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Claudin 18 as a Promising Surrogate Marker for Endocervical Gastric-Type Carcinoma

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Abstract

HIK1083 and TFF2 are known to be expressed in gastric-type carcinoma (GAS) but they do not reliably mark all GASs and focal expression can be missed in biopsy specimens. We aimed to investigate whether Claudin-18 and Alpha-methylacyl-CoA racemase (AMACR) could be surrogate markers to separate GAS from other types of ECA, and to compare their usefulness with that of HIK1083 and TFF2.

Claudin-18 and AMACR immunohistochemistry was performed, and the results were compared with that of TFF2 and HIK1083, using whole sections of 75 ECAs (22 GASs and 53 non-GASs) and 179 ECAs with tissue microarrays (TMAs). TMAs were built to simulate assessment of immunohistochemical stains in small biopsies. Any membranous (Claudin 18) or cytoplasmic/membranous (AMACR, TFF2, HIK1083) staining of more than 5% of tumor cells was considered positive.

Of 75 ECAs with whole sections, Claudin-18 was significantly more frequently expressed in GASs (21/22) compared with non-GASs (8/53) ($p<0.01$). In ECAs with TMAs, Claudin 18 expression was significantly frequent in GASs (15/23, 65.2%) than in non-GASs (3/152, 2.0%; all usual-type) ($p<0.01$). All Claudin-18 positive GASs showed intense staining except one case. Claudin-18 shared the same degree of sensitivity and specificity with HIK1083 and TFF2. Three clear cell carcinomas (CCCs) were positive for Claudin-18, but none showed intense staining.

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AMACR was expressed in a subset of ECAs and showed no impact in distinguishing between GAS and other ECAs.

Our results suggest that Claudin-18 is a promising surrogate marker to separate GAS from other types of ECA, including CCC.

Keywords

endocervical adenocarcinoma; gastric type; immunohistochemical marker; differential diagnosis

INTRODUCTION

The incidence of endocervical adenocarcinoma (ECA) has been increasing and has recently been reported to represent approximately 25% of all invasive carcinomas of the uterine cervix worldwide[1–3]. Accumulated data have revealed that ECA is etiologically divided into HPV-associated and HPV-independent types. Our group established a new histological classification of ECA, International Endocervical Criteria and Classification (IECC), reflecting the importance of HPV in the pathogenesis of all ECAs, and it was subsequently adopted in the fifth edition of World Health Organization (WHO) classification of the tumors[4,5]. The IECC group reported that the most common histotype is usual-type, which is HPV-associated (HPVA)[4]. Gastric-type carcinoma (GAS) is a distinct subtype of ECA, being the second most common histotype and the most frequent HPV-independent type (HPVI), that has aggressive behavior[4,6–9]. Postulated precursors of GAS include atypical lobular endocervical glandular hyperplasia (LEGH)/gastric-type adenocarcinoma in situ[10].

Distinguishing GAS from HPVA-type ECAs as well as from other HPVI-type ECAs, such as clear cell-type, can be difficult in some settings, particularly when one has only limited biopsy material to examine. High-risk HPV status determined by RNA in situ hybridization (HPV-ISH) is a reliable method to distinguish GAS from HPV-associated ECAs, though it may be more costly than immunohistochemistry and not available in most laboratories. HIK1083 has been known to be a positive marker for GAS, but it is not highly sensitive and is not widely available[4]. Recently, TFF2, a mucin-associated peptide expressed in normal gastric glands but not in normal endocervical glands, was reported to be expressed in GAS and LEGH, and less commonly in ECAs of non-GAS types[11–13]. We have validated that TFF2 expression shares the same degree of specificity with HIK1083 to separate GAS from other types of ECA, and that double positivity for TFF-2 and HIK1083 is highly specific for the diagnosis of GAS while it is extremely rare in non-GASs, regardless of specimen size[14]. In our previous study we found that in most GASs positive for TFF2 using whole sections, <50% of tumor cells were stained, and the distribution of staining cells was variable within a tumor[14]. A superior positive marker for GASs with diffuse and strong staining that is easily accessible is still needed.

Claudin 18 gene is a component of tight-junction complexes and has two splice variants, one coding lung-specific and the other coding stomach-specific isoforms[15]. Claudin18 is expressed in various neoplasms including those originating in the stomach and pancreatobiliary tract[16]. A few studies have shown immunohistochemical expression of

Claudin 18 in uterine cervical lesions, such as in GASs, gastric-type adenocarcinoma in situ, LEGH, less often in non-GASs, though the number of cases examined are small[16–18].

Alpha-methylacyl-CoA racemase (AMACR, also known as P504S), is a mitochondrial and peroxisomal enzyme that plays a role in the beta-oxidation of fatty acid derivatives by catalyzing interconversion of several (2R)-methyl branched chain fatty acyl-coenzyme A esters to their (S) stereoisomers[19]. Immunohistochemical AMACR expression has been known to be positive in 83–94% in prostatic adenocarcinoma but less commonly in benign prostatic glands, and it is a commonly used tool for confirming a diagnosis of prostatic adenocarcinoma in challenging cases[19]. It is also expressed in adenocarcinoma of various origins including stomach, colorectum, kidney, salivary gland, and lung[19,20]. One study showed that AMACR was expressed in 80% (40/50) of gastric adenocarcinoma but less often in the adjacent non-neoplastic epithelium[20]. Few studies have examined AMACR expression in ECAs[19,21].

This study was carried out to explore whether Claudin 18 and AMACR could be surrogate markers to separate GAS from other types of ECA, and to compare their usefulness with that of HIK1083 and TFF2.

MATERIALS AND METHODS

Institutional approval for this study was obtained from each of the participating centers.

Patient Selection

Slides from 434 invasive ECAs were collected from 9 international institutions. All Hematoxylin-Eosin (H&E) slides with tumor present (an average of 12 slides per case) were reviewed and a histological consensus was reached in every case with at least two and as many as four expert pathologists reviewing slides at a multiheaded microscope.

Tumors were classified based on the IECC[4]. In situ carcinomas, squamous cell carcinomas, adenosquamous carcinomas, tumors with a neuroendocrine component, carcinosarcomas, and any tumor demonstrating clinical, macroscopic, or microscopic features, suggesting a lower uterine segment, uterine corpus, or adnexal primary origin were excluded. Tumors treated with neoadjuvant chemotherapy and/or radiotherapy were also excluded. Types of specimens included were conizations/trachelectomies/hysterectomies, exenterations with lymph node dissection, while biopsies and LEEP specimens were excluded.

Immunohistochemistry using whole sections and tissue microarray (TMA)

Of 434 cases, 22 GASs and 53 non-GASs from patients treated at two institutions (The Jikei University School of medicine and Memorial Sloan Kettering Cancer Center) between 2001 and 2021 were immunohistochemically analyzed for expression of Claudin 18, AMACR, and the results were compared with that of TFF2 and HIK1083 in matched cases, part of which had been reported in a previous study[14]. Formalin-fixed, paraffin-embedded tumor tissue was sectioned at a thickness of 4 micrometer and the sections were deparaffinized and rehydrated. Rabbit IgG polyclonal antibody Claudin 18 (1:100, Thermo Fisher Scientific, Rockford, USA) was used as a primary antibody for Claudin 18

expression and rabbit monoclonal antibody (clone 13H4, 1:100, DAKO, Santa Clara, USA) for AMACR expression after pretreatment with CC1 (Ventana Medical Systems, Tucson, Arizona, USA) at 100°C for 60 min for both antibodies. Immunostaining was performed in an automated stainer using the BenchMark-ultra with i-View DAB kit. A section of the normal gastric pyloric mucosa was used as a positive control for all antibodies except for AMACAR for which prostatic adenocarcinoma was used as a positive control. When negative for all these markers in a whole section, mouse monoclonal anti Cytokeratin (CAM5.2) (1:1, Becton Dickinson and Company, San Jose, CA USA) was used as a primary antibody to confirm antigen preservation.

In 179 of 434 ECAs, expression of Claudin 18 and AMACR was analyzed immunohistochemically using TMAs and the results were compared with that of TFF2 and HIK1083 in matched cases, which were among those included in previous studies[14,22]. The TMAs were constructed using previously described methods[23,24]. Each tumor was represented by three 0.6mm cores (120 cases) except those from Japan which were represented by a single 3mm core (59 cases).

Stains were scored by one or two study pathologists (T. K. for Claudin 18 and AMACR; T.K. and S.S. for TFF2; R.A.S. and S.S. for HIK1083). The percentage of any membranous staining of tumor cells for Claudin 18 expression, and that of any membranous/cytoplasmic staining of tumor cells for AMACR, TFF2 and HIK1083 was scored as follows: score 0: <5%; score 1+: 5% to 10%; score 2+: 11% to 25%; score 3+: 26% to 75%; score 4+: >75%; and those with score 1+ to score 4+ were considered positive[14,22].

Statistical analysis

Statistical analysis was performed using Fisher’s Exact test.

RESULTS

Expression of Claudin 18, TFF2, HIK1083, AMACR in whole sections

Using whole slide sections, 22 GASs and 53 non-GASs were studied. The non-GASs included 37 usual-type (HPVA), 1 mucinous NOS-type (HPVA), 14 clear cell-type (HPVI), and 1 endometrioid-type (HPVI). Of the 22 GASs, 21were positive for Claudin 18 (95.5%; one score 1+, four score 3+, and 16 score 4+) with all strong staining pattern (Table 1) (Figure 1). Eleven of 22 GASs were positive for TFF2 (50.0%; one score 1+, three score 2+, six score 3+, and one score 4+) and 12 (54.5%; two score 1+, one score 2+, three score 3+, and one score 4+) for HIK108; in most of GASs positive for TFF2 or HIK1083, less than 50% of tumor cells were stained and the distribution of staining cells was variable within a tumor. One GAS was negative for all three markers (triple negative) and was confirmed to be positive for CAM5.2 (Figure 2). The triple negative GAS had few well-formed glands and the majority of the tumor cells showed severe cytological atypia compared with other nine GASs, and occasional signet-ring cells were appreciated. Forty-five percent (10/22) of GASs were positive for all three markers (triple positive). Of 53 non-GASs, eight were positive for Claudin 18 (15.1%; four score1+, and two each score 2+ and score 3+), with five cases being usual-type. Five of 53 non-GASs (9.4%; three score 1+, and one each score 2+ and score 3+)

were positive for TFF2 and 17 (32.1%; seven each score 1+ and score 2+, and three score 3+) for HIK1083. The three non-GAS cases positive for Claudin 18 (one score 1+, and two each score 3+) were clear cell carcinomas and the staining pattern was weak (Figure 3); all these cases were negative for TFF2 while two of three were positive for HIK1083 (one each score1+ and score 3+, weak staining pattern). Two of the five Claudin 18 positive usual-type were also positive for HIK1083 (one each score 1+ and score 2+), but none for TFF2. None of non-GAS cases was triple positive. One mucinous, NOS-type ECA was double positive for TFF2 (score 3+) and HIK1083 (score 1+) but negative for Claudin 18. One usual-type, which was positive for HIK1083 (3+) was negative for both TFF2 and Claudin 18.

Claudin 18 was significantly more frequently expressed in GASs compared with non-GASs ($p<0.01$). Positive and negative predictive values of Claudin 18 for GAS were 72.4% and 97.8%, respectively. Positive and negative predictive values of TFF2 for GAS and those of HIK1083 were 68.8%, 81.4% and 40.6%, 79.1%, respectively. Claudin 18 shared the same degree of sensitivity (95.5%) and specificity (84.9%) with both TFF2 (sensitivity 50%, specificity 90.6%) and HIK 1083 (sensitivity 40.6%, specificity 64.2%) in separating GASs from non-GASs.

One triple positive GAS had atypical LEGH/gastric-type AIS adjacent to invasive carcinoma, which was also triple positive.

AMACR was expressed in six of 22 GASs (27.3%; two each score 1+ and score 3+, and one each score 2+ and score 4+), and 20 of 53 non-GASs (37.7%; fourscore 1+, twoscore 2+, and seven each score 3+ and score 4+); it showed no significant difference in expression between GASs and non-GASs. However, AMACR was significantly more frequently expressed in clear cell carcinomas (11/14; 78.6%) compared with GASs ($p<0.01$), and also compared with non-clear cell ECAs (15/61; 24.6%)($p<0.01$) (Figure 3). The triple negative GAS case mentioned above was also negative for AMACR.

Normal endocervical glands adjacent to carcinoma in the present study showed positivity for none of the four markers examined.

Expression of Claudin 18, TFF2, HIK1083, AMACR in TMAs

TMAs were performed to simulate assessment of immunohistochemical stains in small biopsies. Of 179 ECAs, 175 were considered eligible for scoring Claudin 18 expression; they included 23 GASs and 152 non-GASs; in the latter 126 were usual-type, 7 clear cell-type, 3 endometrioid-type, and 16 other HPVAs (such as 7 invasive stratified mucin producing-type, 3 intestinal mucinous-type, 1 mucinous NOS-type, and 5 adenocarcinomas NOS-type). Our previous study confirmed that GAS, endometrioid carcinoma and clear cell carcinoma were all HPV negative (HPVI)[4].

Of 23 GASs, 15 (65.2%) were positive for Claudin 18, all with score 4+ and strong intensity (Table 2) (Figure 4). Of 152 non-GASs, 3 (2.0%; two score 3+ and one score 4+) were positive for Claudin 18, all of which were usual-type. Claudin 18 was significantly more frequently expressed in GASs compared with non-GASs ($p<0.01$) and showed no significant difference in specificity or sensitivity in separating GASs from non-GASs when compared

with TFF2 or HIK1083 using matched cases. Claudin 18 expression in GASs using TMA was significantly less frequent compared with that using whole section ($p<0.05$).

For scoring AMACR expression, 172 ECAs were considered eligible including 23 GASs and 149 non-GASs (123 usual-type, 7 clear cell carcinomas, 3 endometrioid carcinomas, 7 invasive stratified mucin producing-type carcinomas, 3 intestinal mucinous-type, 1 mucinous NOS-type, and 5 adenocarcinomas NOS-type). AMACR was expressed in four of 23 GASs (17.4%; two each score 2+ and score 3+) and 35 of 149 non-GASs (23.5%; six score 1+, nine score 2+, 15 score 3+, and five score 4+); it showed no significant difference in expression between GASs and non-GASs. Among 35 AMACR positive non-GASs, 32 were usual-type and one each was of clear cell type, mucinous NOS-type and adenocarcinomas NOS-type.

DISCUSSION

The present study has shown that Claudin 18 expression is specific for gastric-type differentiation in GAS and is a promising surrogate marker in separating GAS from other types of ECA regardless of specimen size; it shares the same degree of sensitivity and specificity with TFF2 and HIK1083. Claudin 18 is probably superior to TFF2 or HIK 1083 in separating GASs from non-GASs because the staining pattern of Claudin 18 in GASs was intense and diffuse: in most of GASs showing positivity for TFF2, less than 50% of tumor cells were stained and the distribution of staining cells was variable within a tumor as we found in our previous study[14] and confirmed in the present study; and HIK1083 may be expressed in clear cell carcinomas and is not easy to access. Note that there are rare triple-negative GASs, such that no expression does not entirely exclude GAS or mimickers. The present study has also shown that AMACR is expressed in a subset of ECAs and has no impact in distinguishing between GAS and other ECAs.

GAS has a distinct morphology and a few studies have reported good interobserver reproducibility in its recognition using digital microscopic photographs by general surgical pathologists or biopsy/excision specimens by expert gynecologic pathologists[25,26]. Preoperative biopsy specimens, however, containing limited tumor cells, still cause a diagnostic challenge. Commonly encountered problems in the diagnosis of GAS in routine practice include distinguishing it from the much more frequently encountered HPVA ECAs (i.e. usual-type and mucinous-type ECAs) because of intracytoplasmic mucin and clear cell carcinoma because of frequently similar morphology such as cytoplasmic clearing and crisp cytoplasmic membranes. Compared with usual-type ECA, GAS is significantly associated with a bulky cervical mass, deep stromal invasion, lympho-vascular space invasion, parametrial invasion, ovarian metastasis, positive ascitic fluid cytology, high stage, increased risk for disease recurrence, disappointing disease-specific survival at 5 years, and chemotherapy resistance[6–9,27,28]. Thus, the diagnostic accuracy in recognizing GAS in a preoperative biopsy is especially important as ovarian conservation in patients with GAS may not be recommended and omentectomy should be considered as part of surgical treatment. When the differential diagnosis includes GAS in ECA, Claudin 18 immunohistochemistry should be helpful for assignment of histotype.

Recently, Li et al. immunohistochemically examined Claudin18 expression in 575 primary carcinomas of various organs and their metastatic sites using TMAs and found that Claudin 18 is a sensitive and specific marker for stomach and pancreatobiliary carcinomas and suggested its potential usefulness in identifying the primary site when the tumor was of unknown origin[16]. Their study included 13 primary ECAs, which were not subclassified, and none showed Claudin 18 expression. The results of our present study suggest that ECAs, especially but not restricted to GAS, should be included for differential diagnosis in female patients with Claudin 18 positive metastatic adenocarcinoma with unknown origin.

In the present study, none of the clear cell carcinomas showed intense staining for Claudin 18, indicating that Claudin 18 may be useful to distinguish GAS from clear cell-type ECA. Clear cell carcinoma is a relatively rare histotype and like GAS, it may display clear, foamy, or pale eosinophilic cytoplasm, distinct cellular borders, and rounded nucleoli with a distinct nucleolus. Although GAS is typified by cytoplasmic mucin, clear cell-type ECAs may rarely have similar findings[29]. Because of these features, clear cell carcinomas may be misinterpreted by pathologists as GAS and vice versa, especially when encountered in a biopsy specimen with a paucity of tumor cells. Recent studies have revealed that GAS may express markers typically present in clear cell-type carcinoma, such as HNF-1 beta and napsin A, indicating that they are not useful for this differential diagnosis[6,7,22,30]. It should also be noted that using whole section, more than 40% (6/14) of clear cell-type ECAs expressed HIK1083, the best-known marker for GAS, though the staining intensity was rather weak. Because both clear cell carcinoma and GAS are HPVI and estrogen and progesterone receptors-negative, HPV-ISH and immunohistochemistry for p16, estrogen receptors and progesterone receptors have no diagnostic value in distinguishing between these two types. The results of the present study indicate that a strong staining pattern for Claudin 18 favors GAS over clear cell-type while negativity for Claudin 18 does not exclude it.

The present study has also shown that AMACR is expressed in a subset of ECAs. Although AMACR has no impact in distinguishing between GAS and other ECAs, present study using whole sections suggests that it may be a surrogate marker to distinguish clear cell-type from other types of ECAs, especially from GAS. One study showed significantly more frequent AMACR expression in clear cell-type ECAs (68.8%; 11/16) than in GASs (15.6%; 5/32), although the nature of the specimens (whether they are small biopsy or resected specimens) was not described[21].

The limitation of the present study is the small number of HPVA mucinous carcinoma, which is another potential morphological mimic of GAS, and further studies are needed to compare a greater number of this type of ECA with GASs using TMAs to simulate assessment in small biopsies.

In conclusion, Claudin 18 is a promising “positive marker” of GAS, and its strong and diffuse staining pattern can be useful for distinguishing between GAS and other ECAs regardless of the specimen size when the differential diagnosis includes GAS in ECA. However, Claudin 18 negativity cannot rule out GAS. In addition, AMACR may be useful

as a surrogate marker in separating clear cell carcinoma from other types of ECA including GAS.

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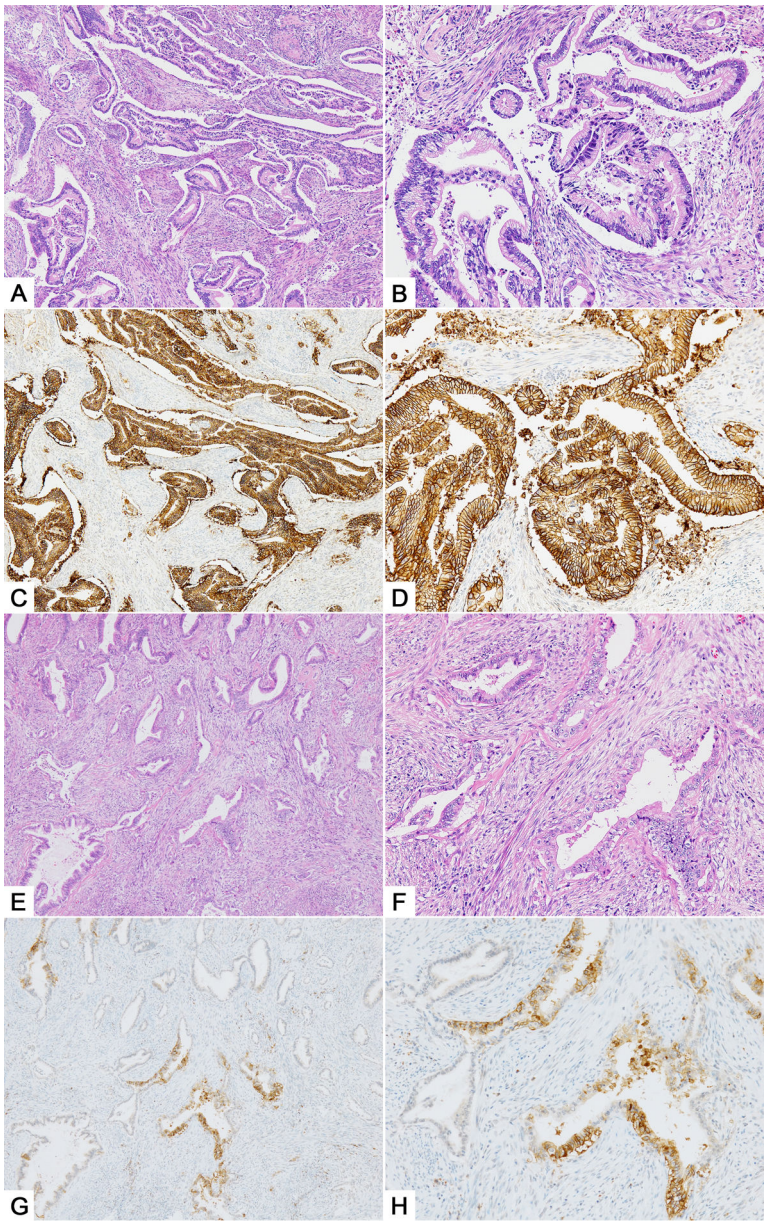

Figure1.

Gastric-type carcinoma in whole section hematoxylin-eosin (A, B); Claudin 18 stainig of score 4 (C, D) (case 1); Gastric-type carcinoma in whole section hematoxylin-eosin (E, F); Claudin 18 stainig of score 3 (G, H) (case 2)

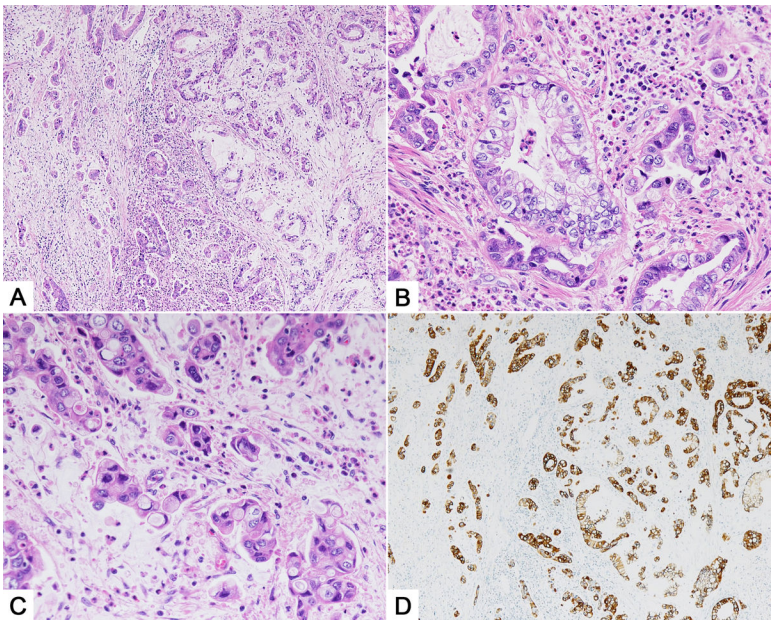

Figure 2.

Gastric-type carcinoma in whole section hematoxylin-eosin (A, B, C). Note less amount of well-forming glands, prominent cytological atypia and occasional signet-ring cells. This case was negative for Claudin 18, TFF2, HIK1083 (triple negative); Diffuse CAM5.2 stainig (D)

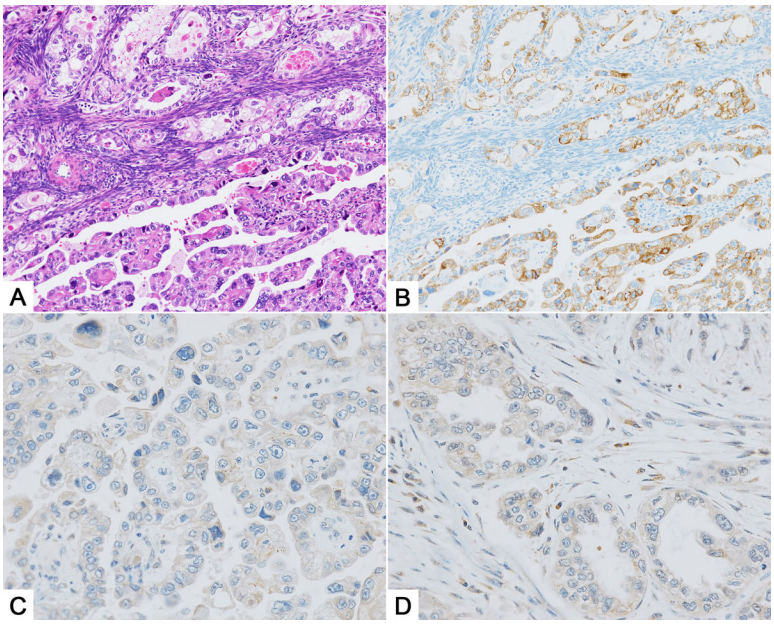

Figure 3.

Clear cell carcinoma in whole section hematoxylin-eosin (A); AMACR stainig of score 4 (B); Claudin 18 stainig with score 3 showing weak membrous stainig (C, D)

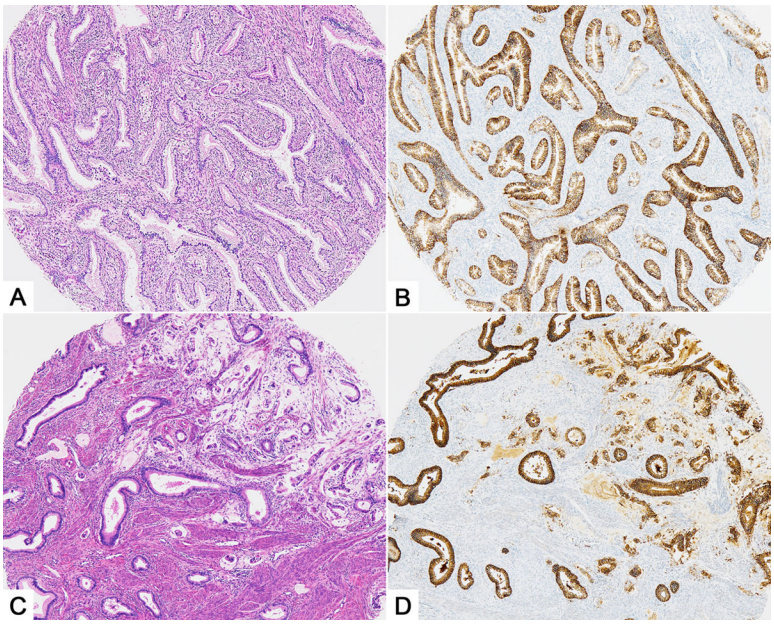

Figure 4.

Gastric-type carcinoma in tissue-microarray hematoxylin-eosin (A); Claudin 18 stainig of score 4 (B) (case 1); Gastric-type carcinoma in tissue-microarray hematoxylin-eosin (C); Claudin 18 stainig of score 4 (D) (case 2)

TABLE 1.

Claudin 18, TFF2, HIK1083, and AMACR Expression of ECAs in Whole Sections

	n/N (%)							
Total	**Claudin 18, N=75**		**TFF2, N=75**		**HIK1083, N=75**		**AMACR, N=75**	
GAS	21/22	(95.5) * (15.1)	11/22	(50.0) * (9.4)	12/22	(54.5) $ (32.1)	6/22	(27.3)
Non-GAS	8/53		5/53		17/53		20/53	(37.7)
Usual	5/37	(13.5)	4/37	(10.8)	10/37	(27.0)	8/37	(21.6)
Mucinous, NOS	0/1	(0)	1/1	(100)	1/1	(100)	0/1	(0)
Clear cell	3/14	(21.4)	0/14	(0)	6/14	(42.9)	11/14	(78.6)
Endometrioid	0/1	(0)	0/1	(0)	0/1	(0)	1/1	(100)
Sensitivity[#]	95.5%		50.0%		40.6%		27.3%	
Specificity[†]	84.6%		90.6%		64.2%		62.3%	

ECAs: Endocervical adenocarcinomas; TMAs:

GAS: gastric-type carcinoma; Non-GAS: Non- gastric-type carcinoma

[*] $p<0.01$

[$] $p<0.05$

[#] Sensitivity in separating GAS from non-GAS

[†] Specificity in separating GAS from non-GAS

TABLE 2.

Claudin 18, TFF2, HIK1083, and AMACR Expression of ECAs in Whole Sections

	n/N (%)							
Total	**Claudin 18, N=175**		**TFF2, N=175**		**HIK1083, N=175**		**AMACR, N=172**	
GAS	15/23	(65.2) (2.0) *	6/23	(26.1) (2.6) *	9/23	(39.1) (1.3) *	4/23	(17.4)
Non-GAS	3/152		4/152		2/152		35/149	(23.5)
Usual	3/126	(2.4)	4/126	(3.2)	1/126	(0.8)	32/123	(26.0)
SMC	0/7	(0)	0/7	(0)	0/7	(0)	0/7	(0)
Mucinous intestinal	0/3	(0)	0/3	(0)	1/3	(33.3)	0/3	(0)
Mucinous, NOS	0/1	(0)	0/1	(0)	0/1	(0)	1/1	(100)
ECA, NOS	0/5	(0)	0/5	(0)	0/5	(0)	1/5	(20.0)
Clear cell	0/7	(0)	0/7	(0)	0/7	(0)	1/7	(14.3)
Endometrioid	0/3	(0)	0/3	(0)	0/3	(0)	0/3	(0)
Sensitivity #	65.2%		60.0%		39.1%		17.4%	
Specificity †	98.0%		97.3%		98.7%		76.5%	

ECAs: Endocervical adenocarcinomas; TMAs: Tissue microarrays

GAS: gastric-type carcinoma; Non-GAS: Non- gastric-type carcinoma

SMC: Stratified mucin-producing carcinoma

* $p<0.01$

Sensitivity in separating GAS from non-GAS

† Specificity in separating GAS from non-GAS