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Lewis y antigen is expressed in oral squamous cell carcinoma cell lines and tissues, but disappears in the invasive regions leading to the enhanced malignant properties irrespective of sialyl-Lewis x

Hiroshi Hotta · Kazunori Hamamura · Kyoko Yamashita · Hidenobu Shibuya · Noriyo Tokuda · Noboru Hashimoto · Keiko Furukawa · Noriyuki Yamamoto · Hisashi Hattori · Shinya Toyokuni · Minoru Ueda · Koichi Furukawa

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Abstract Expression and implication of carbohydrate antigens in squamous cell carcinomas (SCCs) in oral cavity was examined. In the cell lines, type 2H and Lewis y antigens were markedly expressed. In the tissues from SCC patients and benign disorders, type 2H was highly expressed in hyperplasia (96.4 %), displasia (92.9 %) and SCC (100 %). Lewis y was, in turn, expressed mainly in cancer tissues (91.3 %), suggesting that Lewis y is a cancer-associated antigen. Normal oral mucosa showed no expression of these blood group antigens. Surprisingly, Lewis y antigen disappeared in the invasion sites where Ki-67 was definitely stained. Overexpression of Lewis y with manipulation of a fucosyltransferase cDNA resulted in suppression of cell growth and invasion, and knockdown of Lewis y also brought about increased cell growth and invasion. In either situations, no changes in the expression of sialyl-Lewis x could be found. Lowered tumor growth and invasion into surrounding tissues were also shown in Lewis y-positive SCC grafts in nu/nu mice. All these results together with alternative staining between Lewis y and Ki-67 in cancer tissues and *FUT1* transfectants suggested that loss of Lewis y is a crucial event for the late stage of SCCs.

Keywords Lewis y · Squamous cell carcinoma · Oral cavity · Blood group antigen · Hyperplasia · Type 2H

Abbreviations

SCC(s)	Squamous cell carcinomas(s)
DMEM	Dulbecco's modified Eagle's medium
mAb	Monoclonal antibody
EGFR	EGF receptor

Introduction

Squamous cell carcinomas (SCC) are frequent cancers in squamous tissues in skin, lung, esophagus, uterus cervix and oral cavity. Generally, SCCs are resistant to current chemotherapy and radiotherapy [1]. In particular, SCC in oral cavity such

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H. Hotta · K. Hamamura · H. Shibuya · N. Tokuda · N. Hashimoto · K. Furukawa · K. Furukawa (✉)
Department of Biochemistry II, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-0065, Japan
e-mail: koichi@med.nagoya-u.ac.jp

H. Hotta · K. Hamamura · H. Shibuya · N. Yamamoto · H. Hattori · M. Ueda
Department of Maxillo-Facial Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan

K. Yamashita · S. Toyokuni
Department of Pathology and Biological Responses, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-0065, Japan

K. Furukawa
Department of Biomedical Sciences, Chubu University College of Life and Health Sciences, 1200 Matsumoto-cho, Kasugai 487-8501, Japan

as tongue, gingival and oral mucosa are hard to cure, and need very careful consideration in the selection of the treatment, not only for the functional issues but also for cosmetic aspects [2]. Therefore, novel approaches to overcome the desperate situation in the therapy of patients with SCC have been expected for a long time [3].

Representative blood group antigens include A/B/O-type structures and Lewis a, b, x or y antigens. All these carbohydrate structures are derivatives of either core 1 (Galβ1-3GlcNAcβ1-3Galβ1-4 Glc(NAc-)) or core 2 (Galβ1-4GlcNAcβ1Galβ1 4Glc(NAc-)) structures, modified by single or multiple fucosylation [4, 5]. These structures are carried on O-glycans in mucin-type glycoproteins and/or glycosphingolipids [6].

There have been a number of reports on the expression of blood group antigens in human cancers [7–9]. Generally, A/B/O blood group antigens tend to be deleted during transformation, while the normal counterparts express some levels of them [10]. Sometimes, novel blood group antigens were detected in human tumors suggesting evolution of aberrant glycosylation pathway along with malignant transformation [6, 11]. In particular, increased expression of fucosylated structures such as type 1/2 H antigens or difucosylated blood group epitopes such as Lewis b and Lewis y in various cancers have been reported [9, 10, 12, 14]. Furthermore, implication of the blood group antigens in the biological features in individual tumor types have been investigated [7, 15–17].

However, effects of characteristic blood group antigens on the tumor phenotypes, and on the cell signaling as the basis of tumor properties have not been well understood. In this study, we examined expression of blood group antigens in oral cavity-derived SCC cell lines and tissues from patients. Consequently, type 2H and Lewis y were detected commonly in the SCC cell lines and tissues. In particular, Lewis y was expressed in a cancer specific manner, prompting us to investigate the implication of Lewis y in the biological and clinical features of SCCs in oral cavity.

Materials and methods

Cells and cell culture

HSC-2, HSC-3, Sa3, SCCKN and Ca9-22 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 7.5 % fetal bovine serum (FBS) at 37 °C in 5 % CO_2. HSC-4 and HO-1-u-1 cells were maintained in RPMI-1640 (Sigma St. Louis, MO) supplemented with 10 % FBS at 37 °C in 5 % CO_2. All cell lines were purchased from Riken Cell Bank (Tsukuba, Japan).

Antibodies

Anti-human type 2H antibody (BRIC231, mouse IgG1) was purchased from AbD Serotec (Oxford, UK). Anti-human Lewis y antibody (H18A, mouse IgG3), Anti-Lewis a antibody (7LE, mouse IgG1), Anti-Sialyl Lewis a antibody (2D3, mouse IgM), Anti-Lewis b antibody (2-25LE, mouse IgG1), Anti-Lewis x antibody (73-30, mouse IgM), and Anti-Sialyl Lewis x antibody (2H5, mouse IgM) were purchased from SEIKAGAKU CORPORATION (Tokyo, Japan). Anti-human Ki-67 antibody (MIB-1, mouse IgG1) was purchased from Dako (Glostrup, Denmark). Anti-mouse IgG conjugated with HRP was purchased from GE Healthcare (Piscataway, NJ). Alexa Fluor™ 488-conjugated anti-mouse IgG3 and Alexa Fluor™ 555-conjugated anti-mouse IgG1 were purchased from invitrogen (Carlsbad, CA).

Flow cytometry

Cell surface expression of type 2H, Lewis y and other Lewis antigens was analyzed by FACS Caliver™ (Becton Dickinson, Franklin Lakes, NJ). The cells were incubated with primary antibodies for 50 min on ice and then stained with FITC-conjugated goat anti-mouse IgG (H + L)(Cappel, Durham, NC) or FITC-Goat Anti-Mouse IgM (Invitrogen, Carlsbad, CA) for 40 min on ice. Control cells for flow cytometry were prepared using the secondary antibody alone. For quantification of positive cells, CELLQuest™ program was used.

Immunohistostaining of type 2H and Lewis y

Immunohistochemical staining of formalin-fixed, paraffin-embedded sections (5 μm) was carried out. The sections were deparaffinized in xylene and dehydrated through graded alcohols (70 %, 95 %, 95 %, 100 %, 100 %). After transfer of the sections in staining racks into the diluted Immunosaver™ (Nisshin EM Co. Ltd, Tokyo, Japan) 200 time, they were kept at 98 °C for 45 min. After washing in PBS, the sections were blocked with protein blocking agent (Ultra-Tech HRP Streptavidin-Biotin Detection System™, Beckman Coulter, Fullerton, CA) for 15 min to prevent non-specific binding of the antibodies. The sections were first probed with primary antibodies at 15 min using a microwave. After being washed, they were incubated with polyvalent biotinylated secondary antibody (Ultra-Tech HRP Streptavidin-Biotin Detection System™, Beckman Coulter). After being washed, they were incubated with peroxidase-labeled streptavidin (Ultra-Tech HRP Strept- avidin-Biotin Detection System™). After being washed, peroxidase activity was developed using 0.1 mM 3, 3′-diaminobenzidine tetrachloride (DOJINDO, Kumamoto, Japan) with 10 μL of 30 % H_2O_2 in 100 mL of 50 mM Tris–HCl, pH 7.5.

Double immunohistostaining of Lewis y and Ki-67

The sections were deparaffinized in xylene and dehydrated through graded alcohols (70 %~100 %). After transfer of the sections in staining racks into the diluted Immunosaver™ (Nisshin EM Co. Ltd, Tokyo, Japan) 200 time, they were kept at 98 °C for 45 min. After washing in PBS, the sections were blocked with protein blocking agent for 15 min. The sections were first probed with anti-Lewis y antibody for 15 min using a microwave. After washing with PBS, the sections were incubated with polyvalent biotinylated secondary antibody (Beckman Coulter). After being washed, sections were incubated for 5 min with VECTASTAIN™ ABC-AP Reagent (VECTOR LABORATORIES, Burlingame, CA). After washing sections with TBS, the sections were incubated to react with alkaline phosphatase substrates with Histofine™ New Fuchsin chromogen/substrate solution (NICHIREI BIOSCIENCES, Tokyo, Japan) at room temperature for 30–60 min. After adequate coloration, the sections were washed with PBS in order to stop color development. To inactivate antibodies and enzymes, sections were incubated with sodium citrate buffer (pH 6.0) at 95 °C for 15 min. After being washed, sections were incubated with methanol containing 0.3 % H_2O_2 for 30 min to inactivate endogenous peroxidase. The sections were blocked with protein blocking agent for 15 min to prevent non-specific binding of the antibodies. The sections were probed with anti-human Ki-67 antibody at room temperature. After washing with PBS, the sections were incubated with secondary antibody (Histofine™ Simple Stain MAX PO, NICHIREI BIOSCIENCES) at room temperature. After washing with PBS, color was developed with EnVision™ Kits (Dako).

Double immunocytostaining of Lewis y and Ki-67

Cells (1×10^5) were plated in glass base dishes (Iwaki, Tokyo, Japan). After 24 h incubation, serum starved for 48 h with 1 % FBS DMEM. The cells were then fixed with 4 % paraformaldehyde/PBS for 10 min at room temperature, and permeabilized with 0.02 % Triton X-100/PBS for 5 min. Incubate the cells with 2.5 % BSA/PBS for 1 h to block to prevent non-specific binding of the antibodies. The cells were incubated with anti-human Ki-67 antibody at room temperature for 1 h. Wash the cells with PBS. Then, incubated with anti-human Lewis y antibody at room temperature for 1 h. Wash the cells with PBS. The cells were incubated with The secondary antibodies, Alexa Fluor™ 488 conjugated anti-mouse IgG3 at room temperature for 45 min, and Alexa Fluor™ 555 conjugated anti-mouse IgG1 at room temperature for 45 min. Wash the cells with PBS. Cells were counterstained with 4, 6-diamidino-2-phenylindole(DAPI). Confocal microscopy analysis were performed with FV10i-LIV™ (Olympus, Tokyo, Japan).

FUT1 gene expression in oral SCC cells

Tumor tissues and normal tissues were obtained from excised tissues of patients. Expression of *FUT1* (tumor per normal tissue) was analyzed using real time RT-PCR (BioRad, Hercules, CA). The following primers were used: *FUT1* forward, 5′-CTGTCGATCCTGTGTCCAGA-3′, *FUT1* reverse, 5′-TGGCATACTGTCCCATCTGA-3′.

Establishment of Lewis y+ and Lewis y− cells

A HSC-3-21 (type 2H−, Lewis y−) clone was isolated from HSC-3 by limiting dilution. To establish stable Lewis y+ transfectants, HSC-3-21 was transfected with pcDNA3.1 inserted with human *FUT1* cDNA by using Lipofectamine 2000™ (Invitrogen, Carlsbad, CA) and selected by cultivating with G418 (400 μg/mL). Lewis y+ and Lewis y− cells were isolated using FACS Vantage™ SE (BD Biosciences, Franklin Lakes, NJ)

Knockdown of FUT1 with a miR RNAi

An expression system for stably expressing miR RNAi was generated using the BLOCK-iT ™ Pol II miR RNAi expression vector kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In the generation of the miR RNAi vector for human *FUT1* was performed by selecting the target sequence with the forward primer 5′-TGCTGAT CGCCAGCAAACGTCACATCG TTTTGGCCACTG ACTGACGATGTGACTTGCTGGCGAT-3′ and the reverse primer 5′-CCTGATCGCCAGCAAGTCACATCGTCAGT C A G T G G C C A A A A C G A T G T G A C G T T T G CTGGCGATC-3′. A HSC-3-34 (type 2H+, Lewis y+) clone was isolated from HSC-3 by limiting dilution. The vector with *FUT1*-miRNA plasmid was transfected into the HSC-3-34 by Lipofectamine 2000™ (Invitrogen) and selected with blasticidin (5 μg/ml). Lewis y+ and Lewis y− cells were isolated with FACS Aria II™ SE (BD Biosciences)

MTT assay

Cells (4×10^3) were seeded in 96-well plates. An MTT assay was performed as previously described [18].

In vitro invasion assays

Invasion assays were performed with a Boyden chamber. Matrigel™ (BD Biosciences) was diluted with ice-cold PBS (100 μg/ml). Six hundred μl of Matrigel™ was added to each filter (polyethylene terephthalate membrane, 8 μm pore size, 23.1 mm in diameter, BD Falcon, Franklin Lakes, NJ) and left to be polymerized over night. The membrane was reconstituted with serum-free medium. The lower

chamber (6-well plate, BD Falcon) was filled with the culture medium with serum before the chamber was assembled. Cells (2×10^5 cells/well) were added to serum-free medium in the upper chamber and incubated for 24 h. Then the cells on the surface of the filter were stained with Giemsa and the cell numbers was counted under microscopy.

In vivo tumor growth

Seven-week-old female BALB/cAJcl nude mice were obtained from Nippon Clea (Tokyo, Japan). Cells were digested with 0.05 % trypsin 0.5 mM EDTA and resuspended in serum-free DMEM medium. The cell density was then adjusted to five$\times10^7$/mL. Cells were subcutaneously injected into the skin of the abdomen at a volume of 200 μL for each animal. The nude mice in plastic cages on hardwood chips in an air-conditioned, pathogen-free animal room were observed for tumor formation weekly for 13 weeks in a row. The long diameter (a) and short diameter (b) were measured by using a caliper. The tumor size was calculated by using the following formula: Tumor volume $= a \times b^2/2$. All experimental protocols were approved by the animal experimental committee of the Graduate School of Medicine in Nagoya University along the guidelines of Japanese government, and were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (1996).

Results

High expression levels of type 2H and Lewis y in human oral SCC cell lines

To examine the expression of blood group antigens in human oral SCC cell lines, seven oral SCC cell lines were analyzed by flow cytometry. High expression levels of type 2H and Lewis y were detected commonly in the seven cell

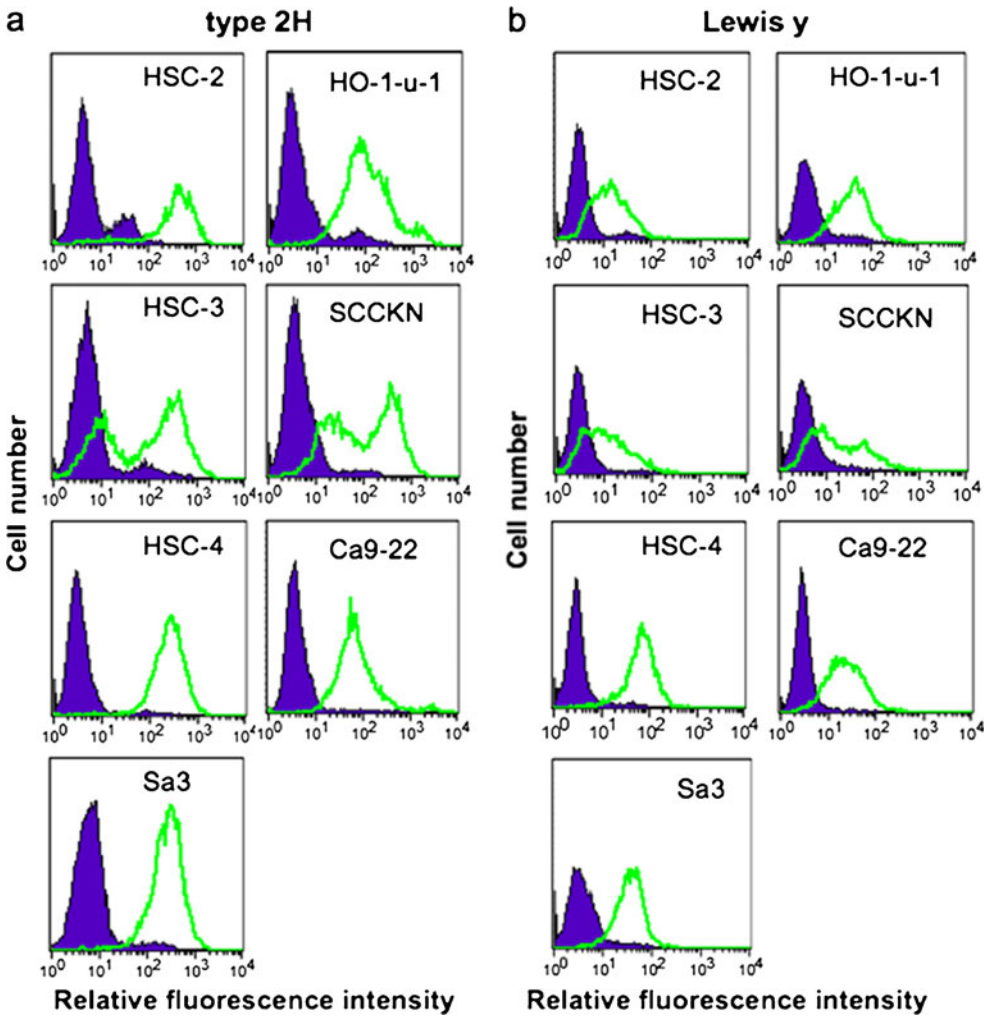


Fig. 1 A flow cytometric analysis of type 2H and Lewis y expressed on oral SCC cell lines. Cell surface expression of blood group antigens was examined by FACS Caliver™, and the results of type 2H (**a**) and Lewis y (**b**) were shown. The cells were incubated with primary antibodies, and then stained with FITC-conjugated goat anti-mouse IgG. The fluorescence intensity was on the horizontal axis, while the number of cells was on the vertical axis

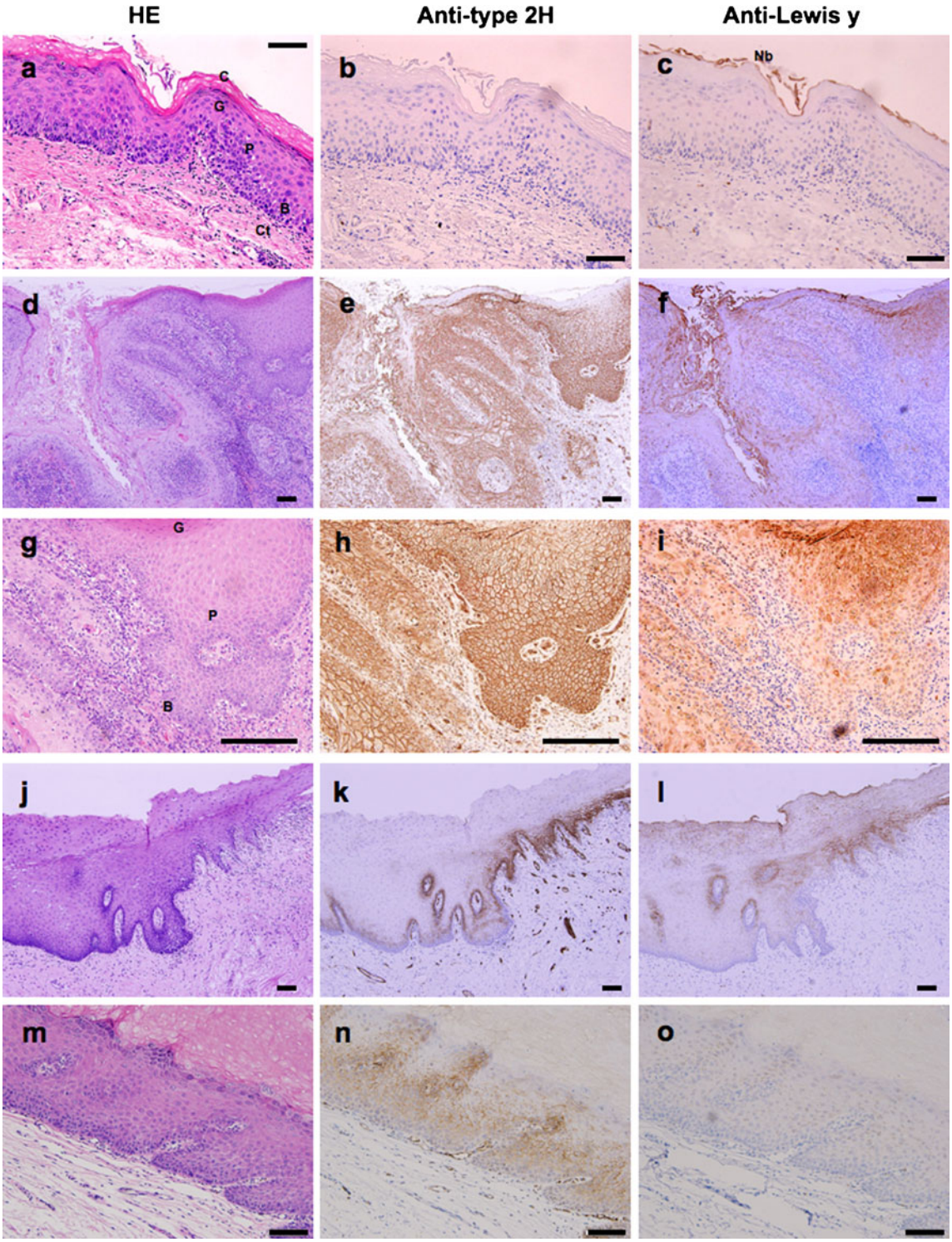


Fig. 2 Expression of type 2H and Lewis y in SCC and related diseases. Normal squamous epithelium (**a**, **b**, **c**), well-differentiated SCC (**d**, **e**, **f**, **g**, **h**, **i**), dysplasia (**j**, **k**, **l**) and hyperplasia (**m**, **n**, **o**) were analyzed. Left panels (**a**, **d**, **g**, **j**, **m**), hematoxylin–eosin stain; center panels (**b**, **e**, **h**, **k**, **n**), type 2H immunostaining; right panels (**c**, **f**, **i**, **l**, **o**), Lewis y immunostaining. The sections were deparaffinized in xylene and dehydrated through graded alcohols, and were transferred into the diluted Immunosaver™. After being blocked with protein blocking agent, the sections were first probed with primary antibodies, then they were incubated with a polyvalent biotinylated secondary antibody. They were incubated with peroxidase-labeled streptavidin followed by development using 3, 3′-diaminobenzidine tetra-chloride. Scale bars :100 μm. C, cornified layer; G, granular cell layer; P, prickle cell layer; B, basal cell layer; Ct, connective tissue; Nb, nonspecific binding

lines (Fig. 1). For other blood group antigens, Lewis b and sialyl-Lewis x were detected in about half of these cell lines (Supplementary Fig. S1)

High expression of type 2H and Lewis y in SCC tissues

Formalin-fixed, paraffin-embedded specimens were prepared from biopsy tissues or surgically resected tissues from patients diagnosed as SCC or related disorders at the Department of Oral and Maxillofacial Surgery in Nagoya University Hospital from 2001 to 2009. Sixty five samples of oral SCC and related disorders were analyzed for expression of blood group antigens with immunohistochemistry (Fig. 2). In the normal squamous epithelia, the expression of type 2H and Lewis y were not observed. But in oral tumor lesions, type 2H was stained from basal cell layer to prickle cell layer, and Lewis y was stained from upper prickle cell layer to granular cell layer. Type 2H was highly expressed not only in oral tumor lesions, but in dysplasia and hyperplasia. On the other hand, as the lesions became definitely malignant, the expression rates of Lewis y increased more (Table 1). Therefore, Lewis y was suspected to be associated with malignant phenotypes.

Poor expression of Lewis y in the invasive regions

Although Lewis y was highly expressed in superficial regions of tumors, it was poorly expressed in the invasive regions (Fig. 3a and b). In double staining of Lewis y and Ki-67, these two antigens were alternatively stained in the invasive regions (Fig. 3c–f). From these results, it was concluded that Lewis y was generally expressed in SCC tissues, but disappeared in highly proliferating and invasive regions.

Table 1 Summary of type 2H and Lewis y expression in SCC and related diseases in oral cavity

Antigen	Histopathological diagnosis	Number of positive/total (% of positive)		Expression scores[a]			
				0	1	2	3
type 2H	Normal	0/8	(0)				
	Hyperplasia	27/28	(96.4)				
	Displasia	13/14	(92.9)				
	SCC	23/23	(100)	0	1	3	19[b]
Lewis y	Normal	0/8	(0)				
	Hyperplasia	14/28	(50)				
	Displasia	11/14	(78.6)				
	SCC	21/23	(91.3)	2	3	5	13[b]

[a] Scores 0, 1, 2 and 3 represent the type 2H or Lewis y positive areas: less than 10 % (0), 10–30 % (1), 30–60 % (2) or more than 60 % (3) of the lesions

[b] Number of cases with SCC corresponding to the individual scores

Increased expression of *FUT1* gene in oral SCC tissues

Five cases of SCC and corresponding normal tissues were analyzed for *FUT1* gene expression by real time RT-PCR. Relative expression levels of *FUT1* were higher in tumor tissues in all of five cases (Table 2).

Effects of Lewis y expression on the cancer phenotypes in isolated clones of HSC-3

A Lewis y-defective mutant line HSC-3-21, Lewis y-high expressing HSC-3-44 and Lewis y-low-expressing HSC-3-AA as well as the parent HSC-3 (moderate expressing) were obtained by limiting dilution (Fig. 4a), and used to analyze effects of Lewis y expression on the cancer properties. In MTT assay, cells with high expression of Lewis y tended to show reduced cell growth (Fig. 4b, *left*). In invasion assay, cells with high expression of Lewis y also tended to show reduced invasion activity (Fig. 4b, *right*). These results suggested that Lewis y expression suppresses malignant phenotypes.

Establishment of Lewis y+ transfectant cells

A Lewis y-defective mutant line HSC-3-21 was obtained by limiting dilution of HSC-3 (Supplementary Fig. S2a and b). Since FUT1 was a major synthetic enzyme of Lewis y (Fig. S2c), and the defect of *FUT1* in HSC-3-21 was confirmed by real time RT-PCR, HSC-3-21 was transfected with a *FUT1* cDNA expression vector. Two transfectant lines (L8 and L15) and two vector control lines (VC7 and VC8) were established by G418 selection followed by cell sorting/limiting dilution (Fig. 4c). After over-expression of *FUT1* cDNA, no marked changes in the expression levels of Lewis x and sialyl Lewis x were detected (Fig. 4d).

Effects of Lewis y expression on cell proliferation and invasion activity

The proliferation and invasion activity of the Lewis y+ transfectant cells and the control cells were compared by MTT assay and the Boyden chamber invasion assay, respectively. In MTT assay, the Lewis y+ cells showed significantly reduced cell growth (Fig. 4e). The cell numbers of these transfectants were 1.25–1.7 folds less than those of the control cells at day 7 of culture. In the invasion assay, transfectant cells showed reduced invasion activity (Fig. 4e). The invaded cell numbers of the transfectants were 4.8–6.3 folds less than those of the control cells.

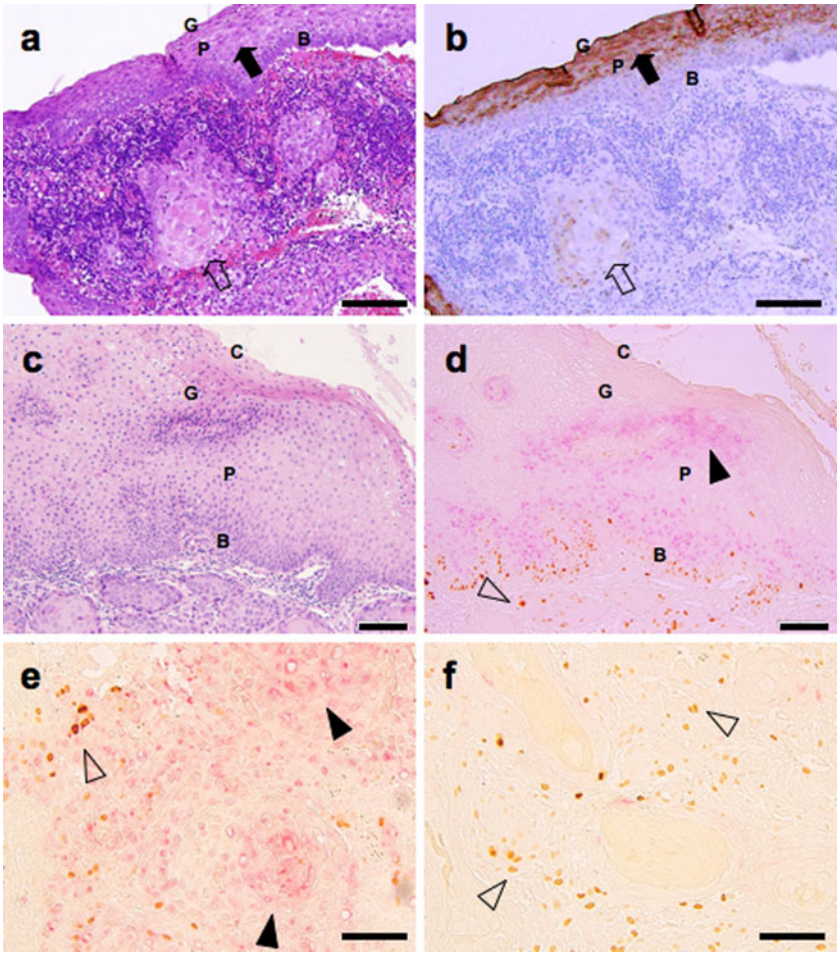


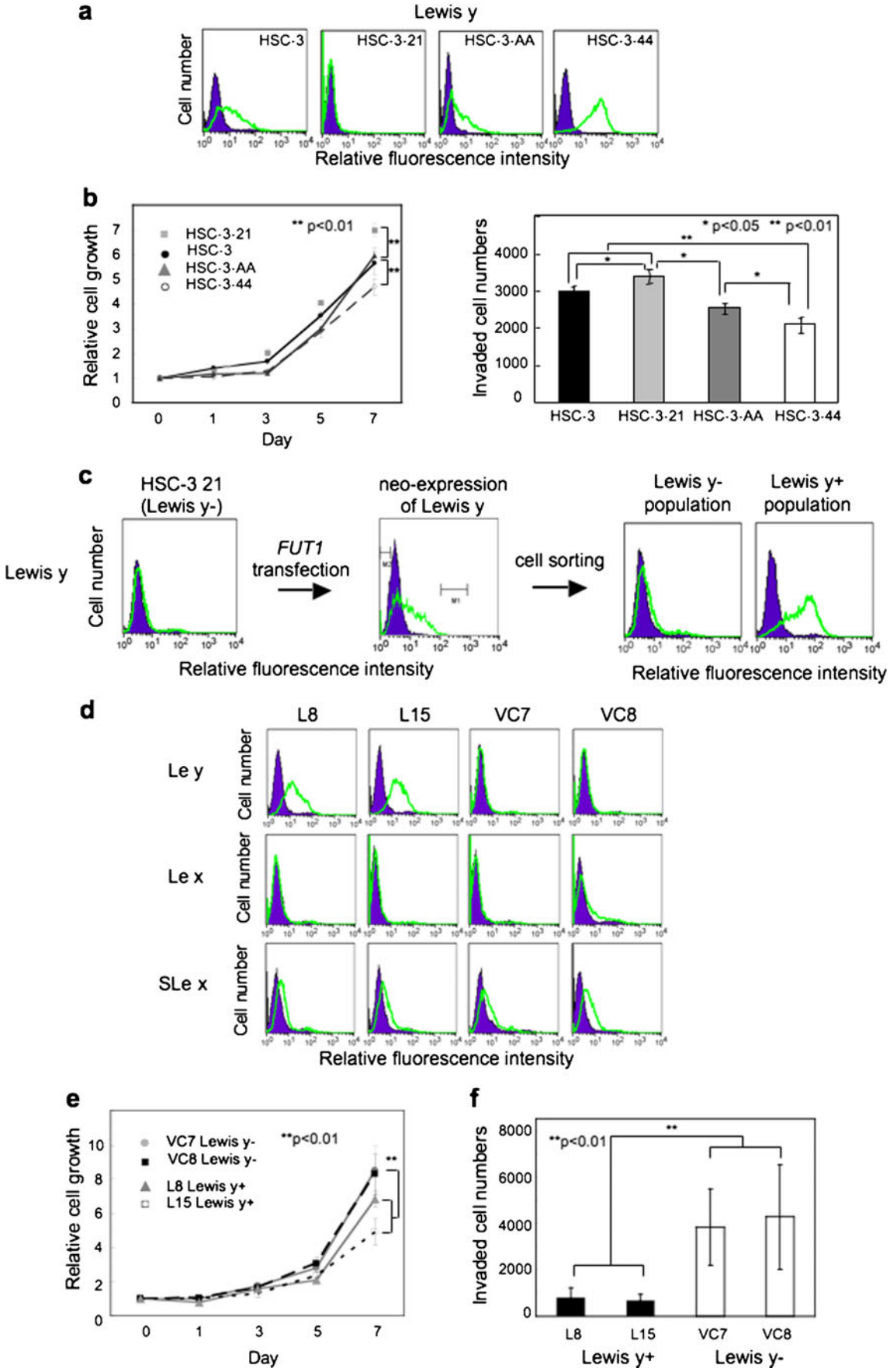
Fig. 3 Lewis y was not stained in the invasive resions of SCC tissues. **a**, **c** Hematoxylin–eosin staining. **b** Immunohistostaining of Lewis y. Lewis y was stained strongly from upper prickle cell layer to granular cell layer (*black arrow*), but stained poorly in invasive regions (*white arrow*). **d**–**f** Double immunohistostaining of Lewis y and Ki-67 performed as described in Materials and Methods. After protein blocking, the sections were first probed with an anti-Lewis y antibody, then incubated with a polyvalent biotinylated secondary antibody. Then, sections were incubated with ABC-AP™ Reagent. The sections were incubated with alkaline phosphatase substrates with Histofine™ substrate solution. After inactivation of antibodies and enzymes, the sections were incubated with methanol containing 0.3 % H_2O_2 to inactivate endogenous peroxidase. The sections were probed with an anti-human Ki-67 antibody, followed by a secondary antibody. Color was developed with EnVision™ Kits. Superficial regions (**d**) and invasive regions (**e** and **f**) were double-stained with Ki-67 (*brown*, *white arrowhead*) and Lewis y (*red*, *black arrowhead*), showing alternative staining between superficial regions and invasive regions in all 8 cases. Scale bars, 100 μm. C, cornified layer; G, granular cell layer; P, prickle cell layer; B, basal cell layer

Effects of knockdown of Lewis y on cell proliferation and invasion activity

To analyze the involvement of Lewis y in cell proliferation and invasion, we generated Lewis y− cells with miR RNAi against *FUT1*. At first, a Lewis y-expressing subline HSC-3-34 was isolated by limiting dilution of HSC-3. Then, we first tested the ability of miR RNAi to knockdown *FUT1* in HSC-3-34 with flow cytometer. *FUT1*-knockdown (*FUT1*-KD) cells and control cells were isolated using a cell sorter (Fig. 5a). To analyze the effects of *FUT1* silencing on cell proliferation, an MTT assay was performed. The *FUT1*-KD cells lacking Lewis y showed markedly increased cell growth (Fig. 5b). The cell numbers of *FUT1*-KD cells were 2.1 folds more than the control cells at day 7 of culture. Invasion assay using Boyden chambers revealed that the cell numbers of *FUT1*-KD cells were 1.7 fold more than the control cells (Fig. 5c).

Table 2 Expression of *FUT1* in oral carcinoma tissues with RT-PCR analysis

FUT1 expression (tumor tissue/normal tissue)	
Case 1	2.31
Case 2	1.56
Case 3	1.20
Case 4	7.89
Case 5	4.99


a
Lewis y
HSC-3
HSC-3-21
HSC-3-AA
HSC-3-44
Cell number
Relative fluorescence intensity
b
HSC-3-21
HSC-3
HSC-3-AA
HSC-3-44
** p<0.01
Relative cell growth
Day
* p<0.05 ** p<0.01
Invaded cell numbers
c
HSC-3 21
(Lewis y-)
Lewis y
Cell number
FUT1
transfection
neo-expression
of Lewis y
cell sorting
Lewis y-
population
Lewis y+
population
Relative fluorescence intensity
d
L8
L15
VC7
VC8
Le y
Le x
SLe x
Cell number
Relative fluorescence intensity
e
VC7 Lewis y-
VC8 Lewis y-
L8 Lewis y+
L15 Lewis y+
**p<0.01
Relative cell growth
Day
f
**p<0.01
Invaded cell numbers
L8
L15
VC7
VC8
Lewis y+
Lewis y-

◀ **Fig. 4** Effects of *FUT1* cDNA transfection on cancer properties. **a** A Lewis y-defective mutant line HSC-3-21, Lewis y-high expressant HSC-3-44 and Lewis y-low-expressant HSC-3-AA as well as the parent HSC-3 were obtained by limiting dilution. **b** Using 4 sublines with different expression levels of Lewis y, cell proliferation and invasion activities were compared. In MTT assay, cells with high expression of Lewis y tended to show reduced cell growth (*left*). In invasion assay, cells with high expression of Lewis y also tended to show reduced invasion activity (*right*). **c** Lewis y− and Lewis y+ populations were obtained from HSC-3-21 (Lewis y−) clone isolated from HSC-3 by limiting dilution using human *FUT1* cDNA and cell sorting. From these populations, stable Lewis y+ transfectants and Lewis y− controls were established. **d** Expression of Lewis y (Le y), Lewis x (Le x) and sialyl-Lewis x (SLe x) on two transfectant lines (L8, L15) and two control lines (VC7, VC8) was analyzed by frow cytometry. (**e**, **f**) Results of the MTT assay and invasion assay using two Lewis y+ sublines and two Lewis y− sublines. Cells (4×10^3) were seeded in 98-well plates, and the MTT assay was performed (**e**) at the time points indicated. The experiments were performed in triplicates, and means ± S.D. are presented. Invasion assays were performed with a Boyden chamber and Matrigel™ (**f**). The lower chamber was filled with the culture medium with serum. Cells (2×10^5 cells/well) were added to serum-free medium in the upper chamber and incubated for 24 h. Then the cells on the surface of the filter were stained with Giemsa and the cell numbers were counted. The experiments were performed in triplicates, and means ± S.D. are presented

Alternative staining of Lewis y and Ki-67 in cultured cells after *FUT1* transfection

Immediately after transfection of *FUT1* cDNA into HSC-3-21, expression patterns of Lewis y and Ki-67 were analyzed by immunocytostaining. Lewis y-positive population sorted by cell sorter showed strong staining of Lewis y, but Ki-67 was scarcely stained as shown in Fig. 6. On the other hand, Lewis y-negative population showed definitely positive staining of Ki-67. These results were summarized at the bottom of Fig. 6.

These results were summarized in Fig. 7. Invasion sites of SCC tumor tissues lost Lewis y where Ki67 was definitely stained. Results of immunocytostaining also supported these data. Manipulation of *FUT1* cDNA in SCC cell lines made it possible to recapitulate implication of Lewis y expression or loss during the progress of SCC.

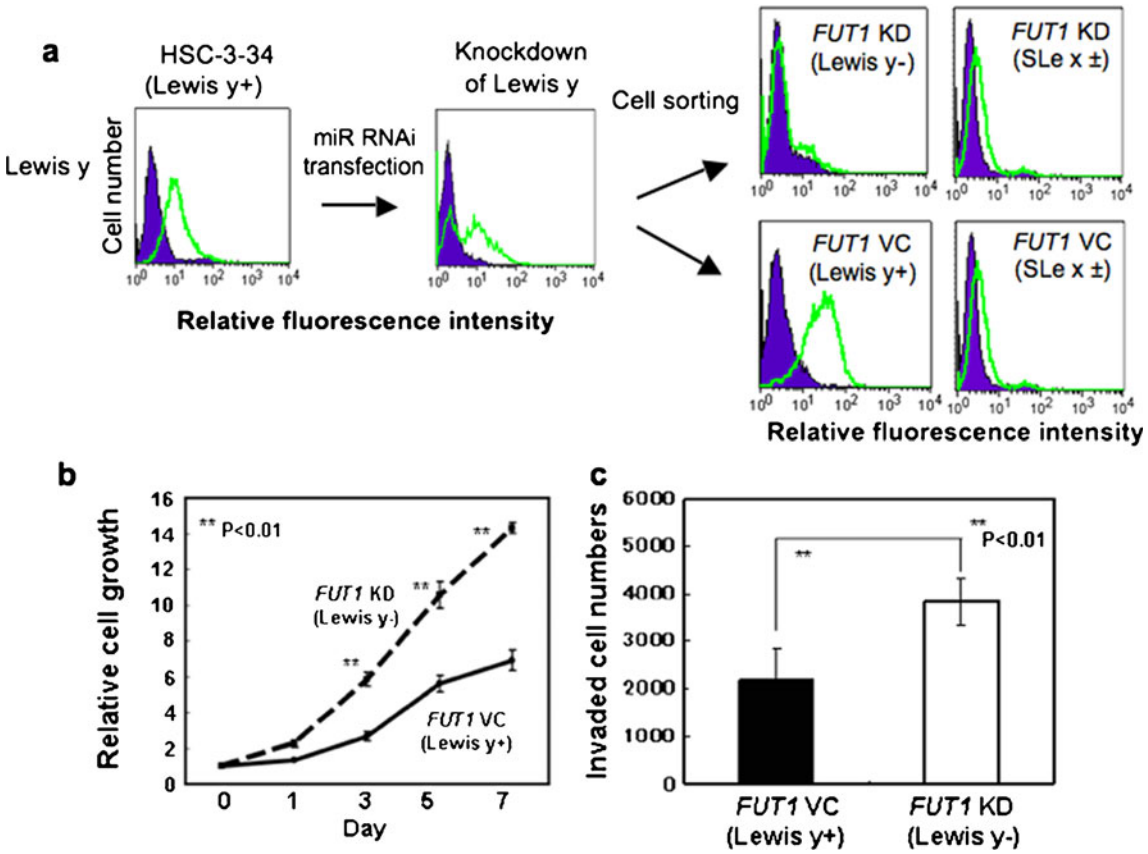
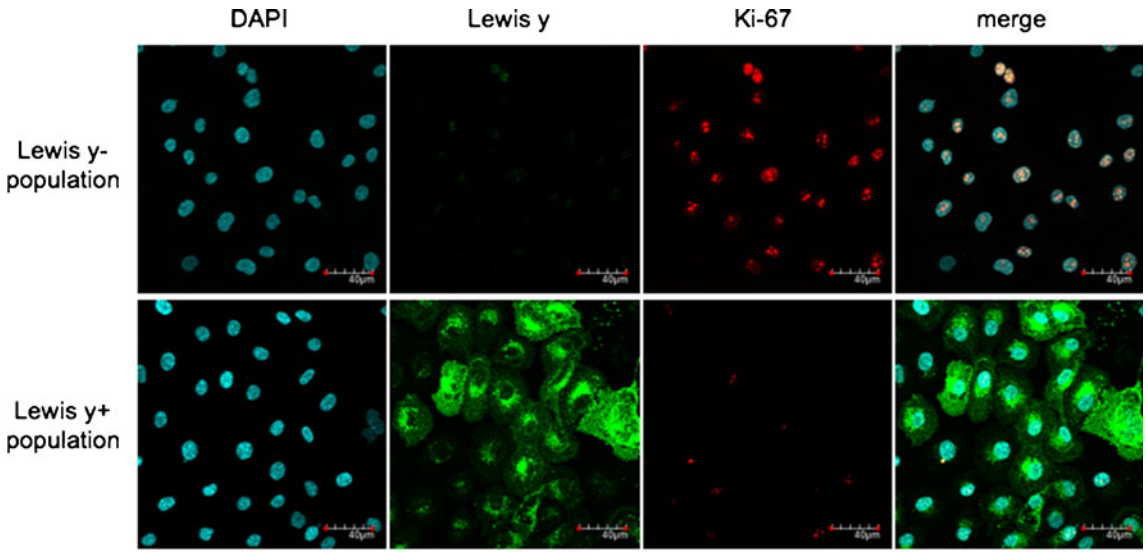


Fig. 5 Knockdown of *FUT1* resulted in the enhancement of cancer properties. **a** A HSC-3-34 (Lewis y+) clone was isolated from HSC-3 by limiting dilution, and transfected with miR RNAi against *FUT1* to establish Lewis y− transfectants. Vector control cells (*FUT1* VC) and *FUT1* knockdown cells (*FUT1* KD) were isolated using a cell sorter. (b, c) Cells (4×10^3) were seeded in 98-well plates, and the MTT assay was performed (**b**) at the indicated time points. The experiments were performed in triplicates, and means ± S.D. are presented. Invasion activity of the *FUT1* KD cells (Lewis y−) was analyzed by a Boyden chamber (**c**). The results in triplicate experiments are presented as means ± S.D. Note that Lewis y+ cells showed reduced invasion compared with Lewis y− cells



Numbers of Ki-67 positive cell after over-expression of *FUT1* cDNA.

Lewis y expression	Numbers of Ki-67 positive cell per 1000 cells
High	23 (2.3%)
Low	64 (6.4%)
Negative	782 (78.2%)

Fig. 6 Alternative staining of Lewis y and Ki-67 in cultured cells. After transfection of *FUT1* cDNA into HSC-3-21, expression patterns of Lewis y and Ki-67 were analyzed by immunocytostaining. As expected, Lewis y-positive population showed strong staining of Lewis y, but scarce staining of Ki-67. Lewis y-negative population showed definitely positive staining of Ki-67

Lewis y+ cells showed reduced tumor growth and peritoneal dissemination in nude mice

To confirm roles of Lewis y in *in vivo* tumors, after subcutaneous injection into nude mice, the tumor sizes of transfectant cells (L8, L15) and controls (VC7, VC8) were measured (Fig. 8a, b). The sizes of L8 and L15 reduced gradually after forming some tumors, while those of control cells increased. Prominent differences between transfectant cells and control cells were observed after 10 weeks (Fig. 8c). Consequently, peritoneal invasion was not observed at all in transfectant cells, whereas it was frequently observed in control cells.

Discussion

There have been a number of studies on the alteration in the expression of carbohydrate antigens with malignant transformation of cells [6]. Although some of them have been used as tumor markers and/or indicators of tumor mass in the bodies during post-operative observation, their implication in cancer phenotypes has scarcely been investigated except for sialyl Lewis x and sialyl Lewis a [19].

Due to the progress in the isolation and analysis of glycosyltransferase genes responsible for the synthesis of cancer-associated complex carbohydrates, roles of individual carbohydrate structures have been investigated by modification of glycosylation patterns in cultured cells [20], and in experimental animals [21]. Indeed, implication of cancer-

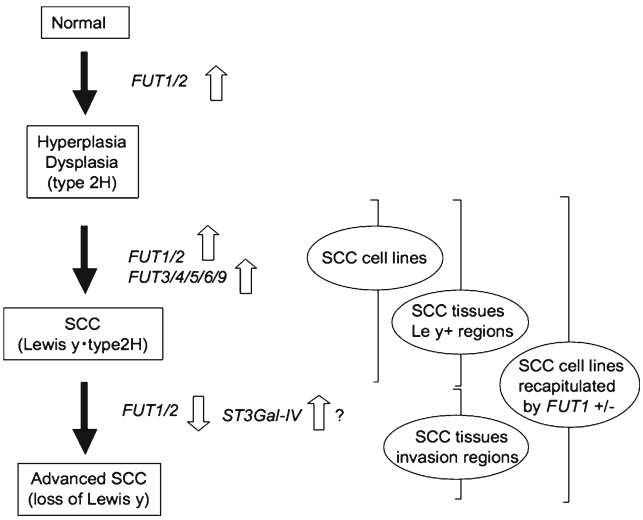
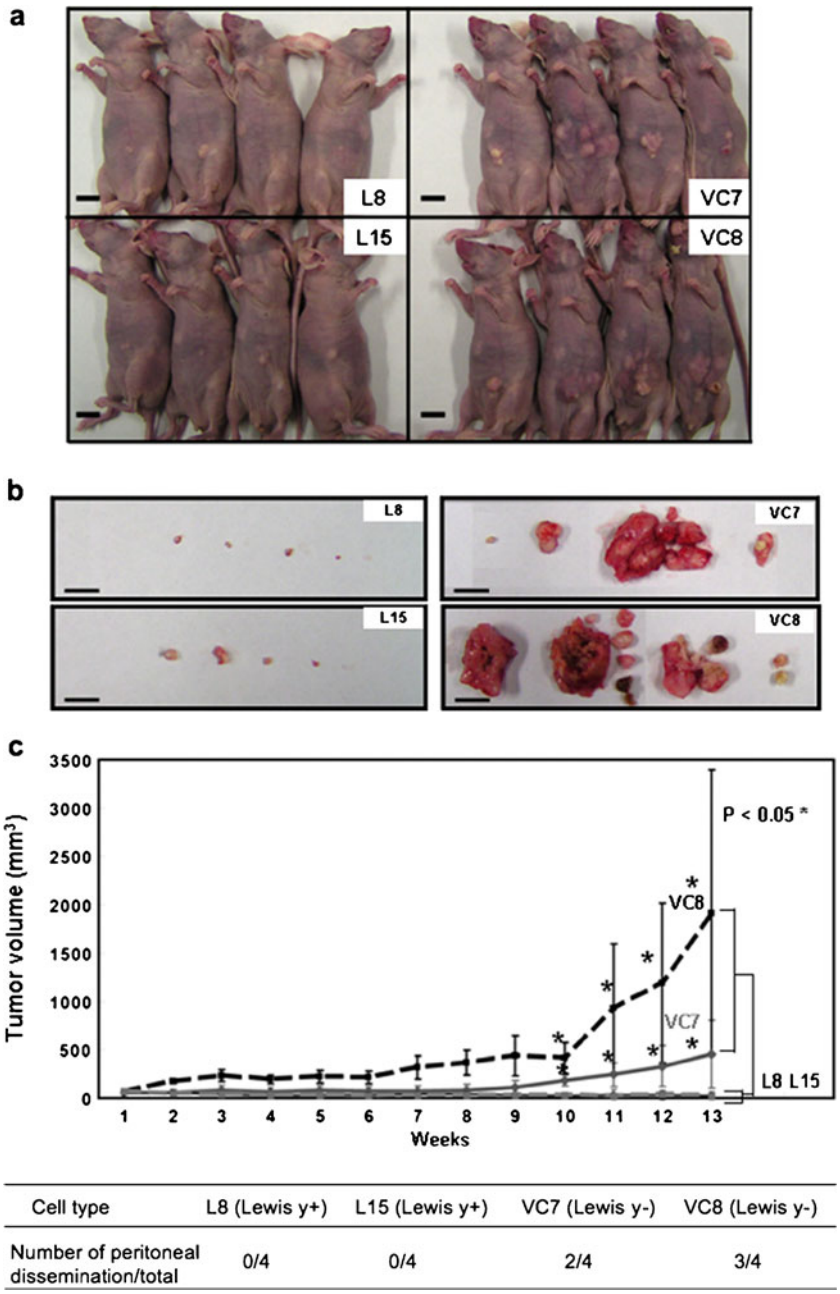


Fig. 7 Summary of the expression of blood group antigens during the development of SCCs in oral cavity. All SCC cell lines examined were Lewis y positive. Majority of cancer tissues of SCC also showed Lewis y-positive. However, invasion sites of SCC tissues were negative for Lewis y where Ki-67 was definitely stained. In this study, manipulation of *FUT1* cDNA in SCC cell lines enabled us to recapitulate whole aspects of Lewis y expression and its implication during the progress of SCC



Cell type	L8 (Lewis y+)	L15 (Lewis y+)	VC7 (Lewis y-)	VC8 (Lewis y-)
Number of peritoneal dissemination/total	0/4	0/4	2/4	3/4

Fig. 8 Reduced tumor growth of Lewis y+ cells transplanted to nude mice. Tumors grown on the abdomens of nude mice were excised at 13 weeks after subcutaneous injection and photographed (**a**). The tumor sizes of transfectant cells (L8, L15) were reduced gradually after once forming tumors, while those of controls (VC7, VC8) increased. The transfectant cells formed scar tissues, while control cells formed definite tumor lumps (**b**). The bar indicates 10 mm. (**c**) Lewis y+ cells showed reduced tumor growth and no peritoneal dissemination in nude mice, while it was frequently observed in control cell lines

associated glycosphingolipids such as gangliosides GD3 and GD2 has been investigated, showing their enhancing effects on cell proliferation and invasion [18, 22, 23]. Apoptosis-inducing function of anti-GD2 mAbs was also reported [24], suggesting crucial roles of GD2 in the regulation of cell signaling.

As for blood group antigens, A/B/H group antigens in cancers have been widely studied [10, 25, 26]. In many cases, A/B antigens were reported to disappear along with malignant transformation in various cancer tissues [27]. Frequent loss of A/B antigens could be sometimes explained by allelic loss or epigenetic regulation such as hypermethylation [13].

In SCCs occurred in oral cavity also showed loss of A/B antigens along with progress of malignant transformation [27]. H antigens and Lewis antigens have been detected in some portions of normal mucosa and abnormal proliferative changes in oral mucous membrane. Lichen, leukoplakia and dysplasia as well as cancers expressed type 2H and Lewis y. In particular, H structures generated by FUT 1 or FUT 2 are frequently expressed in para-basal cells. Furthermore, difucosyl structures such as Lewis y were detected in various cancer tissues [10], and in oral carcinomas with prognostic value [28]. In addition to difucosyl blood group antigens, Lewis x and Lewis a and their sialylated forms were mainly detected in established cancer tissues [27].

As summarized in Fig. 7, either type 2H or Lewis y were not detected in normal tissues in our results, indicating some discrepancy with those studies described above. This might be mainly due to the differences in the specificities of mAbs used, since there is a wide variety of reaction patterns toward similar structures even among mAbs with same name [29].

In our study using clinical samples, high expression rates of type 2 H antigen were detected in a wide range of disorders from hyperplasia to malignant tumor tissues (Table 1), although normal tissues did not show definite expression of type 2H. As for Lewis y, frequency of the expression in the hyperplasia and dysplasia were not so high as type 2H with superficial portion-dominant pattern. Thus, Lewis y expression was more restricted in established cancer tissues, suggesting that Lewis y might be a tumor specific marker. Actually, all SCC cell lines occurred in the oral cavity showed definitely positive expression of Lewis y as well as type 2H (Fig. 1). Generally, many studies reported expression of Lewis y along with increased proliferation [28], and with worse prognosis [10] particularly in ovarian cancers [15], while there was also a report that Lewis y expression predicted better survival in NSCLC [30].

To our surprise, over-expression of Lewis y in a SCC cell line resulted in decreased cell proliferation and invasion activities (Fig. 4). In accordance with results of *FUT1* gene over-expression, gene silencing of *FUT1* resulted in the reduced expression of Lewis y and in the increased invasion and proliferation (Fig. 5). The growth of *in vivo* tumors grafted in nu/nu mice also showed similar results. All these results suggested that Lewis y expression in SCC cells suppresses cancer properties. Pendu *et al*. reported that H/Le b/Le y expression decreased in a late stage (metastatic) of various cancers, and SLe a/SLe x expression increased in stead, suggesting that α1,2-fucosylation was replaced by α2-3-sialylation at the n-n-reducing end of type I/type II core structures [10]. Another report also supported that loss of H antigen is associated with increased tumor invasion [17]. These results well corresponded with our data of Lewis y-overexpression/knockdown experiments.

It has been accepted that glycosylation patterns on key receptors expressed on membrane intensely affect the tumor properties such as cell adhesion, motility and invasion [13]. Although there are a number of glycoproteins and glycolipids which undergo α-3-fucosylation to form Lewis y structure on the cell surface (data not shown), identification of major molecules responsible for the alteration in cancer phenotypes after over-expression and/or knockdown of *FUT1* gene remains to be challenged.

A very intriguing point in the expression of Lewis y antigen is the discrepancy between the prominent expression of Lewis y in the majority of SCC tissues and SCC cell lines examined, and deletion of Lewis y in the invading front of tumors (Fig. 3). Sustained expression of Lewis y might be essential for the SCC cell lines to adapt to the current culture conditions. Namely, Lewis y-positive cell population may be easier to survive in the culture conditions, *i.e*. presence of calf serum, no surrounding cells, or no matrices *etc*. Thus, what kind of roles Lewis y plays seem to be crucial issues. In lung cancer cells, sialylation and fucosylation of EGFR resulted in the suppression of its dimerization and activation [31], suggesting that defucosylation is one of critical factor to promote the aggressive features of cancers. This conclusion is in good accordance with our results. Consequently, loss of Lewis y might be a characteristic features of late phase cancers, and essential for the enhancement of growth factor/receptor-mediated signals to promote invasion and probably metastatic potentials.

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