# Expression of Submaxillary Gland Androgen-regulated Protein 3A (*SMR3A*) in Adenoid Cystic Carcinoma of the Head and Neck

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**Abstract.** *Background: Adenoid cystic carcinoma of the head and neck (ACC) is a rare tumor entity which originates from the salivary glands. The prognosis remains poor, as the tumor tends to exhibit perineural invasion and frequently develops distant metastases. The submaxillary gland androgen-regulated protein 3A (SMR3A) belongs to a gene family producing opiorphin homologs and is physiologically secreted by salivary glands. Expression of SMR3A has been identified as an unfavorable risk factor in survival of patients with squamous cell carcinoma in the head and neck, but its value as a prognostic biomarker for ACC has not been addressed. Materials and Methods: Tissue sections from primary ACC (n=86) and healthy glandular tissue as reference, were stained by immunohistochemistry. SMR3A expression levels were correlated with clinical and pathological features, including overall survival. Results: All patients had undergone surgery and 67 received adjuvant radiotherapy. The median disease-free survival (DFS) was 37 months and the median overall survival (OS) was 75 months. Prominent SMR3A expression in tumor cells was found in 24 of 86 patients (27,9%), and was inversely correlated with a male gender (p=0.009). There was no significant correlation between SMR3A expression and DFS, metastasis-free survival or OS. Conclusion: Our data demonstrate for the first time decreased levels of SMR3A in ACC compared to normal glandular tissue. These data suggest a context-dependent regulation of SMR3A expression in the pathogenesis of distinct subtypes of head and neck tumors, and support the assumption that detection of SMR3A expression serves as a surrogate for aberrant differentiation into mucosal- or glandular-like cells in ACC and head and neck squamous cell carcinoma.*

Adenoid cystic carcinoma of the head and neck (ACC) is a common salivary gland malignancy, but a rare tumor entity with an incidence of only 1% considering all head and neck malignancies (1, 2). Its growth pattern is characterized by infiltrative perineural invasion and early formation of distant metastases (3, 4). These tumors are mostly localized in the major and minor salivary glands of the head and neck area, but also in the oral and the nasal cavity. The standard-of-care remains radical tumor resection together with adjuvant radiotherapy. Current efforts to unravel the biological and genomic characteristics of these tumors have been constrained by the low incidence of ACC (3). Therefore, prognostic biomarkers are urgently needed for better identification of patients at high risk of treatment failure, and to support the establishment of novel targeted therapy.

The family of opiorphins, a class of extracellular pentapeptides, are known to play an important role in physiological processes, such as central pain perception, homeostasis and peripheral inflammatory events (5, 6).

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Table I. *Scoring system used to evaluate percentage of positively stained cells in the stained slides.*

| | Intensity | | |
|---|---|---|---|
| Score | 1 | 2 | 3 |
| Weakly positive=1 | >10% | ≤30% | - |
| Moderately positive=2 | >70% | >30% | ≤30% |
| Strongly positive=3 | - | >70% | >30% |

Table II. *Clinical parameters of the analyzed cohort.*

| Factor | n | % |
|---|---|---|
| Localization | | |
| Glandular | 40 | 46.5 |
| Extraglandular | 46 | 53.5 |
| T-Stage | | |
| T1-2 | 30 | 41.1 |
| T3-4 | 43 | 59.9 |
| Missing data | 13 | |
| N-Stage | | |
| N0 | 58 | 80.6 |
| N+ | 14 | 19.4 |
| Missing data | 14 | |
| M-Stage | | |
| M0 | 67 | 95.7 |
| M1 | 3 | 4.3 |
| Missing data | 16 | |
| Perineural invasion | | |
| Pn0 | 18 | 29 |
| Pn1 | 44 | 71 |
| Missing data | 24 | |
| Therapy | | |
| Surgery | 16 | 19.3 |
| Surgery + radiation | 67 | 80.7 |
| Missing data | 13 | |
| Local recurrence | | |
| None | 41 | 52.6 |
| Present | 37 | 47.4 |
| Missing data | 8 | |
| Distant metastasis during follow-up | | |
| None | 47 | 61.8 |
| Present | 29 | 38.2 |
| Missing data | 10 | |

Opiorphins were first isolated from human saliva and three genes are known to encode opiorphin homologs in humans: Proline rich, lacrimal 1 (*PROL1*), submaxillary gland androgen-regulated protein 3A (*SMR3A*) and *SMR3B*. Recently, we identified an increased expression of SMR3A in recurrent tumors of an orthopic floor-of-the-mouth mouse tumor model after surgery (7). Furthermore, high SMR3A expression correlated with a poor clinical outcome of patients with oropharyngeal squamous cell carcinoma (8). SMR3A expression has not yet been addressed in the pathogenesis and clinical outcome of ACC. Since opiorphins can be determined in human saliva and *SMR3A* seems to be a candidate gene implicated in tumor progression, our aim was to assess SMR3A expression in patients with an ACC of the head and neck.

## Materials and Methods

*Patient material*. Paraffin-embedded tissue from 86 patients who were diagnosed with an ACC of the head and neck region at the University Hospitals of Ulm, Cologne and Pittsburgh were included in this multicenter and retrospective cohort study. Approval was given by the Institutional Review Board (no. 374/13, Ulm). Clinical data were collected and included basic demographic data such as age and gender, date of diagnosis, initial symptoms, time to recurrence, type or combination of therapy, death and last follow-up. The overall survival (OS) was assessed from the time of initial diagnosis (date of pathological report) to last follow-up or date of death. The disease-free survival (DFS) was assessed from the initial curative treatment effort until the time of radiologically or histologically proven recurrence.. Our findings in the immunohistochemical analysis were correlated to the clinical data.

*Immunohistochemical staining*. For immunohistochemical analysis, 3.5 μm sections were cut from paraffin-embedded tissues and stained according to the kit manufacturer's instructions. Heat-induced antigen retrieval was carried out by steaming for 30 min in 10 mM citrate buffer, pH 6 (Multi Gourmet Steamer, Braun, Germany). Immunohistochemistry was conducted with an anti-human SMR3A antibody (ab97942, dilution 1:50; Abcam, Cambridge, UK) using the TSA Amplification Kit (Perkin Elmer, Hamburg, Germany). Counterstaining was carried out with hematoxylin solution modified according to Gill III (Merck Darmstadt, Germany) to visualize tissue integrity.

A semi-quantitative score was used by two independent observers to evaluate the final SMR3A expression level, taking into account the relative proportion of stained tumor cells (%), and the staining intensity (ranging from 1 to 3: score 1=weak staining, score 2=moderate staining, and score 3=strong staining). Negative controls omitting the application of primary antibodies were used in every assay. The resulting scoring is shown in Table I. Patient sub-groups were arranged according to SMR3A expression. Expression was defined as positive if at least 10% of tumor cells showed positivity with an intensity of at least score 1. Tumor samples with a positive immunohistochemical staining rate of fewer than 10% of tumor cells or a staining intensity of 0 were regarded as negative. The expression of SMR3A was correlated to the clinical data of each patient. Normal salivary tissue from the parotid gland served as a positive control and was always stained together with the tumor slides.

*Statistical analysis*. For statistical analysis, SPSS (version 21; IBM, Armonk, NY, USA) statistics software was used. Differences between groups were assessed using Chi square test. Overall survival was calculated as the time of initial diagnosis until the date of cancer-related death within the follow-up interval (event). DFS was calculated from the initial tumor therapy until the date of the first local recurrence, lymph node or distant metastasis, second

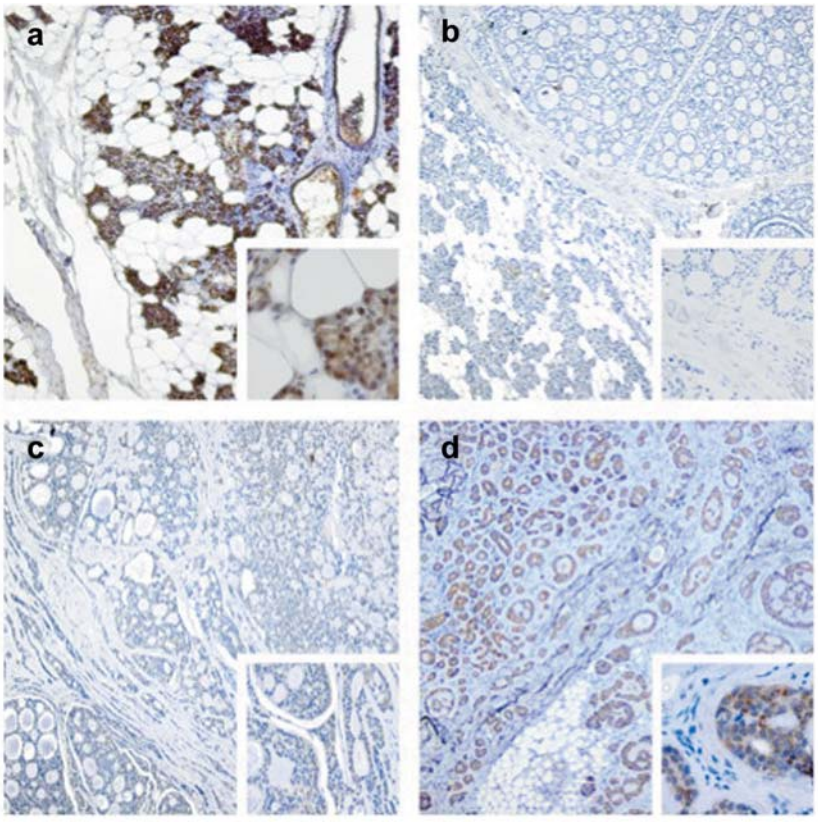


Figure 1. *Microscopic view of tumor samples (magnification 20×/40×): Submaxillary gland androgen-regulated protein 3A (SMR3A) protein expression in adenoid cystic carcinoma of the head and neck (ACC). Representative images of immunohistochemical staining for SMR3A (brown signal) in positive control tissue of normal parotid gland (a), ACC of the submandibular gland without SMR3A expression (b), nasal sinuses with low SMR3A expression (c), and the glandular parotis with high SMR3A expression (d). Counterstaining with hematoxylin was used to visualize tissue architecture.*

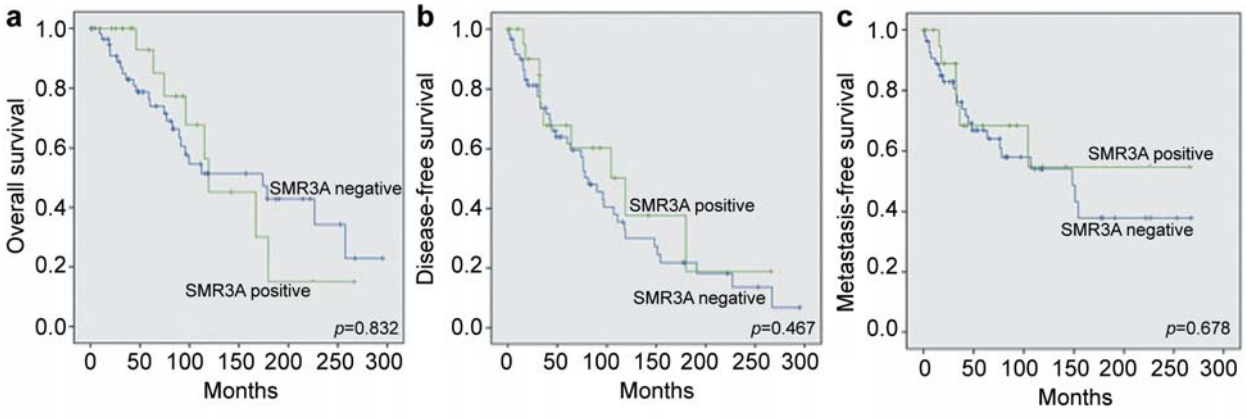


Figure 2. *Kaplan-Meier-analysis of overall survival (OS) (a), disease-free survival (DFS) (b), and metastasis-free survival (MFS) (c) for patients with adenoid cystic carcinoma of the head and neck (ACC) and positive or negative immunohistochemical staining of submaxillary gland androgen-regulated protein 3A (SMR3A). p-Values were calculated by the log-rank test.*

primary carcinoma, or date of cancer-related death within the follow-up period (events). Patients without recurrence (no event) or cancer-unrelated death were censored. Kaplan–Meier curves were calculated to estimate survival distributions and log-rank tests were used to test for statistical significant differences between groups. Univariate analyses were performed using the Fisher's exact or Chi square test. In all statistical tests, a *p*-value of 0.05 or below was considered statistically significant.

*Table III. Submaxillary gland androgen-regulated protein 3A (SMR3A) expression in adenoid cystic carcinoma of the head and neck (ACC) and clinical features. Cross-table analysis (Chi-square test) of different clinical parameters and SMR3A expression status.*

| | SMR3A | | |
|---|---|---|---|
| Factor | Negative | Positive | *p*-Value |
| Age (years) | | | |
| <54.3 | 31 | 11 | 0.729 |
| ≥54.3 | 31 | 13 | |
| Gender | | | |
| Male | 35 | 6 | 0.009* |
| Female | 27 | 18 | |
| Localization | | | |
| Glandular | 29 | 11 | 0.937 |
| Extraglandular | 33 | 13 | |
| Status at end of study | | | |
| Alive | 34 | 15 | 0.529 |
| Dead | 25 | 8 | |
| N-Stage | | | |
| N0 | 42 | 16 | 0.941 |
| N+ | 10 | 4 | |
| T-Stage | | | |
| T1 | 11 | 3 | 0.792 |
| T2 | 12 | 4 | |
| T3 | 7 | 5 | |
| T4 | 22 | 9 | |
| Recurrence status | | | |
| No recurrence | 26 | 14 | 0.171 |
| Recurrence | 30 | 8 | |
| Perineural invasion | | | |
| Positive | 33 | 11 | 0.504 |
| Negative | 12 | 6 | |
| Cervical metastasis | | | |
| Positive | 10 | 4 | 0.941 |
| Negative | 42 | 16 | |
| Distant metastasis | | | |
| Positive | 23 | 6 | 0.288 |
| Negative | 32 | 15 | |

Significant p-values (≤0.05) are marked with an asterisk.

## Results

Among 86 patients, 45 were females and 41 males, and the primary tumors were localized in the parotid gland in 32.6%, submandibular gland in 10.5%, sublingual gland in 3.5%, the small salivary glands of the palate in 9.5% or the base of the tongue in 4.8%, or extraglandular tissue such as the paranasal sinuses (21.4%), or the midface or lacrimal glands (15.5%). The mean age was 54.3 years at initial diagnosis, and 47% had a tumor recurrence during follow-up. All patients had undergone surgery and 80.7% of cases were treated with adjuvant radiotherapy. The Kaplan–Meier survival analysis showed a probability for 1-year OS of 92% and for 5-year OS of 53.7%. The median OS duration was 75 months, while the median DFS was 37 months. Three patients had distant metastases at initial diagnosis and 38.2% developed distant metastases during follow-up. The histopathological analysis reported perineural invasion in 71% of all cases and as expected, patients with perineural invasion or distant metastases during follow-up had a significantly worse clinical outcome ($p=0.007$ and $p<0.0001$, respectively). Clinical parameters of the patients are listed in Table II.

Healthy tissue of parotid glands (n=10) were obtained from adenoma surgery and exhibited consistently strong staining in secretory and ductal cells (Figure 1a). In contrast, cells of connective and fat tissue were negative for SMR3A expression. Immunostaining was specifically found in tumor cells of ACC, but not in any stromal cell of the tumor microenvironment (Figure 1d). Most tumor cells exhibited cytoplasmatic staining, whereas normal glandular cells presented nuclear SMR3A staining (Figure 1a). Positive staining for SMR3A was observed in 29% (25/86) of all ACC analyzed, and 20.9% (18/86) had a moderately or strongly positive result (score 2 or score 3, Table I).

Missing values in the cross-table analysis are due to missing information on the patient's clinical data. Comparison with clinical and pathological features revealed no correlation between positive SMR3A expression and age, localization, status of metastasis, perineural invasion, TNM stage or DFS and OS (Table III). However, SMR3A expression was significantly correlated with gender ($p=0.009$) with only six males having a SMR3A positive staining pattern (Table III). Kaplan–Meier survival analysis was used to evaluate, whether SMR3A serves as a prognostic marker for ACC patients. Our results did not show any significant correlation between overall, disease-free or metastasis-free survival and SMR3A expression (Figure 2). Interestingly, patients with SMR3A over-expression had fewer recurrences.

Adenoid cystic carcinoma of the head and neck represents a rare tumor entity, which tends to form hematogenous metastases. Due to the low efficacy of available systemic therapies and its often poor surgical outcome concerning patient's quality of life, alternatives such as targeted therapy, as well as prognostic biomarkers for patients at high risk of treatment failure, are urgently needed.

In recent years, targeted therapies have been implemented in standard treatment options for many human malignancies. New approaches modulating the immune checkpoint control carry the potential to revolutionize the therapy of advanced and disseminated solid tumors, as shown in malignant melanoma (9). In this context, it seems essential to further investigate key alterations in cellular and molecular principles in the pathogenesis of ACC in order to identify novel drug targets for alternative or adjuvant treatment strategies.

Several publications have addressed SMR3A and its association with erectile physiology, sexual behavior in male rats, and metabolic disorders such as diabetes (10-13). An involvement in recurrent tumor formation was demonstrated for the first time in an orthotopic floor-of-mouth mouse model for tumor relapse after surgery (7). Applying global gene-expression profiling, the murine homolog of *SMR3A* was found to be up-regulated in matched local recurrences compared to primary tumors derived from this animal model (14). Furthermore, high SMR3A expression was identified as an unfavorable risk factor for the survival of patients with oropharyngeal squamous cell carcinoma (8). However, the causual role of SMR3A in tumor development and malignant progression remains poorly understood. In contrast to head and neck squamous cell carcinoma (HNSCC), where SMR3A exhibits only minor expression in normal tissue and strong up-regulation in a substantial proportion of primary tumors, its expression is high in normal glandular tissue and – in our dataset – was found to be lost during the pathogenesis of approximately 70% of ACCs. These data suggest a context-dependent regulation of SMR3A expression and function which is related to the tissue of origin and might be influenced by intrinsic and extrinsic factors. In line with this assumption, SMR3A expression has opposing consequences on the clinical outcome, as high SMR3A levels are associated with unfavorable prognosis of patients with HNSCC (8), whereas this effect was not observed for patients with ACC. This raises an attractive hypothesis in which loss of SMR3A expression is a consequence of de-differentiation from glandular into more epithelial-like cells during pathogenesis of ACC, while its gain in HNSCC might indicate a partial trans-differentiation into a glandular phenotype, reflecting higher resistance to adjuvant systemic therapy such as cisplatin or cetuximab. This trans-differentiation has already been observed and described in ACC with concurrent invasive HNSCC (15). Experimental and clinical validation of this hypothesis in appropriate preclinical models and larger patient cohorts will be a major challenge.

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