The PI3K/AKT/mTOR signalling pathway is active in salivary gland cancer and implies different functions and prognoses depending on cell localisation

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Introduction

The PI3K/AKT/mTOR signalling axis represents an important regulator of cellular proliferation, survival and motility and is involved in different types of cancers with gain or loss of function leading to neoplastic transformation.[1] AKT, also called protein kinase B (PKB), a serine/threonine protein kinase, is a downstream mediator of phosphatidylinositol-3-kinase (PI3K) and is activated by growth factor stimulation and subsequent activation of receptor tyrosine kinases at the cell membrane.[2] There are three family members of AKT, namely AKT-1, AKT-2 and AKT-3 that are ubiquitously distributed. Among various cellular functions, activated AKT-1 can phosphorylate mammalian target of rapamycin (mTOR), which regulates cell proliferation by phosphorylation of p70S6 in the G1/S phase transition of the cell cycle.[3] P70S6 phosphorylates the S6 protein of the 40S ribosomal subunit leading to protein synthesis.[4] As some components, e.g. mTOR, may function as targets for inhibitor therapy, the PI3K/AKT/mTOR pathway has evoked interest during the last years.[5,6]

Salivary gland carcinomas are rare tumours characterised by an enormous morphological diversity between different subtypes going along with variable clinical courses.[7] To date there is little information about the PI3K/AKT/mTOR signalling pathway in these tumours. This study investigates expression patterns of PI3K, phospho-AKT, phospho-mTOR and phospho-S6 ribosomal protein as major components of that pathway in malignant salivary gland tumours by immunohistochemistry. Possible clinical implications and the impact on survival are highlighted.

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Materials and methods

The study comprises 272 major and minor salivary gland carcinomas diagnosed at the Departments of Pathology of Regensburg University, Erlangen University and Nuremberg City Hospital between 1984 and 2008. The medical records were obtained from the clinical tumour registries of Regensburg and Erlangen-Nuremberg. The registries and the related translational research activities are covered by ethical vota of the medical faculties of the Universities of Regensburg and Erlangen-Nuremberg.

Clinical patient characteristics are presented in Table 1. Mean age at diagnosis was 60.4 (11–98) years. Patients were staged according to the TNM system of the Union for International Cancer Control (UICC) 2002.[8] One hundred and forty nine patients (54.8%) presented with advanced (III, IV) UICC tumour stages. All patients underwent primary surgery, concomitant lymph node dissection was carried out in 171 patients (62.9%). Adjuvant radiotherapy (mean dose 58.6 Gy, $n = 121$) or radiochemotherapy (mainly platinum-based with 5-fluorouracil, $n = 40$) was performed in 161 cases (59.2%) with high-grade malignancy, positive margins, lymph node metastases or distant metastases. Single Chemotherapy (mainly taxotere and capecitabine, $n = 10$) was used for palliative metastatic treatment.

The mean follow up of all patients was 4.85 (0.4–24.5) years.

Histology and classification

The haematoxylin–eosin slides from paraffin wax-embedded tumours of all patients have been independently reviewed by two experienced pathologists without knowledge of the initial diagnosis. All tumours were classified according the contemporary World Health Organization's (WHO) classification of salivary gland tumours (Table 2).[7] All cases of squamous cell carcinoma were classified as primitive of the salivary glands after intensive staging procedures (CT or MRI of the head and neck, panendoscopy, X-ray or CT of the chest and ultrasonograpy of the abdomen) and exclusion of a metastasis to the salivary gland. Squamoid variants of mucoepidermoid carcinomas were thoroughly sorted out.[9] The less frequent entities BCAC, EMC, OCC, MMT, UC, CAC and SC were summarised as "others" in Table 3. Grading was based on a three-tiered grading system (Table 2).[10,11] ACCC, BCAC, EMC, CAC and PLGA were considered low grade (G1) with the exception of dedifferentiated tumours which were classified high grade (G3). In contrast, SDC, MMT, OCC, UC and LCC were classified high grade (G3). MECs were graded according to the criteria proposed in the current WHO classification (AFIP).[12] ADCCs were divided into predominantly tubulocribriform (G2) and predominantly solid (G3) tumours.[13] Grading of ACNOS and MYEC was based on nuclear pleomorphism and mitotic activity similar to the Elston and Ellis grading of breast cancer.[14] SQCCs were graded according to the grading principles for SQCC of the head and neck. The 33 cases of carcinoma ex pleomorphic adenoma were classified and graded according to the malignant component of the tumour. From 67 out of the 272 patients normal salivary gland tissue without tumour growth was obtained from sound margins of the excised gland during surgery and evaluated as control tissue (Table 2). This group comprised 36 male and 31 female patients with a mean age of 59.0 (range 11–98) years. The controls were gained from the parotid ($n = 38$, 56.7%), the submandibular ($n = 7$, 10.4%) and the minor glands ($n = 22$, 32.8%). Twenty eight (41.8%) of these patients presented with localised (I–II) tumour stages and 39 (58.2%) with advanced (III–IV) tumour stages. Forty one (61.2%) patients suffered from low and intermediate grade malignancies, 26 (38.8%) patients from high-grade carcinomas.

Immunohistochemistry

A tissue microarray (TMA) with 2.0 mm diameter punch cores was constructed from formalin-fixed paraffin-embedded tissue blocks of all patients as previously described.[15] Haematoxylin–eosin-stained TMA sections were used for reference histology. Immunostaining of P-AKT, P-mTOR, PI3K and p-S6rp was performed on 5 μm sections of the TMAs and applied according to manufacturer's instructions. In brief, following dewaxing, washing and rehydration of the slides through xylene and graded alcohols, microwave heating in citrate buffer was used for antigen retrieval. Endogenous peroxidase was blocked in ChemMate peroxidase-blocking solution (Dako). Antibodies were used as follows: *Phospho-AKT (Ser473, AKT-1)* – Cell Signalling #4060, monoclonal rabbit, dilution 1:50; *Phospho-mTOR (Ser2448)* – Cell Signalling #2976, monoclonal rabbit, dilution 1:50; *PI3Kinase (p110α)* – Cell Signalling #4249, monoclonal rabbit, dilution 1:100; *Phospho-S6 Ribosomal Protein* (*Ser235/236*) – Cell Signalling #4858, monoclonal rabbit, dilution 1:50. EnVision™ Dual Link System (Dako) was used as detection system.

With view to the staining patterns of P-AKT, P-mTOR, PI3K and P-S6rp in the different subtypes, tumours were similarly stained according to their differentiation. In acinic cell carcinomas, expression was mainly found in acinic cells but also in some ductal cells. In adenoid cystic carcinomas, staining was predominantly observed in ductal but also in some myoepithelial cells. Mucoepidermoid carcinomas also predominantly showed ductal expression of the markers while salivary duct carcinomas exclusively presented ductal staining.

Immunohistochemistry was semiquantitatively evaluated based on nuclear and cytoplasmic/membranous reactivity (Fig. 1). An immunoreactive score (IRS) was built as the product of staining intensity (none = 0, weak = 1, moderate = 2, strong = 3) and the percentage of positive tumour cells (0–100%) resulting in an IRS ranging from 0 to 300 points. For proper semiquantitative evaluation of the staining results dichotomisation was performed referring to the mean immunoreactive score of the markers (see Results). In case of P-AKT and P-mTOR, tumours showing any expression (IRS $\geqslant$ 1pts) were considered as positive while tumours completely lacking any staining were assessed as negative. For PI3K and P-S6rp tumours with an IRS ranging from 0 to 99 points were determined as to be negative while those showing an IRS ranging from 100 to 300 points were considered as to be positive. Immunostaining patterns of P-AKT ($n = 67$), P-mTOR ($n = 67$) PI3K ($n = 66$) and P-S6rp ($n = 59$) were also documented in the normal salivary gland control tissues. In the controls, the investigated markers were expressed in both acinic and ductal cells. No significant ($p > 0.05$) associations between immunostaining of the markers and the clinico-pathological parameters age, grade, TNM stage or disease outcome were found. However there were some staining correlations between the controls and the carcinomas with view to histology (see Results).

Statistical analysis

All clinical and immunohistochemical data were analysed with SPSS for Windows, version 15.0 (SPSS, Erkrath, Germany). Relationships between dichotomised immunohistochemical markers and clinicopathologic factors were examined using Fisher's exact probability test ($p < 0.05$). Correlations between immunohistochemical markers were assessed by bivariate analysis using Pearson coefficient. Univariate survival curves were calculated by the Kaplan–Meier method and distributions were compared using the log-rank test. Disease-specific survival (DSS) was calculated from the date of diagnosis until disease-caused death or end of follow up. Cox proportional hazards model (enter method) was used in multivariate analyses.

Table 1
Clinical variables of patient population.

	Tumour localisation				
	Parotid	Submandibular	Minor	Sublingual	Total
Total	190	40	41	1	272
Sex					
Male	97 (51.5%)	14 (35.0%)	20 (48.8%)	1 (100%)	132 (48.5%)
Female	93 (48.9%)	26 (65.0%)	21 (51.2%)	0	140 (51.5%)
Size					
T1–T2	111 (59.0%)	26 (65.0%)	25 (64.1%)	1 (100%)	163 (60.8%)
T3–T4	77 (41.0%)	14 (35.0%)	14 (35.9%)	0	105 (39.2%)
Lymph nodes					
N0	127 (68.3%)	25 (62.5%)	28 (73.7%)	1 (100%)	181 (68.3%)
N1–N3	59 (31.7%)	15 (37.5%)	10 (26.3%)	0	84 (31.7%)
Distant metastases					
M0	169 (89.45)	35 (87.5%)	35 (89.7%)	1 (100%)	240 (89.2%)
M1	20 (10.6%)	5 (12.5%)	4 (10.3%)	0	29 (10.8%)
Grading					
Low/intermediate	92 (48.4%)	19 (47.5%)	35 (85.4%)	0	146 (53.7%)
High	98 (51.6%)	21 (52.5%)	6 (14.6%)	1 (100%)	126 (46.3%)
Surgical treatment					
Local tumour resection	13 (6.8%)	0	41 (100%)	1 (100%)	55 (20.2%)
Surperficial parotidectomy	31 (16.4%)	0	0	0	31 (11.4%)
Total parotidectomy	146 (76.8%)	0	0	0	146 (53.7%)
Submandibulectomy	0	40 (100%)		0	40 (14.7%)
Neck dissection					
No	39 (20.6%)	12 (30.0%)	27 (65.9%)	1 (100.0%)	79 (29.3%)
Yes	150 (79.4%)	28 (70.0%)	14 (34.1%)	0	191 (70.7%)
Residual tumour					
Negative (R0)	147 (79.0%)	31 (81.6%)	28 (71.8%)	0	206 (78.0%)
Microscopic (R1)	29 (15.6%)	6 (15.8%)	10 (25.6%)	1 (100%)	46 (17.4%)
Macroscopic (R2)	10 (5.4%)	1 (2.6%)	1 (2.6%)	0	12 (4.5%)
Radio(chemo)therapy					
No	72 (37.9%)	15 (37.5%)	23 (56.1%)	1 (100%)	111 (40.8%)
Yes	118 (62.1%)	25 (62.5%)	18 (43.9%)	0	161 (59.2%)
Recurrence[a]					
No	134 (74.4%)	31 (79.5%)	28 (70.0%)	0	193 (74.2%)
Yes	46 (25.6%)	8 (20.5%)	12 (30.0%)	1 (100%)	67 (25.8%)

[a] Only curative R0 and R1 resections.

Results

P-AKT

P-AKT staining was observed in both the nuclei and the cytoplasm (Fig. 1, Table 3). One hundred and forty eight tumours (59.9%) showed nuclear expression of P-AKT (IRS $\geqslant$ 1 pts) with a mean immunoreactive score (IRS) of 11.1 points in contrast to an IRS of 1.5 points in normal salivary gland tissue. Nuclear positivity was frequently found in ACCC (74.3%), MEC (70.6%), MYEC (66.7%) and PLGAC (80.0%). SQCC were negative for *n*P-AKT in 66.7%. Absent (negative) P-AKT expression was associated with increased age (>70, $p = 0.45$), high tumour stages ($p = 0.018$) and lymph node metastases ($p = 0.034$). Negative nuclear staining of P-AKT was strongly associated ($p < 0.001$) with lower survival rates (5-year-DSS 63.5%) in comparison to positive *n*P-AKT staining (5-year-DSS 83.6%) in univariate analysis (Fig. 2). Moreover, negative nuclear P-AKT staining demonstrated unfavourable survival in the group of high-grade tumours (5-year DSS 42.0 in comparison to 70.5% for *n*P-AKT positivity, $p = 0.002$, Fig. 2). Absent nuclear P-AKT expression also presented a strong negative predictor in multivariate analysis ($p < 0.001$, Table 4).

Cytoplasmic expression of P-AKT (IRS $\geqslant$ 1 pts) was detected in 45 out of 257 tumours (18.2%, Table 3) with a mean IRS of 7.0 points in contrast to a mean IRS of 0.65 for normal salivary control tissue. In three cases additional membrane staining was recorded.

Table 2
Histology, grading and salivary gland control tissue.

Histology	Grade			Total	Control tissue[b]
	1	2	3		
Acinic cell carcinoma (ACCC)	31	0	6[a]	37	10
Adenoid cystic carcinoma (ADCC)	0	37	11	48	15
Mucoepidermoid carcinoma (MEC)	26	3	11	40	12
Salivary duct carcinoma (SDC)	0	0	29	29	6
Adenocarcinoma NOS (ACNOS)	1	5	28	34	7
Squamous cell carcinoma (SQCC)	0	12	12	24	5
Myoepithelial carcinoma (MYEC)	4	6	8	18	1
Polymorphous low grade adenocarcinoma (PLGAC)	11	0	0	11	6
Basal cell adenocarcinoma (BCAC)	5	0	2[a]	7	1
Epithelial myoepithelial carcinoma (EMC)	2	0	2[a]	4	0
Oncocytic carcinoma (OCC)	0	0	8	8	1
Malignant mixed tumour (MMT)	0	0	5	5	1
Undifferentiated carcinoma (UC)	0	0	4	4	1
Cystadenocarcinoma (CAC)	2	0	0	2	1
Sebaceous carcinoma (SC)	1	0	0	1	0
Total	83	63	126	272	67

[a] Dedifferentiated carcinoma.
[b] Non-tumourous salivary gland control tissue from patients with a salivary gland carcinoma.

Cytoplasmic P-AKT was most frequently expressed in ACCC (22.9%), in SDC (28.6%) and in ACNOS (24.2%). Cytoplasmic P-AKT

Table 3
P-AKT, P-mTOR, PI3K, P-S6rp and clinicopathological parameters.

Histology	P-AKT				P-mTOR				PI3K		P-S6rp	
	Nuclear		Cytopl.		Nuclear		Cytopl.[a]		Cytopl.		Cytopl.	
	neg	pos	neg	pos	neg	pos	neg	pos	0–99	100–300	0–99	100–300
Total	99	148	202	45	196	35	144	87	81	156	127	107
ACCC	9	26	27	8	29	7	8	28	10	26	21	15
ADCC	22	22	36	8	34	8	36	6	13	30	27	12
MEC	10	24	32	2	33	6	31	8	15	21	17	18
SDC	13	15	20	8	16	1	8	9	10	13	7	15
ACNOS	16	17	25	8	21	3	8	16	3	22	14	14
SQCC	14	7	18	3	21	1	15	7	9	12	8	13
MYEC	4	11	12	3	10	4	10	4	7	11	8	9
PLGAC	2	8	10	0	9	0	5	4	6	2	5	4
Others	9	17	21	26	22	5	22	5	8	18	19	7
Age												
<70	55	101	130	26	122	25	90	57	53	98	90	58
>70	44	47*	72	19	74	10	54	30	28	58	37	49**
Grade												
Low/intermediate	46	86	116	16	112	22	81	53	51	82	74	55
High	53	62	86	29**	86	13	64	35	30	74	53	52
Stage												
I–II	35	75	95	15	86	22	64	44	38	72	69	37
III–IV	64*	71	105	30	108	13	79	42	42	83	56	70**
T-stage												
T1–T2	54	97	127	24	118	24	78	64	51	90	85	52
T3–T4	43	49	71	21	74	11	64**	21	28	64	38	55**
N-stage												
N0	59	106	140	25	125	31	96	60	55	110	94	67
N1–3	38*	37	57	18	64**	4	44	24	23	42	29	38*
M-stage												
Mneg	84	135	181	38	167	34	126	75	73	136	109	96
Mpos	15	11	19	7	26	1	16	11	7	18	15	11
Residual tumour												
No	67	118	154	31	143	31	104	70	60	123	98	80
Yes	28	27	43	12	47	4	36	15	18	29	24	24
Recurrence												
No	63	111	146	28	136	31	126	75	58	109	93	74
Yes	30	31	49	12	50	4	16	11	19	40	29	28

[a] Cytoplasmic and/or membrane staining.
* $P \leqslant 0.05$.
** $P \leqslant 0.01$.

expression was rare in MEC (5.9%) but went along with worse survival in this group ($p = 0.010$). In all tumours positivity of cP-AKT was correlated to high-grade malignancy ($p = 0.009$). In univariate analysis positive cP-AKT staining was associated ($p = 0.039$) with unfavourable survival (5-year-DSS 61.7%) in comparison to negative staining (5-year-DSS 78.8%, Fig. 2). Within the subtypes there were no further significant correlations of P-AKT with clinico-pathological parameters.

P-mTOR

Expression of P-mTOR was detected as nuclear, cytoplasmic and membrane staining (Fig. 1). Thirty five tumours (15.2%) demonstrated nuclear staining of P-mTOR (IRS $\geqslant$ 1 pts) with a mean IRS of 3.8 points whereas all normal salivary gland controls were negative for nuclear P-mTOR. ACCC (19.4%), ADCC (19.0%) and MYEC (28.6%) most frequently presented nuclear P-mTOR staining whereas SDC (5.9%) or SQCC (4.5%) nearly completely lacked nuclear P-mTOR expression (Table 3). Those tumours with negative nuclear P-mTOR staining were associated with lymph node metastases ($p = 0.009$). In univariate analysis negative nuclear P-mTOR expression showed lower survival rates (5-year-DSS 74.7%) than positive nuclear P-mTOR (5-year DSS 89.7%) without reaching statistical significance ($p = 0.067$). In high-grade carcinomas negative nuclear P-mTOR staining also indicated ($p = 0.087$) worse survival (5-year-DSS 53.7%) in comparison to nuclear P-mTOR positivity (5-year-DSS 83.1%).

Cytoplasmic and/or membranous expression of P-mTOR was found in 87 (37.7%) tumours with a mean IRS of 13.7 comparable to a mean IRS of 12.4 for salivary gland control tissues. Cytoplasmic and/or membranous staining of P-mTOR was very frequent in ACCC (77.8%) and ACNOS (66.7%) in contrast to ADCC (14.3%) and MEC (20.5%, Table 3). Interestingly, cytoplasmic/membranous P-mTOR was also highly expressed (7/9, 77.8%) in normal salivary gland control tissues of patients with ACCC in comparison to the controls of patients with ADCC (4/14, 28.6%), MEC (5/12, 41.7%) other tumour subtypes. Negativity for cytoplasmic/membranous P-mTOR staining was associated with advanced tumour sizes ($p = 0.003$). Tumours with absence of nuclear P-mTOR expression presented lower survival rates in comparison to tumours expressing P-mTOR in the nucleus ($p = 0.067$, Fig. 2). There was no significant association for cytoplasmic/membranous P-mTOR expression in uni- or multivariate analysis ($p < 0.05$). There were no significant correlations of P-mTOR with clinico-pathological parameters considering the subtypes separately.

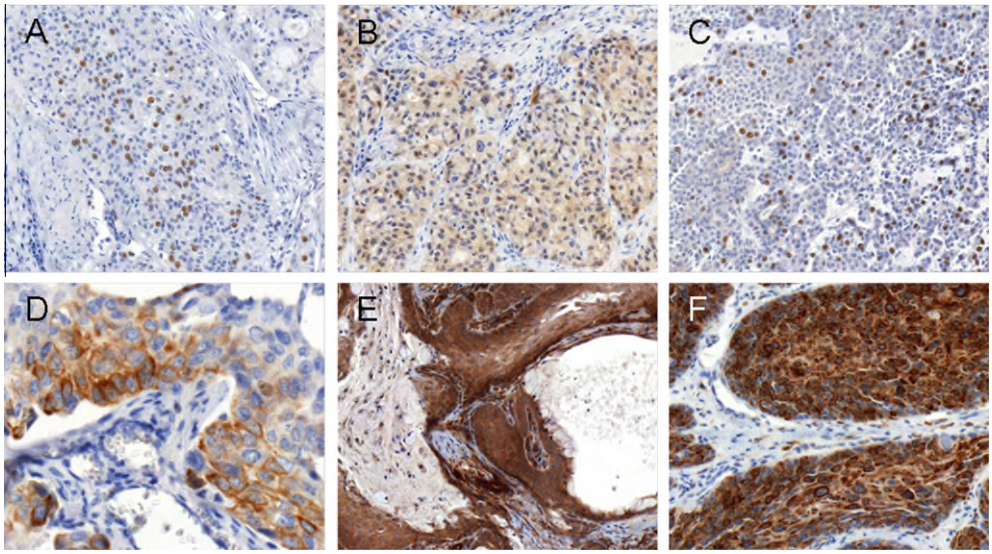

Figure 1 Immunohistochemical staining in mucoepidermoid carcinomas. (A) Positive nuclear AKT (IRS 60 pts, 100×). (B) Positive cytoplasmic AKT (IRS 70 pts, 100×). (C) Positive nuclear mTOR (IRS 90 pts, 100×). (D) Positive cytoplasmic/membrane mTOR (IRS 100 pts, 200×). (E) Positive cytoplasmic PI3K (IRS 300 pts, 100×). (F) Positive cytoplasmic pS6rp (IRS 300 pts, 200×).

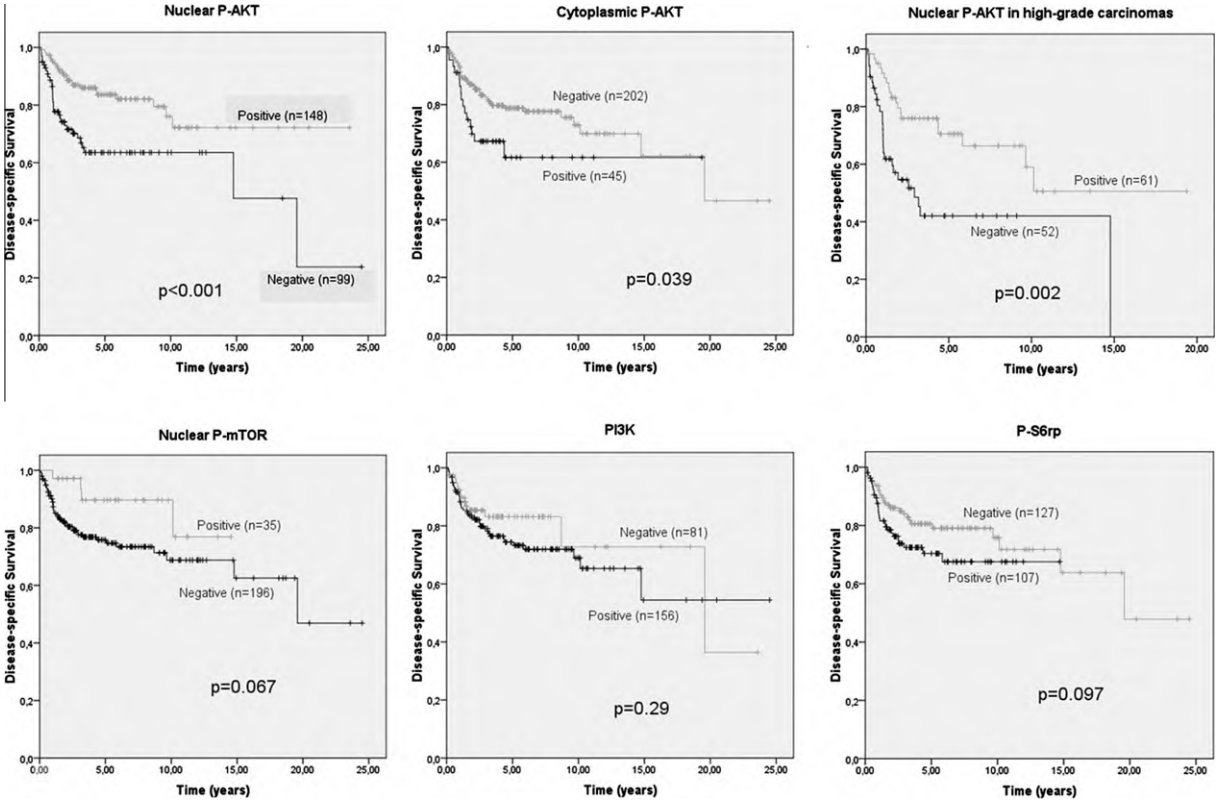

Figure 2 Univariate Kaplan–Meier survival analysis for P-AKT, P-mTOR, PI3K and P-S6rp.

PI3K

PI3K staining was almost exclusively found in the cytoplasm of the tumour cells (Fig. 1). Only seven tumours (3.0%) out of 236 showed nuclear expression of PI3K without any clinical associations. Positive (IRS $\geqslant$ 100pts) cytoplasmic staining of PI3K was observed in 156 (65.8%) tumours. The mean IRS of cytoplasmic PI3K expression in all carcinomas was 136.8 points in contrast to 93.1 for normal salivary gland controls. PI3K positivity was common in different types of salivary gland carcinomas, especially in ACCC (72.2%), ADCC (69.8%) and ACNOS (88.0%) while it was infrequent in PLGAC (25.0%, Table 3). Cytoplasmic PI3K expression was not significantly correlated to any other clinicopathological parameters and did not demonstrate any statistically significant impact on survival. In ADCC PI3K-positivity indicated advanced (T3/T4) tumour sizes (44.8%) in contrast to PI3K-negativity (16.7%, $p = 0.089$).

Table 4
Univariate (Kaplan–Meier – log-rank) and multivariate (Cox regression – enter method) analysis.

Variable (*n*)	Coding	Univariate	Multivariate	
		Log-rank	Sign.	HR (95% CI)
Age (272)	<70y vs. ⩾ 70y	<0.001	0.015	2.32 (1.17–4.58)
Grade (272)	G1/G2 vs. G3	<0.001	<0.001	4.51 (1.98–10.31)
T-stage (268)	1,2 vs. 3,4	<0.001	0.033	2.22 (1.07–4.63)
N-stage (265)	0 vs. 1,2,3	<0.001	0.009	2.39 (1.24–4.60)
R-stage (264)	R0 vs. R1/R2	<0.001	0.001	3.00 (1.55–5.81)
N P-AKT (247)	pos vs. neg	<0.001	<0.001	3.30 (1.72–6.34)
C P-AKT (266)	neg vs. pos	0.039	0.211	1.58 (0.77–3.24)
P-S6rp (234)	0–99 vs. 100–300	0.097	0.901	1.04 (0.54–2.03)

n, number of patients; HR, hazard ratio; sign., significance; *CI*, confidence interval; *R*, residual tumour; *P*, phosphorylated; *N*, nuclear; *C*, cytoplasmic.

P-S6 ribosomal protein

Staining of-P-S6rp was nearly exclusively fount in the cytoplasmic compartment (Fig. 1). Only eight tumours (3.4%) presented nuclear staining with no other clinicopathological associations. Positive (IRS ⩾ 100 pts) cytoplasmic expression of P-S6rp was found in 107 (45.7%) carcinomas. The mean IRS of cytoplasmic P-S6rp staining in all carcinomas was 92.5 points compared to a mean IRS of 64.6 in normal salivary gland tissue. SDC (68.2%) and SQCC (61.9%) showed highest amounts of P-S6rp expression, while the marker was less frequent in ACCC (41.7%) and ADCC (30.8%, Table 3). Positivity for P-S6rp was significantly associated with increased age ($p = 0.010$), high tumour stages ($p = 0.002$), advanced tumour sizes ($p = 0.002$) and lymph node metastasis ($p = 0.042$) in the totality of tumours. Moreover, positive P-S6rp staining was correlated to increased tumour size in MEC ($p = 0.121$), ADCC ($p = 0.067$), MYEC ($p = 0.057$) and SDC ($p = 0.063$) separately. Although not statistically significant ($p = 0.097$), high P-S6rp expression indicated worse survival (5-year-DSS 70.4%) compared to negative or low expression (5-year-DSS 80.6%, Fig. 2).

Correlations between P-AKT, P-mTOR, PI3K and P-S6rp

The statistical correlations between the investigated markers of the AKT pathway are shown in Table 5. In the totality of tumours, cytoplasmic P-AKT expression was associated with expression of PI3K ($p = 0.083$), P-S6rp ($p = 0.052$) and nuclear P-AKT ($p = 0.002$). Increased expression of P-S6rp went along with positivity of PI3K ($p = 0.005$) while it was negatively correlated to nuclear P-mTOR expression ($p = 0.049$). Nuclear P-AKT expression was positively associated with nuclear P-mTOR ($p = 0.004$). In the control tissues, expression of cytoplasmic P-AKT was positively associated with nuclear P-AKT ($p = 0.002$), PI3K ($p = 0.009$) and P-S6rp ($p = 0.050$) expression. Positive cytoplasmic P-mTOR expression correlated with positive P-S6rp ($p = 0.024$).

With view to the subtypes most significant correlations were found for acinic cell carcinomas. In these tumours there was an obvious positive correlation between expression of PI3K, P-AKT, P-mTOR and P-S6rp in the cytoplasm (see Table 5). All ACCCs positive for cytoplasmic P-AKT ($n = 8$) were positive for PI3K ($p = 0.038$). 7/8 ACCCs positive for cP-AKT also expressed cP-mTOR ($p > 0.05$). 6/8 cP-AKT-positive tumours were positive for P-S6rp in contrast to only 7/26 cP-AKT-negative tumours ($p = 0.013$). Moreover, 23/28 ACCCs positive for cP-mTOR were positive for PI3K, too ($P = 0.012$). 13/14 tumours positive for P-S6rp also expressed cP-mTOR ($p = 0.074$). 12/14 tumours positive for PI3K were positive for P-S6rp staining ($p = 0.134$). In contrast, all ACCCs expressing cytoplasmic P-AKT ($n = 8$) were negative for nuclear P-mTOR ($p = 0.106$). Instead, all ACCCs expressing nuclear P-mTOR ($n = 7$) also expressed nuclear P-AKT ($p = 0.106$), while being completely negative for cytoplasmic P-S6rp ($p = 0.028$).

In salivary gland control tissue of patients with ACCC cytoplasmic P-mTOR was positively correlated to PI3K ($p = 0.018$) and P-S6rp ($p = 0.89$), too.

In adenoid cystic carcinomas PI3K was correlated with nuclear P-AKT ($p = 0.078$) and nuclear P-mTOR ($p = 0.040$). Cytoplasmic P-AKT was positively correlated to nuclear P-AKT expression ($p = 0.019$), nuclear P-mTOR ($p = 0.054$) and cytoplasmic P-S6rp (0.011).

In mucoepidermoid carcinomas nuclear P-AKT correlated with nuclear P-mTOR ($p = 0.078$) while cytoplasmic P-mTOR went along with P-S6rp expression ($p = 0.106$).

Table 5
Correlations between P-AKT, P-mTOR, PI3K and P-S6rp in all carcinomas and in ACCC (gray labelled) separately (Bivariate, Pearson coefficient).

		N P-AKT	C P-AKT	N P-mTOR	C P-mTOR	PI3K	P-S6rp
N P-AKT	Correlation		0.193**	0.197**	0.075	0.042	−0.042
	Significance		0.002	0.004	0.277	0.539	0.537
	n		247	210	210	218	217
C P-AKT	Correlation	0.009		−0.043	0.044	0.118	0.132
	Significance	0.960		0.537	0.526	0.083	0.052
	n	35		210	210	218	217
N P-mTOR	Correlation	0.282	−0.282		−0.080	0.039	−0.135*
	Significance	0.106	0.106		0.225	0.572	0.049
	n	34	34		233	217	214
C P-mTOR	Correlation	0.019	0.144	−0.244		0.086	0.083
	Significance	0.914	0.416	0.152		0.209	0.227
	n	34	34	36		217	214
PI3K	Correlation	0.251	0.358*	−0.009	0.414*		0.184**
	Significance	0.153	0.038	0.960	0.012		0.005
	n	34	34	36	36		227
P-S6rp	Correlation	−0.077	0.420*	−0.371*	0.306	0.258	
	Significance	0.667	0.013	0.028	0.074	0.134	
	n	34	34	35	35	35	

P, phosphorylated; *N*, nuclear; *C*, cytoplasmic.
*Significance niveau 0.05 (2-sided); significant correlations marked.
**Significance niveau 0.01 (2-sided).

Discussion

The dysregulation of the PI3K/AKT/mTOR pathway is involved in the pathogenesis of several human carcinomas.[1] The present investigation indicates that this pathway is also activated in salivary gland cancer. Immunohistochemical analysis of the present study revealed an increased protein expression of phospho-AKT, phospho-mTOR, PI3K and phospho-S6rp in different types of salivary gland cancer in comparison to normal salivary gland control tissue. While P-AKT and P-mTOR expression was found in both nuclear and cytoplasmic compartments, PI3K and P-S6rp staining was exclusively detected in the cytoplasm of the carcinomas. This discrimination of the cellular compartments seems important as the function of the protein may vary significantly depending on its localisation.[16]

Activated by PI3K at the plasma membrane, phosphorylated AKT-1 – as investigated in the present study – is supposed to function as a suppressor of apoptosis and stimulates cell cycle, survival, metabolism and migration through phosphorylation of different substrates, e.g. mTOR and S6rp, mainly in the cytoplasm.[17] Investigation of the own salivary gland carcinomas revealed that particularly acinic cell carcinomas display an cytoplasmic interaction between PI3K, P-AKT, P-mTOR and P-S6rp, too, as these proteins were clearly correlated to each others. In contrast, expression of P-AKT in the nucleus went along with absence of cytoplasmic P-S6rp expression and also ACCCs with nuclear P-mTOR expression did not express cytoplasmic P-AKT. Nuclear P-AKT and nuclear P-mTOR however, were correlated to each other. It seems therefore that phospho-AKT and phospho-mTOR possess different interactions and signallings in salivary gland tumours depending on the cell compartment. In the own salivary gland carcinomas, expression of nuclear P-AKT was associated with low-grade histologies like acinic cell carcinomas and polymorphous low-grade adenocarcinomas. Moreover it was correlated to young patients, low tumour stages, absence of lymph nodes and to favourable long-term survival in uni- and multivariate analysis. In contrast, cytoplasmic P-AKT expression was most frequently found in aggressive histologies like salivary duct carcinomas and adenocarcinomas NOS, and was associated with high-grade differentiation of tumours. In univariate analysis cytoplasmic P-AKT expression indicated worse survival whereas nuclear expression of P-AKT went along with more favourable survival rates. With regard to our salivary gland control tissues which displayed absence or weak staining of nuclear and cytoplasmic P-AKT in comparison to the carcinomas, it seems that P-AKT expression in both subcellular compartments contributes to carcinogenesis with different impact on further tumour progress. Cytoplasmic P-AKT expression may indicate ongoing tumour dedifferentiation and progression, whereas nuclear P-AKT stops further tumour dedifferentiation and invasion. There may exist an inactivation/degradation mechanism of the AKT kinase after translocation into the nucleus, at least in certain tumour types.[18] This theory is in accordance with the observation made by Pantuck et al. in renal cell carcinomas where high nuclear P-AKT was associated with limited disease and favourable prognosis, whereas cytoplasmic P-AKT was associated with poor prognosis.[19] Another reason might be that AKT also displays ''tumour suppressive'' roles.[20] Several recent studies have revealed an anti-migratory role for AKT-1 in breast cancer cell lines, in which overexpression of AKT-1 inhibited invasive migration, whereas silencing of AKT-1 enhanced migration.[21–26] The inhibitory effect of AKT-1 on invasive migration is mediated through the proteasomal degradation of the transcription factor NFAT, which is required for breast cancer cell migration.[26] Furthermore, AKT-1 is reported to attenuate ERK/MAPK activity and to downregulate RHO-GTPase activity with degradation of TSC2.[24] Hutchinson et al. reported that AKT-1 can accelerate ErbB-2 mediated mammary tumourigenesis but suppresses tumour invasion.[27] In endometrial carcinomas nuclear P-AKT expression was higher in cancer than in normal endometrium and hyperplasia resembling to our results. However, nuclear P-AKT of moderately- and poorly-differentiated carcinomas was lower than of well-differentiated cancers.[28] From our results it appears likely that the anti-oncogenic role of pAKT is more dependent on nuclear localisation. This shuffle of P-AKT between the nucleus and the cytoplasm as well as missing knowledge of its function in the different compartments may be the reason for the confusing data in the literature referring to the prognostic value of AKT in different types of cancer. For example, P-AKT expression is reported as a poor prognostic factor in oral squamous cell carcinoma,[29] melanoma[30] or pancreatic cancer.[31] On the other hand, P-AKT was found as a favourable prognostic marker in small cell and non-small cell lung cancer,[32–34] in cholangiocarcinoma,[35] in colorectal cancer[36] and in gastric cancer.[37]

Expression of P-mTOR was predominantly found in the cytoplasm (37.7% totally), especially in acinic cell carcinomas (77.8%) and adenocarcinomas NOS (66.7%) in contrast to other subtypes like adenoid cystic carcinomas (14.3%) or mucoepidermoid carcinomas (20.5%). Overall, mean cytoplasmic/membranous P-mTOR expression of all tumours was not significantly upregulated in comparison to the controls and does therefore not serve as a marker of malignancy. Interestingly, cytoplasmic P-mTOR was particularly expressed in the salivary gland controls of patients with acinic cell carcinomas (77.8%) in contrast to controls of patients with adenoid cystic carcinomas (28.6%) or mucoepidermoid carcinomas (41.7%). In a recent mouse model the inactivation of the tumour suppressors Apc and Pten resulted in the development of carcinomas with the histology of acinic cell carcinomas with 100% penetrance. The treatment of the tumour-bearing mice with the mTOR-inhibitor rapamycin led to complete tumour regression in all cases. This group also found cytoplasmic mTOR expression in all investigated human salivary gland acinic cell carcinomas.[38] As a conclusion, acinic cell carcinomas may evolve in patients suffering from defects of the Apc and PTEN tumour suppressor genes leading to increased P-mTOR-levels already in non-tumourous salivary gland tissue and that individualised targeted therapy with mTOR inhibitors may especially be apt for treatment of these tumours.

Nuclear expression of P-mTOR was rare (15.2% of all tumours) and was most frequently detected in acinic cell carcinomas (19.4%) and adenoid cystic carcinomas (19.0%). In contrast, nuclear P-mTOR was often absent in aggressive tumour types like salivary duct carcinomas and squamous cell carcinomas and it was associated with the presence of lymph node metastasis. Analogous to nuclear P-AKT nuclear P-mTOR seems to be activated in less aggressive salivary gland carcinomas as it also showed superior survival rates in comparison to carcinomas lacking any P-mTOR expression in the nucleus, although this observation did not reach statistical significance. In gastric cancer, cytoplasmic expression of P-mTOR was positively correlated with tumour invasion, lymph node metastasis and poor overall survival while nuclear expression of P-mTOR was associated with localised tumour growth, absence of lymph node metastases and favourable overall survival according to our results.[16] The authors also supposed that changes in the localisation of P-mTOR may be involved in tumour progression.

The ribosomal protein S6 is a downstream target of the mTOR effector P70S6K and leads to alterations of mRNA translation when phosphorylated.[4] Cytoplasmic expression of pS6rp is reported to correlate with worse survival in low-grade glioma[39] and in renal cell carcinoma.[19] The own study also indicated worse survival for tumours expressing cytoplasmic P-S6rp without reaching statistical significance. P-S6rp was frequently expressed in high-grade histologies like salivary duct carcinomas and squamous cell

carcinomas and was associated with negative prognostic parameters like increased age, advanced tumour size and lymph node metastasis. Positive correlations of P-S6rp with cytoplasmic P-AKT and PI3K underline the interactions of these components within the PI3K/AKT/mTOR pathway.

PI3K-signalling was also increased in various types of salivary gland cancers in comparison to non-tumourous control tissue, although not showing further associations with clinicopathological parameters or patients' survival.

It has to be critically stated that the above mentioned subcellular signalling patterns do not fit for all investigated subtypes uniquely. For example in adenoid cystic carcinomas, cytoplasmic P-AKT expression went along with P-S6rp expression but also with expression of nuclear P-AKT and nuclear P-mTOR. Also, cytoplasmic PI3K expression was associated with nuclear P-AKT and nuclear P-mTOR expression, thus suggesting a transition of signalling between the cytoplasma and the nucleus at the level of AKT. Anyway, this illustrates the heterogeneity of salivary gland carcinomas and the difficulty of evaluating these tumours together. On the other hand, it is difficult to gain significant data for every single subtype as case numbers are often low due to the rarity of these special tumours.

With view to the control tissues our results indicate expression of the investigated proteins – although at lower levels – in non-neoplastic salivary gland cells, too. All controls were obtained from patients with a salivary gland carcinoma, actually, the controls were closely localised to the tumours. For this reason it is difficult to say, if expression of the markers represents a baseline expression in healthy patients or if early tumour involvement is indicated. Moreover, comparison between salivary gland carcinomas and normal salivary control tissue is difficult in practice, as investigation of the control tissues ideally requires evaluation of the specific cell type (acinic, ductal or myoepithelial cells) corresponding to the tumour.

In conclusion, the PI3K/AKT/mTOR pathway seems to be active in salivary gland cancer. This study supposes that P-AKT and P-mTOR possess distinct molecular functions in these tumours, particularly in acinic cell carcinomas, depending on their subcellular localisation. Expression of cytoplasmic P-AKT and cytoplasmic P-S6rp characterise aggressive tumours with unfavourable prognosis while expression of nuclear P-AKT and nuclear P-mTOR correlates with less aggressive tumour behaviour. Targeted therapy to PI3K/AKT/mTOR signalling may be an alternative in the treatment of certain types of salivary gland carcinomas.

Conflict of interest statement

The authors declare no conflict of interest.

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