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Hämetoja, Hanna

2021-01

Hämetoja , H , Mäkitie , A , Bäck , L , Leivo , I , Haglund , C , Sorsa , T & Hagström , J 2021 , ' Matrix metalloproteinase-7,-8,-9,-15, and-25 in minor salivary gland adenoid cystic carcinoma ' , Pathology, Research and Practice , vol. 217 , 153293 . <https://doi.org/10.1016/j.prp.2020.153293>

<http://hdl.handle.net/10138/337016>

<https://doi.org/10.1016/j.prp.2020.153293>

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

Hanna Hämetoja^{1,2}; Antti Mäkitie^{3,4,5}; Leif Bäck³; Ilmo Leivo⁶; Caj Haglund^{7,8}; Timo Sorsa^{9,10*}; Jaana Hagström^{1,2,7*}

¹ Department of Pathology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland

² Department of Oral Pathology and Radiology, University of Turku and Turku University Hospital, Turku, Finland

³ Department of Otorhinolaryngology - Head and Neck Surgery, University of Helsinki and Helsinki University Hospital, Helsinki, Finland

⁴ Division of Ear, Nose and Throat Diseases, Department of Clinical Sciences, Intervention and Technology, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden

⁵ Research Program in Systems Oncology, Faculty of Medicine, University of Helsinki, Helsinki, Finland

⁶ Institute of Biomedicine, Pathology, University of Turku, Turku, Finland

⁷ Research Programs Unit, Translational Cancer Biology Program, University of Helsinki, Helsinki, Finland

⁸ Department of Surgery, University of Helsinki and Helsinki University Hospital, Helsinki, Finland

⁹ Department of Oral and Maxillofacial Diseases, University of Helsinki and Helsinki University Hospital, Helsinki, Finland

¹⁰ Department of Dental Medicine, Karolinska Institutet, Huddinge, Sweden

*These authors contributed equally to this work.

Corresponding author: Hanna Hämetoja, DDS, Department of Oral Pathology and Radiology, University of Turku – Lemminkäisenkatu 2, 20520 Turku, Finland. Email: hanna.hametoja@helsinki.fi

Word count: 2644

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

27 **Abstract**

28 Knowledge on the role of matrix metalloproteinases (MMPs) in adenoid cystic carcinoma (ACC) is limited.
29 MMPs are capable of degrading almost all extracellular and pericellular components to promote invasion
30 and metastasis. This study aimed to evaluate the immunohistochemical expression of MMP-7, -8, -9, -15,
31 and -25 in ACC and to relate the results with clinicopathological factors and survival.
32 The study included 68 patients with minor salivary gland ACC treated at the Helsinki University Hospital
33 (Helsinki, Finland) in 1974 to 2012. Samples from 52 patients were available, consisting of 44 primary
34 tumours and eight recurrent tumours. We scored immunostaining of MMP-7, -8, -9, -15, and -25 and
35 analysed the immunoscore against clinical and pathological parameters using statistical correlation test.
36 MMP-9 immunoexpression in pseudocysts of ACC and in peritumoural inflammatory cells associated with
37 better survival and fewer treatment failures. High tumoural MMP-7 and -25 associated with better survival.
38 High tumoural MMP-15 associated with poorer survival and high tumoural MMP-9 with advanced stage and
39 regional recurrences. Tumour cells did not show MMP-8 immunopositivity.
40 These results suggest that MMP-9 may contribute to ACC carcinogenesis in different roles. MMP-7, -8, and -
41 9 can stimulate signalling pathways that may promote tissue modulation and metastatic potential. MMP-15
42 and -25 may reflect prognosis.

43
44 **Keywords:** adenoid cystic carcinoma; immunohistochemistry; matrix metalloproteinase, prognosis

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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

79 tumours, such as cancers of the pancreas, lung, cervix, and ovary [5,24]. Furthermore, MMP-9 may also
80 play a suppressive role in cancer progression as observed in colitis-associated colon cancer [25,23].
81 MMP-15 and -25 are membrane-type MMPs (MT-MMPs) responsible for extracellular matrix remodelling
82 and are expressed at the cell surface rather than secreted in a soluble form [26]. MMP-15 has a role in lung
83 cancer progression and recently has been suggested to be a prognostic factor for lung adenocarcinoma
84 [26]. Melanoma cells and surrounding fibroblasts have been shown to secrete MMP-15 during melanoma
85 invasion [27]. MMP-25 secreted by leukocytes and macrophages is an important factor in innate immunity
86 [28] and has been linked to cancer progression [29,30].
87 The aim of our study was to evaluate the immunoexpression of MMP-7, -8, -9, -15, and -25 in a series of
88 ACCs of minor salivary and mucous glands and to compare their immunoexpression with clinical and
89 clinicopathological factors, including patient survival.

90

91 **2. Material and Methods**

92 *2.1 Patients and tumour samples*

93 The study population consisted of 68 (29 [42.6%] men patients and 39 [57.4%] women) with ACC of minor
94 salivary and mucous glands treated at the Helsinki University Hospital (Helsinki, Finland) between the years
95 1974 and 2012. Table 1 shows the main tumour characteristics. Our previous study presented the clinical
96 data of this series [3]. The tumour, node, metastasis (TNM) classification was performed according to the
97 criteria of WHO 2005 and 2017 [31,1]. For immunohistochemical staining, tumour tissues were available
98 from 52 patients, including 44 primary tumours and eight recurrent tumours. The institutional Research
99 Ethics Board approved the study concept (Dnro 31/13/03/02/2010, 01 February 2010).

100 *2.2 Immunohistochemistry*

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

105 Endogenous peroxidase activity was blocked by incubation of the slides with 0.3% Dako REAL Peroxidase-
106 Blocking Solution for 5 minutes. The primary antibody was diluted in Dako REAL Antibody Diluent. The
107 primary antibodies used were mouse monoclonal MMP-7 antibody (1:1000) (EMD Millipore Corporation,
108 Temecula, CA, USA), rabbit polyclonal MMP-8 antibody (1:400) [32], rabbit polyclonal MMP-9 antibody
109 (1:2000) (Calbiochem, Merck KGaA, Darmstadt, Germany), mouse monoclonal MMP-15 antibody (1:250)
110 (EMD Millipore Corporation, Temecula, CA, USA), and rabbit polyclonal MMP-25 antibody (1:300) (Abbexa,
111 Cambridge, UK). The tissues were incubated with primary antibodies for 1 hour except overnight for MMP-
112 8, followed by detection with Dako REAL Detection System (Peroxidase/DAB+, Rabbit/Mouse, Dako,
113 Glostrup, Denmark). Finally, slides were visualized by Dako REAL DAB+ Chromogen or HRP Magenta
114 Chromogen for 10 minutes and counterstained with haematoxylin (Mayer's Hematoxylin Dako, Glostrup,
115 Denmark). As a positive control, we used oral mucosa and pancreas tissue for MMP-7, skin tissue for MMP-
116 8, stomach and oral mucosa tissue for MMP-9, placenta and mammary gland tissue for MMP-15, and colon
117 tissue for MMP-25.

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

124 *2.3 Evaluation of immunostaining*

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

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

136 *2.4 Statistical analysis*

137 The association of MMP-7, -9, -15, and -25 immunoexpression with clinicopathological factors were
138 evaluated by χ^2 test and Fisher's exact test. Overall survival (OS) and disease-specific survival (DSS) between
139 MMPs were analysed by the Kaplan-Meier method and log-rank test. P-values <0.05 were considered
140 statistically significant. Statistical analyses were performed with IBM SPSS Statistics for Windows, version
141 25 (IBM Corp., Armonk, NY).

142

143 **3. Results**

144 *3.1 Gelatinase, MMP-9*

145 MMP-9 immunoexpression was present in 51/52 tumour samples; 30 were scored as negative or very mild,
146 17 as mild, 4 as moderate, and 0 as strong. MMP-9 immunoexpression in ACC tissues was observed in the
147 cytoplasm of the tumour cells. In 24/51 samples MMP-9 was detected in the luminal material of many
148 pseudocysts and in some true glands of ACC. Additionally, MMP-9 was detected in the inflammatory cells
149 (PMN cells, lymphocytes, and plasma cells) surrounding the tumour in 29 samples. MMP-9
150 immunoexpression was negative in most normal salivary gland tissues. Only a few normal salivary gland
151 tissues showed mild MMP-9 immunoexpression in luminal ductal cells.

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

156 inflammatory cells associated with fewer primary recurrences and MMP-9 positivity in pseudocysts (Table
157 2, Figure 1).

158 *3.2 Matrilysin, MMP-7*

159 We evaluated MMP-7 immunoexpression in 50/52 tumour samples; 7 were scored as negative or very mild,
160 15 as mild, 24 as moderate, and 4 as strong. MMP-7 immunoexpression was seen mostly as cytoplasmic
161 positivity in tumour cells. MMP-7 was frequently negative in normal salivary gland tissues as only a few
162 samples showed mild immunopositivity in luminal ductal cells and in acinar structures. In addition, PMN
163 cells, which serve as an internal control for this antibody, were negative.

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

165 *3.3 Collagenase, MMP-8*

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

169 *3.4 MT-MMPs, MMP-15 and -25*

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

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

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

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

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

184

185 **4. Discussion**

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

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

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

208 observed when comparing MMP-9 expression of different salivary gland carcinomas (including ACC) to non-
209 neoplastic salivary gland tissue [39]. This discrepancy could be due to the fact that we used
210 immunohistochemistry while Kayano et al. analysed tumour homogenates by gelatin zymography [39].
211 In the present series, the microenvironment of ACC tissue contained mild to moderate numbers of
212 inflammatory cells. In general, salivary gland carcinomas lack an abundant inflammatory cell stromal
213 reaction, which is more commonly seen in oral SCC. Mosconi et al. have suggested that a low level of
214 immune cell infiltration in the ACC microenvironment may relate to poor prognosis [40]. According to our
215 results, MMP-9 immunoexpression in inflammatory cells seems to have a protective role, as MMP-9
216 positivity in peritumoural inflammatory cells associated with fewer primary treatment failures. Similarly,
217 Pujada et al. have shown that in colitis-associated colorectal cancer, MMP-9 may modulate inflammatory
218 cytokine levels, maintain efficient barrier function and integrity of tight junctions, and act as a tumour
219 suppressor [23]. Interestingly, MMP-9 immunoexpression in pseudocyst structures of ACC had a significant
220 correlation with better survival and fewer primary disease failures. In our study MMP-9 immunoexpression
221 in pseudocyst structures of ACC and in inflammatory cells correlated significantly. We might speculate that
222 when secreted to the pseudocyst structures, MMP-9 might not take an active role in modulating the
223 surrounding tissues. Considering the survival time and lower amount of recurrent tumours, both
224 pseudocyst positivity and peritumoural inflammatory cell positivity showed a protective association in the
225 present ACC series.

226 Increased immunoexpression of MMP-7 in salivary gland acinic cell carcinoma and mucoepidermoid
227 carcinoma has previously been linked to better survival [19]. According to our study, high MMP-7
228 immunoexpression in ACC associated with both longer OS and DSS. Luukkaa et al. suggested that poorly
229 differentiated tumour cells could have diminished MMP-7 expression [19]. In the present study, normal
230 salivary gland tissue was frequently negative for MMP-7, although tumour tissues showed cytoplasmic
231 MMP-7 immunoexpression. In gastric, colorectal, and tongue cancers, earlier studies have shown that high
232 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

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

261

262

263 **Ethical approval**

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

266 **Funding**

267 This study was supported by the Helsinki University Hospital Research Fund.

268 **Declaration of Competing Interest**

269 The authors declare no conflict of interest.

270 **CRedit authorship contribution statement**

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

277 **Acknowledgements**

278 This work was supported by Finnish Dental Society Apollonia (H.H).

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411 **Table Legends**

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

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

416 Table 3. Kaplan-Meier survival analysis.

417

418 **Figure Legends**

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

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. 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.

Table 3. Kaplan-Meier survival analysis.

		<i>OS</i>		<i>DSS</i>	
	<i>Immunoscore</i>	<i>Death (%)</i>	<i>Log-rank, P-value</i>	<i>Death (%)</i>	<i>Log-rank, p-value</i>
<i>MMP-7</i>	0-1	13 (59.1)	0.043	10 (45.5)	0.025
	2-3	9 (32.1)		5 (17.9)	
<i>MMP-9</i>	0-1	26 (55.3)	0.778	18 (38.3)	0.883
	2-3	1 (25.0)		1 (25.0)	
<i>MMP-15</i>	0-1	21 (50.0)	0.232	14 (33.3)	0.041
	2-3	4 (57.1)		4 (57.1)	
<i>MMP-25</i>	0-1	18 (69.2)	0.282	12 (46.2)	0.644
	2-3	10 (40.0)		8 (32.0)	
<i>MMP-9/PA</i>	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.