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Identification and expression of human epiglycanin/MUC21: a novel transmembrane mucin*

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The gene for the human orthologue of mouse epiglycanin, a mucin expressed on mammary carcinoma TA3-Ha cells but not TA3-St cells, was identified by homology search to a mouse epiglycanin cDNA fragment identified by representational difference analysis between TA3-Ha and TA3-St cells. The open reading frame of this gene was cloned from human cervical carcinoma ME-180 cells. It consists of a mucin domain with 28 nonidentical tandem repeats of 45 nucleotides each corresponding to a threonine/serine-rich peptide, a stem domain, a transmembrane domain, and a cytoplasmic tail. The cloned cDNA with a FLAG sequence was expressed in K562 cells. A combination of immunoprecipitation with a polyclonal antibody specific for the cytoplasmic tail and Western blotting analysis with an anti-FLAG antibody and lectins revealed a mucin-like component as the gene product. Analysis by the use of tissue cDNA libraries indicated that the gene is expressed in lung, large intestine, thymus, and testis among 16 normal tissues tested. The polyclonal antibody specific for a synthetic peptide from the cytoplasmic tail, when tested for its reactivity with normal lung tissues, reacted with epithelia of bronchi and bronchioli but not with alveoli. All of 24 lung adenocarcinomas specimens tested were reactive with the antibody, whereas reactivity was observed with only 2 out of 24 squamous and none out of 24 small cell lung carcinomas. This is a novel transmembrane mucin and designated as MUC21.

Keywords: cDNA cloning/epiglycanin/lung carcinoma/mucin

Introduction

Mucins are epithelial defense molecules and clinically used as carcinoma markers and targets of cancer immunotherapy (Hollingsworth and Swanso 2004). Altered expression and glycosylation of mucins were also observed under infectious and other disease conditions (Kawakubo et al. 2004). Their unique molecular functions in the biology of the epithelial tissue largely remain to be elucidated. An important approach to the understanding of the structural diversity and functions of mucins is the cloning of their core polypeptides. In this report we describe the cloning of a mucin whose biochemical and immunological properties have been studied for the last 30 years without clear molecular identity. The mucin has a name epiglycanin, which was first reported in 1975 (Codington et al. 1975). Before this it was known as a cell surface glycoprotein expressed by a subline of TA3 cells (Codington et al. 1972). TA3 cells were isolated as mammary carcinoma cells originating from a spontaneous tumor in A/HeHa mice in 1949 in the laboratory of T.S. Hauschka (Hauschka et al. 1971). The tumor was propagated in solid form in the original mouse strain and later converted to an ascites form on two separate occasions. The two ascites forms were called TA3-St (Klein 1951) and TA3-Ha (Codington et al. 1972). It was reported that TA3-Ha cells could grow in and kill allogeneic mouse strains, rats, and hamsters apparently after acquisition of an additional small chromosome (Friberg 1972a), whereas TA3-St cells grew only in syngeneic hosts. Such unique property of TA3-Ha cells was proposed to be due to the presence of epiglycanin on the cell surface, which was the only identifiable difference between TA3-Ha and TA3-St cells at least in those days. Biochemical and electron microscopic studies revealed that epiglycanin is a large surface molecule with an extended shape (Codington et al. 1972, 1979; Miller et al. 1977). Another subline, TA3-MM, which arose during passage of TA3-St cells in a diseased mouse, could also grow in allogeneic mouse strains (Cooper et al. 1979) and expressed a cell surface glycoprotein with nearly identical amino acid composition and electron microscopic characteristics to that of epiglycanin (Codington et al. 1979). The amino acid composition showed high percentages of serine and threonine. It was proposed that allogeneic growth was possible because epiglycanin sterically hindered cytotoxic T cells from interacting with MHC molecules on the tumor cells (Friberg 1972b; Sanford et al. 1973; Codington et al. 1978). In addition, it was reported that purified epiglycanin derived from TA3-Ha cells elicited an immunosuppressive effect in vivo (Fung and Longenecker 1991). However, there was no specific tool to identify this mucin. Monoclonal antibodies were generated but the predominant specificities of almost all of them were directed toward the carbohydrate portion (Haavik et al. 1992, 1999; Kemperman et al. 1994). Polyclonal antisera against epiglycanin were previously

*The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank/EBI Deta Bank with the accession numbers AB242595 for human epiglycanin/MUC21 cDNA and AB242596 for mouse epiglycanin/Muc21 cDNA.

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raised in rabbits, but were not used to determine the structure of the backbone polypeptide (Codington et al. 1984). Because of this, we started to attempt to clone the mouse epiglycanin gene by the cDNA-representational difference analysis (cDNA-RDA) method (Hubank and Schatz 1994). As a result, we were able to obtain a fragment of a cDNA, which was expressed only in TA3-Ha cells but not in TA3-St cells, containing a segment of mucin-like tandem repeats. We tentatively concluded that this cDNA represented the partial mouse epiglycanin cDNA as discussed below.

The human counterpart of mouse epiglycanin was identified by a homology search for the nontandem repeat domains (i.e., extracellular, transmembrane, and cytoplasmic sequences). A gene that was previously claimed to be a putative transmembrane protein AY358415 (Clark et al. 2003) was identified. However, the claim on AY358415 was solely based on its sequence characteristics. Whether the gene is expressed as a protein, where it is expressed, or any other information was not available. The human epiglycanin, which we tentatively term MUC21, has a structure characteristic of transmembrane-type mucins. Thus, we tested whether the cDNA corresponding to the open reading frame with a FLAG epitope tag at its amino terminus produced a mucin-like molecule when transfected into K562 cells. Mucin-like glycoproteins having an approximate molecular size of 180,000 were expressed as revealed by a combination of immunoprecipitation with a polyclonal antibody, sodiumdodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and Western blotting.

Among organs, mRNA expression was observed in lung, thymus, and colon. Distribution of this molecule in lung and lung carcinoma specimens was investigated by immunohistochemistry by the use of the same polyclonal antiserum used for immunoprecipitation. The results collectively indicate that epiglycanin/MUC21 is a novel transmembrane mucin with a unique organ distribution.

Results

cDNA cloning of mouse epigycanin/MUC21 fragments by cDNA-RDA

Fragments of genes expressed in mouse mammary carcinoma variant TA3-Ha cells but not or at low levels in another variant, TA3-St cells, were identified by cDNA-RDA (Hubank and Schatz 1994). We obtained 205 gene fragments and determined their DNA sequences. Among them 92 (44.9%) turned out to have a very similar sequence of approximately 80 bases. One of these sequences could be translated into PALTTTASSTGSSSTPTPTTTASSTAS. Because this cDNA fragment appeared to be a portion of a mucin-like molecule, it was used as a probe to identify the corresponding mouse mRNA by Northern blotting analysis (Figure 1A). The mRNA was shown to be present in TA3-Ha cells but not in TA3-St cells. Organ distribution and other properties of mouse epiglycanin will be reported in a separate paper. Three prime race reactions were performed by the use of cDNA from TA3-Ha cells as the template and a fragment was obtained. The product was subcloned into a pGEM-T easy vector, and the sequence was determined to be the cDNA fragment shown in Figure 1B. The sequence contained a short mucin-like structure (including six tandem repeats), a sequence similar to the cleavage site of sperm

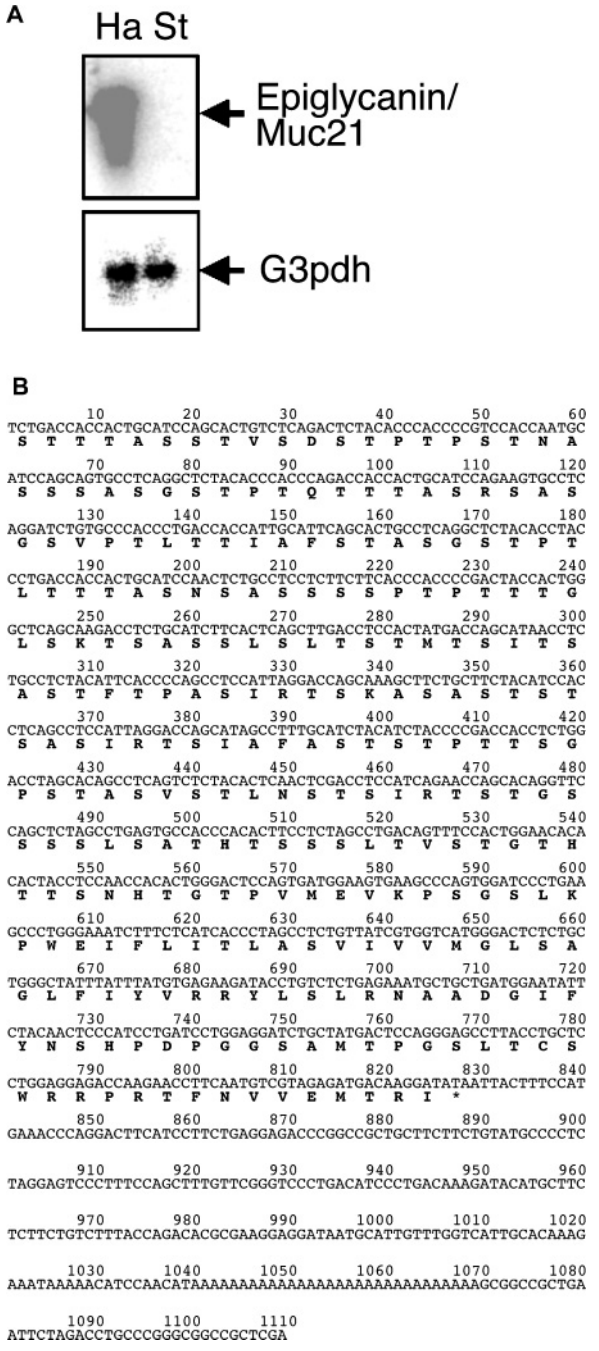

Fig. 1. (**A**) Northern blotting analysis of mRNA from TA3-Ha and TA3-St cells by the use of 3′-race product having a length of 1110 bases. (**B**) cDNA and amino acid sequences of partial cDNA of putative mouse epiglycanin/Muc21 obtained by cDNA-RDA between TA3-Ha and TA3-St cells and the 3′-race reaction.

protein-enterokinase-agrin (SEA) module (SGSL), a putative transmembrane domain (VIVVMGLSAGLFIYV), and a short cytoplasmic tail with 49 amino acids. The genomic sequence corresponding to this sequence was found in the NCBI mouse database and it was shown to be located in the class I major histocompatibility complex on chromosome 17. The amino acid translation of the predicted cDNA (XM_001002195) indicated that there were 60 tandem repeats of 15 amino acids having

Table I. Amino acids compositions (mole percent) of mouse epiglycanin and other known mouse mucins

		Ala (A)	Arg (R)	Asn (N)	Asp (D)	Cys (C)	Gln (Q)	Glu (E)	Gly (G)	His (H)	Ile (I)	Leu (L)	Lys (K)	Met (M)	Phe (F)	Pro (P)	Ser (S)	Thr (T)	Trp (W)	Tyr (Y)	Val (V)
Mouse epiglycanin/MUC21[a]		9.8	1.7	1.1	0.8	0.3	0.3	1.2	6.3	0.4	1.9	3.6	1.1	1.0	0.9	9.0	27.2	30.8	0.3	0.4	2.3
After gel-filtration[b]		13.1	0.1	2.4			3.2		8.1	0.1	0.1	3.3	1.2	ND	0.1	9.3	25.2	30.7			2.6
	Protein ID																				
Muc1	NP 038633	7.6	2	3.5	4.3	0.5	2.6	2.6	5.1	2.8	2.8	5.3	1.8	0.7	2.8	9.2	23.4	12.5	0.2	2.8	7.7
Muc2	CAD 54415	4.9	3.5	3.9	5.9	8.3	3.3	6.5	7.1	3.4	4.3	6.6	4.8	1.5	3.5	4.9	8.3	8.6	1.3	3.9	5.8
Muc3	AAC53 572.1	5.4	2.9	6.1	4.5	4.1	4.1	7.5	7	0.9	4.5	5.7	5.7	1.1	3.8	3.8	6.6	12.0	1.4	2.9	9.5
Muc4	NP 536705	4.3	3.7	3.6	2.8	1.4	6.1	3.3	5.1	3.1	3.9	5.7	2.2	2	2.1	6.6	18.7	19.3	0.8	1.3	4
Muc5AC	Q80 Z21	4.8	2.9	5.2	4.8	8.0	4.5	4.6	7.5	2.5	3.3	6.3	3.3	1.7	3.3	6.9	9.6	9.7	1.0	3.1	6.9
Muc5B	NP 083077	4.4	3	4.1	3.9	6.3	4.2	4.8	6.3	2.3	3.4	5.8	3.4	1.6	2.7	7.6	13.2	13.0	1.3	2.7	6.1
Muc6	NP 859418	5.3	2.2	3.1	2.8	4	4.2	3.5	5.7	3.4	3.2	6.4	2.7	1.7	2.7	9.2	14.1	17.8	0.6	1.9	5.7
Muc10	NP 032670	8.2	3.9	5.5	1.2	0.8	2.4	1.6	1.2	1.2	3.5	2.4	7.8	0.8	3.5	15	7.8	29.0	0.8	2.4	1.6
Muc13	AAA 372391	5.6	2.3	4.2	3.8	3.6	6.7	3.1	8.8	0.7	3.1	4	4	1.3	2.3	7.6	20.0	10.8	0	2.2	6
Muc16	XP 357986	4.2	2.5	4.4	3.2	0.5	3.4	5.5	5.1	3	5.6	7.7	3.6	2.4	2.5	6.8	16.5	15.0	0.8	2	5.4
Muc19	AAO 388511	16	2.8	2.6	1.7	3.7	1.4	3.6	8.6	0.6	1.4	1.8	2.3	0.5	0.9	6.8	20.5	19.9	0.2	1.2	3.3
Muc20	BAD 067191	7.2	3.3	2.7	3.0	0.3	2.7	5.6	3.0	1.9	5.4	9.2	2.4	1.3	2.5	7.6	16.7	19.1	0.3	0.3	5.3

[a]Data were calculated based on the translated sequence of the putative cDNA with 60 tandem repeats.
[b]Data from the published composition of purified mouse epiglycanin (Codington et al. 1972).
The compositions of other known mucins were calculated based on each sequence with the shown protein IDs after removing the signal sequence predicted by SOSUI program (Hirokawa et al. 1998).

a typical but not identical sequence to SNSASSSSPTPTTTG. Because of its large size and the presence of a typical mucin-like structural arrangement, we speculated that this was the cDNA for epiglycanin. The amino acid composition calculated on the basis of the sequence with 60 tandem repeats was very similar to the published composition of purified epiglycanin (Table I) (Codington et al. 1979). Particularly, high contents of serine and threonine were remarkable. The calculated composition of other known mouse mucins did not correspond to that of epiglycanin (Table I).

Identification of the human epiglycanin gene

We performed a BLAST search for human cDNAs by the use of a sequence corresponding to the mouse cDNA fragment mentioned above and found KMQK697, a cDNA clone, GenBank accession number AY358415, the putative human counterpart of mouse epiglycanin without any known biological function (Clark et al. 2003). This gene is located in the MHC class I region as is the mouse gene. Translation of AY358415 yielded a 56.7 kilodalton (kDa) polypeptide characterized by a 5′ signal sequence, a mucin domain, a hydrophobic transmembrane domain, and a highly charged 3′ cytoplasmic tail. This sequence is shown in Figure 2A. This molecule has several characteristics that are essential for it to be included into the mucin family. It contains a typical mucin domain with tandem repeats rich in the amino acid serine and threonine but not proline. The mucin domain is composed of 28 repeats of 15 amino acids containing 60% serine and threonine. A typical sequence of the tandem repeat is SSTTSSGASTATNSE. According to the NetOGlyc server (Julenius et al. 2005), the mucin domain offers 239 *O*-glycosylation sites. This tandem repeat domain is encoded by a single exon, exon 2. The amino acid sequence of this domain is poorly conserved among species as observed with other epithelial mucins, while the homology among the repeats is higher than in most other mucins except for MUC1, 16, and 17. The NCBI SNP database reports 53 amino acid polymorphisms in this domain, which probably reflect tandem repeat sequence differences. In this mucin, there is a high (46.7%) homology in the amino acid sequence immediately downstream of the tandem repeat domain (i.e., stem domain) and in the transmembrane domain among mouse, rat, chimpanzee, and human. In the rat genomic sequence, a highly homologous genomic sequence was found in the MHC class I region and a homologous amino acid sequence was deduced from the nucleotide sequence. In chimpanzee, a homologous gene (LOC462551) was already predicted by the NCBI gene prediction method, GNOMON (Figure 2B). We propose to assign MUC21 to this gene based on the current numbering (Culp et al. 2004; Higuchi et al. 2004; Andrianifahanana et al. 2006). Alignment of the genomic structure and expressed sequence indicated that there are three exons: exon 1 corresponding to the leader sequence, exon 2 containing the tandem repeats, the unique sequence, and the transmembrane domain, and exon 3 containing the cytoplasmic tail (Figure 2C).

cDNA cloning of human epiglycanin/MUC21

Eight human carcinoma cell lines were tested for the expression of the gene by RT-PCR, and a cervical carcinoma cell line ME-180 was shown to express a high level of mRNA. Thus, the full-length open reading frame cDNA of human epiglycanin/MUC21 was cloned by PCR by the use of cDNA from cervical carcinoma ME-180 cells. The amplified cDNA was subcloned into the pCR4Blunt-TOPO vector and sequenced. The results shown in Figure 2A indicate that the sequence (DDBJ/GenBank/EBI accession number AB242595) is almost identical to the predicted sequence from AY358415. The putative translation product is a typical transmembrane mucin consisting of 535 amino acids. It contains a cytoplasmic tail (64 amino acids), a transmembrane domain (23 amino acids), a stem domain (22 amino acids), and 28 tandem repeats with 15 amino acids rich in threonine and serine.

Expression of human epiglycanin/MUC21

A pcDNA3.1 vector containing the full-length human epiglycanin/ MUC21 cDNA FLAG tagged at its amino terminal was generated and transfected into K562 human leukemia cells.

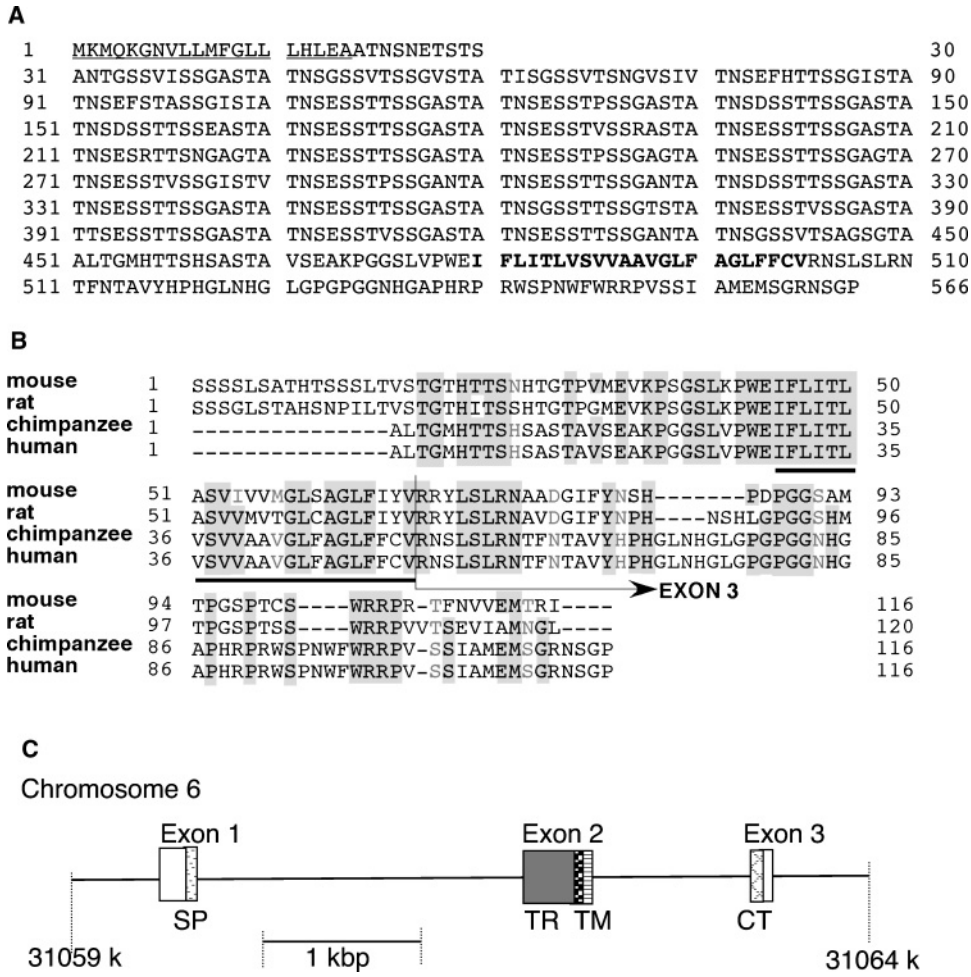

Fig. 2. (**A**) Deduced amino acid sequence of human epiglycanin cloned from ME-180 human cervical carcinoma cells. The 21 amino acid signal peptide is underlined. Twenty-eight tandem repeats of 15 amino acids are aligned. The transmembrane domain is underlined. (**B**) Sequence characteristics of epiglycanin/MUC21 of the mouse, rat, chimpanzee, and human in the nontandem repeat domain, transmembrane domain, and cytoplasmic domain deduced from the homologous genomic or expressed sequences. Dashes indicate gaps introduced in the sequence for alignment purposes. Shades show identical amino acids and grey letters indicate similar amino acids. Bold letters show the transmembrane region. The putative cleavage site is indicated with an arrowhead and the exon/intron boundary is indicated with an arrow. (**C**) Genomic structure of human epiglycanin/MUC21. The gene spans 4.8 kbp and consists of 3 exons coding a 21 amino acid signal peptide (SP), the tandem repeat domain (TR), the transmembrane domain (TM), and the cytoplasmic tail (CYT).

Clones were obtained from the transfected cells by the limiting dilution technique and a clone with high levels of anti-FLAG antibody binding (i.e., N-FLAG-MUC21 cells) was chosen. Figure 3A shows profiles of N-FLAG-MUC21 cells and a clone of mock transfected cells stained with the anti-FLAG antibody. The results revealed that the gene products were localized on the cell surface. The binding of *Vicia villosa* agglutinin isolectin B4 (VVA-B4) and peanut (*Arachis hypogaea*) agglutinin (PNA) was significantly higher to human epiglycanin/MUC21 transfected cells than to mock transfected cells as shown in Figure 3B and C, indicating that the transfected cells displayed many *O*-glycans on their cell surface. The expression of the cloned epiglycanin/MUC21 gene was further confirmed by the binding of the antibody specific for the cytoplasmic tail (termed anti-MUC21CT antiserum) to the permeabilized N-FLAG-MUC21 cells, but not to a mock-transfectant clone (Figure 3D–G). The antibody binding was blocked by preincubation of the antibody with the synthetic CRPVSSIAMEMSGRNSGP-peptide (10 μg/mL) (data not shown).

Cell lysates were prepared from human N-FLAG-MUC21 cells and a mock transfectant clone, immunoprecipitated with anti-MUC21CT antiserum, separated by SDS–PAGE on 8% polyacrylamide gels, and blotted with anti-FLAG antibody or lectins. As shown in Figure 4, the anti-FLAG antibody bound to a major component that migrated at an approximate molecular size of 180 kDa. Bands of similar electrophoretic mobility were also detected by lectin blotting with VVA-B4 and PNA, but not with Con A. Thus, it is likely that many *O*-glycans were attached to the mucin-like gene product precipitated with anti-MUC21CT antiserum. Collectively, the results confirmed that epiglycanin/MUC21 protein was expressed and *O*-glycosylated by these cells, suggesting it functions as a mucin.

Organ distribution of human epiglycanin/MUC21

Human epiglycanin/MUC21 mRNA expression was examined in various normal tissues by PCR screening of human MTC panels (BD Bioscience, Franklin Lakes, NJ) using human

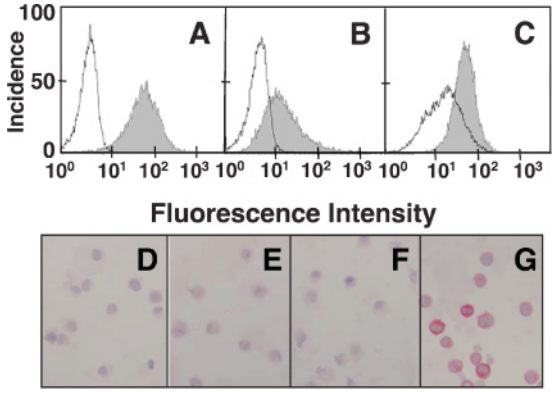

Fig. 3. Expression of epiglycanin gene products in K562 human erythroleukemia cells. A high-expressing clone stably transfected with human epiglycanin cDNA, FLAG-tagged at its amino terminus, were obtained by limiting dilution and screening with an anti-FLAG antibody (K562/MUC21-N-FLAG cells). A clone was also obtained from the mock transfected cells (K562/Mock cells). Flow cytometric analyses with anti-FLAG antibody (**A**), VVA-B4 (**B**), and PNA (**C**) were performed. Profiles of mock transfected cells (unshaded) and cells transfected with human epiglycanin/MUC21 with amino terminal FLAG (shaded) are shown. Binding profiles with K562/MUC21-N-FLAG cells are shown as a solid line. (**D–G**) K562/Mock cells (D and E) or K562/MUC21-N-FLAG cells (F and G) were place on glass slides by Cytospin, permeabilized by saponin treatment, and stained with anti-MUC21CT antiserum. Samples from the D and F were stained with the preimmune rabbit serum obtained from the same rabbit as negative controls, whereas samples E and G were stained with anti-MUC21CT antiserum.

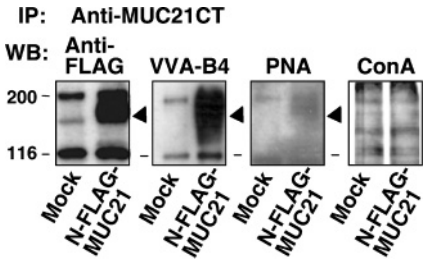

Fig. 4. Western blotting analysis of immunoprecipitated lysate of K562/MUC21-N-FLAG cells after separation by the SDS–PAGE. The lysates were immunoprecipitated with anti-MUC21CT antiserum. The precipitates were treated with SDS and 2-mercaptoethanol and separated by electrophoresis on 8% gels. The proteins were transferred onto PVDF membranes and blotting analysis was performed with an anti-FLAG antibody, VVA-B4, PNA, or ConA.

epiglycanin/MUC21 specific primers. Expression of epiglycanin/MUC21 was demonstrated remarkably in lung, thymus, and colon (Figure 5).

Immunohistological staining of nonmalignant and malignant lung tissue sections

Surgical specimens of normal and diseased tissues were obtained from a total of 55 patients with lung cancer treated at the University of Tokyo Hospital. As shown in Figure 6A and B, the binding of anti-MUC21CT antiserum was observed with normal bronchi, bronchioles, and bronchial glands. Antibody binding showed a diffuse distribution throughout the cytoplasm of epithelial cells. No binding was detected to any cells composing the alveoli. When malignant lung tissues were examined, no antibody binding was detected to small cell carcinomas. Out of 24 cases of adenocarcinomas, 9 showed strong staining as represented in Figure 6C and 15 showed positive staining weaker than that of normal bronchi (Figure 6D). Twenty-two out of 24 squamous cell carcinoma specimens were not stained with anti-MUC21CT antiserum and two specimens showed weak staining (Figure 6E). These results clearly indicated that epiglycanin/MUC21 is expressed by normal and malignant bronchial epithelial cells considering that adenocarcinomas of the lung are derived from these cells.

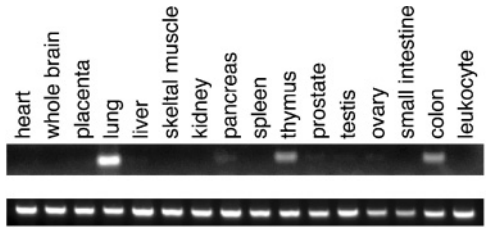

Fig. 5. Human epiglycanin mRNA expression in normal tissues of several organs. Human organ-derived cDNA was examined by PCR analysis. Human MTC panels were used as PCR templates and the organs are listed on the top of the panels.

Discussion

We have identified the mouse epiglycanin/Muc21 gene and the human counterpart. The cDNA corresponding to the open reading frame of human epiglycanin/MUC21 was cloned from ME-180 cells, a cervical carcinoma cell line. The gene is located on chromosome 6 in close proximity to the MHC class I and is mapped within the susceptibility domain for diffuse panbronchiolitis (DPB) a genetically predisposed disease affecting bronchiol observed only among people in the Far East (Keicho et al. 2000). The putative translation product encodes a typical transmembrane mucin with a cytoplasmic tail, a transmembrane domain, a stem domain, and 28 tandem repeats with 15 amino acids each. A sequence (i.e., GSLV) similar to the putative cleavage site associated with the extracellular SEA module (Wreschner et al. 2002) is present immediately upstream of its putative transmembrane domain. However, the mode of processing during the biosynthesis of human epiglycanin/MUC21 is currently unknown. The tandem repeat sequence is relatively conserved and rich in threonine and serine. Proline is present in 3 out of 28 repeats. The nontandem repeat sequence (i.e., transmembrane domain, cleavage site, and cytoplasmic tail) is highly conserved among humans, chimpanzees, rats, and mice.

Mouse epiglycanin/Muc21 was previously identified as a large glycoprotein localized at the surface of TA3-Ha cells. This identification was based on biochemical techniques, lectin binding, and electron microscopic observations (Sanford et al. 1973; Codington et al. 1975). Therefore, at present we have no definite way to prove whether the mouse gene that we identified, is that of epiglycanin/Muc21. However, there are several points that support our conclusion. The amino acid composition calculated from the translated product of the available portion of the gene shows a high degree of similarity to the composition reported by Codington (Codington et al. 1979). The RT-PCR products from mRNA of TA3-Ha and TA3-St cells, using primers for mouse Muc1, 2, 4, 5AC, and 9, were compared. TA3-St cells expressed higher levels of Muc1, 2, and 5AC than

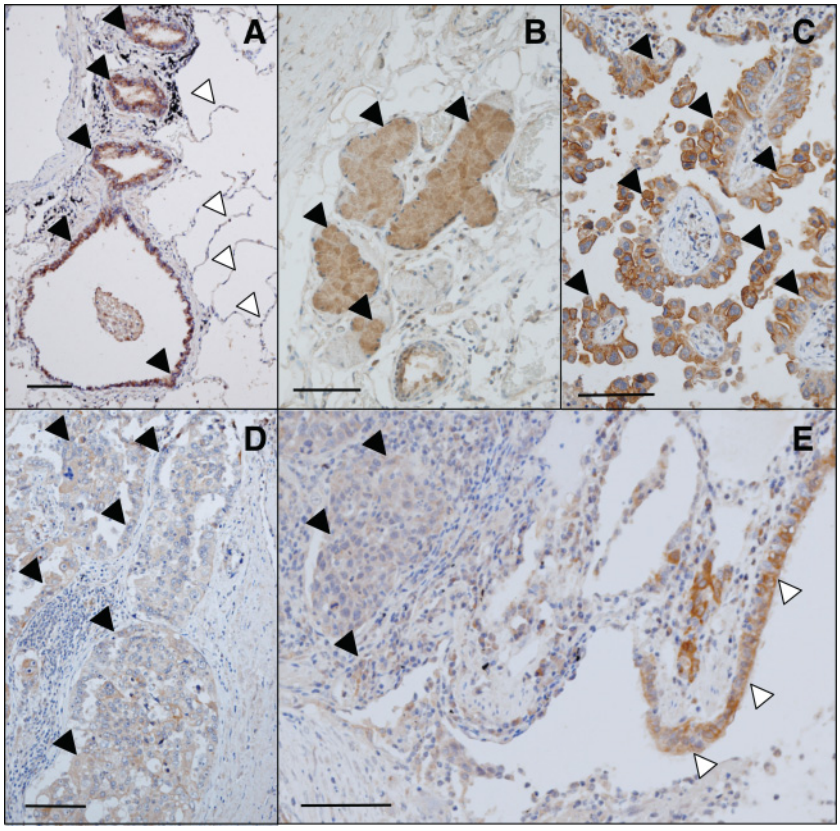

Fig. 6. Immunohistochemical staining of human lung and lung tumors with anti-MUC21CT antiserum. (**A**) A normal lung tissue showing strong antibody reactivity to bronchi and bronchioli (closed triangles). Reactivity was confined to bronchial and bronchiolar epithelial cells. The antibody did not bind alveoli (open triangles). (**B**) A portion of the normal lung tissue showing the bronchial gland stained positively with the antibody (closed triangles). (**C**) An example of lung adenocarcinoma cells strongly stained with anti-MUC21CT antiserum (closed triangles). Membranous and apical expression of tumor cells was noted in the case. (**D**) An example of lung adenocarcinoma cells weakly stained with anti-MUC21CT antiserum (closed triangles). (**E**) An example of squamous cell carcinoma of the lung weakly stained with anti-MUC21CT antiserum (closed triangles). In the right side of the panel shows lung epithelium associated with bronchial epithelia where antibody binding was prominent (open triangles). Bars indicate 100 μm.

TA3-Ha cells, whereas TA3-Ha cells expressed approximately two-fold levels of Muc4 and 9 than St cells (data not shown). However, epiglycanin/Muc21 mRNA was detected at high levels in TA3-Ha cells but was not present in TA3-St cells. Also, epiglycanin/Muc21 was not similar to Muc4 or 9 with respect to their amino acid compositions. Therefore, it is reasonable to conclude that the cDNA-RDA resulted in a fragment of epiglycanin/Muc21. Although the possibility remains that the gene we identified corresponds to another (yet unidentified) mucin different from epiglycanin, it is not possible to prove or disprove as mentioned above. It would be appropriate to name the gene we identified as Muc21 according to the current status of genes for mucin core polypeptides. The cDNA cloning, distribution, and the expression of the mouse epiglycanin/Muc21 gene will be published separately.

The human epiglycanin/MUC21 cDNA was inserted into a plasmid vector with FLAG epitope tagged to the amino terminal, and transfected into K562 cells. The gene products were shown to be expressed on the cell surface. The binding of *O*-glycan-specific lectins, i.e., VVA-B4 and PNA, was observed with these but not with mock transfected cells, suggesting that the expressed protein was *O*-glycosylated. The lysates of these cells were subjected to immunoprecipitation, electrophoretically separated under reducing conditions, and probed with the anti-FLAG antibody or with VVA-B4, PNA, or ConA. The results clearly indicated that the transfected cDNA was translated and the product was *O*-glycosylated. The expressed epiglycanin/MUC21 did not alter the behavior of these cells as far as their motility and adhesion in vitro are concerned (data not shown). Other biological characteristics of the transfected cells remain to be elucidated.

Any association of the expression or mutation of this gene with pathological conditions requires further investigations. The chromosome location of the gene is within the MHC class I locus and the area is within the 200 kb stretch identified as the susceptibility locus of DPB (Keicho et al. 2000). It was also shown recently that another locus, which was linked to susceptibility to this disease and which was identified through comprehensive linkage disequilibrium analysis, was the 5′-upstream regulatory element of the MUC5B gene (Kamio et al. 2005). MUC5B is a secreted mucin and any link between the function of epiglycanin/MUC21 and MUC5B remains to be elucidated. Since the MHC locus is one of the most polymorphic areas in the human genome, polymorphisms of the epiglycanin/MUC21 gene should be further investigated in the context of this disease (Kudoh and Keicho 2003). It should be noted that our results indicated epiglycanin/MUC21 protein expression in bronchial epithelia, which is the affected site in patients with DPB.

Association of altered mucin expression with malignancy is an important issue. Immunohistochemical tests were performed with anti-MUC21CT antiserum using lung and lung carcinoma tissue specimens. This antibody was useful because the binding is independent of the degree of glycosylation. Extensive antibody binding was observed with 9 out of 24 specimens of adenocarcinomas. All of the remaining 15 specimens were stained too, but less intensely than the other 9 cases. Antibody binding was diffusely observed within the cytoplasm of carcinoma cells, suggesting the cytoplasmic portion was not secreted out of the cells. Whether and to which extent secretion or shedding of the extracellular domain of epiglycanin/MUC21 occurs in cancer cells remains to be elucidated. Very low levels of antibody binding to squamous cell carcinoma were observed in 2 out of 24 specimens and no binding was detected to small cell lung carcinomas. Intratumoral heterogeneity was low with adnocarcinomas and squamous cell carcinomas. According to our preliminary studies with mRNA extracted from cancerous tissues, 2 out of 10 cases of lung carcinoma showed high levels of expression of epiglycanin/MUC21 as far as the mRNA levels in the cancer array (BD Biosciences) were concerned (data not shown). These two cases were adenocarcinomas, five squamous carcinomas were negative or weakly positive, and three unknown histological types were weakly positive. Therefore, this mucin is likely to be considered as a marker for adenocarcinomas as far as lung carcinomas are concerned. Thus, this mucin is potentially useful to distinguish between lung metastases of unknown primaries and lung adenocarcinomas. Expressions of this mucin in other malignant tissues including breast will be published separately.

In conclusion, we have obtained the molecular identity of epiglycanin, a mucin previously claimed to possess an immunomodulatory function, and its human counterpart. The gene for this new mucin, MUC21, is located in the MHC class I region. It is a membrane-associated mucin with a typical threonine/serine-rich tandem-repeat domain, a transmembrane domain, and a cytoplasmic domain. The protein was shown to be expressed by normal bronchial epithelial cells and by adenocarcinomas of the lung.

Materials and methods

Cells and cell culture

TA3-Ha cells and TA3-St cells were a kind gift from Dr. Codington. TA3-Ha and TA3-St cells were cultured in a 1:1 mixture of Dulbecco's modified minimum essential medium and Ham's F-12 medium containing 10% fetal calf serum (FCS) in vitro. Human myeloid leukemia K562 cells were purchased from ATCC (Manassas, VA). Human cervical carcinoma ME-180 cells were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer Tohoku University, Sendai, Japan. Cells were cultured in RPMI 1640 medium with 10% FCS at 37°C in a 5% CO_2 atmosphere.

cDNA cloning of a fragment of a mucin-like gene by cDNA-RDA in mice

cDNA-RDA was performed as described by Hubank and co-workers with slight modifications (Hubank and Schatz 1994). mRNA was isolated from TA3-Ha cells and TA3-St cells using μMACS mRNA Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Double-stranded cDNA libraries were synthesized using Timesaver cDNA synthesis kit (Amersham Bioscience, Piscataway, NJ). Double-stranded cDNA libraries were separated on 0.7% Seakem ME agarose gels and DNA corresponding to the size larger than 2 kbp was extracted using Qiaex II Gel extraction kit (Qiagen, Hilden, Germany). About 100 ng of double-stranded cDNA were digested by treatment with *Sau*3AI and ligated to R *Bgl* 24: 5′-AGCACTCTCCAGCCTCTCACCGCA-3′ and R *Bgl* 12: 5′-GATCTGCGGTGA-3′ using Takara DNA Ligation Kit ver.2 (Takara, Kyoto, Japan). One-thirtieth of ligated cDNA mixtures were amplified with the R *Bgl* 24 primers in standard buffer with Ampli Taq DNA polymerase (72°C, 5 min; 20 cycles at 95°C, 1 min; 72°C for 3 min; 72°C, 10 min). PCR products were digested with *Sau*3AI and run on a 3% Nusieve agarose gel (Takara). DNA bands having sizes of 100 bp to 1500 bp were extracted using the Qiaex II gel extraction kit. The cDNA derived from TA3-St cells served as driver amplicons. The cDNAs derived from TA3-Ha cells were ligated with J *Bgl* 24: 5′-ACCGACGTCGACTATCCATGAACA-3′ and J *Bgl* 12: 5′-GATCTGTTCATG-3′, and served as tester amplicons.

For the first RDA, tester amplicons and 100 times the amount of driver amplicons were hybridized in hybridization buffer at 67°C overnight. One-tenth of hybridized cDNA was amplified with the J *Bgl* 24 primers in standard buffer with Ampli Taq DNA polymerase (72°C, 5 min; 20 cycles at 93°C, 1 min; 70°C for 3 min; 70°C, 10 min). First PCR products were treated with mung-bean nuclease (Takara) at 37°C for 30 min, and incubated with 0.05 M Tris (pH 8.9) at 95°C for 5 min. One-fifth of such a treated cDNA was amplified with the J *Bgl* 24 primers in standard buffer with Ampli Taq DNA polymerase (72°C, 5 min; 30 cycles at 93°C, 1 min; 70°C for 3 min; 70°C, 10 min). Second PCR products were digested with *Sau*3AI and run on a 3% Nusieve agarose gel. The DNA bands of 100 bp to 1500 bp were extracted using Qiaex II gel extraction kit. The cDNAs were ligated with N *Bgl* 24 H 5′-AGGCAACTGTGCTATCCGAGGGAA-3′ and N *Bgl* 12; 5′-GATCTTCCCTCG-3′, and served as tester amplicons.

The second and the third RDA were performed by a method identical to the method for the first RDA. The second PCR products after digestion with *Sau*3AI (100 bp to 1500 bp) were ligated with J *Bgl* 24 and J *Bgl* 12, and served as tester amplicons for the third RDA. The second PCR products of the third RDA were separated on a 3% Nusieve agarose gel (Takara). Bands of interest were extracted using Qiaex II gel extraction kit. The extracted DNA fragments were cloned into the pGEM-T Easy vector (Promega, Madison, WI) using the T/A cloning system, and sequenced on both strands using a BigDye terminator v2.0 cycle sequencing kit (Applied Biosystems, Foster City, CA) with T7 and SP6 primers on an ABI 377 DNA sequencer (Applied Biosystems).

Elongation of cDNA fragments obtained by cDNA-RDA

Further characterization was performed focusing on the putative fragment of epiglycanin cDNA. For the isolation of longer cDNAs, the fragment of cDNA derived from cDNA-RDA was elongated by race PCR using the Marathon cDNA Amplification Kit (Clontech, Moutain View, CA). Double-stranded cDNA was ligated to a Marathon cDNA adaptor, and

amplified using primers specific for the adaptor and cDNA fragments. For 3′-race, epi1-5 primer; 5′-TCTGACCACCA-CTGCATCCAGCACT-3′ was used. The race products were run on a 1.2% agarose gel, extracted, cloned into the pGEM-T Easy vector, and sequenced on an ABI 3100 DNA sequencer (Applied Biosystems).

Identification of human epiglycanin

Homologous sequences to the deduced amino acid sequence of a region downstream of the tandem repeat domain of mouse epiglycanin were searched in the human BLAST for expressed sequences. A gene encoding a transmembrane protein with unknown functions was identified (AY358415) (Clark et al. 2003).

Cloning of full-length cDNA for human epiglycanin

mRNA was purified from ME-180 cells with μMACS mRNA isolation kit. First-strand cDNA was synthesized using human epiglycanin specific antisense primer (5′-CCTATAACTGAGT-TCATCTCAGAGC-3′) and Superscript III (Invitrogen, Carlsbad, CA). The resulting single-strand cDNA was used as a template for PCR amplification to obtain a coding sequence of human epiglycanin using 5′-CCCAGGAACACAAACGTAGG-AGACCCACGCTCCTG-3′, and 5′-CAGGATCTCAAACAA-AGCTGGGAGGGGTCTCCTGGG-3′ as primers. The resulting 2.0 kbp products were subcloned into the pCR4Blunt-TOPO vector (Invitrogen) and sequenced with the dye primer method on a LIC 4200 DNA sequencer (Aloka, Tokyo, Japan).

Preparation of polyclonal antisera specific for the cytoplasmic tail of human epiglycanin

A synthetic oligopeptide (C) RPVSSIAMEMSGRNSGP corresponding to the 17 residues at the carboxyl terminal of epiglycanin was prepared by the use of a peptide synthesizer (Pioneer/Applied Biosystems) and purified by high performance liquid chromatography equipped with a C18 reverse-phase column (Nacalai Tesque, Tokyo). The size of the oligopeptide was assessed by MALDI-TOF mass-spectroscopy with Voyager Elite. The peptide (1 mg) was conjugated to keyhole limpet hemocyanin (20 mg: Sigma, St. Louis, MO) with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester through the attached cysteine residue added at the amino terminus. Japanese white rabbits were immunized twice with the conjugate with a 2-week interval. The sera were obtained when the antibody titer was 2.4×10^4 (to reach a half maximum binding to the peptide) in ELISA 2 weeks after the second immunization. This antibody was named as anti-MUC21CT antiserum for reasons stated in the Result section.

Expression of human epiglycanin cDNA in K562 leukemia cells

The coding sequence of human epiglycanin with a FLAG epitope tag added at the amino terminus was inserted into a mammalian cell expression vector, pcDNA3.1(−) (Invitrogen). The plasmid without human epiglycanin was used as a control. K562 cells were transfected with the plasmid by electroporation. After selection with Geneticin (G418 sulfate; Calbiochem, La Jolla, CA), cloning of transfected cells was performed by the limiting dilution method.

Flow cytometric analysis with an anti-FLAG-M2 antibody (Sigma) in a combination with FITC-labeled goat anti-mouse IgG1 (Zymed Laboratories, South San Francisco, CA) was performed on an Epics Coulter XL flow cytometer (Beckmann Coulter, Fullerton, CA) to determine the levels of human epiglycanin expression on these cells. A clonal population was also obtained from the mock transfected cells. To detect specific carbohydrate epitopes on the cell surface, biotinylated VVA-B4 (Vector Laboratories, Burlingame, CA) and biotinylated PNA (Seikagaku, Tokyo, Japan) in combination with phycoerythrin-labeled streptavidin (eBioscience, San Diego, CA) were used. A clone with high expression was designated as N-FLAG-MUC21 cells and used for further investigations.

The cells were loaded onto slides by Cytospin (Thermo, Waltham, MA), fixed with 80% ethanol and permeabilized by treatment with 0.1% Triton X-100 diluted in Dulbecco's phosphate buffered saline (DPBS) for 30 s. The cells were reacted with anti-MUC21CT antiserum (antiserum diluted 1:2000) and with alkaline phosphatase-conjugated anti-rabbit IgG (Zymed).

For the immunoprecipitation and Western blotting analysis, N-FLAG-MUC21 cells were harvested and washed twice with DPBS. After washing, cells were lysed in 10 mM Tris–HCl buffer (pH 7.4) containing 0.5% Nonidet P-40, 0.25 M sucrose, 0.05 mM calcium chloride, 2 mM EDTA, and proteinase inhibitor cocktails (1/1000 dilution, Sigma), and centrifuged at 14,000 rpm at 4°C for 20 min. The supernatants were collected as cell lysates. Protein concentrations in the lysates were determined by BCA protein assay (Pierce, Rockford, IL) using bovine serum albumin as a standard. Immunoprecipitation was performed by mixing the lysates (corresponding to 2 mg protein) with protein G-agarose (20 μL: Amersham Bioscience) previously coated with normal rabbit IgG (5 μg) at 4°C for 2 h to eliminate nonspecific binding. The supernatants were mixed with 5 μL of polyclonal antisera specific for the cytoplasmic tail of epiglycanin (anti-MUC21CT antiserum) conjugated to protein G-agarose (20 μL) and incubated at 4°C overnight. Precipitated material corresponding to 67 μg protein was boiled in SDS–PAGE sample buffer (0.25 M Tris–HCl, pH 6.8, 40% glycerol, 8% SDS, 0.001% bromphenol blue) with 1% (v/v) 2-mercaptoethanol, subjected to electrophoresis on 8% polyacrylamide gels, and transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA). After blocking with 3% BSA in DPBS containing 0.1% Tween 20, the membranes were incubated with a biotinylated anti-FLAG-M2 antibody (1/1000 dilution, Sigma), biotinylated VVA-B4 (2.5 μg/mL), or biotinylated PNA (2.5 μg/mL) diluted with Tris-buffered saline (50 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing 3% BSA and 0.1% Tween 20 at room temperature for 2 h. Negative controls for the staining with antibodies were performed with mouse IgG1 (Zymed) under the same conditions. The binding was detected by horseradish peroxidase-conjugated streptavidin (Zymed) for Western blotting and alkaline phosphatase-conjugated streptavidin (Vector) for lectin blotting diluted 1000 times in Tris-buffered saline containing 3% BSA and 0.1% Tween 20. Bound antibodies and lectins were visualized by using the ECL detection kit (Amersham) or the alkaline phosphatase substrate kit (Vector), respectively.

Examination of epiglycanin/MUC21 mRNA in human tissues

PCR analysis was applied to examine the expression of the epiglycanin gene in human tissues. Human cDNA samples from a variety of organs, human Multiple Tissue cDNA panels I and

II (BD Biosciences), were used as a template in PCR reactions using Ampli *Taq* Gold polymerase (Applied Biosystems). Primers used are 5′-GACCCCTTCATTGACCTCAACTAC-3′ and 5′-CAGTGATGGCATGGACTGTGGT-3′ for human glyceraldehydes-3-phosphate dehydrogenase (G3pdh) and 5′-CTTCCCATAGTGCATCTACTGC-3', and 5′-GAACCAG-TTAGGACTCCACCTGGGCC-3′ for MUC21 corresponding to 983 bp and 269 bp. The cDNA panels contained normalized, first-strand cDNA generated from each of the following human tissues: heart, whole brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and leukocyte.

Immunohistological staining of malignant and nonmalignant lung tissue sections by the use of polyclonal antiserum

Surgical specimens were obtained from a total of 55 patients with lung carcinoma treated at the University of Tokyo Hospital between 1999 and 2003. Twenty-four adenocarcinoma specimens and two independent normal portions of lung tissue were examined. Twenty-four squamous cell carcinomas and five small cell lung cancer specimens were also examined. Tissues were preserved in 10% buffered formalin and embedded in paraffin and were serially sectioned at 4 μm thickness, mounted on silane-coated slides, and deparaffinized. The slides were immersed for 20 min in 0.3% hydrogen peroxide in methanol to inactivate endogenous peroxidase. After washing with DPBS, the slides were incubated with 3% bovine serum albumin to block nonspecific antibody binding at 4°C for 30 min in a humidified chamber, then reacted with anti-MUC21CT antiserum at 1:2000 dilution in DPBS containing 1% BSA overnight. The antibody binding was visualized by the use of peroxidase-conjugated mouse anti-rabbit IgG following incubation with diethylaminobenzidine (50 μg/mL).

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Conflict of interest statement

None declared.

Abbreviations

cDNA-RDA, cDNA-representational difference analysis; DPB, diffuse panbronchiolitis; DPBS, Dulbecco's phosphate buffered saline; FCS, fetal calf serum; kDa, kilodalton; PNA, peanut (*Arachis hypogaea*) agglutinin; SEA, sperm protein-enterokinase-agrin; SDS–PAGE, sodiumdodecyl sulfate-polyacrylamide gel electrophoresis; VVA-B4, *Vicia villosa* agglutinin isolectin B4.

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