

Different expression levels of α 3/4 fucosyltransferases and Lewis determinants in ovarian carcinoma tissues and cell lines

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Received February 22, 2006; Accepted April 14, 2006

Abstract. Ovarian carcinoma is the leading cause of death from gynecological cancers in many countries. Fucosylated glycoconjugates have been associated with various carcinomas. In the present study, we have characterized the expression of α 3/4 fucosyltransferases transcripts and their products, the Lewis carbohydrate determinants, and their *in vitro* specificity towards synthetic acceptors using ovarian carcinoma cell lines OVM, m130, GG and SKOV3. We found different expression patterns: GG cells expressed mostly Lewis^x (Le^x), Lewis^y (Le^y), sLe^a and Le^b, and m130 cells expressed mostly Le^x and Le^y. The detection was on the plasma membrane and in intracellular vesicles. OVM and SKOV3 cells had very low amounts of staining. From RT-PCR studies, enzyme specificity of cellular extracts towards a panel of synthetic carbohydrate acceptors and Western blot analysis we concluded that Le^a, sLe^a and Le^b were synthesised by FUT3, whereas Le^x and Le^y were synthesized by FUT4 and FUT9 in both cell lines. The GG and m130 cell lines are adequate models to investigate the role of Le^x, Le^y, sLe^a and Le^b in ovarian carcinoma development.

Introduction

Ovarian carcinoma is the leading cause of death from gynecological cancers in many Western countries. Primary ovarian cancers can be classified according to the structure of the ovary from which they derived. The majority, 80-90%, develop from epithelial cells (serous, mucinous, endometrium and mixed),

followed by carcinomas originating in granulosa cells or, rarely, in the stroma cells (reviewed in ref. 1).

Fucosylated glycoconjugates have been associated with various cancers. An increase in the carbohydrate determinants H (Fuc α 2Gal) and Lewis^y [Le^y; Fuc α 2Gal β 4(Fuc α 3)GlcNAc] expression concomitant to a decrease of A and B blood group antigens for several tumors, in correlation with a poor prognosis has been observed. Furthermore, increases of sialyl-Lewis^x [sLe^x; NeuAc α 2,3Gal β 4(Fuc α 3)GlcNAc] and sialyl-Lewis^a [sLe^a; NeuAc α 2,3Gal β 3(Fuc α 4)GlcNAc] were also found to be associated with disease progression and bad prognoses. sLe^x and sLe^a are ligands of E- and P-selectin and may aid in hematogenous metastasis (reviewed in ref. 2). Expression of the carbohydrate determinants sialyl Tn, TF, Le^y, Globo H and GM2 have been associated with ovarian cancer, and a vaccine against this type of cancer using as antigen Le^y-keyhole limpet haemocyanin has undergone clinical phase I (reviewed in ref. 3).

The α 3/4 fucosyltransferases (FTs) are key enzymes that regulate the synthesis of Lewis carbohydrate determinants and constitute a group of FTs with a high degree of sequence similarity. α 3/4 FTs catalyse the transfer of fucose from GDP-fucose to one or more acceptor substrates in an α 1,3 or α 1,4 linkage to GlcNAc in Gal-GlcNAc sequences type I, Gal β 3GlcNAc-R (Le^a, Le^b, sLe^a), or type II, Gal β 4GlcNAc-R (Le^x, Le^y, sLe^x). Eight α 3 FTs have been identified in the human genome at present. FUT3, the Lewis enzyme, predominantly synthesizes α 4- and to a lesser extent α 3-fucosylated oligosaccharides with the resulting products Le^a, Le^b, sLe^a, Le^x, sLe^x, Le^y (4). Myeloid FUT4 is an α 3 FT and synthesizes Le^x and Le^y (5). FUT5 and FUT6 synthesize Le^x and sLe^x (6,7) although some reports have shown that FUT5 can also produce Le^a (8). FUT7 can only synthesize sLe^x (9). FUT9 synthesizes Le^x (10). FUT10 and FUT11 have been identified from the human genome since they have a conserved α 3 FT domain (11), however their activity has not been validated (12).

Several reports demonstrated fucosyltransferases neo-expression or upregulation in human tumours that are not normally expressed or only expressed in low levels in the corresponding normal tissue. In lung cancer the expression of

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Key words: ovarian cancer, fucosyltransferase, Lewis determinant

FUT4 and FUT7 genes is related to poor prognosis (13). FUT4 was also observed to be significantly increased in gastric and colorectal carcinoma (14,15). FUT6 mRNA expression was detected specifically in pancreatic cancer tissues (16) and invasive as well as non-invasive bladder carcinoma cell lines (17), but not in normal pancreas or bladder.

Human ovarian carcinomas have been also described with several-fold higher levels of $\alpha 3$ and $\alpha 4$ FTs, compared with normal ovarian tissue (19), however, the identity of the specific enzymes is still not clear.

Ovarian cancer is often asymptomatic in the earlier stages and patients are only diagnosed after metastatic spread within the peritoneal cavity and/or distant organs. The characterization of the Lewis determinants and identification of the FTs associated with carcinoma development might provide means of establishing a specific diagnostic and prognostic.

In the present work, we have determined the expression pattern by RT-PCR of $\alpha 3/4$ FTs in four ovarian carcinoma cell lines, SKOV3, OVM, m130 and GG, and ovarian carcinoma tissues. We also analysed the expression of Le^a, sLe^a, Le^b, Le^x, sLe^x and Le^y carbohydrate determinants, specificities and Western blot analysis of FTs from the different cell extracts. We have found that GG followed by m130 cells expressed the higher amounts of Lewis determinants, and identified that FUT3 synthesized Le^a, sLe^a and Le^b in GG cell line whereas FUT4 and FUT9 synthesized Le^x and Le^y in both cell lines.

Materials and methods

Cell culture. SKOV3, OVM, m130 and GG ovarian carcinoma cell lines, as well as BHK-21B cells, were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma), supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin (Invitrogen). Cells were grown in a humidified incubator at 37°C in a 5% CO₂ atmosphere.

Tissue samples. Patient material was obtained under the approval of the ethics committee of the University of Heidelberg. Diagnoses were established by conventional clinical and histological criteria according to the World Health Organization (WHO). The classification of tumor tissues was as follows: tissue 1, serous ovarian carcinoma pT3c G3 R2; tissue 2, serous papillary ovarian carcinoma pT3c G3 R2; tissue 3, serous papillary ovarian carcinoma pT3c G3 R2; tissue 4, serous papillary ovarian carcinoma pT3c G2 R3; tissue 5, serous ovarian carcinoma pT3c G3 R3; tissue 6, serous ovarian carcinoma pT3c G3 R1; tissue 7, mucinous ovarian carcinoma pT3c G2 R2; tissue 8, serous adenocarcinoma of the fallopian tube pT3c G3 R1; tissue 9, serous ovarian carcinoma pT3c G3 R1; and tissue 10, serous papillary adenocarcinoma of both ovaries pT3c G2 R2. Gx represents tumor grade.

RT-PCR. Total RNA was isolated from cultured cells using the Rneasy mini kit (Qiagen), following the manufacturer's instructions. RNA concentration and quality were determined spectrophotometrically.

The reverse transcription reaction contained 0.1 $\mu\text{g}/\mu\text{l}$ RNA, 60 ng/ μl random hexamers (Invitrogen), 10 mM dNTPs

(Amersham), cDNA synthesis buffer, 0.1 M DTT and 200 units of M-MLV reverse transcriptase (Invitrogen) in 20 μl reaction. RNA and random hexamers were denatured for 5 min at 65°C followed by primer extension at 25°C for 10 min. cDNA synthesis was accomplished at 37°C for 50 min and reaction was inactivated by incubation at 70°C for 15 min.

Frozen sections from ovarian carcinoma tissues contained >90% tumor cells as revealed by hematoxylin/eosin staining of adjacent sections. Fifteen sections (10 μm) were used to isolate mRNA using the Qiagen RNeasy kit as described above.

PCR amplification of all genes studied, was carried out in 25 μl reaction containing buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs (Amersham) and 2.5 units Taq polymerase (Invitrogen). Portions of $\alpha 3/4$ FTs from human ovarian cancer cell lines and tumoral tissues were amplified using specific primers that can discriminate among them, despite their high homology (Fig. 1). PCR conditions were as follows: 1 μl cDNA template for FUT4 to FUT7, FUT9 to FUT11, and β -actin or 2 μl FUT3; 0.25 μM or primers for FT5, FUT11, and β -actin, or 0.4 μM for FUT3, FUT4, FUT6, FUT7, FUT9 and FUT10. For FUT4 5% dimethylsulfoxide was added. The specific PCR conditions consisted of the following steps: pre-denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 45 sec, annealing at 64°C for 1 min for FUT3, FUT4, FUT6 and FUT7, or at 72°C for 2 min for FUT5, or 57°C for 45 sec for FUT9, or 55°C for 45 sec for FUT10 and FUT11, and extension at 72°C for 2 min, and a final extension step at 72°C for 7 min. Positive controls for FUT3, FUT4 and FUT7 were performed using as template the plasmid vectors pCRFT3 (20), pCR3FT4 and pCR3FT7, respectively (21). To ensure primer specificity, all FT sequences were aligned using ClustalW program and cDNA regions with lower similarity were found for primer design. The primers were aligned with known nucleotide sequences using the NCBI BLAST program, and it was confirmed that they were specific for their respective FTs.

In order to control the cDNA integrity and to check for a possible contamination with genomic DNA, β -actin, a house-keeping gene, was amplified. For the reaction 0.25 mM of each primer, forward 5'-gatatgccgctgctgctgctgac-3' and reverse 5'-caggaaggaaggctggaagagtgc-3', were used (22). PCR conditions were as above except that 25 cycles were performed and the annealing temperature was 61°C for 1 min. Reaction products were separated by electrophoresis with 1.5% agarose, stained with ethidium bromide for 10 min and visualized under a UV lamp. In order to confirm the specificity of the amplification a typical fragment from FUT10 of all cell lines was sequenced. The fragments were purified with QIAquick gel extraction kit, from Qiagen, and analysed by automated DNA sequencing.

Immunofluorescence microscopy. Cells grown on glass coverslips to ~80% confluency, were washed with phosphate-buffered saline (PBS) containing 0.5 mM MgCl₂, fixed with 4% (w/v) paraformaldehyde in PBS for 20 min, and non-permeabilized or permeabilized with 0.1% (w/v) Triton X-100 for 15 min. Fixed cells were blocked with 1% of bovine serum albumin (BSA) in PBS for 1 h, followed by incubations at room temperature for 2 and 1 h with primary and secondary antibodies, respectively. Antibodies were diluted in PBS

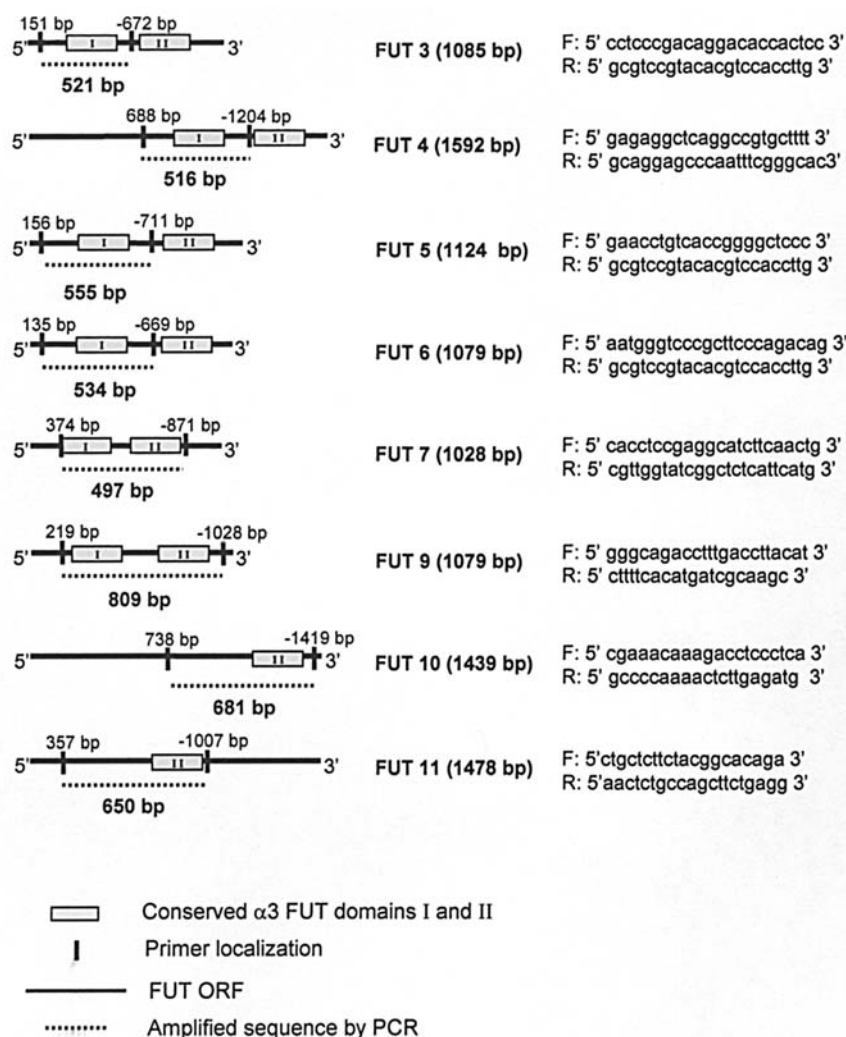


Figure 1. Localization of reverse and forward primers in the indicated $\alpha 3/4$ FTs cDNA sequences. Primer sequences are shown on the right. Primers for FUT3-FUT7 have been described by Numahata *et al* (17), primers for FUT9-FUT11 have been designed by us using the Primer3 software. Sequences have been aligned with ClustalW program and boxes I and II correspond to $\alpha 3$ FT domains as previously defined by Breton *et al* (18). Primers are specific for individual FTs as confirmed by NCBI BLAST program.

containing 1% or 2% BSA, and washes were performed with PBS. The primary antibodies used were: mouse IgG anti-Le^a PR5C5 (Biogenesis) and mouse IgM anti-Le^b BG-6 (Signet Laboratories) at 1:40 dilution in 1% BSA in PBS; hybridoma supernatants mouse IgG anti-sLe^a CA19-9, mouse IgG anti-Le^x SH1 (23), mouse IgM anti-sLe^x FH6 (24) twenty-fold concentrated, mouse IgM anti-Le^y AH6 (25), and mouse IgM anti-Le^x SSEA-1 (MC-480) (DSHB, University of Iowa, USA) at 1:1 dilution in 2% BSA in PBS. The secondary antibodies used were: goat anti-mouse IgM tetramethylrhodamine β -isothiocyanate (TRITC) conjugate (Sigma) and donkey anti-mouse IgG Alexa 594 (Invitrogen) at 1:64 and 1:500 dilution in 1% BSA in PBS, respectively. Coverslips were mounted in Airvol and examined with a Leica DMRB microscope. Images were acquired using a COHU high performance CCD camera coupled to the microscope and Leica QFISH software, with exposure times of 300 to 500 msec and 1000 to 1500 msec.

FACS analysis. The staining of cells with saturating amounts of mAbs, either hybridoma supernatants or purified antibodies, and phycoerythrin-conjugated goat antibodies to mouse immunoglobulins has been described (26). Stained cells were

analyzed with a FACScan cell analyzer (Becton-Dickinson, Heidelberg, Germany) using Cellquest software (Becton-Dickinson).

SDS-PAGE and Western blot analysis. Protein extracts were obtained by solubilization of centrifuged cells in RIPA buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate (DOC), 1% Triton X-100, 1/50 Complete protease inhibitor cocktail tablets (Roche)]. Protein concentration was determined by the bicinchoninic acid method. For fucosyltransferase detection, protein extracts from 1×10^6 cells from each carcinoma cell line were used. Proteins were precipitated with 4 volumes of absolute ethanol, denatured by boiling in SDS-PAGE sample buffer and detected by Western blot analysis. Polyvinylidene difluoride (PVDF) membranes were blocked with 5% defatted dry milk (Nestle) in PBS with 0.1% Tween-20 before the addition of antibodies. The following primary antibodies were used: affinity purified antiserum anti-FUT3 SA-4817 at 0.4 μ g/ml (27), C-20 anti-FUT4 and N-18 anti-FUT5 at 1:1000 dilution, C-20 anti-FUT9 at 1:500 dilution in blocking solution, obtained from Santa Cruz Biotechnology. The secondary antibodies used

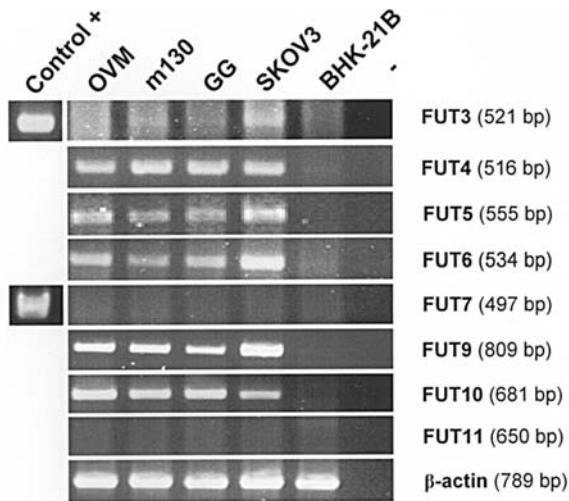


Figure 2. RT-PCR analysis of fucosyltransferases FUT3 to FUT7 and FUT9 to FUT11 from ovarian carcinoma cell lines. β -actin was used as control. cDNA was obtained from ovarian carcinoma cell lines OVM, m130, GG, SKOV3, and the control cell line BHK-21B that does not express peripheral fucosyltransferases. After PCR reaction, products were separated on 1.5% agarose gel and their size estimated relatively to a gene ruler 1 kb and 100 bp DNA ladders. The sizes of the amplified fragments are indicated on the right.

were: anti-rabbit IgG coupled to horseradish peroxidase (Amersham Pharmacia Biotech) at 1:3000 dilution and anti-goat IgG coupled to horseradish peroxidase (Sigma) at 1:15000 dilution in blocking solution. Bands were visualized according to the ECL Plus method (Amersham Pharmacia Biotech).

Glycoprotein detection using lectins. Glycoproteins were stained after transfer to PVDF membrane with lectins. Glycoproteins with affinity for concanavalin A (Con A), peanut agglutinin (PNA) and *Sambucus nigra* lectin (SNA) were detected essentially according to Faye and Chrispeels (28). PNA was covalently linked to horseradish peroxidase (PNA-HRP; Sigma). Con A (Sigma) specifically binds HRP in the presence of Ca^{2+} and Mg^{2+} . SNA was biotinylated (Vector Laboratories). Briefly, the glycoproteins were fixed on the PVDF membrane by incubation with 25% 2-propanol and 10% acetic acid for 5 min. The membrane was blocked for 1 h with TBS containing 0.5 M NaCl and 0.1% Tween-20 (TTBSc), incubated overnight with 10 $\mu\text{g}/\text{ml}$ of lectin PNA-HRP, or 1 h with 25 $\mu\text{g}/\text{ml}$ Con A. The Con A membrane was washed and further incubated for 1 h with HRP type I (Sigma). Incubations with the lectins and washings were performed with TTBSc for PNA-HRP, and with TTBSc containing 1 mM CaCl_2 and 1 mM MgCl_2 for Con A/HRP. Development was performed with 0.5 mg/ml 4-chloro-1-naphthol and $8.3 \times 10^{-3}\%$ H_2O_2 in PBS. For SNA, the membrane was blocked for 1 h with TBS containing 2% bovine serum albumin, incubated with 5 $\mu\text{g}/\text{ml}$ of lectin SNA-biotin, followed by 2 $\mu\text{g}/\text{ml}$ streptavidin-peroxidase (Sigma) in blocking buffer. Development was as above.

Fucosyltransferase activity. Fucosyltransferase activity was measured by the incorporation of radioactive fucose from the GDP-[^{14}C]-Fucose donor (Amersham Pharmacia Biotech) into

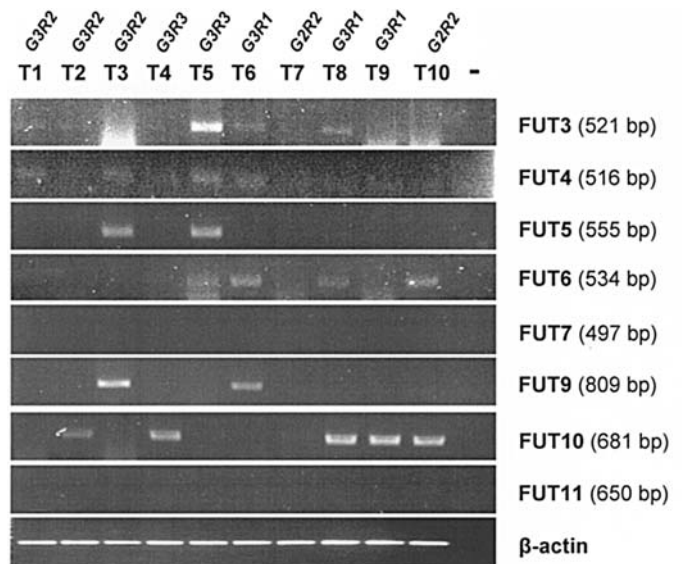


Figure 3. RT-PCR analysis of fucosyltransferases FUT3 to FUT7 and FUT9 to FUT11 from ovarian carcinoma tissues. β -actin was used as control. cDNA was obtained from serous ovarian carcinoma tissues T1, T5, T6 and T9, serous papillary ovarian carcinoma tissues T2, T3 and T4, mucinous ovarian carcinoma tissue T7, serous adenocarcinoma of the fallopian tube tissue T8, and serous papillary adenocarcinoma of both ovaries tissue T10. The progression grade of the tumors is indicated at the top. After PCR reaction, products were separated on 1.5% agarose gel and their size estimated relatively to a gene ruler 1 kb and 100 bp DNA ladders. The sizes of the amplified fragments are indicated on the right.

several acceptors as previously described (27). The acceptors used were: type I, $\text{Gal}\beta 3\text{GlcNAc-R}$, $\text{NeuAca}2,3\text{Gal}\beta 3\text{GlcNAc-R}$ and $\text{Fuca}2\text{Gal}\beta 3\text{GlcNAc-R}$ (H type I); type II, $\text{Gal}\beta 4\text{GlcNAc-R}$ (LacNAc), $\text{NeuAca}2,3\text{Gal}\beta 4\text{GlcNAc-R}$ (sialyl-LacNAc) and $\text{Fuca}2\text{Gal}\beta 4\text{GlcNAc-R}$ (H type II) (Lectinity Holding). The acceptors were linked to a hydrophobic spacer arm where $\text{R} = -(\text{CH}_2)_3\text{-NHCO-(CH}_2)_5\text{-NH-biotin}$. One unit of enzyme activity was defined as the amount of enzyme catalysing the transfer of 1 μmol of Fuc/min to the acceptor.

Results

Fucosyltransferase mRNA expression in ovarian carcinoma cell lines and tissues. RT-PCR analysis was performed on the ovarian carcinoma cell lines SKOV3, OVM, m130 and GG to characterize the expression pattern of the $\alpha 3/4$ FTs in these cells. BHK-21B cells were used as a negative control since they do not express any peripheral FT. Total RNA was isolated from the different cell lines and the cDNA synthesized with M-MLV reverse transcriptase using random hexamers.

We observed the specific amplification of fragments from FUT4 (516 bp), FUT5 (555 bp), FUT6 (534 bp), FUT9 (809 bp), and FUT10 (681 bp) for all cell lines analysed (Fig. 2). Only small amounts of FUT3 fragment (521 bp) were observed predominantly in SKOV3 cells, followed by GG and m130 even if there was a strong amplification in similar conditions from the plasmid pCRFT3 coding for FUT3. These results suggested that a lower amount of FUT3 mRNA was present in those cells. There was no amplification of FUT7 although the primers could amplify the expected band

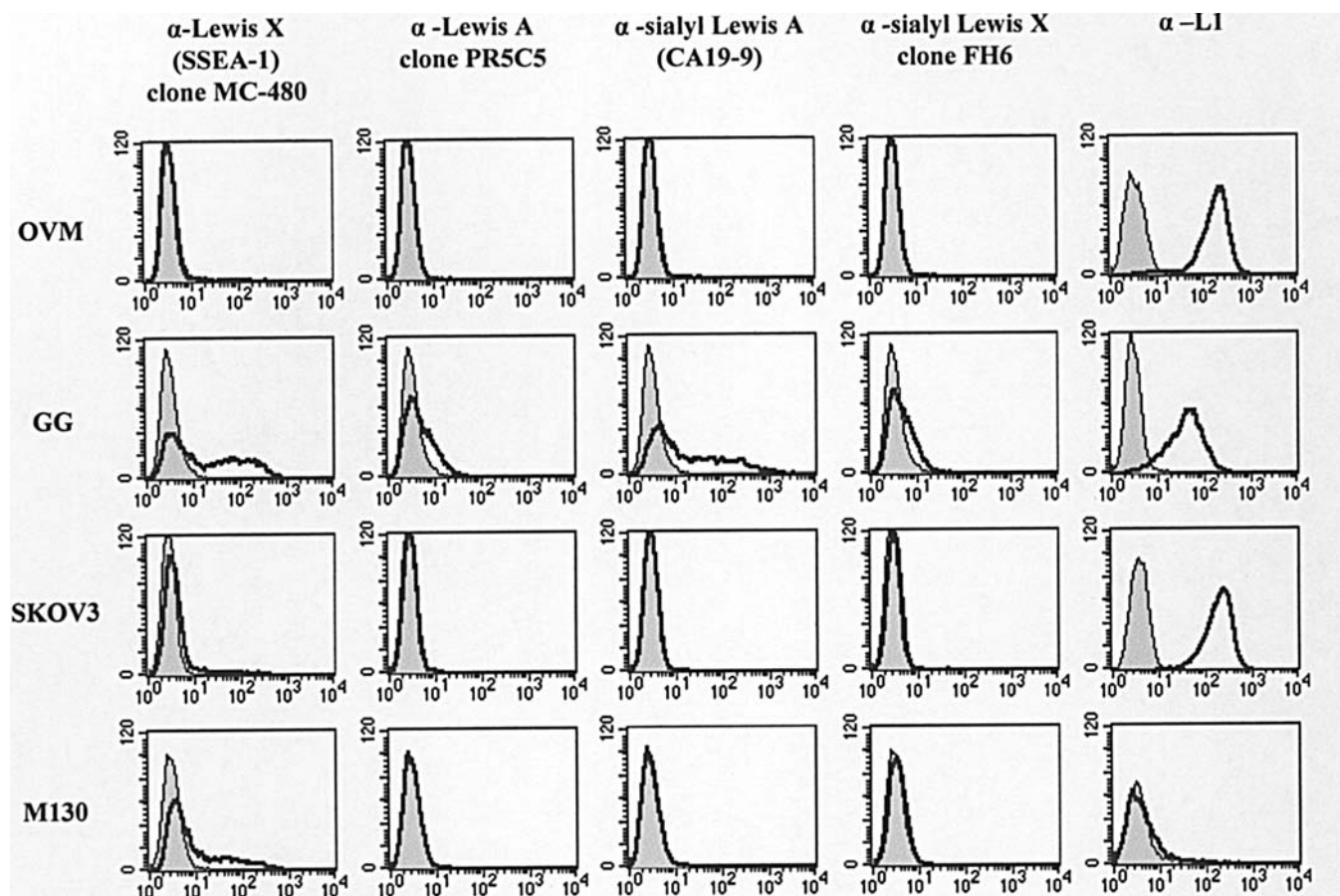


Figure 4. FACS analysis of ovarian carcinoma cells for cell surface expression of carbohydrate epitopes. Cells were incubated with primary antibody followed by phycoerythrin-conjugated secondary antibody.

in similar conditions from plasmid DNA pCR3FT7 coding for FUT7 (21).

No amplification of any of the studied FTs was observed in BHK-21B cells as expected. Amplification of β -actin (789 bp), used as a positive control for the RT-PCR experiment showed similar intensities for all cell lines, suggesting that amplifications were performed on comparable amounts of cDNA except for FUT3 where double the amount of template was used.

We next examined the expression pattern of α 3/4 FTs in ovarian carcinoma tissues of different types: serous ovarian carcinoma (SOC), serous papillary ovarian carcinoma (SPOC), mucinous ovarian carcinoma (MOC), serous adenocarcinoma of the fallopian tube (SAFT) and serous papillary adenocarcinoma (SPA) of both ovaries (Fig. 3). We observed the expression of FUT3 in samples of SOC (T5 and T6) and SAFT (T8). FUT4 was detected in SOC (T1, T5 and T6) and SPOC (T3 and T4). FUT5 was expressed in one sample of SPOC (T3) and another of SOC (T5). FUT6 was found in SOC (T5, T6), SAFT (T8) and SPA (T10). FUT9 was detected in one sample of SPOC (T3) and another of SOC (T6). Finally, FUT10 was found in five samples, two SPOC (T2, T4), one SAFT (T8), one SOC (T9) and one SPA (T10).

Interestingly, from the two tissues examined with a lower grade of progression only one expressed a single FT: FUT6. On the other hand, FUT4 was more extensively represented in tissues with a higher grade of progression.

Expression of carbohydrate Lewis determinants on human ovarian carcinoma cells. The expression of fucosylated carbohydrates on the surface of ovarian carcinoma cells was determined by FACS analysis (Fig. 4). The GG cell line exhibited the higher level of the cell surface carbohydrate epitopes Le^x , Le^a , sLe^a and sLe^x . The cell line m130 followed by SKOV3 had small amounts of surface Le^x . The OVM cell line did not show any of the fucosylated epitopes studied. As a control, the cell surface adhesion molecule L1 was detected for the cell lines studied. L1 was found in high amounts on the surface of OVM and SKOV3 cells, followed by GG cells with lower amounts as expected from previous studies (26).

The expression of intracellular as well as surface Lewis determinants was also determined by immunofluorescence microscopy for all cell lines and additional antibodies anti- Le^b and anti- Le^y were used. Discrimination between plasma membrane and plasma membrane/intracellular staining was accomplished by the comparison of non-permeabilized vs. permeabilized cells. GG cells contained predominantly sLe^a , Le^b , Le^x and Le^y (Fig. 5E, F, A, and C) but almost undetectable amounts of Le^a and sLe^x (Fig. 5D and B). These epitopes were detected on the cell surface (corresponding to FACS detection) where interesting morphological formations consisting of numerous plasma membrane projections were visualized (Fig. 5E, inset). The same epitopes were also detected in intracellular vesicles (Fig. 5A, C, E, and F). Additionally, SSEA-1-stained GG cells showed staining

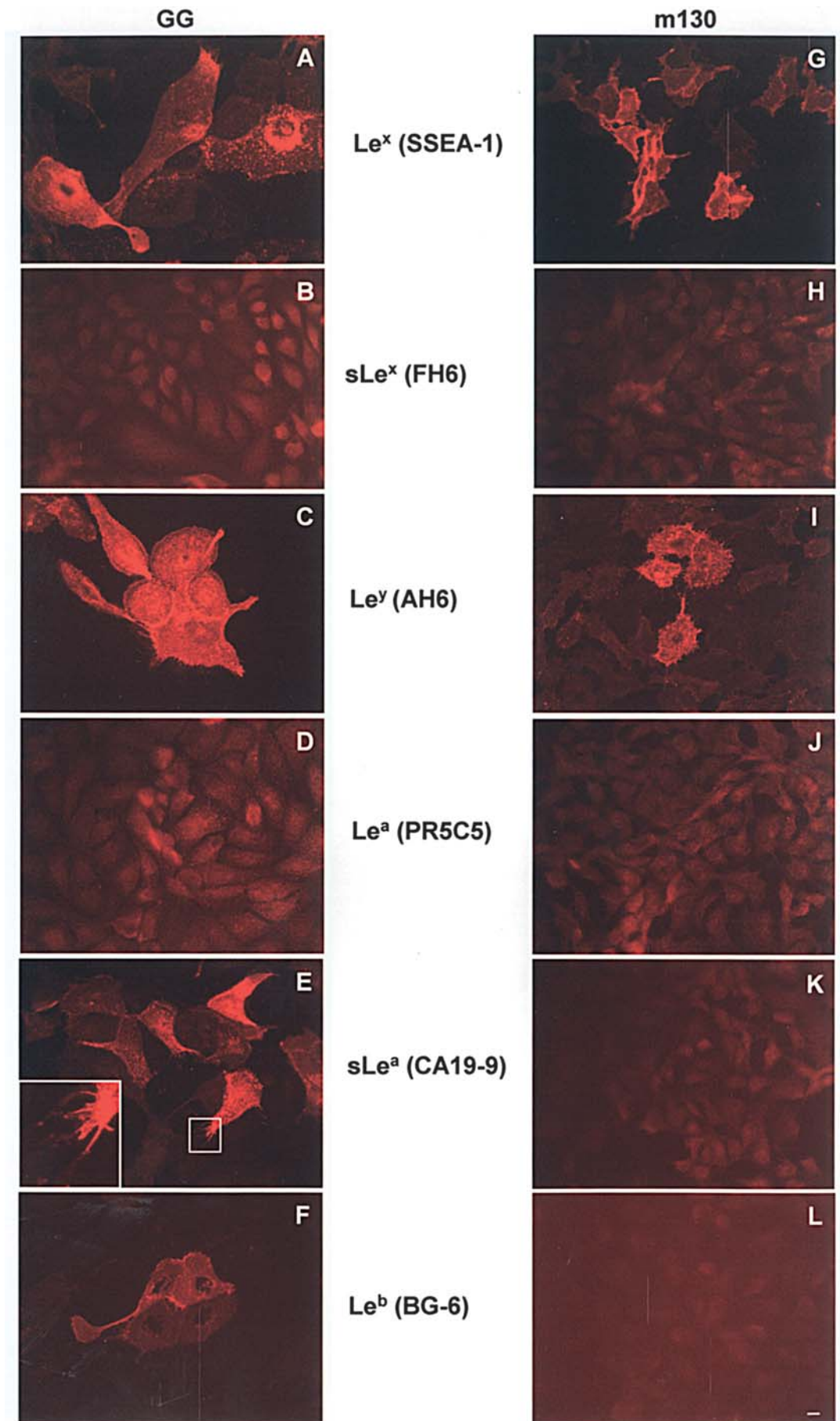


Figure 5. Immunofluorescence microscopy of Lewis carbohydrate determinants from GG and m130 ovarian carcinoma cells. GG and m130 cell lines were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were probed with antibodies anti- Le^a PR5C5, anti-s Le^a CA19-9, anti- Le^b BG-6, anti- Le^x SSEA-1, anti-s Le^x FH6 and anti- Le^y AH6. Secondary antibodies were anti-mouse IgG ALEXA 594 or anti-mouse IgM TRITC conjugate. Images A, C, E, F, G and I were acquired with 300 to 500 msec, images D, H, L, K and J were acquired with 1000 to 1500 msec. of exposure. Scale bar, 10 μm .

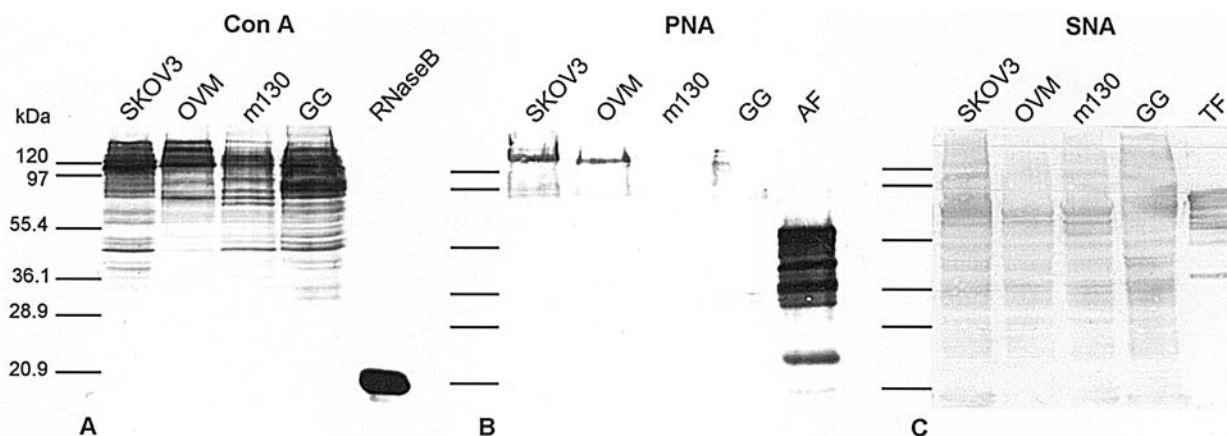


Figure 6. Immunodetection by Western blotting of glycoproteins in ovarian carcinoma cell lines. Sixty μg of protein were applied per lane in a 12% acrylamide gel. Detection was performed using lectin Con A (A), PNA (B) and SNA (C). Two μg of RNaseB, asialofetuin (AF) and 1 μg of transferrin were applied as positive controls (indicated above the lanes).

around the nucleus with a morphology characteristic of the Golgi apparatus (Fig. 5A).

M130 cells showed high amounts of Le^y (Fig. 5I) followed by Le^x (Fig. 5G). Also in these cells stained plasma membrane projections and intracellular vesicles were detected (Fig. 5G and I). The other determinants were only detected in residual amounts.

For both GG and m130 cell lines we have also observed Le^x with the monoclonal antibody SH1 (results not shown). SH1 is capable of recognizing Le^x in short type 2 chains (29) whereas anti-SSEA-1 has a narrower specificity and requires that Le^x is present in a long poly-N-acetylactosamine backbone (30). Therefore, at least a part of the Le^x from GG and m130 cells is present in long poly-N-lactosamine chains.

SKOV3 followed by OVM cells expressed low amounts of intracellular Le^x in few cells, and almost undetectable amounts of Le^a (data not shown).

Lectin-binding properties of human ovarian carcinoma cells. In order to detect glycosylation profiles of ovarian carcinoma cells, we also performed Western blot analysis of cellular extracts followed by lectin binding, and immunofluorescence microscopy with specific carbohydrate-binding antibodies.

We observed that the amount of Con A-binding polypeptides was higher for GG cells and lower for OVM cells (Fig. 6A). Since Con A binds α -mannosyl containing-branched oligosaccharides predominantly from oligomannose-type followed by hybrid- and biantennary complex-type structures (31), the results indicated the predominance of this type of structure in GG cells. As positive control ribonuclease B has been used since it contains oligomannose type oligosaccharides (32).

PNA that is specific for $\text{Gal}\beta 3\text{GalNAc}$ from O-linked oligosaccharides strongly bound one glycoprotein with mass higher than 120 kDa from SKOV3 cells, also found in lower amounts in OVM followed by GG cells but undetectable in m130 cells (Fig. 6B). As positive control for PNA binding asialofetuin was used (33,34).

SNA specific for terminal $\text{NeuAca}2,6\text{Gal}$ or $\text{NeuAca}2,6\text{GalNAc}$, and to a lesser extent terminal $\text{NeuAca}2,3$ -linked, predominantly bound GG and SKOV3 cells followed by

OVM and m130 cells (Fig. 6C). As positive control for SNA binding human plasma transferrin was used that contains peripheral $\text{NeuAca}2,6$ -linked (35). Collectively, the results showed ovarian carcinoma cell type-specific glycosylation.

Fucosyltransferase activities of human ovarian carcinoma cells. The fucosyltransferase activities from the cellular extracts of the four ovarian carcinoma cell lines were assayed using type 1 and type 2 oligosaccharides conjugated to a hydrophobic moiety as previously described (27). m130 and GG had higher FT activities against both type 2 and type 1 oligosaccharides than SKOV3 or OVM cells. These results agreed with the higher amounts of Lewis carbohydrate determinants detected on m130 and GG cells. Substitution of $\text{Gal}\beta 4\text{GlcNAc-R}$ with $\text{Fuca}2$ -linked led to a 2.2-fold increase in $\alpha 3$ FT activity from m130 cells ($11.25 \pm 4.38 \mu\text{U}/1 \times 10^6$ cells to $24.60 \pm 13.10 \mu\text{U}/1 \times 10^6$ cells) (Table I). However, substitution of $\text{Gal}\beta 4\text{GlcNAc-R}$ with $\text{NeuAca}2,3$ -linked led to a 32-fold decrease in $\alpha 3$ FT activity from m130 cells ($11.25 \pm 4.38 \mu\text{U}/1 \times 10^6$ cells to $0.35 \pm 0.14 \mu\text{U}/1 \times 10^6$ cells). These results indicated that the major $\alpha 3$ FT activity present in m130 cells involved in the synthesis of the Le^x and Le^y determinants would possibly consist of FUT9 or FUT4 (36). Although with lower $\alpha 3$ FT activities GG showed the same profile, which suggests that also for these cells FUT4 or FUT9 would be key enzymes for the synthesis of Le^x and Le^y . The low activity observed against the sialylated type 2 acceptor in m130 and GG cells could be due either to FUT5 or FUT6 as detected by RT-PCR (Fig. 2). SKOV3 and OVM cells had only comparably lower $\alpha 3$ FT activity, but followed a similar profile.

Concerning $\alpha 4$ FT activity, we observed that substitution of $\text{Gal}\beta 3\text{GlcNAc-R}$ with $\text{Fuca}2$ -linked as acceptor led to a 3.7-fold increased $\alpha 4$ FT activity from GG cells ($2.90 \pm 0.17 \mu\text{U}/1 \times 10^6$ cells to $10.74 \pm 1.04 \mu\text{U}/1 \times 10^6$ cells) (Table I). However, substitution of $\text{Gal}\beta 3\text{GlcNAc-R}$ with $\text{NeuAca}2,3$ -linked led to a small decrease in GG $\alpha 4$ FT activity ($2.90 \pm 0.17 \mu\text{U}/1 \times 10^6$ cells to $2.67 \pm 0.40 \mu\text{U}/1 \times 10^6$ cells). These results suggested that FUT3 would be the enzyme involved in the synthesis of Le^a , sLe^a and Le^b in GG cells. There is a remote possibility that FUT5 would also participate in the synthesis of these determinants since it has a low activity towards type 1

Table I. Substrate specificity of fucosyltransferases expressed in cancer cell lines.^a

Substrate	Cell line	Activity (μ U/1x10 ⁶ cells)	
Type 1 acceptors			
Gal β 1,3GlcNAc-R	SKOV3	0	0
	OVM	0.04	\pm 0.03
	m130	0.38	\pm 0.09
	GG	2.90	\pm 0.17
NeuAc α 2,3Gal β 1,3GlcNAc-R	SKOV3	0	0
	OVM	0	0
	m130	0.14	\pm 0.30
	GG	2.67	\pm 0.40
Fuc α 1,2Gal β 1,3GlcNAc-R	SKOV3	0	0
	OVM	0	0
	m130	0.05	\pm 0.03
	GG	10.74	\pm 1.04
Type 2 acceptors			
Gal β 1,4GlcNAc-R	SKOV3	0.67	\pm 0.16
	OVM	0.30	\pm 0.24
	m130	11.25	\pm 4.38
	GG	2.27	\pm 0.80
NeuAc α 2,3Gal β 1,4GlcNAc-R	SKOV3	0	0
	OVM	0	0
	m130	0.35	\pm 0.14
	GG	0.15	\pm 0.07
Fuc α 1,2Gal β 1,4GlcNAc-R	SKOV3	0.76	\pm 0.55
	OVM	1.08	\pm 0.17
	m130	24.60	\pm 13.10
	GG	3.79	\pm 0.12

^aThe activity refers to the average of three assays; the standard deviation is represented; R= -(CH₂)₃-NHCO-(CH₂)₅-NH-biotin.

acceptors (6). m130 cells had only a low amount of α 4 FT activity.

Detection of FUT3, FUT4, FUT5 and FUT9 in ovarian carcinoma cells. The results presented above clearly showed that GG and m130 cells expressed higher amounts of Lewis determinants and α 3/4 fucosyltransferase activity. However, it was not clear which enzyme would be expressed and functional in each case. Therefore, we performed Western blot analysis for FUT3, FUT4, FUT5 and FUT9 of cell extracts from GG and m130 cells (Fig. 7). In GG cells, we observed higher amounts of FUT3 and FUT4, followed by FUT9, and FUT5 was almost not detected. The strong band at 42 kDa from GG cells corresponded to FUT3 since heterologous expression of human FUT3 in these cells as well as m130 cells co-migrated with this band by Western

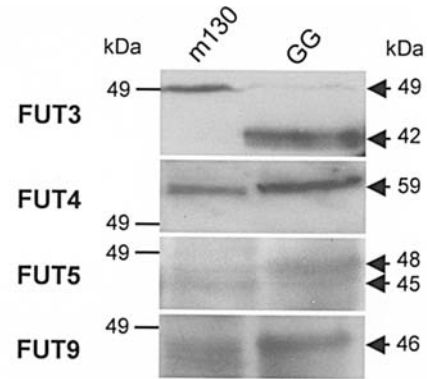


Figure 7. Western blot analysis of FUT3, FUT4, FUT5 and FUT9 in ovarian carcinoma cell lines. Total protein extracted from 1x10⁶ cells of SKOV3, OVM, m130 and GG (indicated above the lanes) was applied in a 12% acrylamide gel. As primary antibodies anti-human FUT3 SA-4817, FUT4 C-17, FUT5 N-18 and FUT9 C-20 polyclonal antibodies were used. The predicted molecular masses from their amino acid sequence of FUT3, FUT4, FUT5 and FUT9 are 42.1, 45.6, 43.0 and 42.0 kDa, respectively. Detection was performed with the ECL method.

blot analysis (data not shown). These results corroborated our previous conclusion that FUT3 was the enzyme involved in the synthesis of Le^a, sLe^a and Le^b in GG cells. FUT4 was detected at 59 kDa that probably corresponded to the long form ELFT-L (predicted molecular mass 59 kDa) identified by Goelz *et al.* (37). FUT9 whose predicted molecular mass from the amino acid sequence is 42 kDa, appeared at 46 kDa. This difference probably corresponded to post-translational modifications, most probably N-glycosylation, since it is known that FUT9 has 3 potential N-glycosylation sites. Due to their relative abundances, we concluded that FUT4 and FUT9 were the enzymes involved in the synthesis of the Le^x and Le^y determinants in GG cells.

For m130 cells, a band at 49 kDa was detected with the anti-FUT3 antibody. This band most probably consisted of non-specific binding of the antibody, since it appeared in all cell lines even when they did not have α 4-FT activity (data not shown). FUT4 and FUT9 were detected in m130 cells, both of the enzymes probably synthesizing Le^x and Le^y in those cells.

Discussion

In the present study, we have characterized the expression of the fucosylated carbohydrate Lewis determinants from four ovarian carcinoma cell lines and identified the α 3/4 FTs that underlie their synthesis. We have found that GG cells expressed high amounts of Le^y, Le^x, sLe^a and Le^b, whereas m130 cells expressed Le^x and Le^y. These cell lines have several glycosylation properties already described for ovarian cancer tissues, where it was found that mucinous tumors homogeneously expressed Le^a and sLe^a whereas serous and endometrioid tumors strongly expressed Le^y and H type 2 antigen (38).

These carbohydrate determinants were abundantly detected on multiple projections of the cell surface (Fig. 5). This localization was likely associated with their role in cell adhesion events occurring during hematogenous metastases where they can serve as ligands for P- and E-selectin (reviewed in ref. 2).

However, we cannot exclude that they might participate in cell adhesion events within the tumor through carbohydrate-mediated homophilic interaction. They were also detected in intracellular vesicles and corresponded to the Lewis containing glycolipids or glycoproteins in transit to the plasma membrane. The high amount observed of those structures indicated the high turnover of the plasma membrane.

The final steps in the synthesis of the Lewis determinants are catalysed by different α 3/4 FTs. Identification of which FUTs are involved in this process provides a means for regulating their expression. We have used three strategies to detect the expression of α 3/4 FTs in ovarian carcinoma cells: RT-PCR, fucosyltransferase activity against a panel of substrate acceptors and Western blotting, since each of these techniques has its own limitations. RT-PCR is not quantitative and very low amounts of mRNA might not be detected; on the other hand, if regulation occurs downstream of transcription, there might be a signal on RT-PCR but no protein will be translated. It is usually possible to identify an FT based on a characteristic profile of activity against an array of acceptors, if the FT is pure; when dealing with cellular extracts the profiles obtained might be altered. Finally, Western blotting is sometimes not sensitive or specific enough.

Our RT-PCR results showed that all ovarian carcinoma cell lines studied expressed FUT4, FUT5 and FUT6 transcripts. FUT3 was expressed at lower levels in SKOV3, followed by GG and m130 cells. Furthermore, several ovarian cancer tissues expressed the same transcripts. However, FUT3, FUT5 and FUT6 were not detected by RT-PCR or Northern blotting on normal ovary tissue (39). Therefore, the expression of FUT3, FUT5 and FUT6 could be a useful indication of an ovarian carcinoma. The fact that only a fraction of the analysed tissues expressed these three FTs may stem from the different origins and stages of the tumours. We have also observed that only one of the tissues analysed with a lower grade of progression (G2x) expressed only FUT6. Furthermore, FUT4 was found in more tissues with a higher grade of progression (G3x). This indicates that concomitantly to carcinoma progression FT transcription, namely FUT3, FUT4, FUT5, FUT6 and FUT9 could be increased. The variation of the profile of FTs expressed by each tumor might be due to a cell heterogeneity of the carcinomas that reflects a variable representation of the cell type.

The RT-PCR of FUT3, FUT4, FUT5, FUT6, FUT9 and FUT10 profiles was very similar for the different cell lines (Fig. 2). However, the Lewis determinants were predominantly detected in GG cells followed by m130 as detected by FACS analysis (Fig. 4) and immunofluorescence microscopy (Fig. 5). These results essentially agreed with those obtained for α 3/4 FT activities where GG and m130 cells had higher values than SKOV3 and OVM cells (Table I). These results indicated that even if the α 3/4 FT mRNA were present in SKOV3 and OVM cells, there was a downstream regulating mechanism that prevented synthesis of the corresponding proteins. Furthermore, SKOV3 and OVM cells probably have a set of acceptors and glycosyltransferases in a distinct organization along the Golgi apparatus that resulted in a distinct glycosylation pattern. For example, SKOV3 and OVM cells contained more Gal β 3GalNAc from O-linked glycoproteins, and SKOV3 cells contained more terminal NeuAc, than the remaining cells

(Fig. 6). These epitopes could respectively correspond to the carbohydrate determinants T sialyl or Tn that are known to be increased in ovarian carcinoma (reviewed in ref. 3).

The results obtained from the *in vitro* α 3/4 FT activities (Table I) together with the Western blot analysis (Fig. 7) suggested that FUT4 and FUT9 could participate in the synthesis of Le^x and Le^y in GG as well as in m130 cells. Furthermore, the results suggested that FUT3 was responsible for the synthesis of Le^a, sLe^a and Le^b in GG cells. Previous studies (19) on the expression of α 3/4 FTs in tumors of ovarian cancer showed that the tumor contained two major identifiable activities. One particular enzyme acted on H-type 2 and its activity was decreased to 20% and 2% when the type 2 acceptors were terminally substituted with sulfo 3- or NeuAc α 2,3-linked, respectively. This activity probably corresponded to FUT4, the myeloid enzyme. The high decrease in α 3 FT activity upon substitution of the type 2 acceptor by sulfate is a characteristic of FUT9 (40), an enzyme that had not been characterized at that time. Therefore, it is possible that besides FUT4, FUT9 could also be found in the ovarian tumor described by those authors. Another activity that was associated with the soluble tumor fraction resembled FUT3, the Lewis enzyme, because it acted preferentially on type 1 acceptors. Interestingly, these authors showed that the ratio in α 4 FT activity towards the 2-methyl Gal β 1,3GlcNAc β -O-benzyl between ovarian tumor and normal ovarian tissue extracts, ranged between 3- and 141-fold. This indicated an association between the Lewis enzyme activity and tumor development. In some of the ovarian cancer tissues analysed in the present work by RT-PCR we also observed the expression of FUT3, FUT4 and FUT9.

In conclusion, we found that GG cells expressed high levels of Le^x, Le^y, sLe^a and Le^b, whereas m130 cells expressed high levels of Le^x and Le^y. Le^x and Le^y were synthesized by FUT4 and FUT9, whereas sLe^a and Le^b were synthesized by FUT3. These have been found to be major activities associated with ovarian carcinoma (19). Therefore, GG and m130 cells constitute adequate models to study the role of Lewis determinants on ovarian tumor progression and metastasis formation, which could be accomplished by silencing the corresponding fucosyltransferases.

Acknowledgements

This work was funded by grant LSHG-CT-2004-503228 from the European Commission. We thank Dr Harald Conradt and Dr Eckart Grabenhorst, GlycoThera, Braunschweig, Germany for the gift of pCRFT4 and pCRFT7 vectors. We thank Professor Henrik Clausen and Dr Ulla Mandel, University of Copenhagen, Denmark, for monoclonal antibodies FH6, AH6, and SH1.

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