

## Expression of EpCAM in adenoid cystic carcinoma

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The mutational landscape of adenoid cystic carcinoma (ACC) is currently being revealed, but further studies are needed to identify biomarkers as therapeutic targets or prognostic factors of ACC. In this study, we investigated the expression of epithelial cell adhesion molecule (EpCAM) in ACCs. We retrospectively collected 83 cases of surgically resected ACCs. Using tissue microarray, we conducted immunohistochemical staining using the anti-EpCAM antibody. EpCAM expression was analysed by intensity score and the total immunostaining score. The positivity was 97.6% (81/83 cases), regardless of the intensity score. A higher histological grade ( $p = 0.006$ ) and specific tumour location (non-salivary gland origin,  $p = 0.02$ ) showed a correlation with higher EpCAM intensity. Higher EpCAM expression by total immunostaining score was associated with histological grade ( $p = 0.004$ ), distant metastasis ( $p = 0.004$ ) and poorer prognosis (overall survival  $p = 0.015$  and progression-free survival  $p = 0.033$ ). We suggest EpCAM as a candidate prognostic marker and a putative therapeutic target in ACC. Also, ACCs arising from salivary gland and non-salivary gland sites, respectively, might display different pathophysiologies in which EpCAM could play a role.

**Key words:** Adenoid cystic carcinoma; EpCAM; Ber-EP4; biomarker; non-salivary gland.

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**INTRODUCTION**

Adenoid cystic carcinoma (ACC) is an uncommon malignancy of the secretory glands and often occurs in the areas of the head and neck, particularly in the salivary glands.<sup>1</sup> It is characterised by indolent, persistent growth and frequent perineural invasion, local recurrence and a poor long-term prognosis.<sup>1,2</sup>

Immunohistochemical staining is often used to distinguish ACC from other malignancies, especially when histological findings are confusing. ACC expresses both ductal and myoepithelial cell markers, such as CK7, CAM 5.2, calponin, SMA, SMMHC, p63, SOX10, and S100, but their variable expressions in ACC and other malignancies have made them not useful for diagnosis.<sup>3,4</sup> Currently, c-KIT is widely used as

a diagnostic marker, because most ACCs are strongly positive for c-KIT regardless of the histological grade.<sup>3,4</sup> Many investigators have focused on this receptor as a therapeutic target.<sup>5,6</sup> However, clinical trials using imatinib, a c-KIT inhibitor, have been unsatisfactory, and several studies have shown that ACCs express wild-type c-KIT.<sup>7–10</sup> Strong nuclear MYB expression, due to MYB-NFIB translocation, is detected in up to 70% of ACCs regardless of the site of tumour origin, and MYB appears to be a valuable diagnostic marker for ACC.<sup>11–13</sup> MYB-NFIB translocation seems to be specific in ACC, and these aberrations may be a critical event in ACC pathogenesis.<sup>14</sup> However, further studies are needed to uncover the application of MYB-associated genes in therapy.

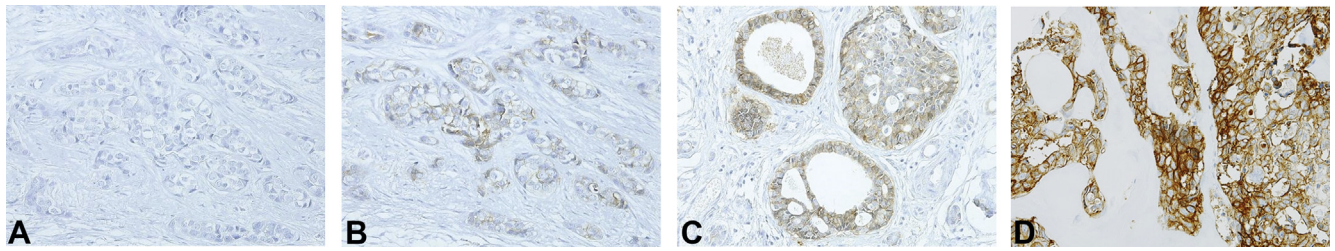
Epithelial cell adhesion molecule (EpCAM) is a 40 kDa cell surface glycoprotein that is overexpressed in epithelial cancers and, at lower levels, in normal epithelium; therefore, it is considered a tumour marker.<sup>15</sup> It consists of a large extracellular domain of 242 amino acids (aa), a single-spanning transmembrane domain of 23 aa and a short cytoplasmic domain of 26 aa. The function of EpCAM includes intercellular adhesion, cell proliferation, signalling, migration and differentiation.

EpCAM is overexpressed in certain carcinomas, including colon, pancreas, and breast carcinomas.<sup>16–18</sup> The high levels of EpCAM in some cancers are related to a poor prognosis, and EpCAM can serve as a prognostic marker. Therefore, the overexpression of EpCAM in tumours makes it an attractive therapeutic target. In the context of skin cancer, EpCAM has been used for diagnostic purposes: the anti-EpCAM antibody Ber-EP4 is a sensitive marker of basal cell carcinoma.<sup>19–21</sup>

Studies regarding EpCAM expression in ACC are relatively scarce. Given the potential diagnostic and therapeutic applications of anti-EpCAM antibodies, we investigated the expression of EpCAM in ACCs. The purpose of this study was to demonstrate the expression of EpCAM in ACC and to verify the clinicopathological features of ACC according to the expression level of EpCAM.

**MATERIALS AND METHODS****Case selection**

We included 83 patients with ACC who underwent surgical resection from 1996 to 2013 at Severance Hospital, Korea. Clinical data, including gender, age at diagnosis, tumour location, disease recurrence, metastasis, and survival of patients, were obtained from the patients' medical records. The histological grades of the tumours and perineural invasion were noted from the review of slides and pathology reports. All slides were retrospectively reviewed by two



**Fig. 1** Examples of the intensity levels of EpCAM in ACC. (A) Negative, 0. (B) Weak, 1+. (C) Moderate, 2+. (D) Strong, 3+.

pathologists (LSJ and KSK), and histological evaluation was conducted on haematoxylin and eosin (H&E) stained slides. Histological grade was evaluated using the grading system of Perzin/Szanto as follows: 1, predominantly tubular and cribriform component, no solid pattern; 2, predominantly cribriform component or mixed, <30% solid component; 3, marked predominance of the solid component.<sup>22,23</sup> All methods and experimental protocols using human tissue [formalin fixed, paraffin embedded (FFPE) tissue] were carried out in accordance with relevant guidelines and regulations approved by the Institutional Review Board of Severance Hospital, Yonsei University Health System (4-2015-0872). The informed consent was waived because the IRB decided that this retrospective study showed minimal risk to the patients (risk level I).

#### Tissue microarray analysis

A representative area was selected on each H&E slide, and a corresponding spot was marked on the surface of the FFPE block. Using a biopsy needle, the selected area was punched out, and a 3 mm tissue core was placed into a 6 × 5 recipient block. Each tissue core was assigned to a unique tissue microarray location number linked to a database containing other clinical data.

#### Immunohistochemistry

FFPE tissue blocks were cut into 4 µm sections. Immunohistochemical staining was performed using a Ventana XT automated stainer (Ventana Corporation, USA) with antibodies against EpCAM (clone VU-1D9, 1:1,000; Calbiochem, USA), according to the manufacturer's instructions. Negative control samples were processed without the primary antibody. Positive control tissue was used as per the manufacturer's recommendation.

The expression of EpCAM was semiquantitatively evaluated by intensity score (IS) analysis and by calculating the total immunostaining score (TIS), the product of the intensity score and proportion score (PS). IS represents the

estimated staining intensity compared with that of control cells (0, no staining; 1, weak; 2, moderate; 3, strong), and PS describes the estimated area of positively stained tumour cells (0, none; 1, <10%; 2, 10–50%; 3, 51–80%; 4, >80%). TIS (IS\*PS) ranges from 0 to 12 with only nine possible values (0, 1, 2, 3, 4, 6, 8, 9 and 12). Using TIS, we defined the low-expression group (TIS 0–8) and high-expression group (TIS 9 and 12).

To see the cellular distribution of EpCAM, the expression pattern (membranous or cytoplasmic) was analysed.

#### Statistical analysis

All statistical analyses were performed using SPSS software, version 21.0 for Windows (IBM, USA). To analyse each clinicopathological parameter, Student's *t*-test, Fisher's exact test and Pearson's  $\chi^2$ -test were used, depending on the purpose. Patient survival statistics were analysed using the Kaplan–Meier method and log-rank test, and uni- and multivariate analyses were performed using the Cox regression model. Hazard ratios (HRs) and corresponding 95% confidence intervals (CIs) were presented. *p* < 0.05 was considered to indicate statistical significance.

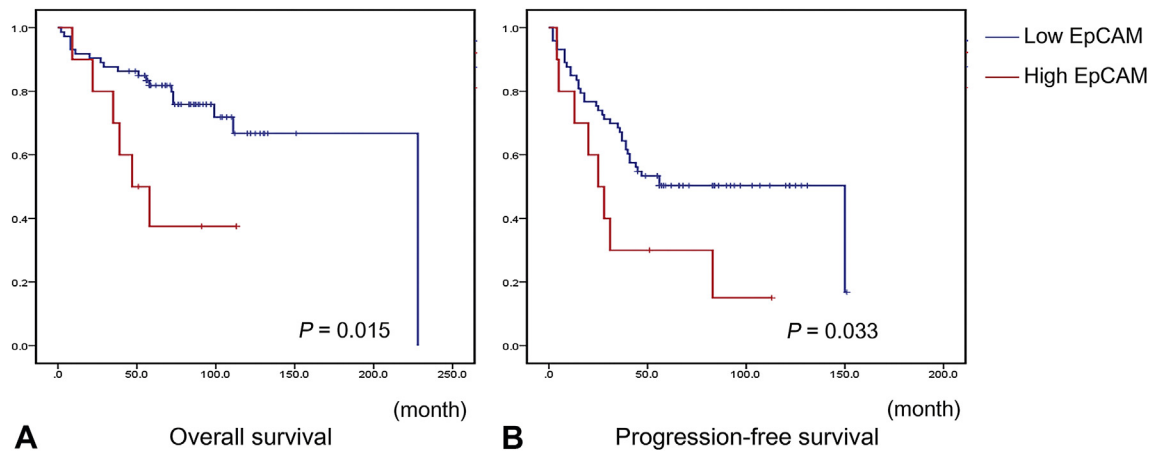
## RESULTS

### Clinicopathological characteristics

This study included 83 patients with ACC: 53 cases (63.9%) arose from the salivary gland (46 cases from the major salivary gland and seven cases from the minor salivary gland), and 30 cases (36.1%) arose from the non-salivary gland site, including the lacrimal gland, orbit, nasal cavity, pharynx, larynx, auditory canal, breast and trachea. The mean age of

**Table 1** Correlation between EpCAM expression by intensity score (IS), total immunostaining score (TIS) analyses and clinicopathological parameters

	<i>n</i>	EpCAM expression (IS)				<i>p</i> value	EpCAM expression (TIS)		<i>p</i> value
		Negative (0) <i>n</i> (%)	Weak (1+) <i>n</i> (%)	Moderate (2+) <i>n</i> (%)	Strong (3+) <i>n</i> (%)		TIS ≤ 8 <i>n</i> (%)	TIS > 8 <i>n</i> (%)	
Age (years)	83								
≤50	31	1 (3.2)	24 (77.4)	3 (9.7)	3 (9.7)	0.464	28 (90.3)	3 (9.7)	0.737
>50	52	1 (1.9)	33 (63.5)	11 (21.2)	7 (13.5)		45 (86.5)	7 (13.5)	
Gender						0.064			0.154
Male	29	2 (6.9)	19 (65.5)	7 (24.1)	1 (3.4)		28 (96.6)	1 (3.4)	
Female	54	0 (0)	38 (70.4)	7 (13.0)	9 (16.7)		45 (83.3)	9 (16.7)	
Location						0.020			0.484
Salivary gland	53	0 (0)	42 (79.2)	6 (11.3)	5 (9.4)		48 (90.6)	5 (9.4)	
Others	30	2 (6.7)	15 (50.0)	8 (26.7)	5 (16.7)		25 (83.3)	5 (16.7)	
Histological grade						0.006			0.004
Grade 1	19	2 (10.5)	14 (73.7)	3 (15.8)	0 (0)		19 (100.0)	0 (0.0)	
Grade 2	48	0 (0)	36 (75.0)	8 (16.7)	4 (8.3)		44 (91.7)	4 (8.3)	
Grade 3	16	0 (0)	7 (43.8)	3 (18.8)	6 (37.5)		10 (62.5)	6 (37.5)	
Perineural invasion						0.769			1.000
Present	61	1 (1.6)	41 (67.2)	11 (18.0)	8 (13.1)		53 (86.9)	8 (13.1)	
Absent	22	1 (4.5)	16 (72.7)	3 (13.6)	2 (9.1)		20 (90.9)	2 (9.1)	
Local recurrence						0.234			0.056
Present	20	0 (0)	12 (60.0)	3 (15.0)	5 (25.0)		15 (75.0)	5 (25.0)	
Absent	63	2 (3.2)	45 (71.4)	11 (17.5)	5 (7.9)		58 (92.1)	5 (7.9)	
Distant metastasis						0.014			0.004
Present	30	1 (3.3)	17 (56.7)	4 (13.3)	8 (26.7)		22 (73.3)	8 (26.7)	
Absent	53	1 (1.9)	40 (75.5)	10 (18.9)	2 (3.8)		51 (96.2)	2 (3.8)	



**Fig. 2** Survival analysis according to the EpCAM expression pattern (TIS ≤ 8 or TIS > 8) in ACC patients. (A) Overall survival (*p* = 0.015). (B) Progression-free survival (*p* = 0.033).

the patients was 53.2 years (age range 20–81 years), and the male-to-female ratio was 1:1.9. According to the histological grade, 19 cases (22.9%) demonstrated grade 1, 48 cases (57.8%) demonstrated grade 2, and 16 cases (19.3%) demonstrated grade 3.

**EpCAM expression in ACC**

The expression of EpCAM in normal salivary gland tissue was negative or weakly positive in acinar and ductal cells, as previously reported.<sup>24</sup> Positive staining of EpCAM occurred primarily on the cell membrane and cytoplasm (Fig. 1). The correlation between EpCAM expression by IS, TIS and clinicopathological parameters is listed in Table 1.

The positivity was 97.6% (81/83 cases), regardless of the intensity score (IS). Negative expression of EpCAM was identified in two cases (2.4%), weak expression in 57 cases (68.7%), moderate expression in 14 cases (16.9%), and strong expression in 10 cases (12.0%). The number in the low-expression group by TIS was 73 (88.0%) and in the high-expression group was 10 (12.0%). No significant difference

was identified between EpCAM intensity and certain clinicopathological parameters such as age at diagnosis, gender, perineural invasion and local recurrence. However, tumour location (non-salivary gland origin, *p* = 0.02), higher histological grade (*p* = 0.006) and distant metastasis (*p* = 0.014) were related to higher expression of IS. By TIS, a higher EpCAM expression level was also related to the histological grade (*p* = 0.004) and frequent distant metastasis (*p* = 0.004).

**Survival analysis**

Kaplan–Meier and Cox regression analyses were performed to discover a link between EpCAM expression in ACC and patient survival. In the Kaplan–Meier analysis, there was a relationship in the patients with higher EpCAM expression (TIS > 8) who had poorer overall survival (OS) and progression-free survival (PFS) than those with a lower TIS level (TIS ≤ 8) (OS, *p* = 0.015 and PFS, *p* = 0.033) (Fig. 2). According to Cox regression analysis, the variables with a significant difference in OS rates were male gender (*p* = 0.009), higher histological grade (*p* = 0.004),

**Table 2** Univariate and multivariate analyses of clinicopathological variables and EpCAM expression in relation to overall survival and progression-free survival in patients with ACC

Risk factors	Univariate analysis			Multivariate analysis		
	HR	95% CI	<i>p</i> value	HR	95% CI	<i>p</i> value
<b>Overall survival</b>						
Age (≤50/>50)	1.248	0.533–2.920	0.610	0.670	0.258–1.741	0.411
Gender (male/female)	0.339	0.151–0.765	0.009	0.277	0.095–0.803	0.018
Location (others/salivary)	0.894	0.410–1.950	0.778	1.840	0.745–4.546	0.186
Histological grade (1+2/3)	3.170	1.431–7.021	0.004	2.146	0.915–5.031	0.079
Perineural invasion (N/P)	4.058	0.953–17.280	0.058	2.164	0.448–10.462	0.337
Local recurrence (N/P)	2.201	0.977–4.959	0.057	1.525	0.555–4.192	0.413
Distant metastasis (N/P)	3.005	1.314–6.873	0.009	2.045	0.873–4.793	0.100
EpCAM IS (0–2/3)	2.998	1.183–7.583	0.021	2.908	0.896–9.440	0.076
<b>Progression-free survival</b>						
Age (≤50/>50)	0.902	0.491–1.658	0.741	0.599	0.299–1.200	0.148
Gender (male/female)	0.467	0.258–0.847	0.012	0.893	0.399–2.000	0.784
Location (others/salivary)	0.876	0.481–1.593	0.664	1.879	0.938–3.766	0.075
Histological grade (1+2/3)	2.131	1.113–4.081	0.023	0.970	0.464–2.030	0.936
Perineural invasion (N/P)	3.013	1.272–7.139	0.012	2.035	0.794–5.219	0.139
Local recurrence (N/P)	3.046	1.671–5.553	<0.001	3.088	1.668–5.717	<0.001
Distant metastasis (N/P)	7.345	3.772–14.301	<0.001	7.611	3.841–15.083	<0.001
EpCAM IS (0–2/3)	2.247	1.039–4.858	0.040	0.956	0.344–2.655	0.932

CI, confidence interval; HR, hazards ratio; IS, intensity score; N/P, negative versus positive

distant metastasis ( $p = 0.009$ ) and strong EpCAM expression ( $p = 0.021$ ) in the univariate analysis (Table 2). EpCAM expression did not affect the OS of ACC patients in the multivariate analysis.

In univariate analysis for PFS, male gender ( $p = 0.012$ ), higher histological grade ( $p = 0.023$ ), perineural invasion ( $p = 0.012$ ), local recurrence ( $p < 0.001$ ), distant metastasis ( $p < 0.001$ ) and strong EpCAM expression ( $p = 0.04$ ) were statistically significant. In the multivariate analysis, EpCAM expression did not affect the PFS. Local recurrence and distant metastasis were identified as independent prognostic factors for PFS. All the statistically significant variables were subjected to the Cox proportional hazards regression model.

## DISCUSSION

Several studies have investigated the expression of EpCAM in various carcinomas. Although EpCAM might be expressed weakly on normal epithelium, it is overexpressed in tumours, including colorectum, oesophagus, liver, prostate, lung, pancreas and breast tumours.<sup>18</sup> EpCAM overexpression is associated with poorer prognosis in carcinomas of the breast, gallbladder, ovary, ampulla and oesophagus because the molecule functions as an inhibitor of E-cadherin; therefore, it is believed that EpCAM plays a role in metastasis.<sup>25–31</sup> On the other hand, EpCAM can participate in cell adhesion, and its overexpression is suggested to be linked to better survival in patients with colorectal carcinoma, gastric carcinoma, clear cell renal cell carcinoma and non-small cell lung cancer.<sup>32–36</sup>

We performed immunohistochemical staining of EpCAM on the tissues of 83 ACC samples. Some reports have suggested that the cellular distribution of EpCAM varies by tumour type and histological differentiation of carcinoma; therefore, it might have diagnostic value.<sup>37,38</sup> However, we identified both membranous and cytoplasmic expression in cancer cells of ACC and it does not have any clinicopathological significance in ACC.

In this study, we found that the overexpression pattern of EpCAM in ACC is associated with a higher histological grade. Furthermore, we could confirm that EpCAM plays a role in tumour distant metastasis in the context of ACC, as previous studies have suggested in other tumours.<sup>25–31</sup>

Phattarataratip *et al.* studied epithelial cell adhesion molecule expression in various salivary gland neoplasms, including mucoepidermoid carcinoma, adenoid cystic carcinoma, pleomorphic adenoma, and polymorphous low-grade adenocarcinoma.<sup>39</sup> They included EpCAM and showed different EpCAM expression patterns among salivary gland neoplasms. They demonstrated that decreased EpCAM expression was associated with aggressive features in mucoepidermoid carcinoma, and the ACCs showed negative or weakly positive immunoreactivity to EpCAM, contrary to our result. However, that previous study possessed some limitations in that the number of ACC cases involved in the study was too small and they found that the solid growth area of ACC showed diffuse and strong immunoreactivity to EpCAM.

By immunohistochemistry, the stronger EpCAM staining intensity was more frequently observed in ACCs arising from non-salivary gland sites. We showed that tumour location tends to affect ACC patients' progression-free survival by multivariate analysis. Lin *et al.* compared the ACCs of salivary and non-salivary origin, and they also found that

sinonasal, lacrimal, and tracheobronchial ACCs had significantly worse outcomes than ACCs of the major salivary glands.<sup>40</sup> The different patients' outcomes between salivary and non-salivary sites of tumour may come from difficulties of diagnosis and complete resection as well as different tumour biologies, but further studies are required to clear these differences according to tumour location.

A previous study investigated the expression of tumour-associated calcium signal transducer 2 (TACSTD2, Trop2), a homolog of EpCAM, in salivary ACC.<sup>41</sup> Similar to our result of EpCAM in ACC, TACSTD2 overexpression was related to a poor prognosis in patients, although the molecule did not reflect the histological subtype.

The possible prognostic significance of EpCAM overexpression in various cancers has been raised by several investigators.<sup>30,42–44</sup> Similarly, overexpression of EpCAM was correlated with higher histological grade and distant metastasis and we confirmed strong EpCAM expression is an independent factor affecting ACC patients' survival in the statistical evaluation.

Several clinical trials using various anti-EpCAM antibodies have been investigated despite the controversy. Catumaxomab was approved in Europe to treat malignant ascites in patients with EpCAM-positive carcinomas.<sup>45,46</sup> Additionally, a few studies using anti-EpCAM antibody showed that it might have an anti-tumour effect.<sup>47–49</sup> However, the validation of EpCAM as a therapeutic target remains unexplored.

In conclusion, we demonstrated the expression of EpCAM in ACC according to the various clinicopathological conditions. A higher histological grade and specific tumour location (non-salivary gland site) showed higher EpCAM expression, and strong EpCAM positivity was associated with distant metastasis and poorer prognosis. We believe that this study will strengthen the basis for understanding the pathophysiology of ACC and suggests EpCAM as a candidate molecule for a prognostic and therapeutic biomarker.

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