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ORIGINAL ARTICLE

The unique luminal staining pattern of cytokeratin 5/6 in adenoid cystic carcinoma of the breast may aid in differentiating it from its mimickers

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Abstract Adenoid cystic carcinoma (AdCC) of the breast is an uncommon but distinct neoplasm composed of a dual cell population polarized around true glandular (luminal) spaces and pseudolumina. The aim of this study was to clarify whether various immunohistochemical markers (CK7, EMA, CD117, p63, calponin, CD10, S100, CK5/6, CK14, vimentin, and type IV collagen) can distinguish between the two cell types in classical AdCC (n = 14) and in collagenous spherulosis (n = 5). The sensitivity and specificity of these 11 markers to distinguish luminal from abluminal cells were evaluated using a curve created by plotting the true-positive rate (sensitivity) against the false-positive rate (1 − specificity) at threshold settings of 0, 10, 50, and 70 %. The most sensitive and specific markers for luminal cells in AdCC were CK7 and EMA; those for abluminal cells were type IV collagen, p63, and vimentin. CD10 and S100 did not act as abluminal markers in AdCC. CK5/6, one of the basal/myoepithelial markers, was expressed more frequently in luminal than in abluminal cells of AdCC. Thus, CK5/6 immunostaining resulted in a reverse expression pattern, analogous to what we recently documented in clear cells in mammary adenomyoepithelioma. In conclusion, compared with myoepithelial/abluminal cells of normal breast or collagenous spherulosis, the neoplastic abluminal cells of classical AdCC are characterized by enhanced vimentin and attenuated CD10 and S100. Furthermore, the luminal cells of AdCC show a unique aberrant staining pattern for CK5/6 that may aid in the differential diagnosis.

Keywords Adenoid cystic carcinoma · Myoepithelial cells · Cytokeratin 5/6 · Vimentin · Collagenous spherulosis

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Introduction

Adenoid cystic carcinoma (AdCC) of the breast is a rare but distinct neoplasm. It has attracted researchers because of its favorable outcome, which is even better than that of its salivary gland counterpart, despite negative hormone receptors and a basal-like phenotype [1–4]. Morphologically, it is composed of luminal and abluminal cells, arranged into characteristic tubular or cribriform architecture. Differentiating classical AdCC from its malignant mimicker cribriform carcinoma is usually straightforward, because the neoplastic cells in the latter are monotonous, positive for hormone receptors, and negative for high molecular weight cytokeratins. However, especially in needle core biopsies, it can be challenging to distinguish AdCC from its benign mimicker collagenous

spherulosis, because both lesions not only show cribriform architecture but also immunophenotypic overlap [2, 5–7].

In order to clarify the nature of the dual cell population of AdCC, various epithelial and myoepithelial/basal cell markers have been used. Previous studies have suggested that myoepithelial markers such as calponin [5, 8], smooth muscle myosin heavy chain [1, 5], CD10 [6, 9], S100 [10–13], and muscle-specific actin [6], which are expressed in normal myoepithelial cells of the breast, are not useful for identifying abluminal cells in AdCC of the breast, because epithelial and myoepithelial marker expression may be modified or altered in AdCC [14, 15]. Recently, our group has found that high molecular weight keratins CK5/6 and CK14 show a unique paradoxical/reverse staining pattern in clear cells of adenomyoepithelioma of the breast, with diffusely positive inner epithelial cells and completely negative outer myoepithelial cells [16]. This prompted us to formally explore the expression of various epithelial and myoepithelial/basal cell markers in AdCC, another mammary neoplasm with a dual cell population. This study differs from previous studies on a similar topic in that the ability of each marker to discriminate between epithelial/luminal and myoepithelial/abluminal cells was assessed using the sensitivity vs 1 − specificity plot, which graphically represents the relationship between sensitivity and specificity over four threshold settings of 0, 10, 50, and 70 %.

Materials and methods

Fourteen cases of mammary AdCC were retrieved from the pathology archives of Nagoya Medical Center (n = 2), Nara City Hospital (n = 2), Iwate Medical University Hospital (n = 2), Kochi Health Sciences Center (n = 2), Tokushima University Hospital (n = 1), Toyota Kousei Hospital (n = 1), and Okayama University Hospital (n = 1). Three cases were retrieved from the breast pathology consultation file of SI. All samples were anonymized prior to the study. The clinicopathological features of AdCC are shown in Table 1. The age of the patients, all female, ranged from 49 to 87 years (mean 65 years). The largest tumor dimension was 5–43 mm (mean 16.1 mm). As to estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor type 2 (HER2), the majority of cases (70 %) was negative for all three markers (triple-negative)(data not shown). The remaining 30 % of cases were luminal A (ER-positive HER2-negative). The range of the MIB-1 index was broad, but there was no difference between solid and classical types.

We reviewed hematoxylin and eosin-stained sections of each case to assess the proportions of morphological components (cribriform, tubular, and solid patterns). AdCC with a solid pattern in more than 90 % of the tumor (n = 7) were excluded from this study. Therefore, the present study included 14 cases of AdCC with classical cribriform or tubular patterns. Five cases of collagenous spherulosis were retrieved from the pathology archives of Nagoya Medical Center. They were non-complicated microscopic foci of collagenous spherulosis incidentally found in a background of malignant or benign breast lesions. The underlying pathologies associated with collagenous spherulosis are listed in Table 2.

Table 1 Clinicopathological features of adenoid cystic carcinoma of the breast

Case	Age (years)	Laterality	Size (mm)	Pattern
1	61	Right	15	TUB
2	68	Right	8	TUB
3	87	Left	35	CR > TUB
4	69	Left	18	CR
5	65	Left	43	CR
6	59	Left	20	CR > TUB
7	52	Right	5	TUB
8	69	Left	10	CR > SOL
9	62	Right	10	CR
10	74	Right	8	TUB
11	49	Right	-(CNB)	CR
12	72	Left	10	TUB > CR
13	66	Right	7	CR
14	56	Left	20	CR

CR cribriform, *TUB* tubular, *SOL* solid, *CNB* core needle biopsy

As immunohistochemical markers, we used CK7, EMA, CD117 (c-Kit), CK5/6, CK14, S100, vimentin, calponin, CD10, p63, and type IV collagen, which are commonly used as markers in cancer. Antibodies, manufacturers, and dilutions used in immunohistochemical staining are listed in Table 3. Representative 4-μm sections of AdCC, normal terminal duct lobular unit (TDLU), normal ducts, and collagenous spherulosis were cut and subjected to immunohistochemical staining using a Leica Bond-Max automated immunostainer (Leica Biosystems, Tokyo, Japan).

Marker expression in AdCC was evaluated by focusing on the two landmark structures of the tumor, namely a true lumen and a false lumen. The former is small and contains neutral periodic acid-Schiff-positive mucin. The latter is of varying shape, mostly round, and contains a myxoid acidic stromal substance that stains with Alcian blue or straps of collagen with small capillaries [7]. The definitions of epithelial and myoepithelial cells in adenoid cystic carcinoma (AdCC) are not as clear as in normal TDLU and ducts. In this study, therefore, topographical terms, luminal and abluminal, rather than epithelial and myoepithelial cells, were adopted to describe the cells. Luminal cells in AdCC were defined as the cells facing the true lumina, and abluminal cells as the cells facing the false lumina in cribriform structures. Similarly, luminal

Table 2 Underlying pathology in collagenous spherulosis

Case	Age (years)	Specimen type	Primary diagnosis
1	54	Mastectomy	Invasive ductal carcinoma, NST
2	46	Wide local excision	Intraductal papilloma
3	52	Mastectomy	Invasive ductal carcinoma, NST
4	53	Mastectomy	Ductal carcinoma in situ, high grade
5	44	Mastectomy	Ductal carcinoma in situ, high grade

NST no special type

cells in collagenous spherulosis were defined as the cells facing the true lumina, and the abluminal cells were defined as the cells rimming the round spaces containing eosinophilic, hyaline, acellular spherules.

The proportion of epithelial/luminal or myoepithelial/abluminal cells that were positive for a marker was scored into five categories as follows: completely negative (0), less than 10 % (1+), 10–49 % (2+), 50–69 % (3+), and 70 % or more (4+) as previously described [16].

Expression of the 11 markers for epithelial/luminal and myoepithelial/abluminal cells was evaluated in 12 TDLUs and 14 ducts observed in the background of AdCC (Supplemental Tables 1 and 2), 5 cases of collagenous spherulosis (Supplemental Table 3), and 14 of AdCC (Supplemental Table 4). The sensitivity and specificity for detecting epithelial/luminal or myoepithelial/abluminal cells were calculated for each marker at four cut-off values 0, 10, 50, and 70 %. Expression was assessed using a curve created by plotting the true positive rate (sensitivity) against the false positive rate (1 − specificity) at four threshold settings of 0, 10, 50, and 70 %. Online Resource 1 shows how the curves were generated based on the sensitivity and the specificity of each marker expression in normal TDLU/duct, collagenous spherulosis, and AdCC. This curve is a comparison of the true positive rate and the false positive rate as the criterion for epithelial/luminal marker changes. It depicts relative trade-offs between true and false positives. The best possible prediction method for epithelial/luminal markers would yield a point in the upper left corner or coordinate (0,1) of the sensitivity vs 1 − specificity space, representing 100 % sensitivity and 100 % specificity (called a perfect classification). Absolutely no classification ability would give a point along a diagonal line from the left bottom to the top right corner. It is important to note that the result of a consistently poor predictor for epithelial/luminal markers could simply be inverted to obtain a good predictor for myoepithelial/abluminal ones. In this study, therefore, the best myoepithelial/abluminal markers would yield a point in the lower right corner or coordinate (1, 0) of the sensitivity vs 1 − specificity space, representing 100 % sensitivity and 100 % specificity for myoepithelial/abluminal cells.

Table 3 Antibodies used in this study

Antibody	Clone	Dilution	Antigen retrieval	Source
CK5/6	D5/16 B4	×150	Heat	DC
CK7	OV-TL 12/30	×200	Heat	Novo
CK14	LL002	×40	Heat	Novo
CD10	56C6	×50	Heat	Novo
CD117	Polyclonal	×200	Heat	DC
Calponin	26 A11	×10	Heat	Novo
EMA	E29	×1200	Heat	Novo
p63	7JUL	×100	Heat	Novo
S100	Polyclonal	×2	Heat	DC
Vimentin	V9	×4000	Heat	DC
Type 4 collagen	PHM-12	×1600	Enzyme	Novo

DC Dako Cytomation, Carpinteria, CA, USA, *Novo* Novacastra Laboratories Ltd., Newcastle upon Tyne, UK

Results

Normal breast

Expression of 11 markers was assessed in normal TDLUs (n = 12) and ducts (n = 14) present in the 14 cases of AdCC (Supplemental Tables 1 and 2). Between TDLU and ducts, there was no significant difference in sensitivity and specificity of each marker for detecting epithelial or myoepithelial cells. The most sensitive and specific epithelial markers for normal TDLU/ducts were CK7 and EMA, with perfect sensitivity and specificity (Supplemental Tables 1 and 2; Figs. 1 and 2). CD117 (c-Kit) also showed excellent, albeit not perfect, specificity and sensitivity for detecting epithelial cells of normal TDLU/ducts. The most sensitive and specific myoepithelial markers for detecting normal TDLU/duct were p63 and CD10 (Supplemental Tables 1 and 2; Figs. 1 and 2). Type IV collagen, vimentin, CK14, calponin, and S100 were also useful markers, although they were less sensitive or less specific than p63 and CD10. CK5/6 was unsatisfactory both in terms of specificity and sensitivity to differentiate between epithelial and myoepithelial cells of normal TDLU/ducts.

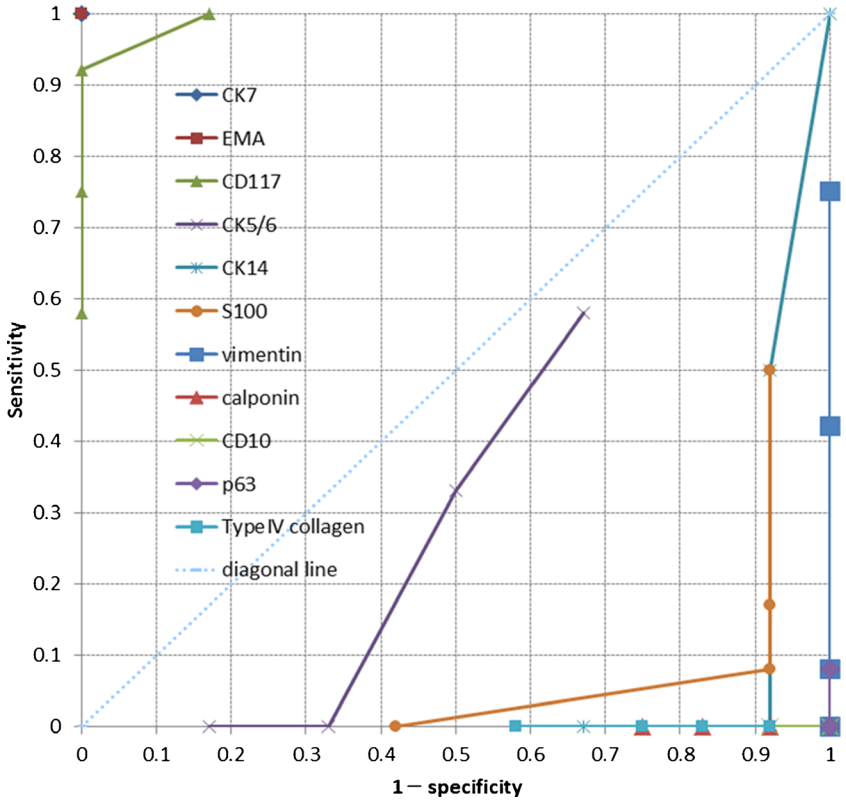

Fig. 1 Sensitivity vs (1 − specificity) plot of 11 markers in normal terminal duct lobular units (TDLUs) of the breast. The *Y axis* is sensitivity, and the *X axis* is 1 − specificity for luminal epithelial cells. CK7 and EMA are located in the *upper left corner*, signifying 100 % sensitivity and specificity for luminal epithelial cells. P63 and CD10 are located around the *lower right corner*, indicating excellent classification ability for abluminal cells. Vimentin is also a highly sensitive abluminal marker but less specific than p63 and CD10. In contrast, calponin and type IV collagen are highly specific abluminal markers but less sensitive than p63 and CD10. S100 and CK14 are informative abluminal markers with similar sensitivity and specificity. CK5/6 approaches the diagonal line from the *left lower corner* to the upper right corner, suggesting a suboptimal luminal and abluminal marker in normal TDLUs

Collagenous spherulosis

Expression of the 11 markers in 5 cases of collagenous spherulosis is shown in Supplemental Table 3. Figure 4 shows that in collagenous spherulosis, the best marker for luminal cells was CK7, with perfect sensitivity and specificity (Fig. 3b). Although EMA and CD117 showed 100 % specificity for luminal cells in collagenous spherulosis (Fig. 3a), their sensitivity was less than that of CK7. The best markers for abluminal cells in collagenous spherulosis were p63 (Fig. 3c), CD10, and type IV collagen (Fig. 3d). Calponin and vimentin were less sensitive than p63, CD10, and type IV collagen, but they showed perfect specificity for abluminal cells in collagenous spherulosis. CK14 and CK5/6 were also informative but suboptimal in their sensitivity and specificity.

Adenoid cystic carcinoma

Expression of the 11 markers for luminal and abluminal cells in 14 AdCC cases is shown in Supplemental Table 4. Figure 6 indicates that EMA (Fig. 5b) and CK7 (Fig. 5c) were the most sensitive and specific markers for luminal cells in AdCC (Fig. 5a). CD117 was also a highly sensitive marker for luminal cells, but its specificity was less than that of EMA and CK7. The most sensitive and specific markers for abluminal cells in AdCC were type IV collagen (Fig. 5d), p63 (Fig. 5e), and vimentin. The present data also indicate that CK5/6 (Figs. 5f and 6) is a marker for luminal rather than abluminal cells in AdCC. Expression of CD10 and S100 was, unlike that in normal TDLU/ducts and collagenous spherulosis, approaching a diagonal line from the left bottom to the top right corners, indicating almost no classification ability (Fig. 6).

Discussion

Recognizing two histological characteristics within the tumor is essential for a correct diagnosis of AdCC [1]. One is a true lumen containing neutral PAS-positive mucin, lined by luminal cells. The other is called a false lumen and contains amorphous glycosaminoglycans, believed to be surrounded by myoepithelial/basaloid cells. In order to identify these two

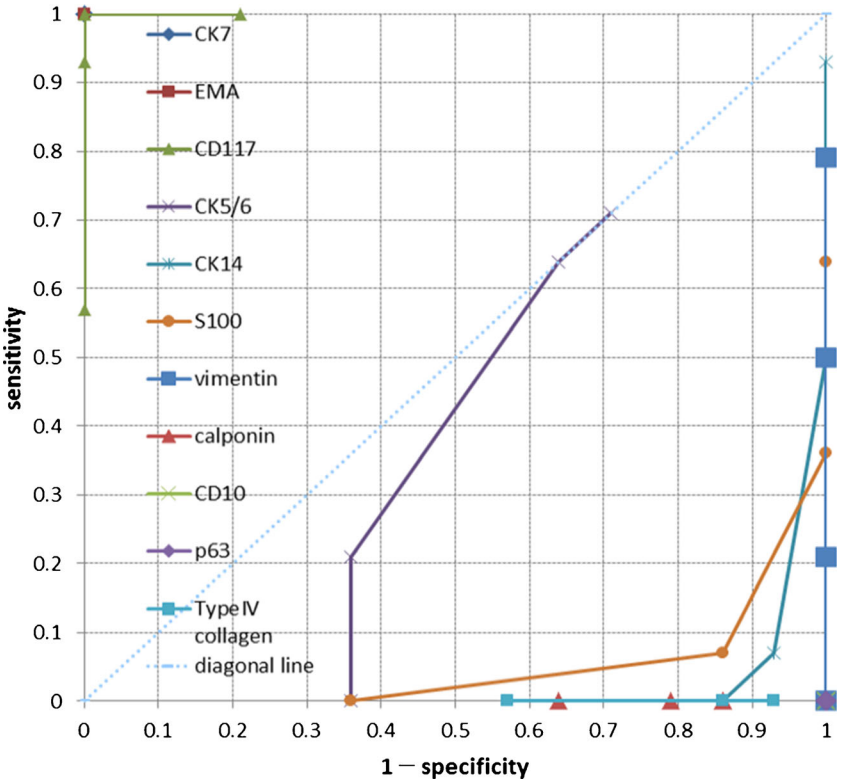

Fig. 2 Sensitivity vs (1 − specificity) plot of 11 markers in normal ducts of the breast. The *Y axis* is sensitivity, and the *X axis* is 1 − specificity for luminal cells. CK7 and EMA are located in the upper left corner, signifying 100 % sensitivity and specificity as luminal markers. P63, calponin, and CD10 are located at the lower right corner, indicating perfect classification as abluminal markers. Vimentin and type IV collagen show excellent specificity but less sensitivity for abluminal cells than p63, calponin, and CD10. The status of the other markers is similar to those in normal TDLUs

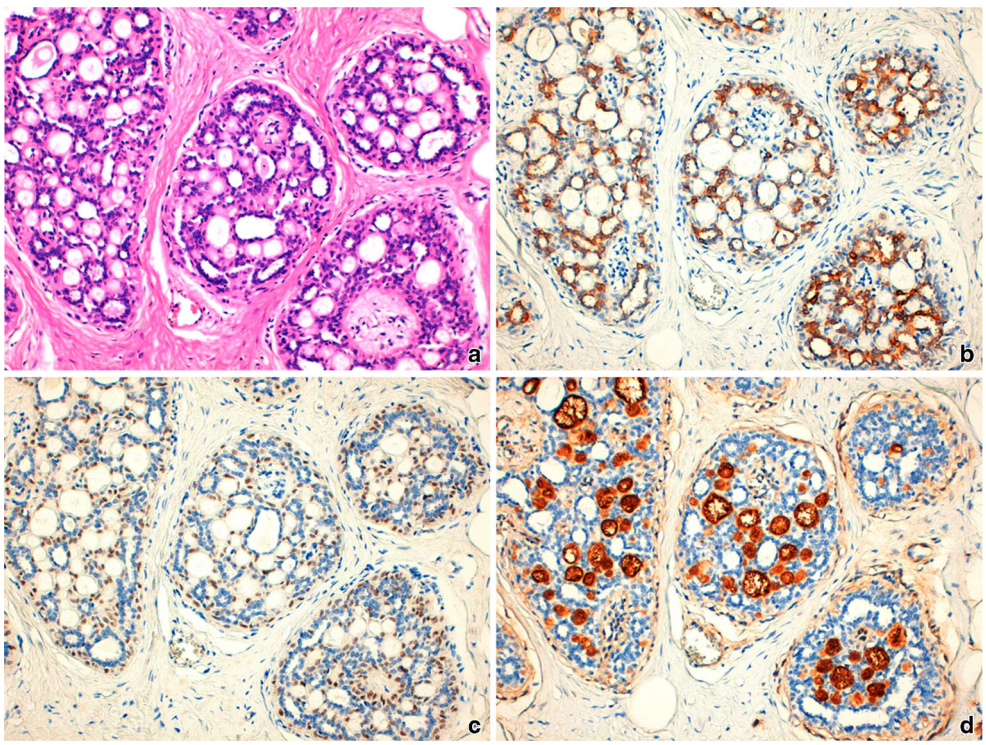

Fig. 3 Hematoxylin and eosin (HE)-stained section of collagenous spherulosis (**a**). Luminal cells around the true glandular spaces are positive for CK7 (**b**), while the abluminal cells rimming the round spaces containing acellular spherules are positive for p63 (**c**) and collagen type IV (**d**)

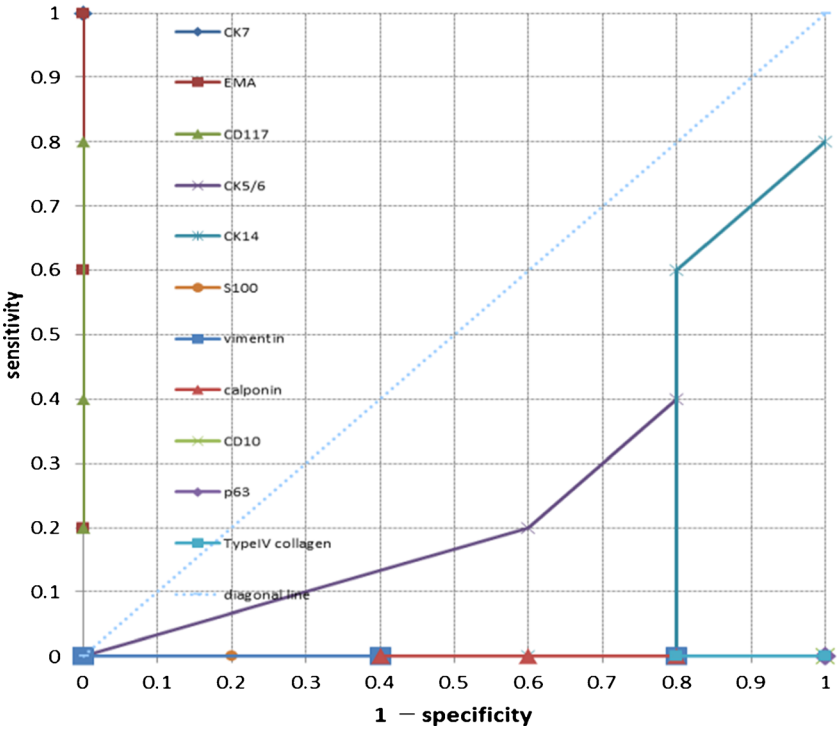

Fig. 4 Sensitivity vs (1 − specificity) plot of 11 markers in collagenous spherulosis of the breast. The *Y axis* is sensitivity, and the *X axis* is 1 − specificity for luminal cells. CK7 is located in the *upper left corner*, indicating perfect classification for luminal cells. EMA and CD117 are less sensitive than CK7 as luminal markers of collagenous spherulosis. P63 and CD10 are located at the *lower right corner*, signifying perfect sensitivity and specificity for abluminal cells. Type IV collagen, calponin, vimentin, S100, CK14, and CK5/6 are also informative abluminal cell markers of collagenous spherulosis

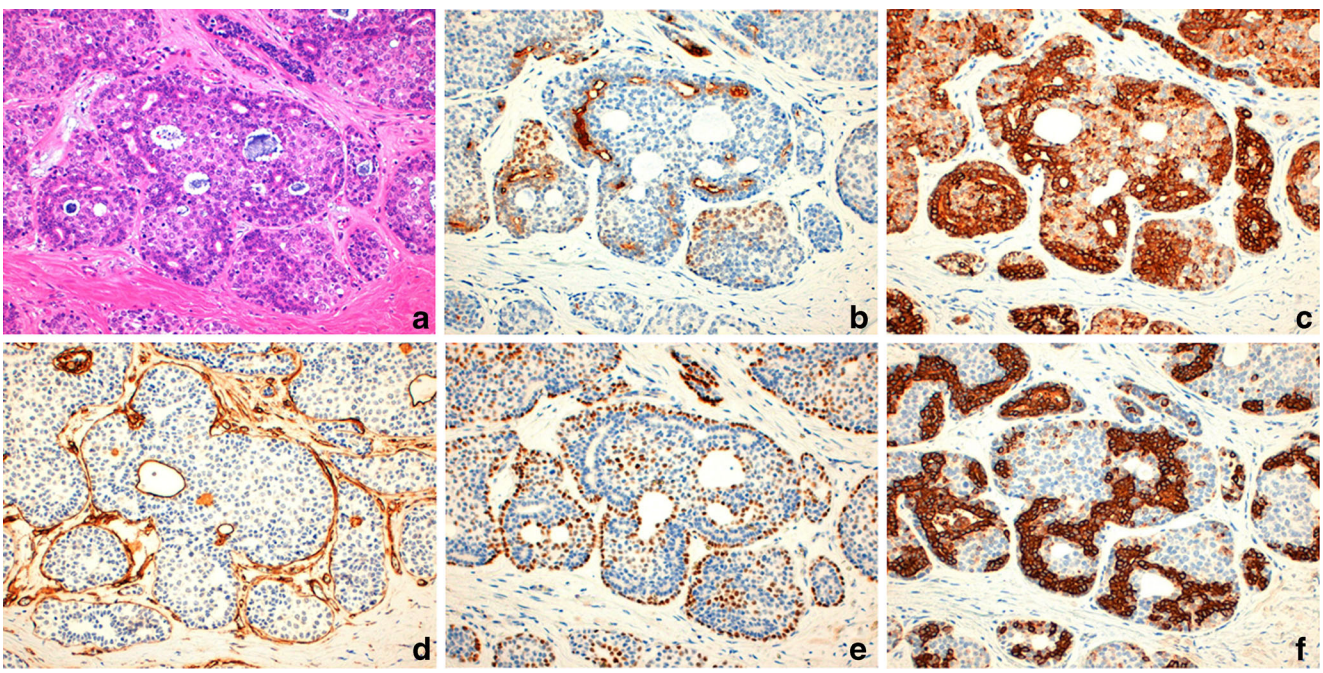

Fig. 5 Hematoxylin and eosin (HE)-stained section of adenoid cystic carcinoma with a cribriform pattern (**a**). There are two types of structures: true glandular spaces and pseudolumina. Luminal cells around the true glandular spaces are positive for EMA (**b**), CK7 (**c**), and CK5/6 (**f**), while the abluminal cells around the pseudolumen are positive for p63 (**e**) and collagen type IV (**d**). Note that CK5/6 is a marker for luminal rather than abluminal cells in adenoid cystic carcinoma

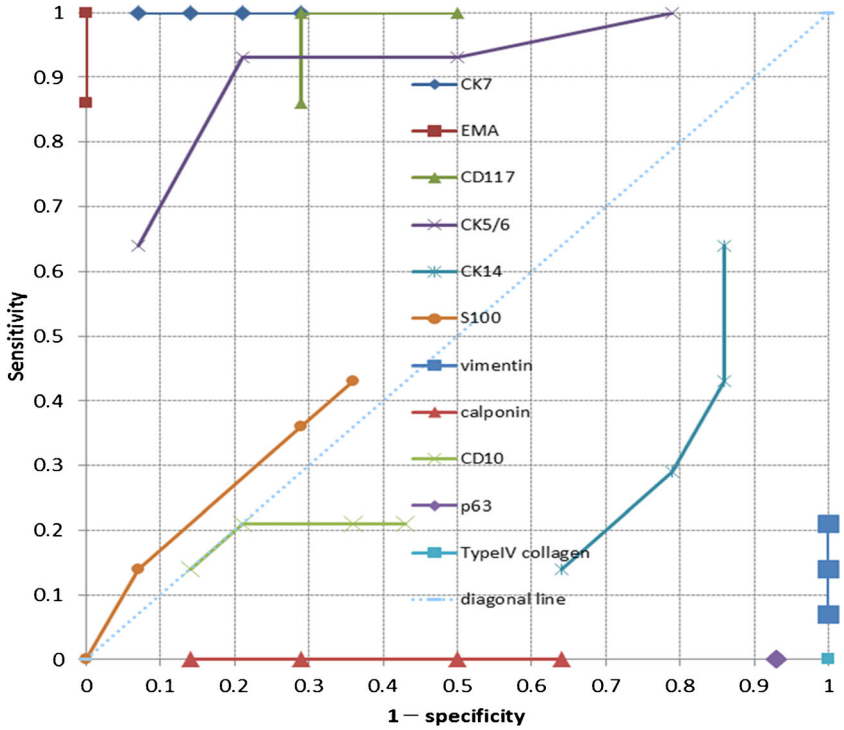

Fig. 6 The sensitivity vs (1 − specificity) plot of 11 markers in Adenoid cystic carcinoma (AdCC) of the breast. The *Y axis* is sensitivity, and the *X axis* is 1 − specificity for luminal cells. EMA and CK7 are located around the upper left corner, implying that they are excellent luminal cell markers in AdCC. P63, type IV collagen, and vimentin are located around the *lower right corner*, indicating that these are excellent abluminal cell markers in AdCC. Note that CK5/6 acts as a luminal cell marker in AdCC. CD10 and S100 are almost along the *diagonal line* from the *left lower corner* to the *upper right corner*, indicating they do not act as luminal or abluminal cell markers in AdCC

types of lumen, expression of various markers has been tested. To the best of our knowledge, systematic appraisal of various immunohistochemical markers for AdCC using a sensitivity vs 1 − specificity plot has not been performed. The results of the present study are summarized in Table 4.

To detect myoepithelial/basaloid cells, a panel approach including antibodies directed against basal epithelial cytokeratins and myofilaments has been recommended [7]. The recent reviews on mammary AdCC, as well as the latest version of the WHO classification of tumors of the breast, state that the myoepithelial/basal cells of AdCC are immunoreactive for basal cytokeratins (CK5, CK5/6, CK14, CK17), myoepithelial markers (p63, actin, calponin, S100 protein), vimentin, and epidermal growth factor receptor (EGFR) [4, 7, 17]. We found, however, that S100 is not useful as a marker for myoepithelial cells in AdCC and that CK5/6-positive cells in AdCC are more frequently around true AdCC lumina, indicating that CK5/6 is a marker for luminal rather than abluminal cells in AdCC (Figs. 5f and 6).

A meticulous bibliographic survey identified sporadic descriptions of CK5/6 expression in AdCC, although it was rarely specified whether the expression was around pseudolumina or true lumina [3, 11, 18, 19]. Azoulay et al. assessed immunohistochemical staining for CK5/6, CK8/18, and p63 in 18 cases of AdCC of the breast. In cribriform and tubular areas of AdCC, the cells around glandular lumina were CK8/18- and CK5/6-positive, consistent with our findings. However, this phenomenon is not further disscussed. We present the first formal report on this reverse staining pattern of CK5/6 in AdCC of the breast. We have recently reported a similar paradoxical phenomenon in clear cells in adenomyoepithelioma of the breast [16]. A difference in the paradoxical expression of high molecular weight keratins between adenomyoepithelioma and AdCC needs to be emphasized: in adenomyoepithelioma the reverse staining pattern was observed for both CK5/6 and CK14, but in AdCC only for CK5/6. Recognition of this difference would be useful to distinguish between AdCC and adenomyoepithelioma.

With regard to the origin of salivary gland-like breast tumors, including AdCC and adenomyoepithelioma, Boecker et al. speculated that CK5/CK14-positive progenitor cells have a potential to differentiate to glandular and myoepithelial lineages and also to generate heterogeneous cell differentiation such as squamous and mesenchymal progenies [20]. It would be interesting to know molecular mechanisms driving reverse expression of CK5/6 in AdCC and both CK5/6 and CK14 in adenomyoepithelioma, since this may reflect differences in tumorigenesis between these closely related mammary neoplasms.

CK7, EMA, and CD117 have been used as marker for luminal epithelial cells in AdCC [4, 7, 21]. We show that CK7 and EMA are stable, sensitive, and specific markers for

Table 4 Summary of immunohistochemical markers that distinguish between luminal and abluminal cells in collagenous spherulosis and adenoid cystic carcinoma

	Topology	Highly recommended	Informative	Not recommended
Collagenous spherulosis	Luminar	CK7	EMA CD117	
	Abluminar	p63	S100	
		CD10	Calponin	
		Type IV collagen	CK14	
			Vimentin	
			CK5/6	
Adenoid cystic carcinoma	Luminar	CK7 EMA	CD117 CK5/6	
	Abluminar	p63	CK14	CD10
		Vimentin	Calponin	S100
		Type IV collagen		

luminal epithelial cells in AdCC, as well as in normal TDLU/ducts. Our data are consistent with those of Nikitakis et al., who noted that of 25 AdCCs, 14 were diffusely CK7-positive and 11 of 25 focally positive [22]. In focally positive AdCCs, CK7 immunoreactivity was limited to luminal cells, while expression in abluminal cells was very weak or negative. CD117 (c-Kit) has also been proven an excellent luminal epithelial marker in normal breast, which is consistent with other reports on CD117 expression in normal tissues [23]. However, the specificity of CD117 for detecting luminal cells in AdCC is lower because abluminal cells express it in half of the cases (Supplementary Table 4). This is consistent with other studies on CD117 expression in AdCC [3, 19, 24–26]. All AdCCs examined by Azoulay et al. expressed CD117 [3], and over 80 % of AdCCs expressed CD117 in more than 50 % of tumor cells in another report [25]. In AdCC with a classic or solid cystic pattern, CD117 expression was localized to the inner cell layer, while in the solid basaloid pattern expression was seen in all cell layers [25].

We show that p63 is a stable, sensitive, and specific myoepithelial/abluminal marker in normal breast and in both collagenous spherulosis and AdCC. Our data furthermore suggest that expression of some myoepithelial markers is modified or altered depending on the type of myoepithelial lesion. Both CD10 and S100 are useful myoepithelial markers in normal breast and collagenous spherulosis, but their sensitivity and specificity for detecting abluminal cells is lower in AdCC. These results are consistent with those reported by Neves et al., who examined CD10 expression in 20 cases of AdCC of the salivary glands [9]. According to their report, CD10 is expressed in less than 10 % of the neoplastic cells. The authors suggest that CD10 staining could be a useful adjunct to separate epithelial myoepithelial carcinoma from AdCC, because the former showed CD10 expression in 83 % of cases. With regard to S100, Morice et al. reported that 80 % of AdCCs were positive, but with low to moderate intensity [27].

Surprisingly, vimentin appeared to be a very sensitive and specific abluminal/myoepithelial marker for AdCC and normal breast, but not for collagenous spherulosis. This was also reported by Morinaga et al., who observed that myoepithelial cells of AdCC are always positive for vimentin while epithelial cells are negative but strongly positive for keratin [15].

Collagenous spherulosis is an incidentally discovered benign myoepithelial lesion, often observed in intraductal papillomas, as well as in usual ductal hyperplasia, adenosis and other breast lesions. It features intraluminal eosinophilic, hyaline, acellular spherules rimmed by myoepithelial cells, histologically mimicking cribriform ductal carcinoma in situ or AdCC [7]. According to Rabban et al., AdCCs are CD117 positive and calponin negative, whereas collagenous spherulosis lesions are CD117 negative and calponin positive. This is an oversimplification, because we found that 64 % of AdCCs express calponin in abluminal cells (Supplemental Table 4), and 80 % of collagenous spherulosis cases express CD117 in abluminal cells (Supplemental Table 3), although with low scores. Our data suggest that aberrant expression of myoepithelial/basal markers, including reverse pattern of CK5/6, increased expression of vimentin and attenuated expression of S100 and CD 10 favors a diagnosis of AdCC over that of collagenous spherulosis.

In conclusion, based on systematic evaluation of 11 markers using sensitivity and 1 − specificity plots, we recommend in AdCC as markers for luminal cells CK7 and EMA and for abluminal cells type IV collagen, p63, and vimentin. S100 and CD10 are not appropriate as markers in AdCC. Although CK5/6 and CK14 are generally believed to be myoepithelial/basal markers, our data indicate that CK5/6 is a luminal epithelial marker in AdCC, which may aid in excluding its mimickers, including collagenous spherulosis, adenomyoepithelioma, and cribriform carcinoma.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

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