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HISTOCHEMICAL AND MORPHOMETRIC STUDIES OF ENDOCERVICAL TYPE ADENOCARCINOMA IN THE CERVIX UTERI

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SUMMARY

The histochemical analyses of glycoproteins were performed on specimens of endocervical type adenocarcinoma of uterine cervical carcinoma (EA) (40 cases) and normal cervices (19 cases) by the following methods: (1) Modified Schiff stains for sialates, including both Periodic Acid-Thionin Schiff/Potassium hydroxide/Periodic Acid-Schiff stain (PAT/KOH/PAS) and Mild Oxydation-Thionin Schiff/Periodic acid Borohydride/Potassium hydroxide/Periodic Acid-Schiff stain (MOT/PBT/KOH/PAS), (2) Immuno-histochemical stains with biotinylated lectins, including Dolichos Biflorus Aggulutinin (DBA), Griffonia Simplicifolia I (GS-I), Ulex Europaeus Agglutinin (UEA-I), Peanut Aggulutinin (PNA), Concanavalin A (Con A), Ricinus Communis Agglutinin I (RCA 120), Soybean Aggulutinin (SBA), and wheat germ aggulutinin (WGA). In the modified Schiff stains, tumor cells contained o-acetylated sialates or frequently lost the ability to produce sialates, while the normal endocervical cells produced predominantly non-acetylated sialates. Abrupt transition was also confirmed by these stainings. Based on the lectin study, the frequency of the positive reaction to PNA and Con A was statistically high in EA ($p<0.005$). The nuclear stratification index (NSI) was calculated positive in the cancerous glands for histochemical reactions, and the NSI of EA's was higher than that of normal cervices ($P<0.05$). These histochemical reactions were statistically significance and indicated both abrupt transition and NSI as important atypisms for cancer diagnosis.

INTRODUCTION

Although the light microscopic and histochemical characteristics of EA have been well defined[4,7,9,13,15], diagnostic difficulties have remained, particularly in the well-differentiated type of EA[13], as suggested in our last paper[9]. In the histochemical examinations of mucins, modified Schiff stains, including the PAT/KOH/PAS stain[1,2,11] and the MOT/PBT/KOH/PAS stain[1,2,7,11,14], differentiated the location of o-acetylated side chains in sialates. Normal colonic sialates consisted mainly of o-acetylated type sialic acids, but sialates had reduced o-acetylated side chains in tumor cells. Conversely, the sialates of normal endocervical cells were of the non-acetylated type, and the EA cells contained o-acetylated sialates. The application of this staining for pathological diagnosis, however, was limited because in these tumor cells the conversion of sialic acetylations frequently failed to occur (Table 1-A, B). The immunohistochemical stainings with lectins binding to the oligosaccharides[8,15] were used for both the histological diagnosis of malignancies[4,8,13] and the prediction of their prognosis[3]. On the other hand, it was speculated in our report that both the severity of nuclear stratification (NSI) and abrupt structural transition from the normal epithelium to the cancerous one were the criteria for the histopathological diagnosis of EA[9]. However, there have been few reports discussing correlations among the above histochemical results and the histopathological atypism of tumor cells[4].

In this study, EA was analyzed histologically by both modified Schiff stains and immunohistochemistry with lectins. Furthermore, the significance of both the NSI and abrupt structural transition of the histological atypism of EA was reassessed in cancerous foci with positive histochemical stainings.

MATERIALS AND METHODS

Materials

Forty subjects with EA, aged 27 to 73 years (mean 50.1 ± standard deviation 32.0), were available. Specimens were obtained from extirpated uteri, and the EA consisted histologically of 27 cases of well-differentiated types, including four cases of minimal deviation adenocarcinoma, six cases of a moderately differentiated type, and seven cases of a poorly differentiated type. As controls, 19 normal cervices, which were obtained because of endometriosis, myoma uteri, and prolapsus uteri, were used. The subjects in the control group were from 35 to 73 years (54.2±12.0), and there was statistically no difference between the ages of the above two groups. Furthermore, the surgical specimens of the stomach, small intestine, and colon were simultaneously used as staining controls.

In the immunohistochemical technique with lectins, Biotinylated Lectin KIT I (Vector, USA) and biotinylated GS-I lectin (EY Lab., USA) were employed as the first reagents. The endocervical cells, reacting to lectins[4], had blood group isoantigens

(BGIs)[13] and oligosaccharides. Because the antigenic characteristics of BGI consisted of oligosaccharides, the staining with several lectins (DBA, GS-I, UEA-I, and PNA) was identical with that of BGI[8]. Based on these findings and the significance of BGI[3,13], the examined lectins were allotted to two groups: BGI-related ones and others (Appendix 2). The absorbed lectins, incubated with specific polysaccharides (Sigma, USA) in the proportion of 50 μg/ml for 30 min at 37℃, were simultaneously used as negative controls (Appendix 2)[11]. Vectastain ABC Kit (Vector, SA) was used for the avidin-biotin-peroxidase complex method (ABC method)[10].

All specimens in this study were fixed in 10% formalin, processed routinely, and embedded in paraffin. Sections were cut at 3 μm and attached to glass slides, which had been coated with 0.01% aqueous Poly-L-Lysin (Sigma, USA) to prevent the tissue desquamation during procedures.

Histochemical Techniques

Modified Schiff Stain: In each staining procedure, the specimens, including small intestinal mucosa and colonic mucosa, were stained simultanuously as the staining control. The staining methods are shown in Appendixes 1, 3, and 4[1,2,7,14]. The staining results are shown in Tables 1-A and B.

Immunohistochemical Stain with Biotinylated Lectins: The staining methods are shown in Appendixes 2 and 5[3,5,6,8,10,12]. The pretreatment with enzymes to unmask antigens was done before the first reagent reaction: (i) digestion with trypsin before UEA-I and Con A reaction, i. e., incubating in 0.1% trypsin (Wako Pure Chem., Japan) in 0.05M Tris buffer, pH 7.6, with 0.1% calcium chloride for 30 min at 37℃, (ii) digestion with neuraminidase before staining with PNA: i. e., incubating in 0.015% neuraminidase, type V (Sigma, USA) in 0.1M acetate buffer, pH 5.5, with 0.01% calcium chloride for 60 min at 37℃. Furthermore, in the Con A stain, the additional pretreatment with 1% diastase (Hoeiyoko, Japan) in 0.01M PBS, pH 7.2, for 60 min at 37℃ was done to remove glycogen cross-reacting with Con A, and the simultaneous staining of the gastric mucosa was performed as the positive control staining.

Measurement of NSI

The areas for the NSI were selected from both the central zone of the carcinoma, which also revealed cancer-staining patterns when subjected to the above histochemical procedures, and the deep-seated normal endocervical epithelium. Each section was viewed under the x40 objective lens, and measurements were taken of ten consecutive views. The NSI represented nuclear stratification per unit of the epithelial area and was taken as the ratio obtained from the total number of nuclei divided by the sum of the lining nuclei seated on the basement membrane.

RESULTS

Modified Schiff Stain

Normal cervical mucous cells always stained blue with PAT/KOH/PAS stain and purple with MOT/PBT/KOH/PAS stain similar to the small intestinal mucous cell in the control specimens. This showed that the normal cervical mucous cells contained only nonacetylated (C0) sialic acids (Table 1-A, C). Tumor cells usually turned blue when stained with PAT/KOH/PAS (Fig. 1-A), but unstained or red-stained tumor cells increased in number with the severity of the cellular atypism. With the MOT/PBT/KOH/PAS stain, half the tumor cells stained red (Fig. 1-B), and several were clearly stained blue (Fig. 1-C). In minimal deviation adenocarcinoma, most cells were stained purple, but several tumor cells with nuclear atypism were either unstained or stained red (Fig. 1-D). These findings revealed that the mucus cancerous cells contained predominantly o-acetylated sialic acids (Table 1-A, C). The abrupt transition between the cancerous epithelium and the neighboring normal epithelium (so-called front formation) became easily confirmed with a modified Schiff stain. This front of the intraepithelial invasion formed a convex face, the convexity facing toward normal cervical epithelium (Fig. 1-A, B). The crenated budding pattern as a structural atypism was frequently confirmed in cancerous epithelium, furthermore, o-acetylated sialic acids were revealed (Fig. 1-B).

Immunohistochemical Stain with Biotinylated Lectins

In lectins with an affinity for BGI, the reaction products were restricted mainly to the cell membrane (Fig. 2-A). The preservation of the appropriate BGI, i. e., the patient's own BGI, was histologically observed in 63% and 20% of benign tumors and carcinoma, respectively. The cancerous cases, showing an inappropriate BGI, i. e., other than the patient's own BGI, reached 43%, and this result was statistically significant ($p<0.05$, chi-square test, Table 2). The PNA product, reacting to T antigen, was significantly identified in tumor cells ($p<0.005$, Table 2).

As for lectins with little affinity for BGI, Con A stained both the cytoplasm and the cell membrane (Fig. 3), and the number of positive cases was high in the cancer group ($p<0.005$, Table 2). The cell membrane exhibited intense immunoreaction to RCA-1, SBA, and WGA. The SBA-positive cases were quite numerous in the cancer group ($p<0.025$, Table 2).

Analysis of NSI

EA consisted of two types of tumor cells. One was a clear-cell type tumor cell, which contained abundant intracytoplasmic mucus and a large round vesicular nuclei with macronucleoli. These cells were frequently found in well-differentiated adenocarcinoma and tended to lose their nuclear polarity. The other was a dark cell type characterized by decreased mucus and an ellipsoid nuclei with marked coarse hetero-

chromatin. There was no clear histochemical difference between these two cell types. The NSI's of the normal control group, the clear-cell type carcinoma group, and the dark-cell type carcinoma group were 1.24 ± 0.18, 1.74 ± 0.22, and 2.46 ± 0.50, respectively, and these indices were statistically significant ($p < 0.05$, Student t test).

DISCUSSION

In the histological identification of EA, both the pattern of crenated budding and the cribriform pattern were the most reliable structural atypisms[9]. On the other hand, there remained a degree of indistinctness about the criteria of cancerous atypisms, which were frequently dependent on each pathologist's judgment, so that the well-differentiated type of EA was difficult to diagnose from small biopsied specimens because of their lesser atypism. We examined the effectiveness of both the modified Schiff stains for sialates and the lectin stainings to distinguish the EA from the benignancy. Furthermore, the atypisms (the NSI and the abrupt transition), which we took as criteria for malignancies[9], were reappraised by these histochemical results.

In the histochemical analysis of the o-acetylated side chains of sialates by the modified Schiff stains, nomal cervical mucous cells contained non-acetylated sialates (C0). The tumor cells of EA, however, produced o-acetylated sialates (C7, 8, 9)[7] or ceased to produce sialates. This was seen in all EA's without regard to histological differences. Furthermore, the discontinuous chage in histochemical stainability from normal to cancerous epithelium corresponded with the abrupt structural transition.

As for immunohistochemical stains with lectins, both PNA and Con A statistically reacted to tumor cells of EA ($p < 0.005$)[4]. As in transitional cell carcioma of the urinary bladder[3], there was the tendency in EA for tumor cells to show the defect of the immunoreactivity to the appropriate BGI on one hand and the immunoreaction of the inappropriate BGI on the other.

In the histopathological analysis of tumor cells, two types were differentiated by hematoxylin and eosin stain. On the one hand, the clear-cell type adenocarcinoma contained abundant intracytoplasmic mucus, a large round nucleus with a thickened nuclear rim, and macronucleoli, and there was a pronounced loss of nuclear polarity. On the other hand, the dark-cell type adenocarcinoma contained less mucus, had a rod-shaped or oval nucleus containing more coarse hetero-chromatin[8] and marked stratified nuclei, which frequently came up to the luminal surface. No histochemical difference between these two types of tumor cells could be shown. However, the NSI's for the clear-cell type adenocarcinoma group and the dark-cell type adenocarcinoma group were 1.74 ± 0.22 and 2.46 ± 0.50, respectively, and these values were statistically significant for cancer diagnosis ($p < 0.05$).

These histochemical methods are valuable to substantiate a diagnosis of EA. NSI and abrupt transition are reconfirmed as the reproducible, simple, and plain indices of cancerous atypisms.

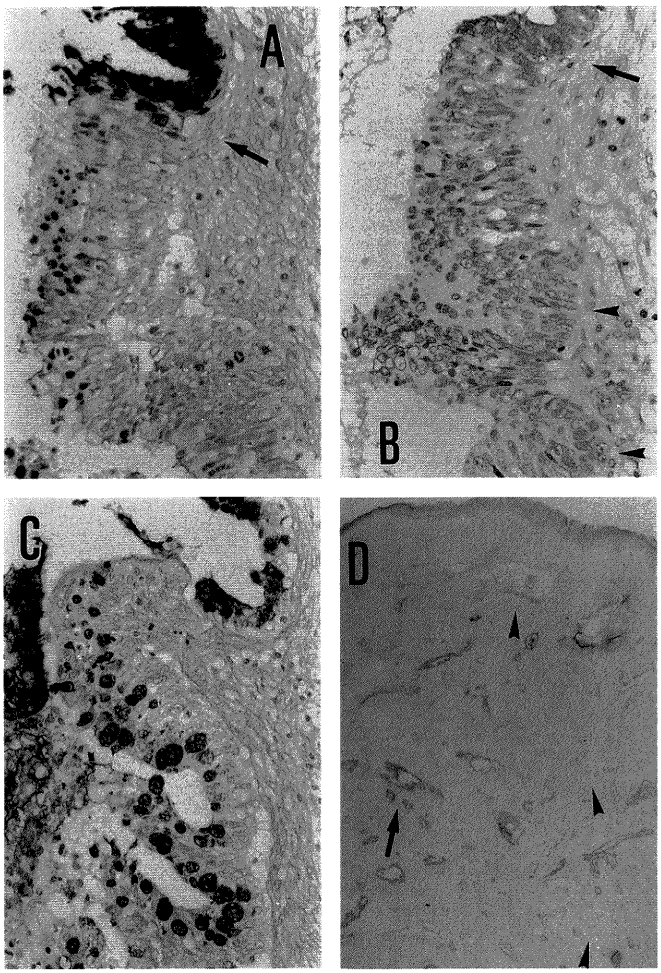

Fig. 1

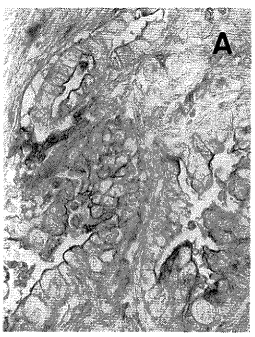

Fig. 2

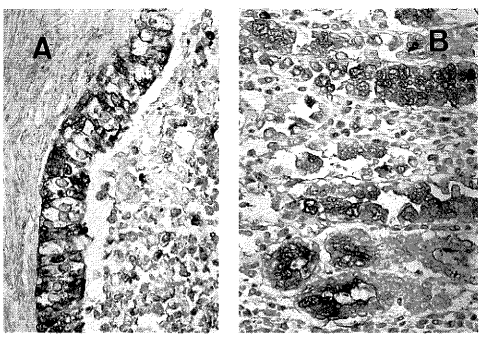

Fig. 3

Appendix 2. Specificity of lectins, concentration for usage, and sugar for each lectin as negative controls

Lectins	Affinity for BGI	Concentration for use (μg/ml)	Sugar for negative controls
Biotinylated Lectin Kit I			
DBA	A	15	N-Acetyl-D-Galactosamine
UEA - 1	O (H)	15	L-Fucose
PNA	T	15	D (+) - Galactose
Con A		50	Methyl a-D-Mannopyranoside
RCA 120		15	D (+) - Galactose
SBA		15	D (+) - Galactose
WGA		15	N-Acetylglucosamine
Biotinylated GS - I Lectin	B	15	D (+) - Galactose

Table 1. A.Histochemical identification of side chain O - acetyl sialic acids[1)2)11)]

Side chain substituent		PAT/KOH/PAS	MOT/PBT/KOH/PAS
C0		blue	purple
C7		blue	red
C9		blue	N
C8		red	red
B. Histochemical stainings in mucous cells of intestines[1)2)11)]			
Small intestine	NED	blue	purple
large intestine	NED	red	red
	carcinoma	blue	purple
C. Histochemical stainings in mucous cells of uterine endocervical glands			
	NED	blue	purple
	carcinoma	blue/N (red)	red/N (purple)

N: unstained, ( ): sometimes stained in parenthese
NED: no evidence of disease

Table 2. Immunohistochemical study of lectins in normal cervices and endocervical type adenocarcinoma

Lectins		Control (19 cases)	Adenocarcinoma (40 cases)	P
Lectins with affinity for BSI	Preservation of own BSI	12	8	
	Production of other BSI	0	17	0.05
	PNA	6	31	0.005
Others	Con A	6	32	0.005
	RCA 120	16	34	
	SBA	6	28	0.025
	WGA	18	39	

P, chi-square test

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Appendix 1.

1. 1 g Thionin Crestain (Merck, Germany) in 100 ml of distilled water, heating.
2. Bottle with 0.75% thionyl chloride (Wako Pure Chem., Japan), 4℃, overnight.
3. Shake with 2 g activated charcoal.
4. Filter and store in a sealed dark bottle, 4℃.

cf. This solution is available only for 7 days or for one usage.

Appendix 3. PAT/KOH/PAS technique

PAT:
1. place in 1% aqueous periodic acid, 30 min.
2. wash in running tap water, 10 min.
3. rinse in distilled water.
4. place in Thionin Schiff reagent, 30 min.
5. wash in running tap water, 10 min.

KOH/PAS:
6. rinse in 70% ethanol.
7. place in 0.5% potassium hydroxide in 70% ethanol, 30 min.
8. wash in running tap water, 10 min.
9. rinse in distilled water.
10. place in 1% aqueous periodic acid, 15 min.
11. wash in running tap water, 10 min.
12. rinse in distilled water.
13. place in Schiff reagets (Merck, Germany), 30 min.
14. place in 0.5% aqueous sulfurous acid, 3 min. ×3.
15. wash in running tap water, 10 min.
16. dehydrate, clean, and mount.

Appendix 4. MOT/PBT/KOH/PAS technique

MOT:
1. rince in 0.1M acetate buffer, pH 5.5, 0℃.
2. place in 10% aqueous ethylane glycol, 10 min., 0℃.
3. rinse in acetate buffer.
4. place in Thionin Schiff reagent, 50 min.
5. rinse in 1% aqueous thionyl chloride, 3 min. ×3.

PBT:
6. rinse in acetate buffer.
7. place in 1% aqueous periodic acid, 90 min.
8. rinse in acetate buffer.
9. place in 0.05% sodium borohydride in 10% aqueous disodium hydrogen phosphatfte, 10 min.
10. rinse in acetate buffer.

KOH/PAS: following procedures same as those in Table 3.

Appendix 5. ABC method

1. place in 0.3% hydrogen peroxide in absolute methanol, 30 min.
2. rinse in distilled water.
3. rinse in 0.01M HEPES buffer, pH 7.5, with 0.15M sodium chloride and 0.1mM calcium chloride (HEPES buffer).
4. incubate with each biotinylated lectin dilated in HEPES buffer, overnight, 4℃; simultaneously substitute the absorbed lectin for each biotinylated lectin for control studies.
5. rinse in HEPES buffer.
6. place in ABC complex.
7. react with diaminobenzidine fluid (DAB).
8. dehydrate, clean, and mount.

ILLUSTRATIONS

Fig. 1-A: Endocervical type adenocarcinoma stained by PAT/KOH/PAS technique. Normal cervical cells stain blue (upper side). Tumor cells also stain blue unevenly (lower side). The abrupt transition is easily identified (arrow). ×100., B: Serially sectioned specimen of A, staining by the MOT/PBT/KOH/PAS technique. Normal cervical cells stain blue-purple (upper side). Tumor cells mainly stain red (lower side). The abrupt transition is easily identified (arrow). The crenated buddings are found (arrowhead). ×100., C: Same section in B. Red-and blue-stained tumor cells coexist in same gland. ×100., D: Minimal deviation adenocarcinoma stained by MOT/PBT/KOH/PAS technique. Carcinomatous glands without atypism stain purple (arrow), and others with slight nuclear atypism remain unstained (arrowhead) or show red. ×2.

Fig. 2: Endocervical type adenocarcinoma stained by the immunohistochemical stain; A, PNA stain; B, absorbed-PNA stain. Note: PNA-positive material is distributed in the cell membrane of tumor cells. ×100.

Fig. 3: Immunohistochemical stain with Con A; A, endocervical type adenocarcinoma; B, fundic glands of stomach. Note: Con A positive material is distributed in the cell membrane and the cytoplasm. × 100.