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N-Acetyllactosamine and sialosyl-*N*-acetyllactosamine in normal and malignant human endometrium

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We have evaluated using immunohistochemistry the expression of the type 2 chain histo-blood group precursors *N*-acetyllactosamine (Lac), sialosyl-Lac (S-Lac) and binary-sialosyl Lac (DS-Lac) in epithelial cells of normal non-secretory, gestational and malignant human endometrial tissues ($n = 120$). Staining was assessed in relation to genetic (ABO, Lewis blood group and secretor status), morphologic and hormonal factors (serum levels of estrogens). The staining pattern for Lac, S-Lac and DS-Lac showed great variation and was not related to blood group or the secretor status. Staining for Lac showed a limited distribution in both normal and malignant endometrium and was most frequenly found in gestational and atrophic endometrium. S-Lac was strongly expressed, but only infrequently as DS-Lac structures, in normal endometrium. Staining for both S-Lac and DS-Lac was most widespread in proliferating endometria. Endometrial carcinomas showed an increased staining for DS-Lac and a varied, and in most cases a reduced, staining for S-Lac, a pattern like that previously found in secretory endometrium. Staining scores for S-Lac showed a weak correlation with serum levels of free estradiol. Thus, the increased expression of DS-Lac in contrast to S-Lac structures in endometrial carcinomas is probably unrelated to both hormonal and genetic factors and may be considered a 'tumor-associated' but not a tumor-specific change in endometrial cell glycosylation.

Keywords: *N*-acetyllactosamine, endometrium, human, sialylation

Introduction

Malignant transformation of epithelial cells is associated with aberrant glycosylation, which includes accumulation of precursor glycoconjugates [1]. These tumor-associated changes often involve blood group-related antigens, which because of their tissue distribution are termed histo-blood group antigens [2]. Carbohydrates with blood group specificity are bound to lipids or proteins by an inner core composed of either unbranched or branched glycoconjugates. The peripheral core structures of these carbohydrate chains form highly immunogenic structural isomers, the type 1, 2, 3 and 4 chains of blood group antigens, on which synthesis of carbohydrates with blood group specificity takes place [3].

The expression of histo-blood group antigens is different in different tissues [4]. Recent studies have suggested that normal human endometrial glands express mainly type 2 and 3 carbohydrate chains. The glycosylation seems to be both genetically and hormonally influenced in the normal endometrium. 'Tumor-associated' changes in glycosylation have also been demonstrated in endometrial carcinomas [5–9].

The glycosylation pattern of normal human endometrium differs in many ways from that of other normal tissues. Human endometrial glands

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throughout the menstrual cycle express the type 2 chain precursor, *N*-acetyllactosamine, which is strongly masked by sialic acid [6]. However, this structure is not found in most other normal epithelia, but tends to accumulate in carcinomas and is, therefore, regarded in these tissues as a tumor-associated structure [10]. The aim of the present study was to characterize further the expression of *N*-acetyllactosamine (Lac), sialosyl-*N*-acetyllactosamine (S-Lac) and binary sialosyl-*N*-acetyllactosamine (DS-Lac) in normal endometrium in relation to genetic and hormonal factors, and to characterize the possible tumor-associated changes in expression of these carbohydrates in the endometrium.

Materials and methods

Normal and malignant endometrical tissues were obtained from hysterectomies ($n = 110$) and gestational endometrium from early legal abortions ($n = 10$). Formalin-fixed paraffin-embedded material was available in all cases, and frozen tissue in 87 of the cases (Table 1). The histological diagnosis was based on conventional criteria [11–13]. The carcinoma group contained endometrioid endometrial carcinomas except for one grade 3 serous and one grade 3 adenosquamous carcinoma. Fifty carcinomas were pure endometrioid, but in 13 cases there were focal areas of secretory or mucinous differentiation; endometrioid plus serous/clear cell carcinoma components were present in four cases. Six carcinomas were stage 1A, 29 stage 1B, seven stage 1C, eight stages 2A + B and the remaining six stages 3–4.

The blood group status (ABO and Lewis) was determined by routine procedures [5]. The secretor status, *i.e.* the ability to secrete ABH substances in saliva, was determined in 71 of the 120 patients. When saliva was not available the secretor status was determined from the Lewis phenotype; erythrocyte Le^{a-b+} individuals were regarded as secretors, and Le^{a+b-} individuals as non-secretors [14–16]. The secretor status was not known in five Le^{a-b-} erythrocyte individuals with endometrial carcinoma.

The results of hormone analysis (estrone, 17β-estradiol, estrone sulfate, sex hormone-binding globulin, non-protein-bound E_2 and non-SHBG-bound E_2) were available on 56 of the patients. The methods used have been described in detail previously [8]. Hormone analysis was performed on seven women receiving hormonal treatment

Table 1. Morphology and blood group status of endometrial tissues investigated

Morphology[a]	No. para/froz[c]	Blood group status[b]				Hormone		
		O		A/B		Analysis	Treatment[d]	Free E_2 (pmol/l) median (range)[e]
		Se	se	Se	se			
Atrophic/inactive	16/12	6	1	9	0	13	2(5)	1.0 (0.9–2.2)
Weakly proliferative	10/8	4	0	5	1	6	0(6)	1.1 (0.9–3.0)
Proliferative	7/6	2	0	5	0	4	1(5)	10.0 (10.0–26.0)[g]
Irregularly proliferative	8/6	3	0	4	1	5	2(4)	12.0 (6.2–23.5)
Gestational tissue	10/0	3	1	4	2	0	0(7)	Not determined
Adenomatous hyperplasia[f]	13/11	1	2	6	3	6	2(6)	1.5 (1.0–5.9)
Adenocarcinoma grade 1	33/25	13	2	13	3	13	11(10)	1.2 (0.9–3.1)
Adenocarcinoma grade 2	16/12	4	2	6	3	6	2(8)	1.0 (0.9–1.0)
Adenocarcinoma grade 3	7/7	1	0	2	2	3	1(2)	0.9 (0.7–1.1)[g]
All morphologies	120	37	8	54	15	56	21(53)	

[a]All morphological classification was based on standard criteria [11–13].
[b]Blood group secretor status (Se, secretors; se, non-secretors) was determined as described in the text; five carcinomas were of unknown secretor status.
[c]Para/froz = paraffin-embedded material/frozen tissue.
[d]Number of women who had received hormone treatment within the previous month or more than 1 month before (in parenthesis).
[e]Interquartile range.
[f]Adenomatous hyperplasia group contained all grades.
[g]Total range.

(median level of free E_2 = 2.2 pmol/l; interquartile range 0.9–10). In women with adenomatous hyperplasia or endometrial carcinoma who had never taken hormones ($n = 8$), the median serum level of free E_2 was 1.1 pmol/l (1.0–2.6), and in a corresponding group on previous hormonal treatment ($n = 16$), the median level of free E_2 was 1.0 pmol/l (0.8–1.2).

Immunohistochemistry reagents

Mouse monoclonal antibodies (MAbs) with specificity for *N*-acetyllactosamine (Lac) (MAb 1B2) and binary sialosyl-*N*-acetyllactosamine (DS-Lac) (MAb NUH2) were used. The specificity, immunoglobulin isotype and reference for their production are given in Table 2 [17, 18]. The MAbs were used undiluted. Neuraminidase type X from *Clostridium perfringens* (0.1 unit per ml in a 0.1 M acetate buffer for 2 h at 37°C; Sigma) was used to remove sialic acid residues [19] and to estimate expression of *N*-acetyllactosamine with substitution of sialic acid (S-Lac) [5]. Peroxidase-conjugated rabbit anti-mouse immunoglobulin (Ig) P260 was purchased from Dako, Denmark.

Immunohistochemical staining

Because staining with MAb NUH2 was considerably lower in formalin-fixed than in frozen tissue sections, this MAb was exclusively used on acetone-fixed sections of frozen tissue. MAb 1B2 was used on sections of formalin-fixed, paraffin-embedded material, as previous studies have shown no difference in staining with frozen sections [6]. An indirect two-layer standardized peroxidase staining reaction was used as previously described [6]. Serially cut tissue sections untreated or pretreated with neuraminidase were incubated overnight at 4°C with the primary MAbs, and for 1 h with peroxidase-conjugated rabbit anti-mouse Ig diluted 1:20. Staining was developed using 0.04% 3-amino-9-ethyl-carbazole (AEC) as the chromagen.

Controls

Staining was controlled by replacing the primary antibody with (a) diluent buffer, (b) an irrelevant MAb of the same isotype and (c) culture supernatant. Uterine cervical tissue was used as positive tissue controls for staining with MAb 1B2, and villous syncytiotrophoblast for the NUH2 MAb [18]. Neuraminidase treatment was controlled by (a) incubating with 0.1 M acetate buffer, pH 5.5, alone; (b) staining with MAb 1B2 in endothelial cells after neuraminidase pretreatment; and (c) abolishing staining with MAb NUH2 after neuraminidase pretreatment [18].

Evaluation

Staining was evaluated semiquantitatively using an arbitrary staining score based on subjective estimates of the fraction of stained cells. This was scored as follows: 0, no cells stained; 1, less than 10% of cells stained; 2, between 10% and 25%; 3, between 25% and 75%; and 4, > 75% of the cells stained [5].

Statistics

Spearman's rank correlation test and the Mann–Whitney test were performed using the computer programme Medstat (Astra, Copenhagen, Denmark). A P-value < 0.05 was chosen as the level of significance.

Results

In epithelial cells the expression of Lac, S-Lac, and DS-Lac varied, but distinct patterns of staining related to the morphology were evident. Staining was very heterogeneous, particularly in carcinomas. Variations in staining showed no relationship

Table 2. The mouse monoclonal antibodies reacting with type 2 chain structures, their antigen determinants, their isotype and reference

Type 2 chain	Antigen determinant	Antibody/isotype	Reference
N-Lac	Galβ1-4GlcNAcβ1 → R	1B2/IgM	17
DS-Lac	NeuAcα2-3Galβ1-4GlcNAcβ1 → 6 Galβ1-4GlcNAcβ1 → R NeuAcα2-3Galβ1-4GlcNAcβ1 → 3	NUH2/IgM	18

N-Lac, *N*-acetyllactosamine; DS-Lac, binary sialosyl-*N*-acetyllactosamine. Sialosyl-lactosamine (S-Lac) was demonstrated by using MAb 1B2 on neuraminidase-pretreated sections. MAb 1B2 is also reactive with desialylated binary *N*-acetyllactosamine [18].

to the blood group (ABO and Lewis type) or to the secretor status. Stromal cells were unstained, except for staining of endothelium and lymphocytes, which stained for *N*-acetyllactosamine in neuraminidase-pretreated sections. The findings for epithelial cells are now described in more detail.

N-*Acetyllactosamine*

Normal endometrium Lac was demonstrated at the apical membranes of a few scattered epithelial cells in less than half of the normal and irregular proliferative endometria, but showed a more frequent expression in atrophic, weakly proliferating and, especially, gestational endometrium (Figure 1 & Table 3). Staining of the cytoplasm was infrequent, and was mainly seen in a few scattered cells in atrophic endometria.

Neoplastic endometrium Staining for Lac varied, and not all specimens were stained (Figure 1 & Table 3). The staining pattern was very similar to that observed in normal non-decidual endometrium. As in normal endometrium, Lac was expressed at the apical membranes. In about half of the cases, the apical or apical–basal cytoplasm also stained for Lac.

*Sialosyl-*N*-acetyllactosamine*

Normal endometrium All specimens were stained for Lac in neuraminidase-pretreated sections. Nearly all cells stained at apical membranes except for the atrophic endometrial glands, where slightly fewer cells were stained (Figure 1 & Table 3). Compared with untreated sections, more cells were stained after neuraminidase pretreatment in weakly proliferating, normally proliferating and irregularly proliferating endometrium. The increase in staining was less pronounced in atrophic and gestational endometrium (Table 3). The cytoplasm was stained in scattered cells in more than half the cases.

Neoplastic endometrium All but one (grade 3) carcinoma stained for Lac after neuraminidase pretreatment. Staining showed pronounced heterogeneity, but, in general, fewer cells than in the normal endometrium stained (Figure 1 & Table 3). In addition to staining of apical membranes, which was the predominant localization of staining in grade 1 carcinomas and adenomatous hyperplasias, the cytoplasm of scattered cells was also stained in most carcinomas. Staining of the cytoplasm was predominant in grade 3 carcinomas.

*Binary sialosyl-*N*-acetyllactosamine*

Normal endometrium In most cases, scattered cells (score 0–2) stained for DS-Lac at apical membranes and in the apical cytoplasm (Figure 1 & Table 3). The fraction of stained cells varied. In a few (3/14) weakly or normally proliferative endometria approximately one-third of the cells were stained. Except for this, no variation in staining scores in relation to morphology was evident. Gestational endometrium was not investigated owing to lack of frozen decidual tissues.

Neoplastic endometrium Adenomatous hyperplasias stained very similarly to normal non-secretory endometria (Figure 1 & Table 3). All endometrial carcinomas stained, and the staining scores were higher than found in normal non-secretory endometrium (*i.e.* score > 2) in 16 of 44 (36%) endometrial carcinomas. The staining was heterogeneous and the fraction of stained cells varied from a few to nearly all the cells. Both the apical membrane and the cytoplasm stained in all carcinomas. Staining showed no relationship to the histological grade.

Expression of N*-acetyllactosamine and sialylated derivatives in relation to FIGO stage*

There was no relation between staining scores for Lac, S-Lac and DS-Lac and the FIGO stage.

Expression of N*-acetyllactosamine and sialylated derivatives in relation to serum estrogen levels*

Except for a weak correlation between S-Lac expression (*i.e.* the difference in staining scores between neuraminidase-pretreated and untreated sections) and serum levels of free E_2, no correlation between staining scores and serum estrogen levels was found.

Discussion

In the present study we found that sialylated forms of Lac are strongly expressed by normal human

Figure 1. Immunohistochemical staining for *N*-acetyllactosamine (A, D & G), sialosyl-*N*-acetyllactosamine (B, E & H, neuraminidase-pretreated sections) and binary sialosyl-*N*-acetyllactosamine (C, F & I frozen tissue) in endometrial tissues. A–C, atrophic endometrium; D–F, proliferative endometrium; G–I, grade 1 endometrial carcinoma.

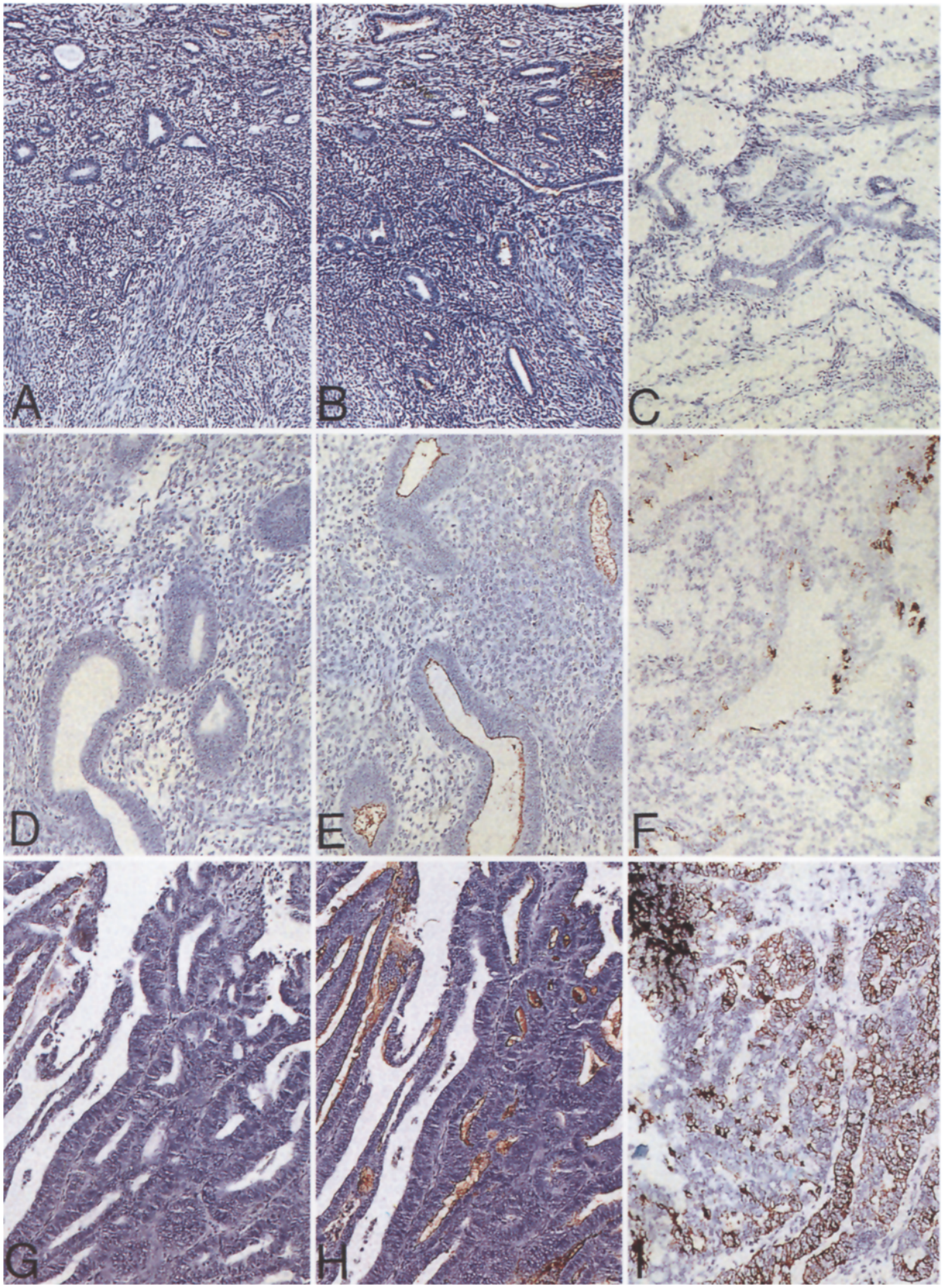
A
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Table 3. Immunohistochemical staining for Lac, S-Lac and DS-Lac in human endometrium

Morphology	Lac		S-Lac		DS-Lac	
	Positive (%)[a]	Score[b]	Positive (%)	Score	Positive (%)	Score
Atrophic/inactive	82	1 (0–3)	100	3 (2–4)	100	1 (1–2)
Weakly proliferative	82	1 (0–2)	100	4 (3–4)	100	1 (1–2)
Normally proliferative	43	0 (0–1)	100	4 (3–4)	83	2 (0–3)
Irregularly proliferative	38	0 (0–2)	100	4 (3–4)	100	1 (1–2)
Gestational endometrium	100	3 (1–3)	100	4 (3–4)	Not determined	
Adenomatous hyperplasia	69	1 (0–3)	100	3 (1–4)	91	1 (0–3)
Adenocarcinoma grade 1	70	1 (0–3)	97	2 (0–4)	100	3 (1–4)
Adenocarcinoma grade 2	63	1 (0–3)	100	3 (1–4)	100	3 (1–4)
Adenocarcinoma grade 3	71	1 (0–1)	100	1 (1–4)	100	2 (1–3)

[a]Percentage of specimens stained.
[b]Median staining score and ranges.

endometrial glands, and that the proportion of DS-Lac structures was increased in endometrial carcinoma cells compared with expression in normal non-secretory endometrium.

The genetic background of an individual (*i.e.* ABO, Lewis blood group and secretor status) did not influence expression of Lac and S-Lac in the endometrium. This agrees with findings in other normal epithelial tissues, which show a limited expression of these structures [3, 10, 20–22]. Gestational and atrophic endometrial glands expressed Lac, whereas other normal endometria and endometrial carcinomas expressed Lac inconsistently and generally in a few cells only [6, 23]. In other tissues, Lac is regarded a tumor-associated antigen. However, our data show that Lac does not accumulate significantly in endometrial carcinoma cells.

Most cells stained for Lac in neuraminidase-pretreated sections of normal and malignant endometria. This indicates a pronounced expression of sialylated *N*-acetyllactosamine structures in human endometrium, and that sialyltransferases may be regulating the synthesis of fucosylated derivatives of Lac, *i.e.* the expression of Le^X, H and Le^Y antigens [24–26]. S-Lac expression was expressed very similarly to the type 3 chain, the sialosyl-T antigen [5, 24]. Also, the S-Lac staining showed a low, albeit significant, correlation with E_2 levels in serum, as previously demonstrated for the S-T antigen. The varied expression of S-Lac found in endometrial carcinomas may thus in part be related to differences in hormonal stimulation, *i.e.* estrogen receptor content and/or serum estrogen levels.

The distribution of biantennary S-Lac antigen seemed to be limited in other normal tissues. In the normal endometrium, DS-Lac is maximally expressed by endometrial epithelial cells in the luteal phase, which is characterized by high progesterone levels. This hormone may, therefore, be affecting DS-Lac expression [6]. However, our data do not support any relationship between DS-Lac expression and serum estrogen levels in normal non-secretory and malignant endometrium.

DS-Lac showed an increased expression in endometrial carcinomas, as previously demonstrated for other sialylated carbohydrate antigens such as the sialosyl-Tn and sialosyl-Le^a antigens. However, because of the concomitant expression in luteal endometria, all these carbohydrates can be considered 'tumor-associated' but not tumor-specific antigens of endometrial tissue [5, 7, 24, 27, 28].

The significance of DS-Lac expression in the endometrium and other tissues is largely unknown. It is, however, worth noting that some other normal cells express DS-Lac, *i.e.* trophoblast, megakaryocytes and sperm, and those cells are characterized by their ability to move and invade [18]. This attribute is also essential for malignant cells. The MAb NUH2 caused an immobilization of ejaculated sperm in the presence of complement [29]. Similar mechanisms could be involved in regulation of trophoblast invasiveness and may influence invasiveness of neoplastic cells expressing DS-Lac. However, the present study did not show any relation between DS-Lac expression and the FIGO stage, *i.e.* degree of myometrial invasion, and any prognostic significance of DS-Lac expression in endometrial carcinomas remains to be elucidated. The increased expression of DS-Lac in malignant endometrium seems to reflect a tumor-

associated but not a tumor-specific change in endometrial cell glycosylation, which could have biological significance.

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