Human Monoclonal Antibody (HMST-1) against Lacto-Series Type 1 Chain and Expression of the Chain in Uterine Endometrial Cancers

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ABSTRACT

A human monoclonal antibody termed HMST-1 was produced by fusing lymphocytes from segments of human pelvic lymph nodes from an endometrial cancer patient with murine myeloma cells. The epitope recognized by HMST-1 was determined to be lacto-series type 1 chain-containing glycosphingolipid (Galβ1-3Galβ1-3Galβ1-4Glcβ1-1Cer) by isolating the antigen from endometrial cancer cell line SNG-II and analyzing with fast atom bombardment mass spectrometry, permethylation analysis, and exoglycosidase treatment.

By the immunohistochemical avidin-biotin-peroxidase complex method, no normal endometrium and benign endometrial hyperplasia were stained with HMST-1, but HMST-1 reacted with about 35% of endometrial cancer cases. These facts indicate that the rate of expression of the antigen increases along with the course of malignancy in the endometrium. By slalidase treatment of the section, the positive rate increased to 57% in endometrial cancers and to 13% in normal endometrium, indicating that the antigen was masked with sialic acid and exposed by neuraminidase treatment.

Immunohistochemistry also revealed that the antibody reacted with human fetal alimentary tract epithelium and mesothelium, indicating the oncodevelopmental nature of Galβ1-3Galβ1-3Galβ1-4Glcβ1-1Cer.

INTRODUCTION

Uterine endometrial adenocarcinoma is gradually increasing, but its cell biological characteristics have not yet been fully investigated. Recently it was reported from immunohistochemical-application of newly generated murine monoclonal antibodies that the phenotypic expression of blood group carbohydrate antigens of endometrial cells changed in association with malignant transformation (1, 2). Along with the transformation-associated change in the blood group, the expression of fetal carbohydrate antigen is frequently observed in transformed cells (3). Although the functional significance of cell surface carbohydrates is not fully understood, recent observations (4, 5) strongly indicate that they actively regulate cellular proliferation and differentiation. In addition, they are an important target molecule for the immune system to suppress unregulated cell growth. In fact, the rate of incidence of cancer is well correlated negatively with the activity of the immune system, with high rates in the immature stage of young people and in the declining stage of old people. Therefore, it is quite important to know what molecule is responsible for distinguishing cancer cells from normal cells. To prove the humoral immune response to the cancer cells and to clarify the target antigen, we attempted to fuse lymphocytes from segments of human pelvic lymph nodes from an endometrial cancer patient with murine myeloma cells to obtain human-mouse hybridomas against endometrial cancer cells that synthesize human monoclonal antibody. This paper reports the production of a human monoclonal antibody which recognizes LeOse4Cer4 (lacto-series type 1 chain-containing glycoconjugate) and expression of this antigen in endometrial cancer cells.

MATERIALS AND METHODS

Cell Cultures. The murine myeloma cell line P3/X63-Ag.8.U1 was grown in RPMI 1640 supplemented with 10% FCS. Human cell lines established from uterine cervical epidermoid cancers [SK-G-1 (6), SK-G-3 (7), SK-G-H1a, and -Hb (8)], endometrial adenocarcinoma [SN-M-I (9), SN-M-II (10), SN-M-IIIo], uterine leiomyosarcoma [SKN (11)], ovarian rhabdomyosarcoma [KRN (12)], choriocarcinoma [NJG (13)], and ovarian clear cell adenocarcinoma [RMG-I (14)], all of which were established in our laboratory, were grown in Ham’s F-12 medium supplemented with 10% FCS. SN-G-IIo was established from the surgical specimen of the patient whose lymph nodes were used for the present study. HeLa, WiDr (colonic adenocarcinoma), CCRF-CEM (leukemia), and CCD-118Lu (lung normal fibroblast) were supplied from the American Type Culture Collection.

Production of Hybridomas. Left obturatoric lymph nodes were obtained from a 56-year-old Japanese housewife with Stage II uterine endometrial cancer at radical hysterectomy. Pathological examination revealed that the endometrial lesion was a well-differentiated adenocarcinoma and the lymph nodes used for the cell suspension were negative for metastatic carcinoma. A cell suspension was made from the lymph nodes and incubated in RPMI 1640-10% FCS medium supplemented with 5 µg/ml lipopolysaccharide (Sigma, St. Louis, MO) at 37°C for 4 days. Cell fusion and hybridoma selection were carried out by the method of Cote et al. (15) with a slight modification. In brief, lymphocytes were isolated with Ficoll-Paque (Pharmacia, Uppsala, Sweden) from the incubated cell suspension and fused with murine myeloma P3/X63-Ag.8.U1 cells in the presence of 42.6% (w/v) polyethylene glycol 1540. Hybridomas were first selected by the production of human immunoglobulin and subsequently by their reactivity to SN-G-II cell extract. Human immunoglobulins were measured by a sandwich EIA with anti-human immunoglobulin and horseradish peroxidase-labeled anti-human immunoglobulins (Dakopatts, Glostrup, Denmark).

Reactivity of the antibody to SN-G-II cell extract was tested by indirect EIA as reported previously (16). A human antibody designated as HMST-I, which reacted with cancer cell lines but not with normal fibroblasts, was selected. HMST-1 reacted only with anti-human immunoglobulin antibody, but not with anti-mouse IgM or IgG by the sandwich EIA, and the HMST-1 subclass was determined to be IgM.

Isolation of Glycolipid Antigen. Total lipids of SN-G-II cells were extracted with organic solvents and fractionated into neutral and acidic lipids by DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden) column chromatography (17, 18). Neutral and acidic glycolipids were applied to a thin layer plate precoated silica gel plate (Merck, Darmstadt) with a slight modification. In brief, lipids were extracted with organic solvents and fractionated into neutral and acidic lipid systems formed with chloroform:methanol:water (70:30:4 and 55:45:10), and chloroform:methanol:0.3% CaCl2 in water (55:45:10). The isolation of glycolipid antigen was carried out by a Latrobeads column (H-8060; Latron, Tokyo, Japan) with a gradient solvent system formed with chloroform:methanol:water (70:30:4 and 90:10:4).

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and by high-performance liquid chromatography (Shimadzu, Kyoto, Japan) (16).

Immunostaining on TLC Plates. TLC immunostaining was performed according to the method described previously (19). In brief, after the chromatography of glycolipids on a TLC plate, the plate was incubated with HMST-1 (10 µg/ml) and the antibody bound on the TLC plate was then detected with peroxidase-labeled goat anti-human IgM (Kirkegaard & Perry Lab., Inc., Gaithersburg, MD). The plate was also incubated with serum before the surgical procedure (1:20 dilution) from the endometrial cancer patient whose lymphocytes were used for fusion to detect the antibody with the same specificity as HMST-1.

Exoglycosidase Treatment. β-Galactosidases from jack bean (Sigma) and from Diplococcus pneumoniae (20) and N-acetyl-β-D-glucosaminidase (bovine kidney; Sigma) were used for the structural determination of the glycolipid antigen. About 5 µg of glycolipid were treated with 0.5 unit of either of the two β-galactosidases or with 5 units of N-acetyl-β-D-glucosaminidase at 37°C for 18 h in 500 µl of 50 mM sodium citrate buffer containing 1 mg/ml sodium taurocholate at pH 4.0, 6.8, or 5.0, respectively, and the products were examined by TLC with visualization by orcinol-H₂SO₄ and HMST-1 staining.

Negative Ion FABMS Analysis. About 5 µg of the isolated glycolipid dissolved in 2 µl of chloroform:methanol (1:1, by volume) were mixed with 2 µl of triethylamine and 1 µl of tetramethylurea and analyzed with a negative ion FABMS (JMS-HX-100, JEOL Co., Ltd., Tokyo, Japan) (18).

Methylation Analysis. The purified glycolipid antigen was permethylated with methyl sulfanyl carbanion and methyl iodide in dimethyl sulfoxide, and the product was purified by solvent partition and Latrobeads column chromatography. The permethylated glycolipid was used for the analysis of linkage and sugar composition as the partially permethylated aldonexitil acetates. Identification of each product was performed by comparing its retention time on a gas-liquid chromatography (Shimadzu, Kyoto, Japan) (16). Followed by immersion in 3,3′-diaminobenzidine-tetrahydrochloride solution, in order to exclude the false positive signals which may result from the direct binding of non-specifically bound antibody to endogenous human immunoglobulins in tissues, control experiments without HMST-1 were performed on all positive cases. Serial tissue sections were also treated with 0.2 unit/ml sialidase (Boehringerwerke AG, Marburg, West Germany) at 37°C for 2 h prior to avidin-biotin-peroxidase complex staining, and the changes in the staining patterns were compared. For electron microscopic immunocytochemistry, SNG-II cells and cancer tissues were stained by the preembedment indirect immunostaining method as published previously (1).

Enzyme Immunocytochemistry. Ethanol-fixed cultured cells and formalin-fixed human tissues were incubated with HMST-1 and subsequently treated with biotinylated goat anti-human IgG antibody and avidin-biotin complex reagent (Vector Laboratories, Inc., Burlingame, CA) (16), followed by immersion in 3,3′-diaminobenzidine-tetrahydrochloride solution. In order to exclude the false positive signals which may result from the direct binding of non-specifically bound antibody to endogenous human immunoglobulins in tissues, control experiments without HMST-1 were performed on all positive cases. Serial tissue sections were also treated with 0.2 unit/ml sialidase (Boehringerwerke AG, Marburg, West Germany) at 37°C for 2 h prior to avidin-biotin-peroxidase complex staining, and the changes in the staining patterns were compared. For electron microscopic immunocytochemistry, SNG-II cells and cancer tissues were stained by the preembedment indirect method as published previously (1).

RESULTS

Establishment of Human Monoclonal Antibody HMST-1. In 93% (1704 of 1824) of all the wells seeded, colonies of hybridomas were observed, and human immunoglobulins were detected in 99% (1692 of 1704) of these wells by the sandwich EIA. In 18% (306 of 1692) of the wells containing cells producing human immunoglobulins, strong reactivity with SNG-II cell extract was detected by the indirect binding EIA; and one of the antibodies, designated HMST-1, showed high, specific reactivity toward endometrial cancer cell line SNG-II (Fig. 1). Immunocytochemical avidin-biotin-peroxidase complex staining with HMST-1 revealed almost the same results; i.e., the incidence and the intensity of positive cells were the highest in three kinds of uterine endometrial adenocarcinoma cell line (SNG-M, SNG-II, and SNG-IIo), SKG-III and RMG-I were moderately positive, and the other cell lines were negative (Fig. 1). The concentrations of human immunoglobulins in spent media during cell culture were measured by the sandwich EIA.

Stable and constant antibody production was observed for at least 3 months at a concentration of 80 µg/ml.

Structural Characterization of HMST-1 Antigen. As shown in Fig. 2, SNG-II contained several neutral glycolipids (Lane A), and TLC immunostaining of the plate with HMST-1 revealed the existence of one immunoreactive neutral glycolipid which had almost the same mobility as nLeOse₄Cer. However, nLeOse₄Cer, asialo-GM₁, and globoside did not react with HMST-1 at all. After isolation of the antigen, the negative ion FABMS spectrum was obtained to elucidate the structure, as shown in Fig. 3. Molecular and fragment ions were detected at m/z 1225 (Cer–Hex–Hex–HexNa–Hex, m/z 1063 (Cer–Hex–Hex–HexNa), m/z 860 (Cer–Hex–Hex), m/z 698 (Cer–Hex–HexNa).
HUMAN MONOCLONAL ANTIBODY AGAINST LeCer

Fig. 3. Negative ion FABMS spectrum of HMST-1 antigen from SNG-II. M.W., molecular weight.

Fig. 4. Thin-layer chromatogram of the products of HMST-1 antigen cleaved sequentially with exoglycosidases (orcinol-H$_2$SO$_4$ staining). Lane 1, HMST-1 antigen (a); Lane 2, the product (b) obtained by hydrolysis of a with $\beta$-galactosidase (jack bean); Lane 3, the product (c) obtained by hydrolysis of b with $\beta$-N-acetylglucosaminidase (bovine kidney); Lane 4, the product (d) obtained by hydrolysis of c with $\beta$-galactosidase (jack bean); Lane 5, lactosylceramide as standard glycolipid; Lane 6, glucosylceramide as standard glycolipid; arrow, position of sodium taurocholate.

Hex), and $m/z$ 536 (Cer), indicating that the carbohydrate chain of the HMST-1 antigen contained three hexoses and one N-acetyltalosaminine. Molecular ion species were given at $m/z$ 1337, 1335, 1309, 1253, and 1225, corresponding to the glycolipid with C24:0, C24:1, C22:0, C18:0, and C16:0 fatty acid-containing ceramides, respectively.

Next, the carbohydrate sequence and the anomeric configuration were determined by sequential hydrolysis of the HMST-1 antigen with exoglycosidases such as $\beta$-galactosidase and $\beta$-N-acetyl-$\beta$-D-glucosaminidase, as shown in Fig. 4. The HMST-1 antigen (a) was susceptible to $\beta$-galactosidase treatment to give a glycolipid that closely migrated with LeCer (b), and b was converted by treatment with N-acetyl-$\beta$-D-glucosaminidase to a glycolipid with the same position as lactosylceramide (c). Furthermore, c was cleaved by $\beta$-galactosidase hydrolysis to a product (d) which had the same mobility as glucosylceramide. These results indicate that the carbohydrate structure of the antigen is Gal$\beta$-GlcNAc$\beta$-Gal$\beta$-Glc$\beta$-Cer.

TLC of the glycolipid antigen hydrolyzed with two kinds of $\beta$-galactosidases having different specificities is shown in Fig. 5. Terminal galactose of the glycolipid antigen was cleaved with jack bean $\beta$-galactosidase (Fig. 5A, Lane 2), which hydrolyzes both Gal$\beta$1-3 and Gal$\beta$1-4 linkages, but not with diplococcal $\beta$-galactosidase (Fig. 5A, Lane 3), which does not hydrolyze the Gal$\beta$1-3 linkage, indicating that the terminal galactose is linked with the $\beta$1-3 linkage. Accordingly, the immunoreactivity with HMST-1 was deleted by jack bean $\beta$-galactosidase (Fig. 5B, Lane 2), but not by diplococcal $\beta$-galactosidase (Fig. 5B, Lane 3).

Partially methylated aldohexitol acetates from the purified glycolipid antigen were analyzed by gas-liquid chromatography, and peaks of each product are shown in Fig. 6. 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylgalactitol (a), 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylgalactitol (b), 1,4,5-tri-O-acetyl-2,3,6-O-methylglucitol (c), and 1,3,5-tri-O-acetyl-2-deoxy-2-N-methylacetamido-4,6-di-O-methylglucitol (d) were detected, indicating that the carbohydrate structure of HMST-1 antigen is Gal$\beta$1-3GlcNAc$\beta$1-3Gal$\beta$1-4Glc$\beta$1-Cer (LeCer or lacto-series type 1 chain-containing glycosphingolipid).

Immunohistochemical Staining of HMST-1 Antigen in Human Tissues. Staining patterns of each endometrial specimen were...
evaluated on the basis of both staining intensity and incidence of positive cells. The intensity was graded on an arbitrary scale into (+), (++), (+++), and the percentage distribution of positive endometrial cells in each section was classified into three grades, less than 10%, 10 to 50%, and more than 50%. By combination of intensity and percentage, the reactivity of each specimen was divided into weakly, moderately, and strongly positive. Fig. 7 summarizes the immunohistochemical reactivity of various endometrial lesions with HMST-1. None of 24 cases of normal endometrium and none of 7 of benign endometrial hyperplasia was positive. In borderline lesions, in which atypical endometrial hyperplasia and the early stage of adenocarcinoma localized only in endometrium were included, 1 of 8 cases were positive. In adenocarcinoma (Fig. 8, a, c), 15 of 43 cases were positive (35%), and the rate of positive cases increased in proportion to the degree of histological differentiation: 10 of 23 well-differentiated cases (44%), 4 of 12 moderately differentiated cases (33%), and 1 of 8 poorly differentiated cases (13%). As shown in Table 1, in normal tissues other than endometrium, glandular cells of uterine cervix, gastrointestinal tract, and renal tubule were also positive. In malignant tumors aside from endometrial cancers, not only adenocarcinomas of uterine cervix, stomach, pancreas, colon, and ovary but also squamous cell carcinoma of uterine cervix were positive. Gastrointestinal mucosa and mesothelium of fetuses of 9 to 11 weeks of gestation were also stained. By sialidase treatment of endometrial cancer specimens (Fig. 8, b and d), the percentage of positive cases increased: well-differentiated type, 82% (strong, 4%; moderate, 61%; weak, 17%); moderately differentiated type, 67% (moderate, 50%; weak, 17%); and poorly differentiated type, 38% (weak, 38%) (Fig. 7). A similar tendency was observed not only in other cancers but also in normal tissues including endometrium. The most striking change was observed in kidney and pancreas; none of the glomeruli (Fig. 8e) in 7 cases and none of the islands of Langerhans (Fig. 8g) in 6 cases were positive, but all of them became strongly positive after the sialidase treatment (Fig. 8, f, g). By sialidase treatment, positive cells increased in number, and glomeruli and islands of Langerhans became positive. Existence of HMST-1 in the Patient's Serum. As shown in Fig. 10, the serum of the patient whose lymphocytes were used for establishment of monoclonal HMST-1 antibody reacted with LcOse4Cer on TLC.

**DISCUSSION**

This paper demonstrates the establishment by utilization of lymph nodes from a patient with uterine endometrial cancer of human monoclonal antibody (termed HMST-1, IgMX) which recognizes LcOse4Cer. In general, the stable production of human antibody from heterohybridoma clones is difficult, because human chromosomes tend to be lost. Nonetheless we have been able to obtain HMST-1 continuously for over 4 years by repeated subculture. As shown clearly in this paper, the antigen of HMST-1 antibody was purified from cultured SNG-II cells established from an endometrial adenocarcinoma, and the structure was proved to be Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1Cer, lacto-series type 1 chain-containing glycosphingolipid by TLC immunostaining, exoglycosidase hydrolysis, FABMS analysis, and permethylation analysis. A mouse monoclonal antibody, designated as F10.2, that
Table 1 Immunohistochemical (avidin-biotin-peroxidase complex method) reactivity of HMST-1 toward formalin-fixed sections of human fetal and adult tissues and toward some cancers, with or without sialidase treatment

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<th>Tissue</th>
<th>Sialidase(−)</th>
<th>Sialidase(+)</th>
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<tr>
<td>I. Fetal tissue</td>
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<tr>
<td>Gastrointestinal tract</td>
<td>4/4</td>
<td>3/3</td>
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<tr>
<td>Kidney</td>
<td>1/1</td>
<td>1/1</td>
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<tr>
<td>Renal tubule</td>
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<td>1/1</td>
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<tr>
<td>Glomerulus</td>
<td>0/2</td>
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<tr>
<td>Mesothelium</td>
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<td>0/3</td>
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<td>Placenta</td>
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<tr>
<td>II. Adult tissue</td>
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<tr>
<td>Gastrointestinal tract</td>
<td>8/12</td>
<td>5/8</td>
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<td>Glomerulus</td>
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<tr>
<td>Pancreas</td>
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<td>Islands of Langerhans</td>
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<tr>
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<tr>
<td>III. Cancer</td>
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<tr>
<td>Squamous cell carcinoma</td>
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<tr>
<td>Renal cell carcinoma</td>
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Fig. 9. Immunocytochemical electron micrograph of SNG-II cells stained with HMST-1. Antigen recognized by HMST-1 is present on the cell surface.

Fig. 10. TLC immunostaining of LeOse4Cer with monoclonal antibody HMST-1 (Lane 1) and human serum from a patient whose lymphocytes were used for the production of HMST-1 (Lane 2).

Because this study was undertaken under the assumption that B-lymphocytes in the pelvic lymph nodes of a patient with endometrial cancer would produce antibodies against some tumor antigens, the immunoreactivity of HMST-1 with endometrial cancer tissues was investigated in order to confirm the localization of HMST-1 antigen in cancer cells. Immunohistochemical staining revealed that a certain number of cancer cells in 35% of the endometrial cancer patients examined (including the patient whose lymphocytes were used for fusion) reacted with HMST-1, but no cells in normal endometrial tissues did. The rate of positive cases in borderline lesions was greater than that in benign endometrial hyperplasia. These facts indicate that the HMST-1 antigen (LeOse4Cer) increases in quantity during the course of malignant changes in the endometrium. As we reported earlier (1), the amount of blood group carbohydrate antigen Leb is increased in endometrial cancer cells, and an increase in other blood group antigens such as Leα (2) and H (3) were also reported. Because LcOse4Cer is the precursor of H and Leα blood group antigens, the increase seen in LcOse4Cer in endometrial cancers is quite plausible. HMST-1 may be a very useful tool in the understanding of the abnormal expression of blood group antigens in various cancers, because antibodies against lacto-series type 2 chain (23) have mainly been used thus far in this connection. Further, the existence of anti-LcOse4Cer antibody in the serum of this patient was also confirmed by TLC immunostaining, as was already reported in the case of serum from a patient with bronchogenic carcinoma (24). These facts seem to indicate that the human immune system has a potential to recognize LcOse4Cer as a tumor-associated antigen. In addition, just as Fukuda et al. (25) pointed out that blood group type 1 sequences were expressed in human embryonal carcinoma cells, the oncodevelopmental nature of LcOse4Cer is also suggested from the fact that HMST-1 reacted with glandular cells of the fetal gastrointestinal tract and with human meconium (26). However, immunohistochemical staining with HMST-1 was also positive in normal adult tissues such as gastrointestinal and uterine cervical glandular cells, and renal tubes, indicating that LcOse4Cer is also expressed in noncancerous cells. Therefore, LcOse4Cer serves as a cancer-associated antigen in uterine endometrium. Another interesting phenomenon is that cells that stained positively with HMST-1 increased in number after sialidase treatment in both cultured SNG-II cells and formalin-fixed thin sections of endometrial lesions, especially in well-differentiated adenocarcinoma, indicating that sialylated LcOse4Cer is often expressed in endometrial cancer cells. Because our investigation of glycolipids in uterine endometrial cancers has indicated that LcOse4Cer is rich in mono or disialosyl derivatives rather than...
being in native form, and because Fukuda et al. (25) reported that mono- and disialosyl glycolipids with the blood group type 1 sequence were present in human embryonal carcinoma cells, the occurrence of sialylated Le\textsubscript{a}Ose\textsubscript{4}Cer was found to be well correlated with the morphological observations.

This monoclonal antibody will be useful in the investigation of the cell biological characteristics of human endometrial carcinoma.

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