

A novel anti-MUC1 antibody against the MUC1 cytoplasmic tail domain: use in sensitive identification of poorly differentiated cells in adenocarcinoma of the stomach

Suguru Yonezawa · Shinichi Kitajima · Michiyo Higashi · Masahiko Osako · Michiko Horinouchi · Seiya Yokoyama · Sho Kitamoto · Norishige Yamada · Yukihiro Tamura · Takeshi Shimizu · Mineo Tabata · Masamichi Goto

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Abstract

Background Isolated cancer cells of non-solid type poorly differentiated adenocarcinoma (por2) or signet-ring cell carcinoma (sig) are frequently seen in scirrhous gastric cancers with a very poor prognosis. These cells are often scattered in granulation tissue or desmoplastic fibrotic tissue and tend to be overlooked in routine pathological

examination. We aimed to raise a novel antibody that can identify the isolated cancer cells easily.

Methods Because the MUC1 cytoplasmic tail domain (CTD) has many biological roles including tumor progression and cell adhesion disturbance and is expected to be expressed in isolated cancer cells, we raised a novel monoclonal antibody (MAb) MUC1-014E against an intracellular nonrepeating 19-amino-acid sequence (RY-VPPSSTDRSPYEKVSAG: N-1217-1235-C) of the MUC1 CTD, using a synthetic peptide including the 7-amino-acid epitope (STDRSPY: N-1223-1229-C).

Results In the immunohistochemical staining of 107 gastrectomy specimens including 48 por2 and 31 sig lesions, the MAb MUC1-014E showed high rates of positive staining ($\geq 5\%$ of carcinoma cells stained) for por2 (100%) and sig (97%), and of the highest intensity staining (4+, $\geq 75\%$ of carcinoma cells stained) for por2 (100%) and sig (90%). In the 89 biopsy specimens including 82 por2 and 38 sig lesions, the MAb MUC1-014E showed high rates of positive staining for por2 (100%) and sig (100%) and of 4+ staining for por2 (87%) and sig (84%). All the rates were significantly higher than those with cytokeratins (AE1/AE3 or CAM5.2).

Conclusions The MAb MUC1-014E is very useful for accurate detection of isolated cancer cells in scirrhous gastric cancers.

Keywords MUC1 · Cytoplasmic tail domain · Monoclonal antibody · Scirrhous gastric cancer · Accurate detection

Abbreviations

por2	Non-solid type poorly differentiated adenocarcinoma
sig	Signet-ring cell carcinoma

S. Yonezawa (✉) · M. Higashi · S. Yokoyama · S. Kitamoto · N. Yamada · Y. Tamura · M. Goto
Department of Human Pathology, Field of Oncology,
Kagoshima University Graduate School of Medical and Dental
Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
e-mail: syoneza@m2.kufm.kagoshima-u.ac.jp

S. Kitajima
Department of Surgical Pathology, Kagoshima University
Hospital, Kagoshima, Japan

M. Osako · M. Tabata
Department of Surgery, Kagoshima-shi Medical Association
Hospital, Kagoshima, Japan

M. Horinouchi · T. Shimizu
Department of Pathology, Kagoshima-shi Medical Association
Hospital, Kagoshima, Japan

N. Yamada
Division of Reproductive Sciences, Department of Pediatrics,
Cincinnati Children's Hospital Medical Center,
Cincinnati, OH, USA

Y. Tamura
Department of Internal Medicine, Ohsumi-Kanoya Hospital,
Kanoya, Kagoshima, Japan

M. Goto
National Sanatorium Hoshizuka-Keiaiien,
Kanoya, Kagoshima, Japan

CTD	Cytoplasmic tail domain
MAb	Monoclonal antibody
JCGC	Japanese Classification of Gastric Carcinoma
H&E	Hematoxylin and eosin
TR	Tandem repeat
MAb MUC1-014E	MAb MUC1-common clone 014E
aa	Amino-acid
KLH	Keyhole-limpet hemocyanin
PAb	Polyclonal antibody
CK	Cytokeratin
PBS	Phosphate-buffered saline
pap	Papillary adenocarcinoma
tub1	Well-differentiated tubular adenocarcinoma
tub2	Moderately differentiated tubular adenocarcinoma
por1	Solid type poorly differentiated adenocarcinoma
muc	Mucinous adenocarcinoma
WHO	World Health Organization

Introduction

Isolated cancer cells of non-solid type poorly differentiated adenocarcinoma (por2) and signet-ring cell carcinoma (sig) of the stomach in the Japanese Classification of Gastric Carcinoma (JCGC) [1] are frequently seen in scirrhous gastric cancers with a very poor prognosis [2]. These cells are often scattered in granulation tissue or desmoplastic fibrotic tissue, which makes likely to be overlooked in a routine histopathological examination using hematoxylin and eosin (H&E) staining. The aim of this study is to raise a novel antibody that can identify the isolated cancer cells easily.

Mucins are high molecular weight glycoproteins with oligosaccharides attached to serine or threonine residues of the mucin core protein backbone by *O*-glycosidic linkages. These proteins are produced by various epithelial cells. Human mucins are categorized into membrane-associated mucins and secreted mucins [3–6]. In 1993, we reported the first evidence that pancreatic or biliary invasive carcinomas with aggressive biological behavior usually show expression of MUC1 [7, 8]. Subsequently, we have shown that MUC1 expression is associated with the aggressive behavior of various human neoplasms and with a poor outcome [4]. MUC1 was the first cloned membrane-associated mucin that has been studied in most detail. Full-length human MUC1 synthesized as a single polypeptide is processed into two subunits by proteolytic cleavage: a

larger subunit containing most of the extracellular domain including a tandem repeat (TR) domain, and a smaller subunit containing a shorter extracellular domain, a transmembrane domain, and a cytoplasmic tail domain (CTD) (Fig. 1a) [3, 9]. In our previous studies and in most other studies of MUC1 expression in human neoplasms, anti-MUC1 antibodies were raised against the TR region [4, 7, 8].

Although the large extracellular domain of MUC1 containing TRs has many functions, recent studies have also suggested that MUC1 CTD has many biological roles including tumor progression and cell adhesion disturbance, although this region contains only 69 amino acids [3, 9–11]. Thus, we expected that MUC1 CTD plays an important role in isolated cancer cells in scirrhous gastric cancers. In the present study, we raised a novel anti-MUC1 monoclonal antibody (MAb), which we designated as MAb MUC1-common clone 014E (abbreviated as MAb MUC1-014E), against an intracellular nonrepeating 19 amino-acid (aa) sequence (RYVPPSSTDRSPYEKVSAG: N-1217-1235-C) in the CTD (Fig. 1a, b). The 19 aa sequence is common in most human isoforms of MUC1 (thus we named this region MUC1-common), but an antibody against this sequence has not been reported.

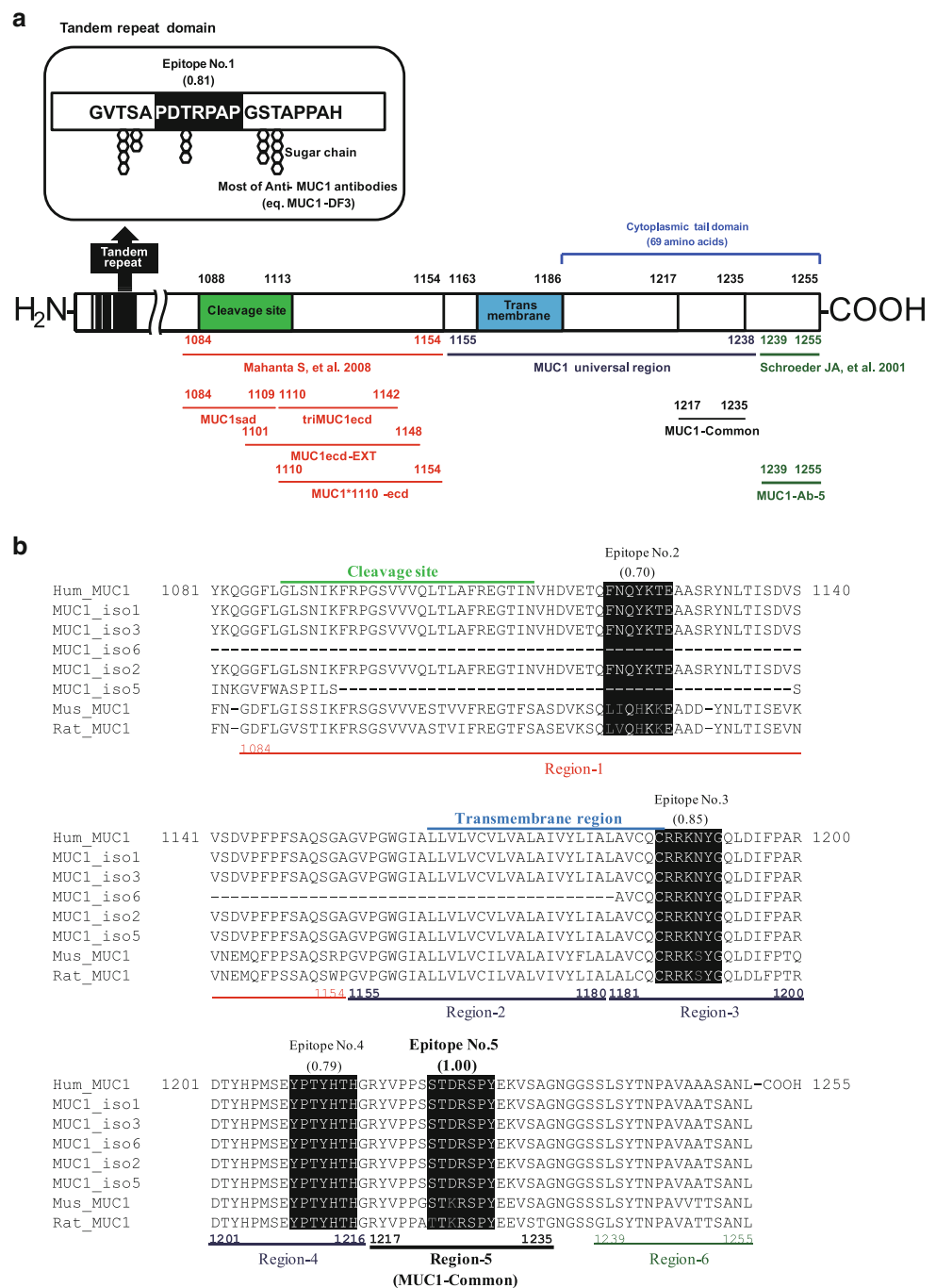
In an immunohistochemical study of human gastrectomy and biopsy specimens with gastric cancer, we found that MAb MUC1-014E was able to identify isolated cancer cells in por2 and sig of the stomach. Therefore, MAb MUC1-014E may be of particular value for identifying these cells in resected specimens and biopsy specimens of stomach.

Patients and methods

Selection of the antigen epitope

We aimed to select a specific antigen epitope, to establish a novel anti-MUC1 antibody on the C-terminal side of the cleavage site in MUC1 (Fig. 1a, b), although most of the anti-MUC1 antibodies were raised against the TR region containing Epitope No. 1 on the N-terminal side (Fig. 1a). Four antibodies had already been raised (Fig. 1a) against the aa sequence N-1084-1154-C (Region-1) containing Epitope No. 2 (Fig. 1b) in the short extracellular domain from the cleavage site to the N-terminus of the transmembrane domain (Fig. 1a) [12]. Another commercially available MAb MUC1-Ab-5 (Fig. 1a) binds to the N-1239-1255-C sequence in the C-terminal region of the MUC1 CTD (Region-6) (Fig. 1b) [13]. Thus, we focused on the sequence N-1155-1238-C, which we refer to as the MUC1 universal region (Fig. 1a) because it is highly conserved in isoforms of human MUC1.

Fig. 1 Antigen epitopes in MUC1. Most anti-MUC1 antibodies are raised against the tandem repeat (TR) region containing Epitope No. 1 on the N-terminal side (a). Four antibodies are reported against the amino-acid (aa) sequence in Region-1 (N-1084-1154-C) containing Epitope No. 2 in the short extracellular domain from the cleavage site to the N-terminus of the transmembrane domain (a, b). (Cited from Mahanta et al. [12].) Another commercially available MAb MUC1-Ab-5 is targeted to the aa sequence of Region-6 (N-1239-1255-C) at the C-terminal region in the MUC1 CTD (a, b). (Cited from Schroeder et al. [13].) Thus, we focused on aa sequence N-1155-1238-C (MUC1 universal region) (a). The MUC1 universal region was divided into four regions (Region-2 to -5) (b). In three predicted B-cell epitopes, No. 3, No. 4, and No. 5 of the MUC1 universal region, Epitope No. 5 in Region-5 has the highest score (1.00), and the 19 aa sequence of Region-5 is a very common MUC1 sequence that has the strongest antigenicity to make a mouse monoclonal antibody (MAb) (b). Therefore, Region-5 (N-1217-1235-C) was selected as the antigen to raise the MAb, and was named MAb MUC1-common clone 014E (MAb MUC1-014E), because the aa sequence is common to most isoforms of human MUC1 (a, b)



In the universal region, three predicted B-cell epitopes, No. 3 (CRRKNYG: N-1186-1192-C), No. 4 (YP-TYHTH: N-1209-1215-C), and No. 5 (STDRSPY: N-1223-1229-C), had high scores in an evaluation based on physicochemical parameters including hydrophilicity, polarity, accessibility and flexibility (Fig. 1b). Region-2 had no predicted B-cell epitope. Region-4 carried Epitope No. 4, but has an aa sequence identical to that of MUC1 in the mouse, indicating poor antigenicity in mouse. Thus, these two regions were not suitable

candidates for an antigen. Epitope No. 5 had the highest score (1.00). In addition, homology evaluation using BLAST for isoforms of human MUC1 showed that the 19 aa sequence of Region-5 was identical in 45 isoforms, whereas the 19 aa sequence in Region-3 was identical in 35 isoforms [14]. Thus, the Region-5 containing Epitope No. 5 was selected as the antigen to raise MAb, and was named MUC1-common (RYVPPSSTDRSPYEKVSAG: N-1217-1235-C) because of its occurrence in most human MUC1 isoforms (Fig. 1b).

Immunization and screening for MUC1-positive hybridomas

The MUC1-common peptide with a cysteine attached to the N-terminus was synthesized by the Fmoc solid-phase method, followed by conjugation of maleimide-activated keyhole-limpet hemocyanin (KLH). C57BL/6 mice were immunized subcutaneously with 200 mg of the KLH-conjugated MUC1-common peptide emulsified in Freund's complete adjuvant, and MAbs were produced according to the conventional method as described before [15].

As the result, five clones were isolated and continuously subcloned by the conventional limiting dilution method. After secondary screening by immunohistochemistry, a promising clone (clone 014E) was established, and the raised MAb was designated as MAb MUC1-014E.

Comparison with other anti-MUC1 antibodies

MAb MUC1-014E was compared immunohistochemically with the following anti-MUC1 antibodies (Fig. 2): MAb MUC1-Ab-5, raised against the C-terminal region of the MUC1 CTD [13] (Thermo Scientific, Fremont, CA, USA);

polyclonal antibody (PAb) anti-MUC1*1110-ecd_P, which binds to the extracellular domain (45 aa: GTINVHD VETQFNQYKTEAASPYNLTISDVSVDVPPFSAQS GA: N-1110-1154-C) [12]; and PAb anti-MUC1*1110-ecd_R, which also binds to the extracellular domain (45 aa: GTINVHDVETQFNQYKTEAASRYNLTISDVSVDVPPFSAQSGA: N-1110-1154-C) (we note that the underlined amino acid at position N-1131 was P in Mahanta et al. [12], but R in the BLAST/NCBI nonredundant protein database [14]; thus, we made PABs against both antigens by immunization of rabbits with synthetic peptides with KLH conjugated at the C-terminus); and MAb MUC1-DF3, which was raised against the TR region of MUC1 (TFB, Tokyo, Japan) [16–18].

Comparison with anti-cytokeratin antibodies (AE1/AE3, CAM5.2)

MAb MUC1-014E was compared immunohistochemically with the MAbs against cytokeratin (CK), CK-AE1/AE3 (NCL-AE1/AE3; Leica Biosystems Newcastle, Newcastle, UK) or CK-CAM5.2 (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA).

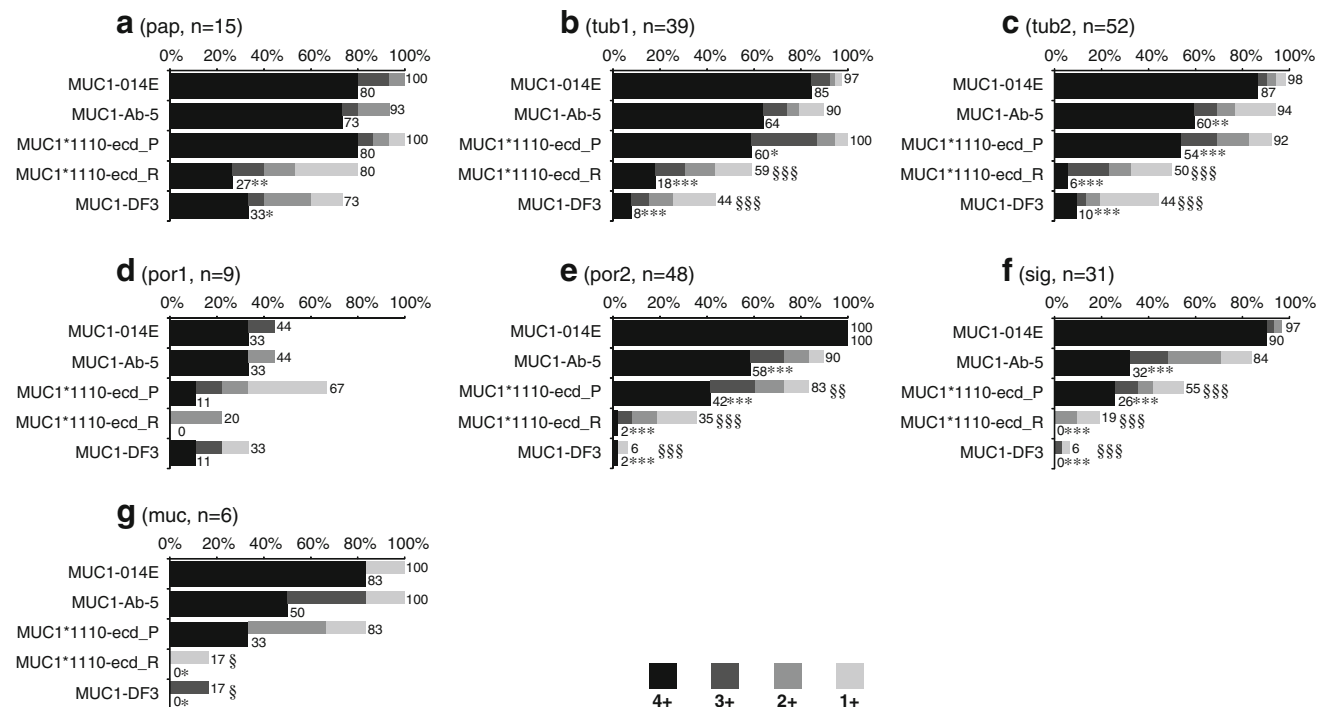


Fig. 2 Comparison of MUC1-014E expression with that of MUC1-Ab-5, MUC1*1110-ecd_P, MUC1*1110-ecd_R, and MUC1-DF3 for each histological type in gastrectomy specimens: pap (a), tub1 (b), tub2 (c), por1 (d), por2 (e), sig (f), and muc (g). The results for MUC1-014E are compared with those for other antigens using positive staining (total of 1+, 2+, 3+ and 4+) (a–g, upper bars) and 4+ staining (highest intensity staining) (a–g, lower bars). Numbers at the right sides of each bar are percentages of the positive staining or

4+ staining. MUC1-014E shows the highest positive staining (upper bars) and the highest 4+ staining (lower bars) for most histological types. For por2 and sig, MUC1-014E shows significantly higher staining than other MUC1 antigens, particularly for 4+ staining (e, f, lower bars). §*P* < 0.05; §§*P* < 0.01; §§§*P* < 0.001 (upper bars); **P* < 0.05; ***P* < 0.01; ****P* < 0.001 (lower bars) for MUC1-014E staining versus that for each of the other antigens

Tissue samples

MAB MUC1-014E stained isolated cancer cells of scirrhous gastric cancers of por2 very well, in the preliminary immunohistochemical staining of the formalin-fixed paraffin-embedded tissue sections of human cancers of the stomach, colon, rectum, pancreas, biliary tract, lung, and ovary. Then, we examined gastrectomy specimens of 107 early gastric cancers (pT1b2). In the cases in which more than two histological types were mixed in one lesion, each histological pattern was evaluated independently, according to the JCGC [1].

Subsequently, we also examined 89 biopsy specimens with histological types of por2 and/or sig.

All specimens were obtained from the files of Kagoshima University Hospital and Kagoshima-shi Medical Association Hospital. The study was approved by the ethical committees of both hospitals.

Immunohistochemistry

Immunohistochemistry was performed by the immunoperoxidase method as follows. Antigen retrieval was performed using CC1 antigen retrieval buffer (Ventana Medical Systems, Tucson, AZ, USA) for all sections. Following incubation with the primary antibodies (MAB MUC1-014E diluted 1:5; PAb anti-MUC1*1110-ecd_P diluted 1:3,000; PAb anti-MUC1*1110-ecd_R diluted 1:1,500; MAB MUC1-DF3

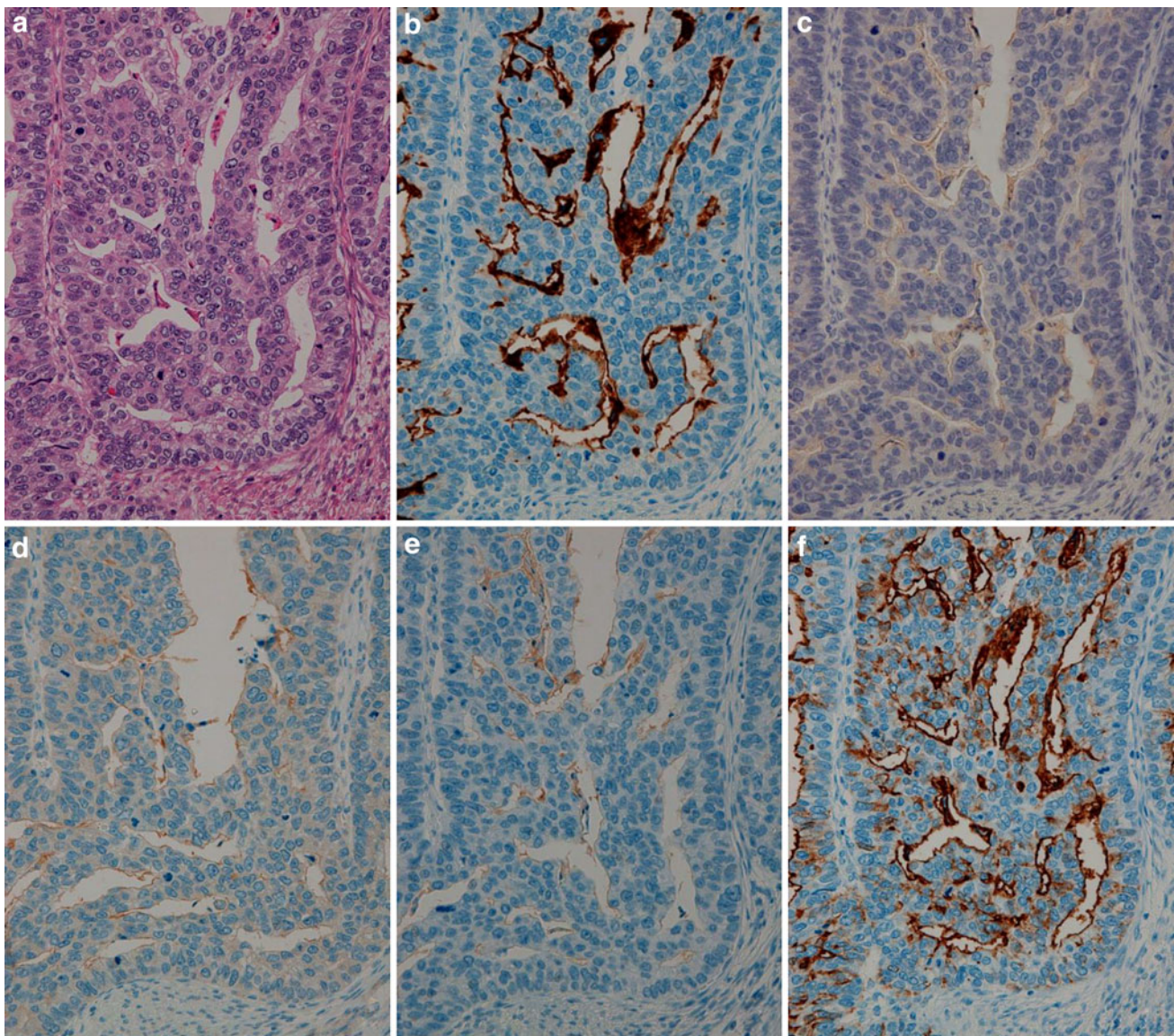


Fig. 3 In tub1 (a H&E staining), MUC1-014E (b) shows intense staining mainly at cell apices. MUC1-Ab-5 (c), MUC1*1110-ecd_P (d), and MUC1*1110-ecd_R (e) show very weak staining at cell

apexes, but MUC1-DF3 (f) gives intense staining mainly at cell apices in this case. $\times 400$

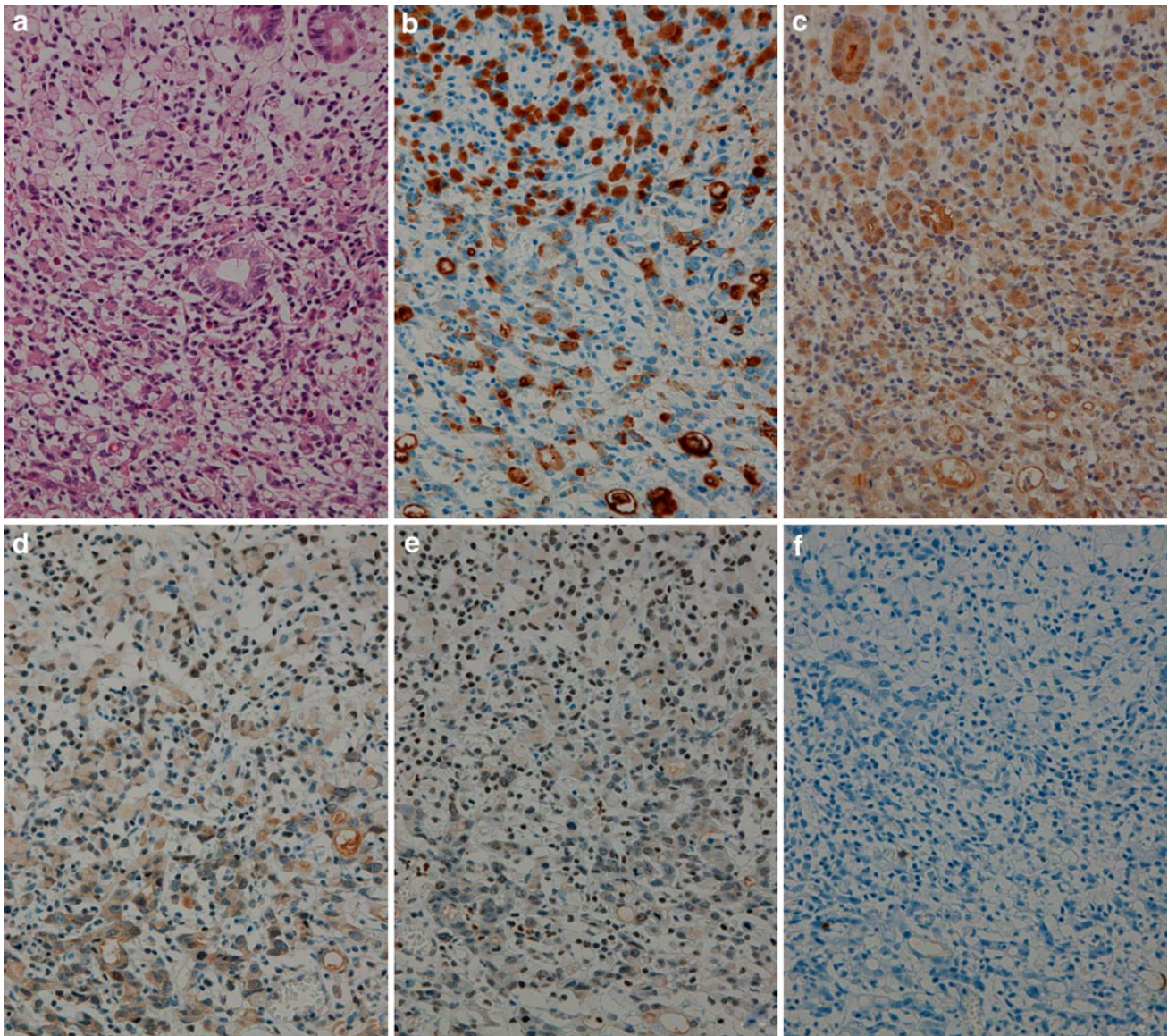


Fig. 4 In por2 (**a** lower area, H&E staining), MUC1-014E (**b**) gives intense staining in the cytoplasm (**b** lower area). In sig (**a**, upper area, H&E staining), MUC1-014E (**b**) strongly stains intracellular pooled mucin (**b**, upper area). MUC1-Ab-5 (**c**) gives weak staining in the cytoplasm of por2 (**c**, lower area) and intracellular pooled mucin of sig (**c**, upper area), and also stains inflammatory cells among the carcinoma cells (**c**). MUC1*1110-eed_P (**d**) gives weak staining of the cytoplasm in

por2 (**d**, lower area) and very weak staining of the intracellular pooled mucin of a small number of carcinoma cells in sig (**d**, upper area). MUC1*1110-eed_R (**e**), and MUC1-DF3 (**f**) do not show staining in por2 or sig. Note that MUC1-014E (**b**) stained clearly the carcinoma cells intermingled with fibrous tissue or inflammatory cells, whereas MUC1-Ab-5 (**c**) and MUC1*1110-eed_P (**d**) stained not only carcinoma cells but also inflammatory cells. $\times 400$

diluted 1:10; MAb CK-AE1/AE3 diluted 1:500; MAb CK-CAM5.2 diluted 1:25) in phosphate-buffered saline (PBS) pH 7.4 with 1% bovine serum albumin, sections were stained on a Benchmark XT automated slide stainer using a diaminobenzidine detection kit (Ventana Medical Systems). MAb MUC1-Ab-5 (diluted 1:200 in PBS pH 7.4 with 1% bovine serum albumin), which was raised in hamster [13], was incubated with sections, followed by anti-hamster sheep serum diluted 1:4,000, and stained using an avidin-

biotinylated horseradish peroxidase complex kit (Dako Japan, Tokyo, Japan) [19]. MAb MUC1-014E absorbed by MUC1-common (RYVPPSSTDRSPYEKVSAG: N-1217-1235-C) peptide showed negative staining. Reaction products were not present when non-immune serum or PBS was used instead of the primary antibodies. For simplicity, MUC1-014E, MUC1-Ab-5, MUC1*1110-eed_P, MUC1*1110-eed_R, MUC1-DF3, CK-AE1/AE3, and CK-CAM5.2 are used to indicate the antigens detected by each antibody.

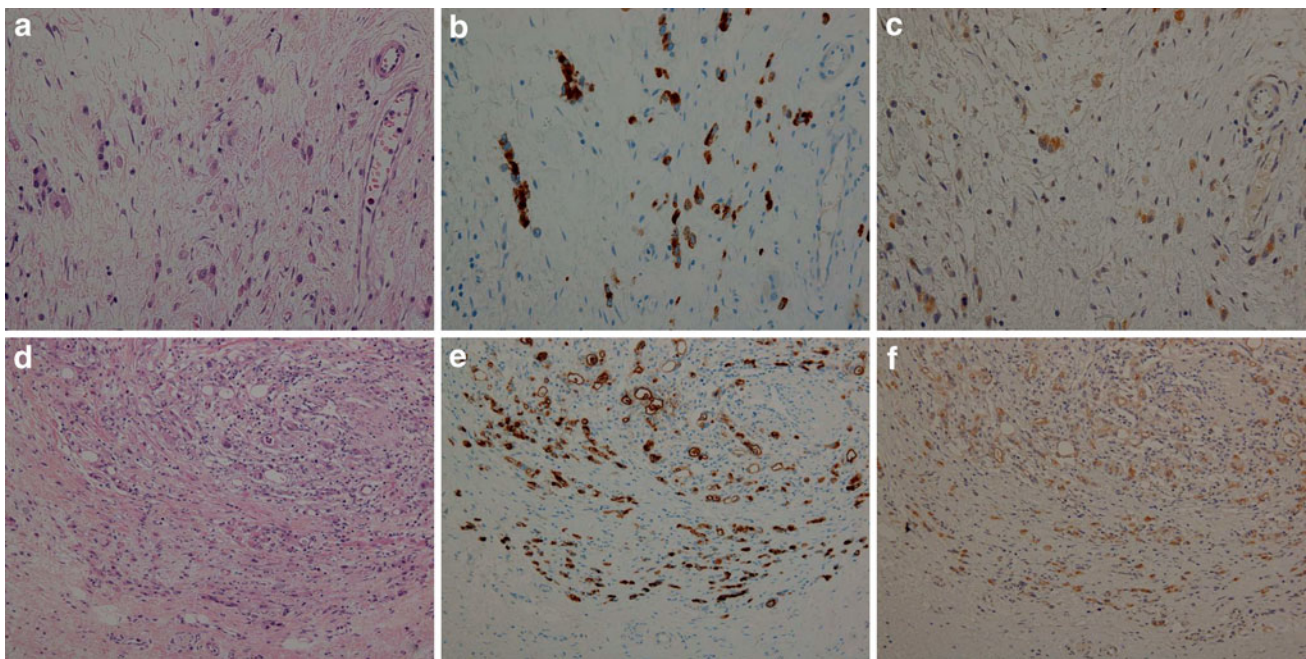


Fig. 5 For por2 carcinoma cells scattered in fibrous tissue (**a**, H&E staining), MUC1-014E is able to sharply and specifically detect isolated carcinoma cells (**b**). MUC1-Ab-5 weakly stains the

cytoplasm of por2 (**c**). The deepest margin of invasion of por2 (**d**, H&E staining) is clearly demonstrated by MUC1-014E (**e**), and is fairly evident with MUC1-Ab-5 (**f**). **a–c** $\times 400$; **d–f** $\times 200$

Scoring of the results and statistical analysis

Three blinded investigators (S.Y., K.S., and M.G.) evaluated the immunohistochemical staining data independently. When the evaluation differed among the three, a final decision was made by consensus. The results were evaluated based on the percentage of positively stained carcinoma cells, using the following grading system: 0, $<5\%$ of carcinoma cells stained; 1+, ≥ 5 to $<25\%$; 2+, ≥ 25 to $<50\%$; 3+, ≥ 50 to $<75\%$; and 4+, $\geq 75\%$ stained. Cases with $\geq 5\%$ of carcinoma cells stained were considered positive. The results for MUC1-014E were compared with those for other antigens using positive staining (total of 1+, 2+, 3+, and 4+) and 4+ staining (the highest intensity staining), as 4+ staining is required to avoid overlooking cancer cells in tissue examinations. Statistical analysis was performed by the Fisher exact test using SPSS 18 software.

Results

Immunohistochemical staining of MAb MUC1-014E and other anti-MUC1 antibodies in gastrectomy specimens

We examined gastrectomy specimens of 107 early gastric cancers (pT1b2) because we wished to avoid the major degenerative changes that are frequently seen in advanced

cancer tissues. When more than two histological types were mixed in one lesion, each histological pattern was evaluated independently, according to the JCGC [1]. Therefore, in the 107 gastrectomy specimens, we could evaluate 200 carcinoma foci of various histological types in total (Fig. 2a–g).

Among the 200 lesions, there were 15 lesions of papillary adenocarcinoma (pap), 39 of well-differentiated tubular adenocarcinoma (tub1) (Fig. 3a), 52 of moderately differentiated tubular adenocarcinoma (tub2), 9 of solid type poorly differentiated adenocarcinoma (por1), 48 of por2 (Figs. 4a-lower, 5a, d), 31 of sig (Fig. 4a-upper), and 6 of mucinous adenocarcinoma (muc), based on the JCGC [1]. The por1, por2, and sig were classified into “poorly cohesive carcinoma (including signet-ring cell carcinoma and other variants)” in the World Health Organization (WHO) classification of tumours of the stomach [20]. The results of immunohistochemical staining of the lesions with each antibody were as follows.

MAb MUC1-014E

MAb MUC1-014E showed positive staining of carcinoma cells at very high rates (97–100%) for most histological types, i.e., pap, tub1, tub2, por2, sig, and muc (Fig. 2a–c, e–g: upper bars), with the exception of por1 (44%) (Fig. 2d: upper bar). MAb MUC1-014E showed 4+ staining for all 48 por2 lesions (100%) (Fig. 2e: lower bar) and 4+ staining for high percentages (80–90%) of most other histological

types (Fig. 2a–c, f, g: lower bar), again with the exception of por1 (33%) (Fig. 2d: lower bar). MUC1-014E was present mainly at cell apexes in pap, tub1 (Fig. 3b), and tub2, and mainly at apexes of floating carcinoma cells in mucin in muc. In contrast, MUC1-014E was present in the cytoplasm of por1 and por2 (Figs. 4b-lower, 5b, e) and in intracellular pooled mucin of sig (Fig. 4b-upper). MAb MUC1-014E detected isolated carcinoma cells sharply and specifically, even for por2 carcinoma cells scattered in fibrous tissue (Fig. 5b, e), and clearly showed the deepest margin of invasion (Fig. 5e).

MAb MUC1-Ab-5

MAb MUC1-Ab-5 also showed positive staining of carcinoma cells at very high rates (84–100%) for most histological types (Fig. 2a–c, e–g: upper bars), with the exception of por1 (44%) (Fig. 2d: upper bar), with no significant differences in positive staining rates compared with MAb MUC1-014E (Fig. 2a–g: upper bars). However, the 4+ staining rates with MAb MUC1-Ab-5 were significantly lower than those with MAb MUC1-014E for tub2, por2, and sig (Fig. 2c, e, f: lower bars), but not significantly lower for other histological types (Fig. 2a, b, d, g: lower bars). The staining patterns of MAb MUC1-Ab-5 were similar to those of MAb MUC1-014E. MUC1-Ab-5 was expressed weakly mainly at cell apexes in pap, tub1 (Fig. 3c), and tub2; mainly at apexes of floating carcinoma cells in mucin in muc; in the cytoplasm in por1 and por2 (Figs. 4c-lower, 5c, f); and in intracellular pooled mucin in sig (Fig. 4c-upper). MAb MUC1-Ab-5 also stained por2 carcinoma cells scattered in fibrous tissue (Fig. 5c, f), but this staining was neither clear nor sharp compared with MAb MUC1-014E staining (Fig. 5b, e). In addition, MAb MUC1-Ab-5 also showed nonspecific immunostaining of inflammatory cells (Fig. 4c).

*PAb anti-MUC1*1110-ecd_P*

PAb anti-MUC1*1110-ecd_P showed positive staining of carcinoma cells at significantly lower rates than MAb MUC1-014E for por2 and sig (Fig. 2e, f: upper bars), but not for other histological types (Fig. 2a–d, g: upper bars). It also showed significantly lower rates of 4+ staining for tub1, tub2, por2, and sig (Fig. 2b, c, e, f: lower bars), but not in others (Fig. 2a, d, g: lower bars). The staining patterns for PAb anti-MUC1*1110-ecd_P were similar to those for MAb MUC1-014E, but weak compared with those for MAb MUC1-014E, mainly at cell apexes in pap, tub1 (Fig. 3d), and tub2; mainly at apexes of floating carcinoma cells in mucin in muc; and in the cytoplasm in por1 and por2 (Fig. 4d-lower); and a very weak presence in intracellular pooled mucin in sig (Fig. 4d-upper). PAb anti-

MUC1*1110-ecd_P also stained por2 carcinoma cells scattered in the fibrous tissue, but much less clearly and sharply compared with MAb MUC1-014E. Nonspecific immunostaining of inflammatory cells was also seen with PAb anti-MUC1*1110-ecd_P (Fig. 4d).

*PAb anti-MUC1*1110-ecd_R*

PAb anti-MUC1*1110-ecd_R showed positive staining of carcinoma cells at significantly lower rates than MAb MUC1-014E in many histological types (tub1, tub2, por2, sig, and muc) (Fig. 2b, c, e–g: upper bars), but not in others (Fig. 2a, d: upper bars). It also showed significantly lower rates of 4+ staining in most histological types (Fig. 2a–c, e–g: lower bars), except for por1 (Fig. 2d: lower bar). PAb anti-MUC1*1110-ecd_R showed no or very weak staining of por2 and sig (Fig. 4e) compared with PAb anti-MUC1*1110-ecd_P (Fig. 4d), despite a difference of only one amino acid (P vs. R at position N-1131 in MUC1) in these antigens.

MAb MUC1-DF3

MAb MUC1-DF3 showed positive staining of carcinoma cells at significantly lower rates than MAb MUC1-014E in many histological types (tub1, tub2, por2, sig, and muc) (Fig. 2b, c, e–g: upper bars), but not in pap and por1 (Fig. 2a, d: upper bars). It also showed significantly lower rates of 4+ staining in most histological types (Fig. 2a–c, e–g: lower bars), except for por1 (Fig. 2d: lower bar). Although several cases of pap and/or tub1 showed intense and clear staining by MAb MUC1-DF3 at the apexes of carcinoma cells (Fig. 3f), most cases of por2 and/or sig showed no staining by MAb MUC1-DF3 (Fig. 4f). MAb MUC1-DF3 did not show nonspecific immunostaining of inflammatory cells (Fig. 4f), in contrast to that seen in staining with MAb MUC1-Ab-5 (Fig. 4c) and PAb anti-MUC1*1110-ecd_P (Fig. 4d).

Summary of immunohistochemical staining in gastrectomy specimens

Overall, in the gastrectomy specimens, MAb MUC1-014E showed the highest rates of positive and 4+ staining in most histological types. Particularly in 4+ staining of por2 (Fig. 2e: lower bar) and sig (Fig. 2f: lower bar), MUC1-014E had significantly higher rates than those for other MUC1 antigens. These tendencies were also seen for tub2 (Fig. 2c), followed by tub1 (Fig. 2b), muc (Fig. 2g), and pap (Fig. 2a).

MAb MUC1-014E showed intense, sharp, and specific staining of carcinoma cells, but never stained stromal cells such as fibroblasts, endothelial cells, and inflammatory cells (Figs. 3b, 4b, 5b, e) compared with the other MUC1-related antibodies (Figs. 3c–e, 4c–f, 5c, f).

Immunohistochemical staining in normal gastric mucosa

In the normal gastric mucosa, MUC1-014E, MUC1-Ab-5, MUC1*1110-ecd_P, and MUC1*1110-ecd_R were always present in the surface mucous epithelium and in the fundic and pyloric glands. MUC1-DF3 was present in the surface mucous epithelium in several cases, and always in the fundic glands, but not in the pyloric glands. CK-AE1/AE3 and CK-CAM5.2 were expressed in the surface mucous epithelium. However, there is no problem because of the simple morphological recognition of the normal gastric mucosa.

Immunohistochemical staining of MAb MUC1-014E and other anti-MUC1 antibodies in biopsy specimens, in comparison with cytokeratin

In the gastrectomy specimens, MUC1-014E showed significantly higher rates than those for other MUC1 antigens, particularly in the 4+ staining of por2 (Fig. 2e: lower bar) and sig (Fig. 2f: lower bar). Thus, we examined 89 biopsy specimens with histological types of por2 and/or sig to examine the utility of MAb MUC1-014E for pathological diagnosis of por2 and sig in biopsy specimens, because these types are frequently seen in scirrhous gastric cancers with a very poor prognosis [2]. In the 89 biopsy specimens, we could evaluate 82 por2 lesions and 38 sig lesions (Fig. 6a, b).

For por2, the MAb MUC1-014E positive rate (100%) and 4+ rate (87%) were significantly higher than the other MUC1-related antibodies (Fig. 6a: upper and lower bars). For sig, the MAb MUC1-014E positive rate (100%) did not differ significantly from that for MUC1-Ab-5, but was

significantly higher than those for MUC1*1110-ecd_P, MUC1*1110-ecd_R and MUC1-DF3 (Fig. 6b: upper bar), whereas the MAb MUC1-014E 4+ rate (84%) was significantly higher than the other MUC1-related antibodies (Fig. 6b: lower bar).

In the comparison of the MAb MUC1-014E staining with CK stainings in por2 and sig, the MUC1-014E positive rates (100% in por2 and 100% in sig) and 4+ rates (87% in por2 and 84% in sig) were significantly higher than the CK-AE1/AE3 positive rates (72% in por2 and 68% in sig) and 4+ rates (15% in por2 and 16% in sig), or than the CK-CAM5.2-positive rates (88% in por2 and 82% in sig) and 4+ rates (34% in por2 and 34% in sig) (Fig. 6a, b).

MAb MUC1-014E detected carcinoma cells of por2 (Fig. 7a) and sig sharply and specifically (Fig. 7b), compared with the other MUC1-related antibodies (Fig. 7c–f), without showing the nonspecific staining of inflammatory cells seen with MAb MUC1-Ab-5 (Fig. 7c) and PAb anti-MUC1*1110-ecd_P (Fig. 7d). The immunostaining of MAb MUC1-014E for por2 (Fig. 7b) and sig was much clearer than that of CK-AE1/AE3 (Fig. 7g) or CK-CAM5.2 (Fig. 7h).

Discussion

In the present study, we raised a novel antibody, MAb MUC1-014E, that showed high rates of detection of carcinoma cells in several types of gastric cancer. In particular, MAb MUC1-014E clearly and sharply detected carcinoma cells in por2 and sig lesions, which are frequently seen in scirrhous gastric cancers and are associated with a very poor prognosis [2], in both gastrectomy and biopsy specimens. The por2 and sig cells tend to be

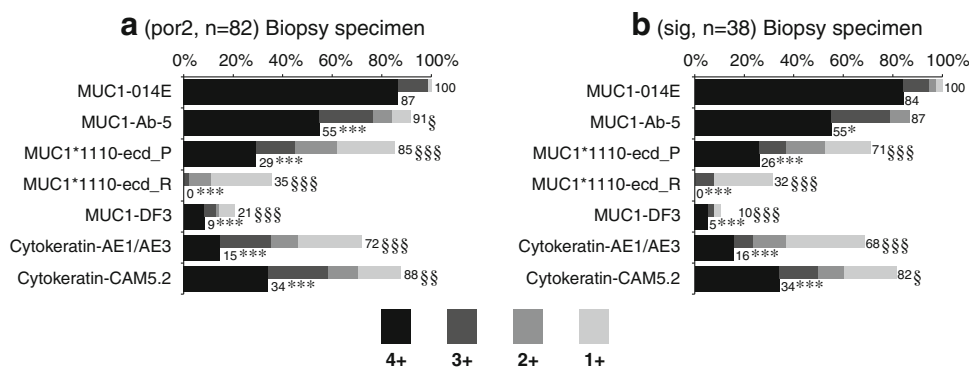


Fig. 6 Comparison of MUC1-014E expression with that of MUC1-Ab-5, MUC1*1110-ecd_P, MUC1*1110-ecd_R, MUC1-DF3, CK-AE1/AE3 and CK-CAM5.2 for por2 (a) and sig (b) in biopsy specimens. Results for MUC1-014E are compared with those for other antigens using positive staining (total of 1+, 2+, 3+, and 4+) (a, b, upper bars) and 4+ staining (highest intensity staining) (a, b, lower bars). Numbers at the right sides of each bar are percentages of the positive staining or 4+ staining. MUC1-014E shows the highest

positive staining (upper bars) and the highest 4+ staining (lower bars). For por2 and sig, MUC1-014E shows significantly higher staining than the other MUC1 antigens, particularly for 4+ staining (a, b, lower bars). MUC1-014E shows significantly higher rates than CK-AE1/AE3 and CK-CAM5.2 for both positive and 4+ stainings (a, b, upper and lower bars). § $P < 0.05$; §§ $P < 0.01$; §§§ $P < 0.001$ (upper bars); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (lower bars) for MUC1-014E staining versus that for each of the other antigens

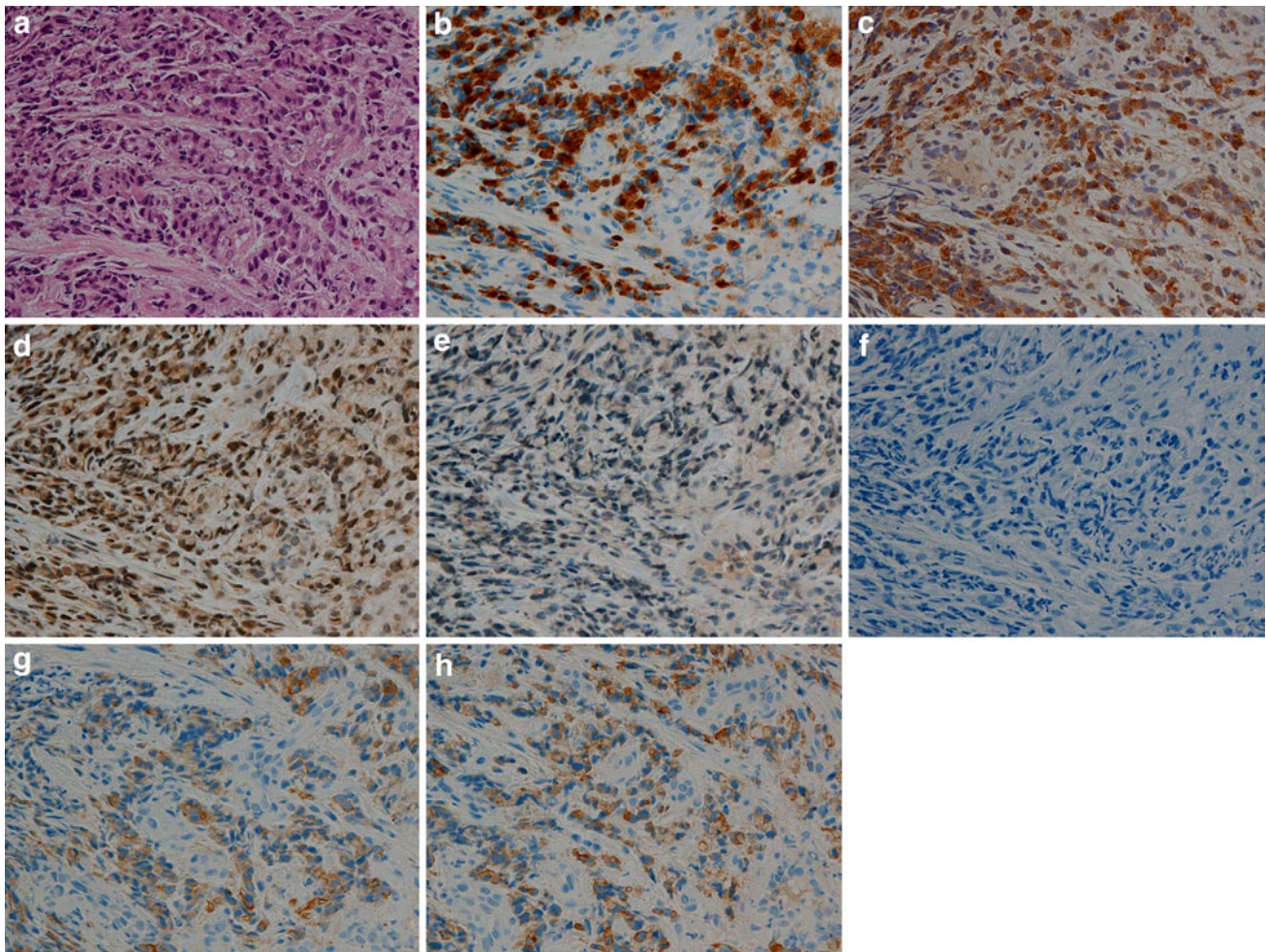


Fig. 7 In a biopsy specimen with por2 carcinoma cells mixed in fibrotic tissue (**a**, H&E staining), MUC1-014E (**b**) sharply and specifically detects the isolated carcinoma cells. MUC1-Ab-5 (**c**) detects the isolated carcinoma cells, but also stained inflammatory cells among the carcinoma cells. MUC1*1110-ecd_P (**d**) stains the isolated carcinoma cells weakly, but staining is not sharp compared

with MUC1-014E, and also stains inflammatory cells. MUC1*1110-ecd_R (**e**) and MUC1-DF3 (**f**) are unable to detect isolated carcinoma cells. In this biopsy specimen, CK-AE1/AE3 (**g**) and CK-CAM5.2 (**h**) are also observed in isolated carcinoma cells, but less clearly than MUC1-014E. $\times 600$

scattered in granulation tissue or desmoplastic fibrotic tissue, and are often difficult to detect with routine H&E staining. Among all tested antibodies, MAb MUC1-014E showed 4+ and clear staining for por2 and sig, indicating that the new antibody will be very useful for detection of por2 or sig. MAb MUC1-014E staining was also highly positive in other histological types of gastric cancer, except for por1. Nevertheless, the por1 is easy to identify based on its solid pattern in routine H&E staining.

MAb MUC1-Ab-5 and PAb anti-MUC1*1110-ecd_P also stained carcinoma cells in por2 and sig lesions at relatively high rates, but at rates lower than with MAb MUC1-014E. The sharpness and specificity of immunohistochemical staining using MAb MUC1-014E, which stained cancer cells but not inflammatory cells, was also much better than with MAb MUC1-Ab-5 or PAb anti-

MUC1*1110-ecd_P, which reacted with not only cancer cells but also inflammatory cells.

PAb anti-MUC1*1110-ecd_R and MAb MUC1-DF3 showed no or low staining in por2 and sig lesions. The positive rates were higher for MUC1*1110-ecd_P than for MUC1*1110-ecd_R in all histological types of gastric cancer, even though there is a difference of only one amino acid at position N-1131 of MUC1 (P vs. R) between the two antigens. It is unclear how this relatively small difference gives rise to such a large difference in antigenicity. Staining with MAb MUC1-DF3 was significantly lower than with MUC1-014E in most histological types, except for pap and por1. MAb MUC1-DF3 is an anti-MUC1 antibody against the TR region of the extracellular domain [16–18], and the low MUC1-DF3 expression may be related to loss of this domain caused by cleavage [3, 9]. In

contrast, MUC1-014E (N-1217-1235-C) and MUC1-Ab-5 (N-1239-1255-C) are located side by side in the MUC1 CTD. Because both antigens may be present in carcinoma cells even after the cleavage of the MUC1 molecule, they may have high levels in many types of gastric cancers.

The CK-AE1/AE3 or CK-CAM5.2 immunostaining was used to be effective to detect the carcinoma cells in por2 or sig [21]. The present study in the biopsy specimens demonstrated that the MUC1-014E-positive rate and 4+ rate were significantly higher than those for CK-AE1/AE3 or CK-CAM5.2, and the MAb MUC1-014E staining was generally clearer than the CK-AE1/AE3 or CK-CAM5.2 staining. For accurate detection of the carcinoma cells in por2 or sig, MAb MUC1-014E is useful as well as CK-AE1/AE3 or CK-CAM5.2.

Our immunohistochemical results are also of interest in the context of common histological classification of gastric cancer in JCGC [1], in which poorly differentiated adenocarcinoma is classified into por1 (solid type) and por2 (non-solid type), as well as in the context of WHO classification of tumours of the stomach, in which por1 and por2 were classified into “poorly cohesive carcinoma (including signet-ring cell carcinoma and other variants)” [20]. There is a marked difference in expression of MUC1 between por1 and por2 lesions, with the MUC1 expression pattern in por2 being very similar to that of sig, and also similar to that of tub2. Coexistence of por2 and sig is frequently observed, as shown in Fig. 4, and coexistence of tub2 and por2 and/or sig is also common. A relatively high frequency of por1 lesions are found in elderly patients and in cases with specific microsatellite instability, expansive growth, and lymphocytic infiltration [22]. In contrast, por2 tends to affect younger patients and frequently has hereditary characteristics [23, 24]. The marked difference in MUC1 expression between por1 and por2 may also be an area for future study.

In conclusion, the novel MAb MUC1-014E is very useful for accurate identification of por2 or sig carcinoma cells that are frequently seen in scirrhous gastric cancer.

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Conflict of interest The authors declare that they have no conflict of interest regarding the work in the study.

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