross-linking. Binding of EGF to EGFR results in its dimerization and activation. Therefore, the dimerization of EGFR was evaluated using bis (sulfosuccinimidyl) suberate (BS^3), a reagent used for cross-linking amino groups. Four $\times 10^5$ cells were seeded in a 6-cm dish. After serum starvation for 24 h, the cells were stimulated with 2.5 ng/ml rhEGF for 60 min. Then, the cells were washed with PBS immediately and then cross-linked using 1 mM BS^3 in PBS at 4˚C for 30 min (15). The cross-linking reaction was terminated with 0.1M Tris-HCl, and the cells were washed and lysed.

Binding kinetics. Cells were suspended in a binding buffer (Hanks' containing 1 mg/ml each of glucose and BSA) at 5×10^4 cells/0.2 ml and incubated at room temperature for 30 min with 10 pM to 0.3 nM ^{125}I-EGF. The reaction mixture was added in 0.32 M sucrose

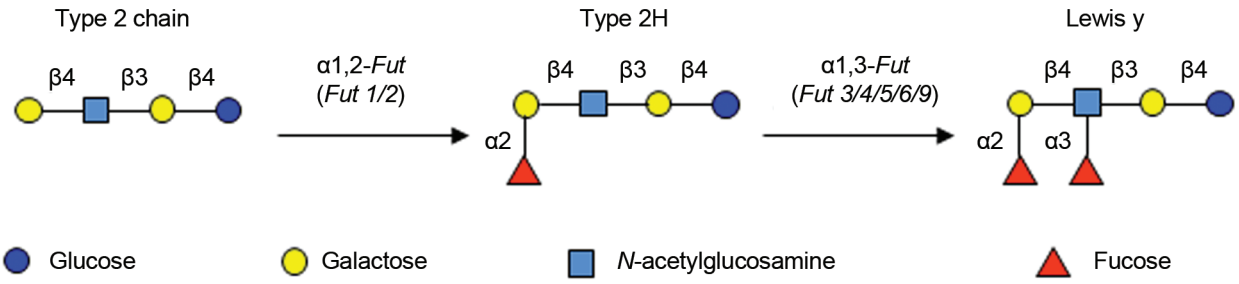


Figure 1. *Synthetic pathway of Lewis y. Type 2 chain has core 2 (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) structures. Type 2H is synthesized by α1,2 fucosyltransferase (α1,2-Fut) from type 2 chain. Furthermore, Lewis y is synthesized by α1,3 fucosyltransferase (α1,3-Fut) from type 2H. blue circle; glucose, yellow circle; galactose, blue square; N-acetylglucosamine, red triangle; fucose.*

containing 5% BSA and centrifuged at 10,000 × *g* for 1 min at 4˚C. After collecting the supernatants, the cell pellets were used to measure bound radioactivity. Nonspecific binding was evaluated using a 1,000-fold excess of unlabeled EGF. The radioactivity was measured by a γ-counter. Bmax (maximum binding that represents receptor density) was measured by regression analysis on the basis of a Scatchard plot.

Cell adhesion analysis by real-time cell electronic sensing (RT-CES). The intensity of adhesion of Lewis y+ and Lewis y– cells was measured by the RT-CES system (Wako Pure Chemical, Osaka, Japan) (8, 16). ACEA E-plates (ACEA Biosciences, San Diego, CA, USA) were coated with collagen type I or fibronectin for 1 h at 37˚C. After being washed with PBS, the plates were blocked with PBS containing 0.5% BSA for 20 min at 37˚C and 1×10^4 cells were added on the plates. The adhesion of cells was observed continuously by RT-CES for 24 h.

Statistical analysis. All data are expressed as the mean±S.D. Statistical significance was evaluated using one-way or two-way analysis of variance (ANOVA) followed by Tukey *post-hoc* test and mixed design ANOVA followed by Shaffer *post-hoc* test. Statistical significance was analyzed using R software (version 3.6.3). Significance was set at $p<0.05$, and single and double asterisks indicate $p<0.05$ and $p<0.01$, respectively.

Results

Induction of phosphorylation of EGFR by EGF treatment. Type 2 chain has core 2 (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) structures. Type 2H is synthesized by α1,2 fucosyltransferase (α1,2-*Fut*) from type 2 chain. Furthermore, Lewis y is synthesized by α 1,3 fucosyltransferase (α1,3-*Fut*) from type 2H with a type 2 core chain (Figure 1). The levels of Lewis y on EGFR as well as the tyrosine phosphorylation levels of EGFR were examined in seven OSCC cell lines. The levels of Lewis y on EGFR were higher in HSC-2, HSC-4, Sa3, and SCCKN than those in HSC-3, HO-1-u-1, and Ca9-22 (Figure 2A). The phosphorylation levels of EGFR were higher in HSC-3, HO-1-u-1, and Ca9-22 than in HSC-2, HSC-4, Sa3, and SCCKN (Figure 2B).

Effects of Lewis y levels on tyrosine-phosphorylation levels of EGFR. The time-course of tyrosine-phosphorylation in EGFR in Lewis y+ transfectant cells (Lewis y positive cells; L8 and L15) and the control cells (Lewis y negative cells; VC7 and VC8) were analyzed by immunoprecipitation (IP)/immunoblotting (IB) (Figure 3A and B). Phosphorylation levels of EGFR after EGF treatment were weaker in Lewis y+ transfectant cells than in controls (Figure 3B). Furthermore, the time-course of tyrosine-phosphorylation levels of EGFR in *Fut1*-knockdown (KD) cells (Lewis y–) (HSC-3-34; Lewis y+) and control cells (Lewis y+) (HSC-3-34; Lewis y+) were analyzed. Increased phosphorylation of EGFR after EGF treatment in *Fut1*-KD cells (Lewis y–) compared with *Fut1*-VC cells (Lewis y+) was observed (Figure 3C). Of note, *Fut1*-KD cells have been established previously, and almost none of them expressed Lewis y (14).

Decreased dimerization of EGFR with EGF treatment in Lewis y+ cells. EGFR were cross-linked with BS^3 following EGF stimulation, as described in "Materials and Methods." After cross-linking, lysates were immunoblotted with the anti-EGFR antibody. The immunoblotting showed high molecular mass bands corresponding to EGFR dimer after treatment of control cells with EGF (Figure 4A). On the other hand, EGFR dimers were barely found in Lewis y+ cells. The ratios of the intensities of dimerized EGFR/monomeric EGFR in Lewis y– cells were higher than those in Lewis y+ cells (Figure 4B).

Altered binding kinetics of EGF. Using gradually diluted ^{125}I-labeled EGF, EGF binding to Lewis y+ cells and control cells was examined, as described in "Materials and Methods." As shown in Figure 4C, there was no difference in the dissociation constant (Kd values) of EGF between Lewis y+ cells and control cells. Kd values of EGF binding in L8, L15, VC7, and VC8 cells were 0.725, 0.446, 0.641, and 0.834 nM, respectively. B_{max} values calculated from the binding curves

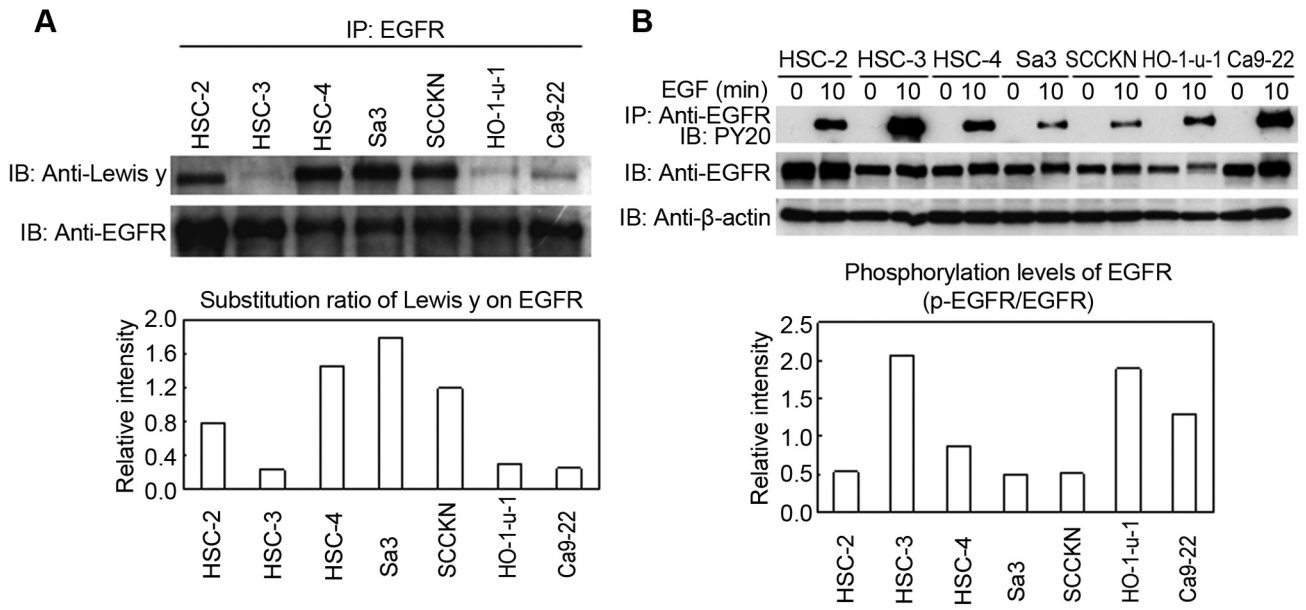


Figure 2. *Inverse correlations between phosphorylation levels of EGFR and expression levels of Lewis y. (A) Ratio of Lewis y on EGFR in OSCC cell lines as measured by the intensities of bands in immunoblotting. Cell lysates were immunoprecipitated by an anti-EGFR antibody, then immunoblotted with an anti-Lewis y or EGFR antibody (upper). The intensities of bands in upper panels were quantified using Image J. Relative intensities of bands of Lewis y were normalized by those of EGFR bands (lower). IP: Immunoprecipitation, IB: immunoblotting. (B) Comparison of tyrosine phosphorylation in EGFR following EGF stimulation among the cell lines. After serum starvation, OSCC cell lines were stimulated with EGF (50 ng/ml) for 10 min. Cell lysates were immunoprecipitated by an anti-EGFR antibody, then immunoblotted with an anti-phosphotyrosine antibody (PY20) (upper). The intensities of bands in the upper panels were quantified using Image J. Relative intensities of bands of phosphorylated EGFR (p-EGFR) were normalized by those of the EGFR bands (lower).*

were expressed as fmol/5×10^4 cells. B_{max} values of EGF binding in L8, L15, VC7, and VC8 cells were 35.65, 23.95, 46.74 and 60.02 fmol/5×10^4 cells, respectively. Thus, B_{max} values for Lewis y+ cells were 1.3-2.5-fold lower than those for control cells (Figure 4C), suggesting that EGF-binding sites on Lewis y+ cells were decreased compared with control cells.

Effects of knockdown of Lewis y on morphology, invasion, and adhesion. Rounding of cell morphology induced by EGF was more rapid in *Fut1* KD cells (Lewis y–) (HSC-3-34; Lewis y+) than in control cells (Lewis y+) (HSC-3-34; Lewis y+) (Figure 5A). Invasion activities of *Fut1* KD cells (Lewis y–) and control cells (Lewis y+) following EGF stimulation were examined by invasion assay (Figure 5B). The numbers of invading *Fut1* KD cells were 2.4-fold higher than those of control cells in the absence of EGF. The numbers of invading *Fut1* KD cells were 2.6-fold higher than those of control cells in the presence of EGF. Furthermore, we analyzed the differences in the intensity of adhesion and spreading on collagen type I or fibronectin between *Fut1* KD cells (Lewis y–) and *Fut1* VC (Lewis y+) cells using the RT-CES system (Figure 5C). The cell adhesion and spreading of *Fut1*-KD cells to collagen type I and fibronectin were weaker than those of control cells.

Discussion

In this study, we demonstrated the mechanisms of how Lewis y attenuates the malignant properties of OSCC. Phosphorylation levels of EGFR after EGF treatment were lower in Lewis y+ cells than in Lewis y– cells in OSCC. Dimerization of EGFR after EGF treatment in Lewis y+ cells was decreased compared with that in Lewis y– cells. There was no significant difference in Kd values of EGF binding between Lewis y+ and Lewis y– cells. On the other hand, B_{max} values of EGF binding to Lewis y+ cells were lower than those of its binding to Lewis y– cells. Consistent with these results, Lewis y– cells had increased malignant properties.

It is known that EGFR is over-expressed in head and neck (including oral cavity) SCC (17, 18). The over-expression of EGFR has been correlated with decreased survival and resistance to radiation (19). Therefore, we considered that investigating the regulatory mechanisms of EGFR signaling by Lewis y in OSCC is important. EGFR and its family members are important receptors involved in the development (20) and malignant properties of various cancers (21). Upon the binding of EGF, EGFR forms a dimer and its kinase activity is increased, leading to the activation of downstream signaling (22).

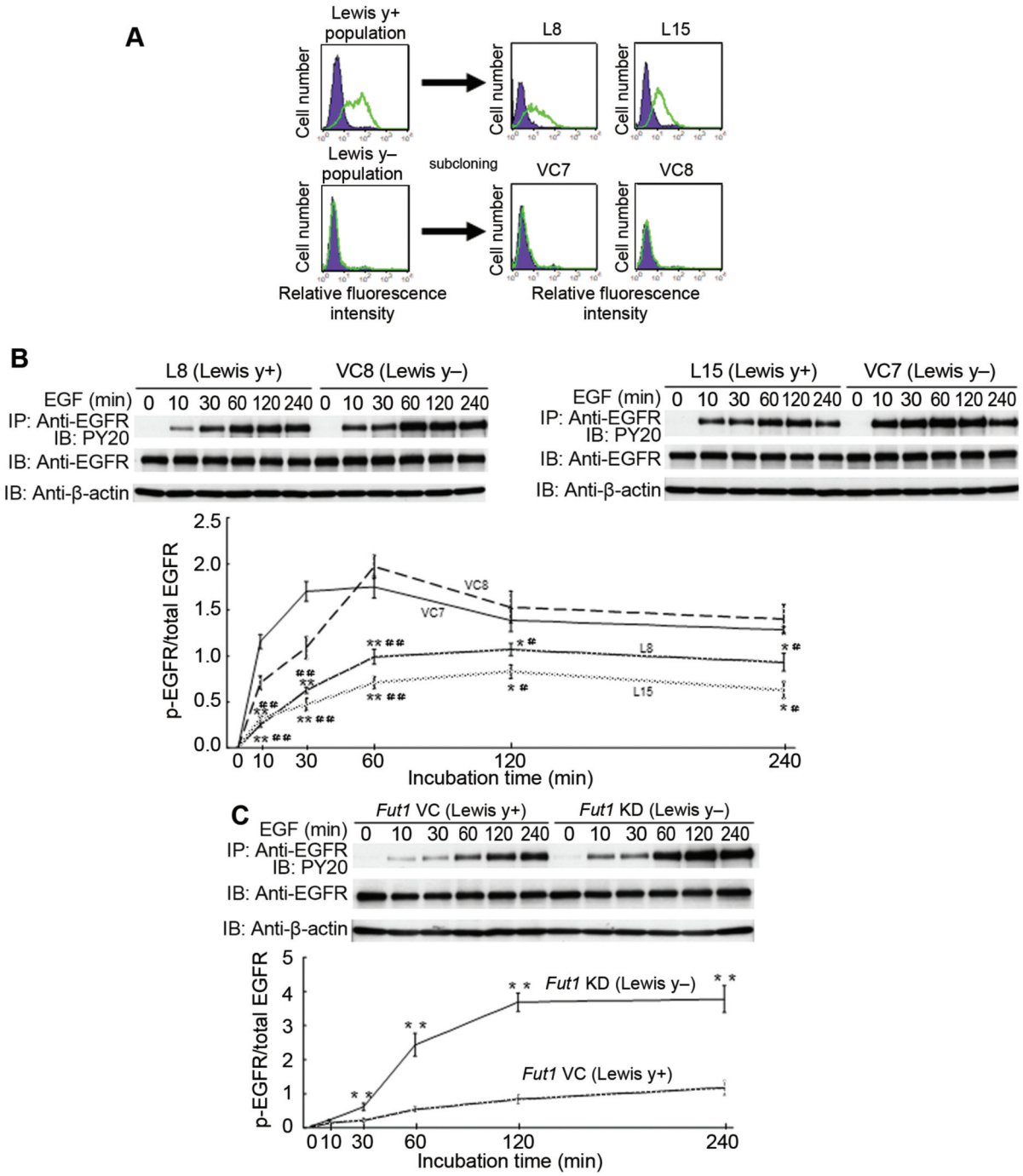
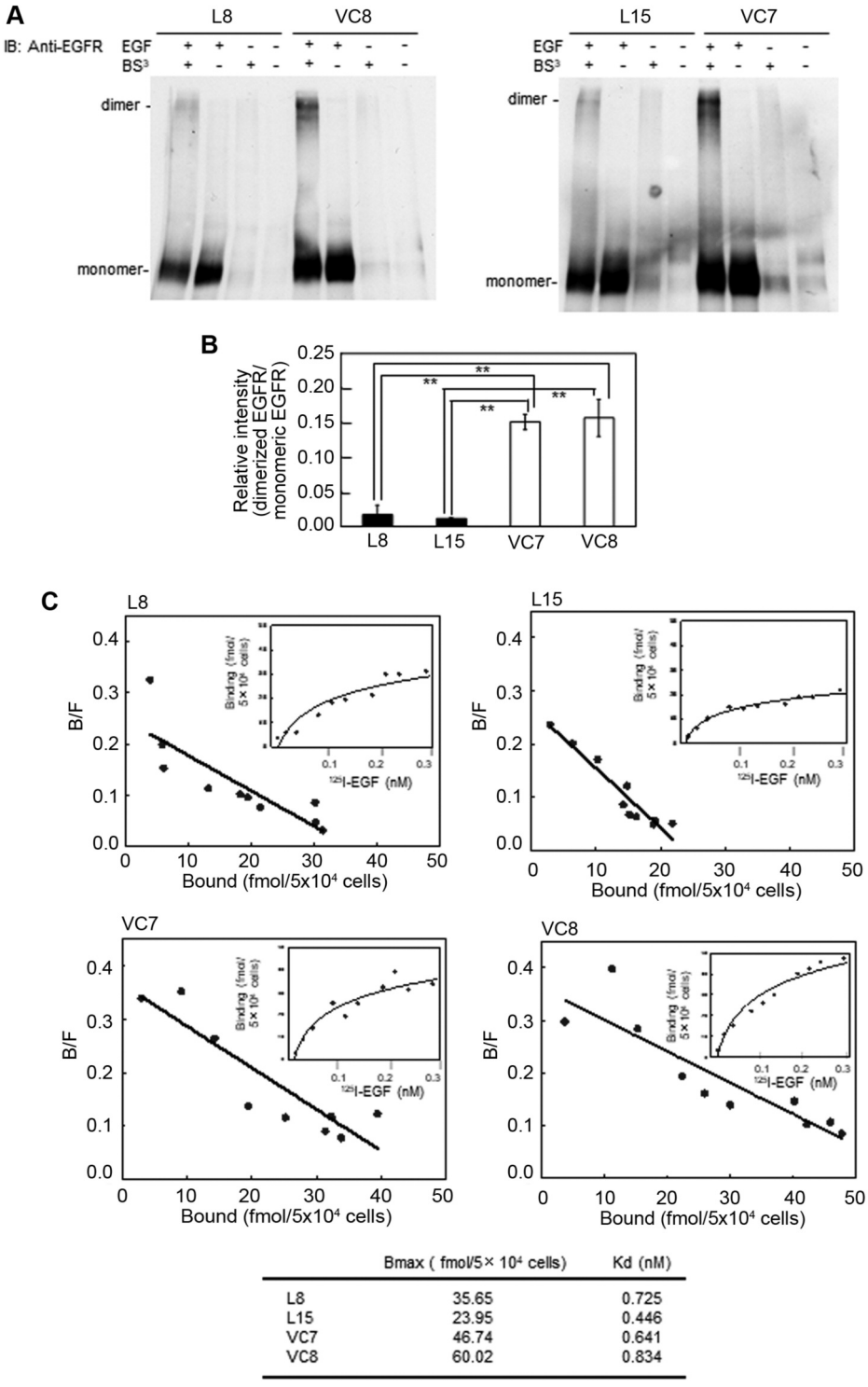


Figure 3. *Increased levels of Lewis y resulted in the suppression of EGF/EGFR signals. (A) Analysis of the levels of Lewis y in two lines stably transfected with Fut1 cDNA (L8 and L15) and two control lines (VC7 and VC8) using flow cytometry. (B) The time-course of tyrosine phosphorylation levels of EGFR after EGF treatment of Lewis y+ cells (L8 and L15) and Lewis y– cells (VC7 and VC8). After serum starvation for 24 h, the cells were treated with EGF (2.5 ng/ml) for 0-240 min. Cell lysates were immunoprecipitated by an anti-EGFR antibody, and the immunoprecipitates were analyzed by western blotting using the anti-PY20 antibody (upper). The intensities of bands in the upper panels were quantified using Image J. The intensities of bands of phosphorylated EGFR (p-EGFR) were normalized by EGFR (lower). Data are expressed as the mean±S.D. *p<0.05 and **p<0.01 vs. VC7. #p<0.05 and ##p<0.01 vs. VC8. (C) The time-course of tyrosine phosphorylation levels of EGFR in Fut1-KD cells (Lewis y–) and control cells (Lewis y+) were analyzed. After serum starvation for 24 h, the cells were treated with EGF (2.5 ng/ml) for 0-240 min. Cell lysates were immunoprecipitated by an anti-EGFR antibody, and then the immunoprecipitates analyzed by western blotting using the anti-PY20 antibody (upper). The intensities of bands in the upper panels were quantified using Image J, as described above. Data are expressed as the mean±S.D. **p<0.01 vs. FUT1 VC (Lewis y+). Statistical significance was evaluated using two-way ANOVA followed by Tukey post-hoc test.*



	Bmax (fmol/5× 10^4 cells)	Kd (nM)
L8	35.65	0.725
L15	23.95	0.446
VC7	46.74	0.641
VC8	60.02	0.834

Figure 4. *Dimerization of EGFR after EGF treatment and EGF binding kinetics to EGFR in Lewis y+ cells (L8 and L15) and Lewis y– cells (VC7 and VC8). (A) After serum starvation for 24 h, cells were stimulated with EGF (2.5 ng/ml) for 60 min. Following a cross-linking reaction, the dimeric as well as monomeric forms of EGFR were immunoblotted with anti-EGFR antibody. (B) The relative intensity of bands of dimerized EGFR/monomeric EGFR was measured using Image J. Statistical significance was evaluated using one-way ANOVA followed by Tukey post-hoc test. (C) EGF binding to Lewis y+ cells and Lewis y– cells was investigated using gradually diluted ^{125}I-labeled EGF. Scatchard plot analysis was conducted, and the results of individual samples are presented. Dissociation constants (Kds), and Bmax values calculated from the binding curves in Lewis y+ cells and Lewis y– cells are shown at the bottom. B/F: Bound EGF/Free EGF.*

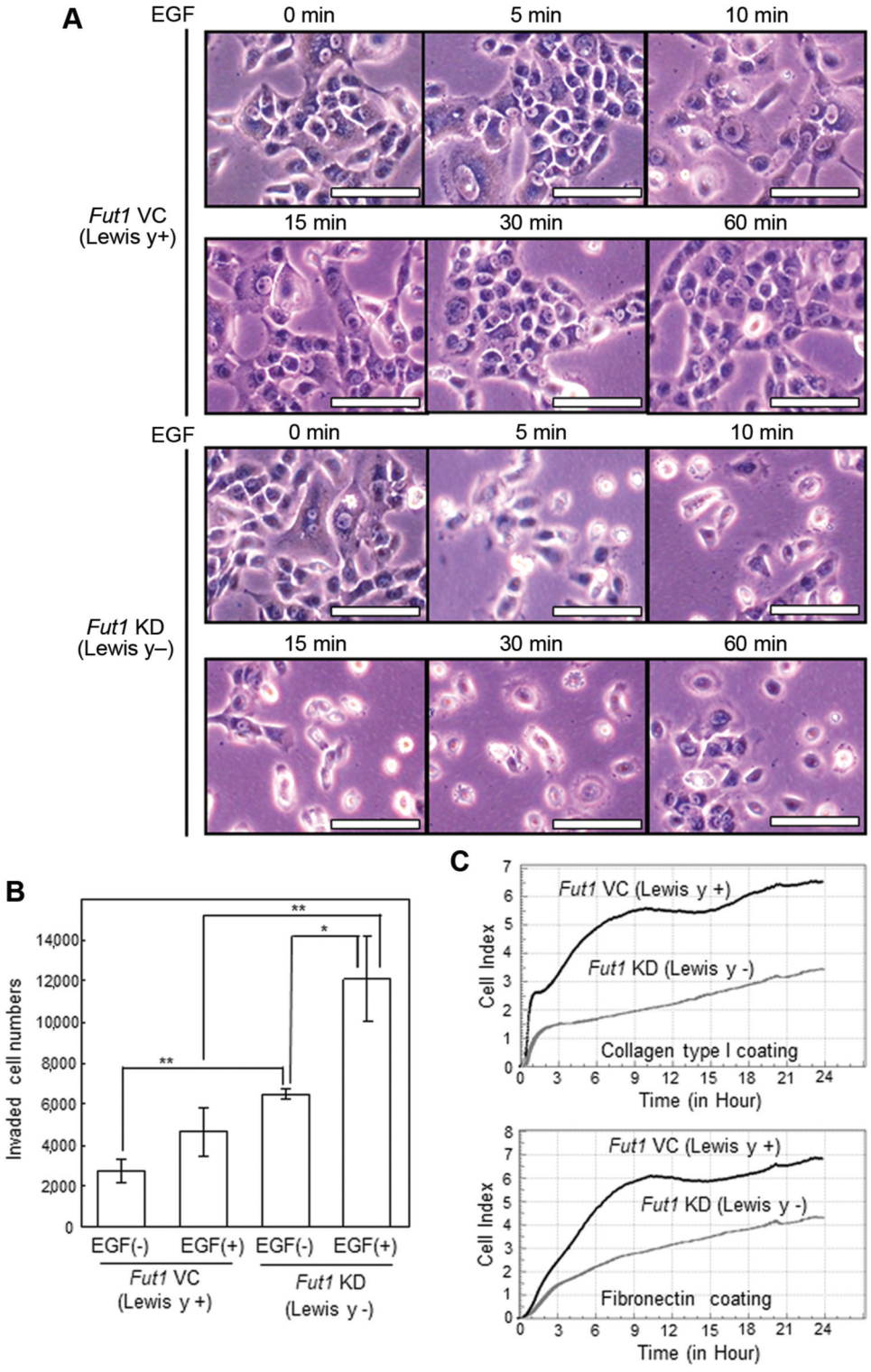


Figure 5. *Changes in morphology, invasion activity, and adhesion following knockdown of Fut1. (A) Fut1 KD (Lewis y–) and Fut1 VC (Lewis y+) cells stimulated with EGF (2.5 ng/ml) for 0-60 min were observed under a microscope. Scale bars: 100 μm. (B) Invasion activities of Fut1 KD (Lewis y–) and Fut1 VC (Lewis y+) cells were examined by invasion assay following EGF stimulation. Statistical significance was evaluated using mixed design ANOVA followed by Shaffer post-hoc test. (C) Intensities of adhesion and spreading of Fut1 KD and Fut1 VC cells on collagen type I or fibronectin were analyzed by real-time cell electronic sensing (RT-CES). ACEA E-plates were coated with collagen type I or fibronectin. After protein blocking, cells were added on ACEA E-plates. The adhesion of cells was observed continuously under the RT-CES system for 24 h.*

EGFR is known to be *N*-glycosylated and *O*-glycosylated (2, 23-25). The glycosylation affects the functions of EGFR (9, 10, 26-30). For example, *N*-glycosylation plays crucial roles in the structural arrangement of the ligand-binding EGFR ectodomain (27). Furthermore, fucosylation on *N*-glycans is essential for the binding of EGF to its receptor (9), and increased fucosylation of EGFR significantly promotes the EGF-mediated growth of cancer cells (10). Also, *N*-linked GlcNAc termini of EGFR have been shown to modulate EGFR tyrosine kinase activity through the interaction of ganglioside GM3 (28). As for *O*-glycan, *O*-glycans on EGFR can be modified by Core 1 β1,3-galactosyltransferase (C1GALT1) in head and neck SCC cells, and the glycosylation on EGFR increases EGF-EGFR binding affinity, resulting in the activation of its signaling (29). These reports showed that certain glycosylations such as fucosylation by α1,6-linkage (*Fut8*) and C1GALT1-modified *O*-glycans on EGFR promote malignant properties. In addition, it has also been reported that sialylation and fucosylation of EGFR attenuated its dimerization and activation in lung cancer cells (30). These results are similar to ours. Thus, differences in EGFR glycosylation result in the activation or suppression of EGFR signaling depending on the cancer cells and type of glycosylation.

In contrast to our results, it has been reported that Lewis y on EGFR increased the migration of the OSCC cell line OC-2 (31). The differences between their results and ours may be explained by the different cell lines used. In our study, although the levels of Lewis y were higher in the superficial areas of tumors, Lewis y was not observed in the invasion areas. Furthermore, inverse correlations between the phosphorylation levels of EGFR and the levels of Lewis y on EGFR were detected in seven OSCC cell lines. The experiments using Lewis y transfectant and knockdown cells also revealed that Lewis y in OSCC cells suppressed EGFR phosphorylation. These results strongly suggest that Lewis y negatively regulates the phosphorylation of EGFR.

To clarify the mechanisms by which Lewis y in the carbohydrate chains suppresses EGFR dimerization and signaling, a binding assay with radio-labeled EGF was performed. It was revealed that EGF-binding sites on Lewis y+ cells were clearly decreased compared with Lewis y– cells. The extracellular sequence of EGFR is divided into I, II, III, and IV domains (32). Among these domains, the space between domains I and III comprises the binding pocket to EGF (33). Thus, the addition of Lewis y on EGFR might physically disturb domains I and III, resulting in a decrease in the number of EGF-binding sites.

It is known that EGF treatment leads to rapid rounding of human epidermoid carcinoma cells (34). It has been reported that EGF promoted rapid dephosphorylation of FAK and cell invasion and metastasis in various cancer cells (35). Our results also revealed that EGF treatment of Lewis y– cells (with high phosphorylated EGFR by EGF treatment) induced quick rounding, promoted invasion activity, and attenuated adhesion compared to Lewis y+ cells (with low phosphorylated EGFR by EGF treatment). These results suggest that the down-regulation of Lewis y may promote the dephosphorylation of adhesion-related molecules such as FAK and paxillin via activation of EGFR signaling following treatment of OSCC cells with EGF, resulting in the promotion of malignant properties.

In summary, we demonstrated that decreases in the levels of Lewis y on EGFR resulted in the phosphorylation and activation of EGFR, leading to the enhancement of the malignant properties of OSCC cells. Thus, the levels of Lewis y may be used for the prognosis and selection of an appropriate therapeutic strategy for OSCC patients.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

H.H., K.H., K.F. (Keiko Furukawa), and K.F. (Koichi Furukawa) designed the research. H.H., K.H., H.S., and Y.O. conducted the experiments and analyzed the data. K.H. and K.F. (Koichi Furukawa) wrote the manuscript. All Authors read and approved the final manuscript.

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