Matrix metalloproteinase-7, -8, -9, -15, and -25 in minor salivary gland adenoid cystic carcinoma

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Abbreviations: ACC, Adenoid cystic carcinoma; MMP, matrix metalloproteinase; SCC, squamous cell carcinoma; PMN, polymorphonuclear; TIMP-1, tissue inhibitor of metalloproteinase 1; MT-MMP, membrane-type matrix metalloproteinase; T, tumour; N, node; M, metastasis; EMA, Epithelial membrane antigen; CEA, carcinoembryonic antigen; OS, overall survival; DSS disease-specific survival

Abstract

Knowledge on the role of matrix metalloproteinases (MMPs) in adenoid cystic carcinoma (ACC) is limited. MMPs are capable of degrading almost all extracellular and pericellular components to promote invasion and metastasis. This study aimed to evaluate the immunohistochemical expression of MMP-7, -8, -9, -15, and -25 in ACC and to relate the results with clinicopathological factors and survival.

The study included 68 patients with minor salivary gland ACC treated at the Helsinki University Hospital (Helsinki, Finland) in 1974 to 2012. Samples from 52 patients were available, consisting of 44 primary tumours and eight recurrent tumours. We scored immunostaining of MMP-7, -8, -9, -15, and -25 and analysed the immunoscore against clinical and pathological parameters using statistical correlation test.

MMP-9 immunoexpression in pseudocysts of ACC and in peritumoural inflammatory cells associated with better survival and fewer treatment failures. High tumoural MMP-7 and -25 associated with better survival. High tumoural MMP-15 associated with poorer survival and high tumoural MMP-9 with advanced stage and regional recurrences. Tumour cells did not show MMP-8 immunopositivity.

These results suggest that MMP-9 may contribute to ACC carcinogenesis in different roles. MMP-7, -8, and -9 can stimulate signalling pathways that may promote tissue modulation and metastatic potential. MMP-15 and -25 may reflect prognosis.

Keywords: adenoid cystic carcinoma; immunohistochemistry; matrix metalloproteinase, prognosis

1. Introduction

Adenoid cystic carcinoma (ACC) is the second most common salivary gland malignancy worldwide according to the World Health Organization (WHO) [1]. ACC has a heterogenous histopathological growth pattern, a predilection to perineural invasion, and an ability to develop metastatic disease even after prolonged dormancy [1,2,3].

Matrix metalloproteinases (MMPs) form a multigene family that consists of 25 structurally related and genetically distinct secreted or cell surface-associated proteolytic enzymes [4,5,6]. MMPs are capable of degrading almost all extracellular, pericellular, and basement membrane components, such as collagens and non-matrix bioactive substrates such as interleukins, chemokines, cytokines, and cell-surface receptors [4,5]. MMPs thus participate in angiogenesis, bone formation, wound healing, and immunomodulation [5]. In cancer tissue, MMPs have an essential role in degrading the basement membrane and extracellular matrix, thus enabling invasion and metastases [7]. Previous studies on MMPs in ACC have shown that MMP-2, -9, -14, and -15 participate in invasion and metastasis, and MMP-9 expression is associated with advanced tumour stage and decreased survival time [8-17].

MMP-7, or matrilysin-1, is expressed by different tumour types such as salivary gland carcinoma and oral squamous cell carcinoma (SCC), especially at the invasive front [7,18,19]. In normal conditions, MMP-7 is expressed by non-injured and non-inflamed exocrine epithelium e.g. in salivary glands [19]. MMP-7 can activate defensins and other MMPs, including MMP-8 and -9, and can process extracellular matrix components and cell-surface molecules [19] but is not expressed by inflammatory cells.

In humans, MMP-8, or neutrophil collagenase or collagenase-2, is mainly produced and released by mature polymorphonuclear (PMN) cells. MMP-8 has been shown to have an anti-inflammatory defensive role against the spread of skin and tongue cancer [20,21]. In colorectal cancer, elevated serum levels of MMP-8 and TIMP-1 (tissue inhibitor of metalloproteinase 1) are related to poor prognosis [22].

MMP-9, or gelatinase B, is upregulated during inflammation and in cancer [23,24]. MMP-9 can degrade type-IV collagen of basement membranes and thus promote tumour invasion and metastasis [24]. Consequently, increased MMP-9 production and activation have been detected in many malignant

tumours, such as cancers of the pancreas, lung, cervix, and ovary [5,24]. Furthermore, MMP-9 may also play a suppressive role in cancer progression as observed in colitis-associated colon cancer [25,23]. MMP-15 and -25 are membrane-type MMPs (MT-MMPs) responsible for extracellular matrix remodelling and are expressed at the cell surface rather than secreted in a soluble form [26]. MMP-15 has a role in lung cancer progression and recently has been suggested to be a prognostic factor for lung adenocarcinoma [26]. Melanoma cells and surrounding fibroblasts have been shown to secrete MMP-15 during melanoma invasion [27]. MMP-25 secreted by leukocytes and macrophages is an important factor in innate immunity [28] and has been linked to cancer progression [29,30].

The aim of our study was to evaluate the immunoexpression of MMP-7, -8, -9, -15, and -25 in a series of ACCs of minor salivary and mucous glands and to compare their immunoexpression with clinical and clinicopathological factors, including patient survival.

2. Material and Methods

2.1 Patients and tumour samples

The study population consisted of 68 (29 [42.6%] men patients and 39 [57.4%] women) with ACC of minor salivary and mucous glands treated at the Helsinki University Hospital (Helsinki, Finland) between the years 1974 and 2012. Table 1 shows the main tumour characteristics. Our previous study presented the clinical data of this series [3]. The tumour, node, metastasis (TNM) classification was performed according to the criteria of WHO 2005 and 2017 [31,1]. For immunohistochemical staining, tumour tissues were available from 52 patients, including 44 primary tumours and eight recurrent tumours. The institutional Research Ethics Board approved the study concept (Dnro 31/13/03/02/2010, 01 February 2010).

2.2 Immunohistochemistry

For immunohistochemistry, formalin-fixed and paraffin-embedded blocks were cut into 4-µm thick sections, which were deparaffinized in xylene and rehydrated in graded ethanol and distilled water. Tissue slides were heated in a PreTreatment module (Agilent Dako, Santa Clara, CA, US) in Tris-EDTA buffer, pH 9.0 (MMP-7, MMP-8, MMP-9) and Tris-HCl buffer, pH 8.5 (MMP-15 and MMP-25) for 20 min at 98°C.

Endogenous peroxidase activity was blocked by incubation of the slides with 0.3% Dako REAL Peroxidase-Blocking Solution for 5 minutes. The primary antibody was diluted in Dako REAL Antibody Diluent. The primary antibodies used were mouse monoclonal MMP-7 antibody (1:1000) (EMD Millipore Corporation, Temecula, CA, USA), rabbit polyclonal MMP-8 antibody (1:400) [32], rabbit polyclonal MMP-9 antibody (1:2000) (Calbiochem, Merck KGaA, Darsmstadt, Germany), mouse monoclonal MMP-15 antibody (1:250) (EMD Millipore Corporation, Temecula, CA, USA), and rabbit polyclonal MMP-25 antibody (1:300) (Abbexa, Cambridge, UK). The tissues were incubated with primary antibodies for 1 hour except overnight for MMP-8, followed by detection with Dako REAL Detection System (Peroxidase/DAB+, Rabbit/Mouse, Dako, Glostrup, Denmark). Finally, slides were visualized by Dako REAL DAB+ Chromogen or HRP Magenta Chromogen for 10 minutes and counterstained with haematoxylin (Mayer's Hematoxylin Dako, Glostrup, Denmark). As a positive control, we used oral mucosa and pancreas tissue for MMP-7, skin tissue for MMP-8, stomach and oral mucosa tissue for MMP-9, placenta and mammary gland tissue for MMP-15, and colon tissue for MMP-25.

Immunohistochemistry for epithelial membrane antigen (EMA) and carcinoembryonic antigen (CEA) was performed in a routine laboratory with a Ventana Benchmark Ultra instrument (Roche, Tucson, AZ, USA). Tissue slides were heated in a Ventana Cell Conditioning Solution (CC1) for 64 min for EMA and 92 min for CEA. The primary antibodies used for EMA was clone E29, 790-4463 (ready-to-use), Ventana, and clone II-7, M7072, (1:25), Dako for CEA. Incubation times were 40 and 60 minutes, respectively, followed by detection with an Ventana Ultraview DA and counterstaining with haematoxylin.

2.3 Evaluation of immunostaining

Two independent researchers scored the slides without knowledge of clinical data (H.H and J.H). We analysed the location of immunoexpression at the cellular level (cytoplasm, nucleus, cell membrane, cell type). In addition, immunoexpression of MMP-9 in tumour-surrounding inflammatory cells (PMN cells, lymphocytes, and plasma cells) and pseudocysts of ACC was recorded. We estimated the percentage of positively stained cells and grouped the immunoscores as follows: 0 for negative or very mild (0-10%), 1 for mild (11-40%), 2 for moderate (41-70%), and 3 for strong (71-100%) positivity. The scoring was modified

from our previous publication [18]. To validate the expression in normal tissue, we immunoscored normal salivary gland tissues found on tumour slides. We performed EMA and CEA immunostainings to ascertain the localization of MMP-9 immunopositivity in ACC tissues, particularly in relation to pseudocysts and true glands. It is known that EMA and CEA do not stain pseudocysts, but are often positive in the true glands of ACC [33].

2.4 Statistical analysis

The association of MMP-7, -9, -15, and- 25 immunoexpression with clinicopathological factors were evaluated by χ^2 test and Fisher's exact test. Overall survival (OS) and disease-specific survival (DSS) between MMPs were analysed by the Kaplan-Meier method and log-rank test. P-values <0.05 were considered statistically significant. Statistical analyses were performed with IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, NY).

3. Results

3.1 Gelatinase, MMP-9

MMP-9 immunoexpression was present in 51/52 tumour samples; 30 were scored as negative or very mild, 17 as mild, 4 as moderate, and 0 as strong. MMP-9 immunoexpression in ACC tissues was observed in the cytoplasm of the tumour cells. In 24/51 samples MMP-9 was detected in the luminal material of many pseudocysts and in some true glands of ACC. Additionally, MMP-9 was detected in the inflammatory cells (PMN cells, lymphocytes, and plasma cells) surrounding the tumour in 29 samples. MMP-9 immunoexpression was negative in most normal salivary gland tissues. Only a few normal salivary gland tissues showed mild MMP-9 immunoexpression in luminal ductal cells.

High tumoural MMP-9 immunoexpression associated with regional metastases by Fisher's exact test (P=.035), although the number of cases was only four. Additionally, cytoplasmic MMP-9 positivity associated with more advanced tumour stage. Interestingly, MMP-9 positivity in pseudocysts of ACC associated with better survival and with fewer primary recurrences. Moreover, MMP-9 positivity in

inflammatory cells associated with fewer primary recurrences and MMP-9 positivity in pseudocysts (Table 2, Figure 1).

3.2 Matrilysin, MMP-7

We evaluated MMP-7 immunoexpression in 50/52 tumour samples; 7 were scored as negative or very mild, 15 as mild, 24 as moderate, and 4 as strong. MMP-7 immunoexpression was seen mostly as cytoplasmic positivity in tumour cells. MMP-7 was frequently negative in normal salivary gland tissues as only a few samples showed mild immunopositivity in luminal ductal cells and in acinar structures. In addition, PMN cells, which serve as an internal control for this antibody, were negative.

High immunoexpression of MMP-7 in ACC was associated with longer OS and DSS (Table 3, Figure 1).

3.3 Collagenase, MMP-8

The samples containing PMN cells close to tumour showed MMP-8 immunopositivity. Normal salivary gland or salivary tumour tissues did not show MMP-8 immunopositivity (Figure 1). Statistical analysis related to MMP-8 immunoexpression was not performed.

3.4 MT-MMPs, MMP-15 and -25

We evaluated MMP-15 immunoexpression in 49/52 tumour samples; 32 were scored as negative or very mild, 10 as mild, 5 as moderate, and 2 as strong. MMP-15 immunoexpression was seen mostly as cytoplasmic positivity in tumour cells. Inflammatory cells did not express MMP-15. In normal salivary gland tissues, MMP-15 immunoexpression varied from negative immunoexpression to mild immunoexpression in ductal and in acinar structures.

High MMP-15 immunoexpression in ACC predicted poorer DSS (Table 3, Figure 1).

We evaluated MMP-25 immunoexpression in 51/52 tumour samples; 4 were scored as negative or very mild, 22 as mild, 17 as moderate, and 8 as strong. MMP-25 positivity was detected in a granular pattern in the cytoplasm of tumour cells. MMP-25 immunoexpression was observed in inflammatory cells (solely PMN cells) in some tumour samples. MMP-25 immunoexpression was negative in normal salivary gland tissues.

High MMP-25 immunoexpression in ACC associated with longer OS by Fisher's exact test, although no correlation was detected by Kaplan-Meier analysis (Table 2 and 3, Figure 1).

Gender, age, T, N, and M categories of TNM classification, neural invasion, or distant metastasis did not correlate significantly with immunoexpression of the studied MMPs.

4. Discussion

In this study, we observed that MMP-9 immunoexpression in pseudocysts of ACC tissue and in peritumoural inflammatory cells associated with better survival and fewer primary recurrences. In comparison, high tumoural MMP-9 immunoexpression associated with advanced tumour stage and nodal disease involvement of the neck, although the number of regional metastases in this study was limited. In addition, high tumoural MMP-7 and -25 immunoexpression associated with better survival. Further, high tumoural MMP-15 associated with poorer survival.

Due to its ability to degrade basement membrane, MMP-9 is among the important factors for tumour behaviour, particularly regarding invasion and occurrence of metastases [24,34]. Moreover, MMP-9 has a role in angiogenesis and processing of the tumour microenvironment [24,34]. Based on immunohistochemical studies, MMP-9 has previously been suggested to act as a tumour biomarker in various types of adenocarcinomas, such as oesophageal, lung, and thyroid gland adenocarcinomas [35-37]. In adenocarcinomas of the oesophagogastric junction and thyroid gland, MMP-9 expression is associated with lymph node metastasis and advanced tumour stage [38,37]. Similarly, in our study, MMP-9 immunoexpression associated with regional metastasis, although the number of these cases was reduced as mentioned before. In lung adenocarcinoma, high tumourous MMP-9 expression is associated with poorer survival [36].

Cell-line studies in ACC have shown that MMP-2, -9, and -14 are upregulated, leading to a high incidence of invasion and lung metastases [15,13]. Previous studies have demonstrated that increased MMP-9 expression in ACC is associated with advanced stage and decreased survival time [9,16]. In the present study, advanced tumour stage and occurrence of neck metastases similarly correlated with elevated tumoural cytoplasmic MMP-9 immunoexpression. The normal salivary gland was frequently negative for MMP-9. However, an earlier study by Kayano et al. reported contradictory results as no differences were

observed when comparing MMP-9 expression of different salivary gland carcinomas (including ACC) to non-neoplastic salivary gland tissue [39]. This discrepancy could be due to the fact that we used immunohistochemistry while Kayano et al. analysed tumour homogenates by gelatin zymography [39].

In the present series, the microenvironment of ACC tissue contained mild to moderate numbers of inflammatory cells. In general, salivary gland carcinomas lack an abundant inflammatory cell stromal reaction, which is more commonly seen in oral SCC. Mosconi et al. have suggested that a low level of immune cell infiltration in the ACC microenvironment may relate to poor prognosis [40]. According to our results, MMP-9 immunoexpression in inflammatory cells seems to have a protective role, as MMP-9 positivity in peritumoural inflammatory cells associated with fewer primary treatment failures. Similarly, Pujada et al. have shown that in colitis-associated colorectal cancer, MMP-9 may modulate inflammatory cytokine levels, maintain efficient barrier function and integrity of tight junctions, and act as a tumour suppressor [23]. Interestingly, MMP-9 immunoexpression in pseudocyst structures of ACC had a significant correlation with better survival and fewer primary disease failures. In our study MMP-9 immunoexpression in pseudocyst structures of ACC and in inflammatory cells correlated significantly. We might speculate that when secreted to the pseudocyst structures, MMP-9 might not take an active role in modulating the surrounding tissues. Considering the survival time and lower amount of recurrent tumours, both pseudocyst positivity and peritumoural inflammatory cell positivity showed a protective association in the present ACC series.

Increased immunoexpression of MMP-7 in salivary gland acinic cell carcinoma and mucoepidermoid carcinoma has previously been linked to better survival [19]. According to our study, high MMP-7 immunoexpression in ACC associated with both longer OS and DSS. Luukkaa et al. suggested that poorly differentiated tumour cells could have diminished MMP-7 expression [19]. In the present study, normal salivary gland tissue was frequently negative for MMP-7, although tumour tissues showed cytoplasmic MMP-7 immunoexpression. In gastric, colorectal, and tongue cancers, earlier studies have shown that high MMP-7 expression relates to poor prognosis [41,42,18].

Compared to the above MMPs that have been studied extensively, the functions of MMP-15 and -25 are poorly understood. Previously, MMP-15 has been shown to be upregulated in colorectal cancer and it has been related to potential metastatic ability in laryngeal SCC [43,44]. In lung cancer, Chen et al. observed a correlation between increased MMP-15 level, presence of lymph node metastasis, and advanced tumour stage [45]. However, MMP-15 did not impact prognosis [45]. Similarly, in oesophageal cancer MMP-15 immunoexpression had no prognostic significance [46]. In the present study, tumourous MMP-15 immunoexpression correlated with poorer survival. However, in colorectal cancer, high expression of MMP-15 has been shown to be related to positive prognostic factors such as longer disease-free survival [47]. MMP-25, first identified from leucocytes [48], is a neutrophil-specific protease that regulates chemotaxis of neutrophils and monocytes [49]. We observed MMP-25 positivity in inflammatory cells that might reflect the role of MMP-25 in inflammation [49]. Due to the biochemical structure and location on cell membrane, MMP-25 has a low ability to promote tumour migration and invasion [30]. Increased MMP-25 expression has been observed in brain, colon, urothelial, and prostate cancer tissues [30]. In our earlier study of tongue SCC, tumourous MMP-25 immunoexpression did not correlate with clinical parameters [18]. In contrast, the present study showed that MMP-25 immunoexpression in ACC associated with better prognosis. To understand the exact role of MMP-25 in ACC needs further studies.

Certain malignant cells, such as skin and tongue cancer cells, have been shown to express MMP-8 *in vivo* [20,21]. MMP-8 might have a protective role against carcinogenesis according to MMP-8 knockout mouse experiments [20,21]. Nevertheless, we did not observe tumoural MMP-8 immunopositivity. However, serum MMP-8 levels have been related to prognosis in hepatocellular and gastric cancer [50,51]. In this study, we detected MMP-8 immunopositivity only in a few stromal PMN cells and thus statistical analysis was not performed.

5. Conclusions

Overall, the present data revealed that MMP-9 in particular may directly contribute to ACC carcinogenesis by tissue modulation. MMP-7, -8, and -9 can promote tissue modulation and metastatic potential by

activating different signalling pathways and by the immunomodulation. Membrane-type MMP-15 and -25 are related to prognostic factors.

Ethical approval

The institutional Research Ethics Board (Dnro 31/13/03/02/2010, 01 February 2010) approved the study concept. The study was performed in accordance with the Declaration of Helsinki.

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Declaration of Competing Interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

H. Hämetoja: Methodology, Formal analysis, Investigation, Writing –original draft. **A. Mäkitie:** Formal analysis, Writing – review and editing, Funding acquisition, Project administration. **L. Bäck:** Formal analysis, Writing – review and editing. **I. Leivo:** Formal analysis, Investigation, Writing – review and editing. **C. Haglund:** Methodology, Writing – review and editing, Funding acquisition. **T. Sorsa:** Formal analysis, Writing – review and editing **J. Hagström:** Methodology, Formal analysis, Investigation, Writing –original draft, Supervision.

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Table Legends

Table 1. Tumour characterization of the 68 patients with an adenoid cystic carcinoma of minor salivary and mucous glands. Trachea excluded from the TNM classification.

	n	**%**
Tumour site		
Oral cavity	41	60.3
Paranasal cavities	6	8.8
Trachea	6	8.8
Nasopharynx	5	7.4
Oropharynx	3	4.4
Ear	4	5.9
Larynx	2	2.9
Oesophagus	1	1.5
T class		
T1	18	26.5
T2	12	17.6
T3	6	8.8
T4	22	32.4
N/A	4	5.9
N class		
N0	54	79.4
N1	1	1.5
N2	3	4.4
N/A	4	5.9
Stage		
I	16	23.5
II	12	17.6
III	7	10.3
IV	23	33.8
N/A	4	5.9

Abbreviations: TNM, tumour, node, metastasis; N/A, not available.

Table 2:

Table 2. Evaluation of the association between MMP-7, -9, and 25 immunoexpression with clinicopathological factors by χ^2 test and Fisher's exact test.

	MMP-7		MMP-9						MMP-25			
	Localization of immunopositivity				Localization of immunopositivity				Intensity of granular cytoplasmic immunopositivity			
	N	C	N	C	PA/Neg	PA/Pos	IC/Neg	IC/Pos	0	1	2	3
Stage I	2 (50.0)	9 (29.0)	5 (50.0)	0 (0)					1 (33.3)	5 (31.3)	3 (21.4)	2 (25.0)
Stage II	0 (0)	5 (16.1)	0 (0)	3 (33.3)					1 (33.3)	3 (18.8)	2 (14.3)	1 (12.5)
Stage III	0 (0)	5 (16.1)	2 (20.0)	1 (11.1)					1 (33.3)	3 (18.8)	2 (14.3)	0 (0)
Stage IV	2 (50)	12 (38.7)	3 (30.0)	5 (55.6)					0 (0)	5 (31.3)	7 (50.0)	5 (62.5)
P-value		0.594		**0.026***								0.760
MMP-9-PA												
PA/Neg							11 (84.6)	13 (43.3)				
PA/Pos							2 (15.4)	17 (56.7)				
P-value								**0.012**				
Primary recurrence												
No	4 (100)	26 (66.7)	9 (90.0)	9 (75.0)	17 (58.6)	19 (86.4)	6 (46.2)	24 (80.0)	3 (75.0)	14 (63.6)	11 (64.7)	8 (100.0)
Yes	0 (0)	13 (33.3)	1 (10.0)	3 (25.0)	12 (41.4)	3 (13.6)	7 (53.8)	6 (20.0)	1 (25.0)	8 (36.4)	6 (35.3)	0 (0)
P-value		0.297*		0.594*		**0.031**		**0.037***				0.244
Regional metastasis												
No	3 (75.0)	36 (92.3)	9 (90.0)	9 (75.0)	28 (96.6)	19 (86.4)	13 (100)	26 (86.7)	3 (75.0)	20 (90.1)	17 (100)	6 (75.0)
Yes	1 (25.0)	3 (7.7)	1 (10.0)	3 (25.0)	1 (3.4)	3 (13.6)	0 (0)	4 (13.3)	1 (25.0)	2 (9.1)	0 (0)	2 (25.0)
P-value		0.334*		0.594*		0.303*		0.297*				0.172
OS												
Alive	3 (75.0)	18 (46.2)	6 (60.0)	8 (66.7)	7 (24.1)	17 (77.3)	4 (30.8)	17 (56.7)	1 (25.0)	7 (31.8)	8 (47.1)	7 (87.5)
Deceased	1 (25.0)	21 (53.8)	4 (40.0)	4 (33.3)	22 (75.9)	5 (22.7)	9 (69.2)	13 (43.3)	3 (75.0)	15 (68.2)	9 (52.9)	1 (12.5)
P-value		0.345*		1.000*		**<0.001**		0.119				**0.043***
DSS												
Alive	3 (75.0)	25 (64.1)	7 (70.0)	10 (83.3)	12 (41.4)	20 (90.9)	7 (53.8)	19 (63.3)	2 (50)	12 (54.5)	10 (58.8)	7 (87.5)
Deceased	1 (25.0)	14 (35.9)	3 (30.0)	2 (16.7)	17 (58.6)	2 (9.1)	6 (46.2)	11 (36.7)	2 (50)	10 (45.5)	7 (41.2)	1 (12.5)
P-value		1.000*		0.624*		**<0.001**		0.559				0.395

P-values were evaluated by chi-square test or Fisher exact test*. Abbreviations: N, nucleus; C, cytoplasm; PA/Neg, Immunonegative pseudocyst areas; PA/Pos, Immunopositive pseudocyst areas; IC/Neg, Immunonegative inflammatory cells; IC/Pos, Immunopositive inflammatory cells; OS, overall survival; DSS, disease-specific survival.

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Table 3. Kaplan-Meier survival analysis.

		OS		*DSS*	
	Immunoscore	***Death (%)***	***Log-rank, P-value***	***Death (%)***	***Log-rank, p-value***
MMP-7	0-1	13 (59.1)	**0.043**	10 (45.5)	**0.025**
	2-3	9 (32.1)		5 (17.9)	
MMP-9	0-1	26 (55.3)	0.778	18 (38.3)	0.883
	2-3	1 (25.0)		1 (25.0)	
MMP-15	0-1	21 (50.0)	0.232	14 (33.3)	**0.041**
	2-3	4 (57.1)		4 (57.1)	
MMP-25	0-1	18 (69.2)	0.282	12 (46.2)	0.644
	2-3	10 (40.0)		8 (32.0)	
MMP-9/PA	Positive	5 (22.7)	**0.020**	2 (9.1)	**0.012**
	Negative	22 (75.9)		17 (58.6)	

Abbreviations: OS, overall survival; DSS, disease-specific survival; PA, pseudocyst areas.

Figure Legends

Figure 1. A: Cytoplasmic immunopositivity of MMP-7 in ACC (arrow). B: MMP-8 immunopositivity in polymorphonuclear leukocytes (arrowhead). C: MMP-9 positivity in the luminal material of pseudocysts of ACC (arrow). D: Cytoplasmic MMP-9 positivity in ACC (arrow). E: MMP-9 positivity in peritumoural inflammatory cells (PMN-cells, lymphocytes, and plasma cells) (arrowhead). F: EMA positivity in true glands of ACC (arrow). G: Cytoplasmic MMP-15 positivity in ACC (arrow). H: Granular cytoplasmic MMP-25 positivity in ACC (arrow). Magnification A and H x400; B, C, D, E, F, and G x200. Abbreviations: MMP, matrix metalloproteinase; ACC, adenoid cystic carcinoma; EMA, epithelial membrane antigen.