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Prognostic value of survivin expression in parotid gland cancer in consideration of different histological subtypes

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ABSTRACT

Background: Cancer of the major salivary glands comprises a morphological diverse group of rare tumours of largely unknown cause. Survivin, an inhibitor of apoptosis has shown to be a significant prognostic indicator in various human cancers. The aim of this study was to assess the long-term prognostic value of survivin in a large group of histological different salivary gland cancers.

Methods: We analysed the survivin expression in 143 patients with parotid gland cancer by means of immunohistochemistry and tissue micro array. Survivin expression was categorised into a low and a high expressing group. The experimental findings were correlated with clinicopathological and survival parameters. The mean follow-up time was 54.8 months.

Results: A positive cytoplasmic expression of survivin was found in 61.5%, a high expression in 25.9% of all specimens. In the whole group, high cytoplasmic survivin expression significantly indicated a poor 5-year disease-free and overall survival rate ($p < 0.0001$, $p = 0.003$). This applied for all adeno-, adenoid cystic and undifferentiated carcinomas whereas in mucoepidermoid carcinomas an analogical non-significant trend could be observed. A high cytoplasmic survivin expression significantly indicated a poor survival in high grade but not in low grade tumours. A multivariate analysis revealed that high cytoplasmic survivin expression was the only significant negative prognostic indicator for a poor 5-year disease-free survival rate in all patients ($p = 0.042$).

Conclusion: The correlation between cytoplasmic survivin expression and survival probabilities of salivary gland cancer might make this an effective tool in patient follow-up, prognosis and targeted therapy in future.

1. Introduction

Salivary gland cancer accounts for 6% of all head and neck cancers and 0.3% of all human malignancies.[1] The annual incidence varies from 0.4 to 1.2 new cases per 100,000 persons.[2,3] The majority of these tumours arise in the parotid glands.[4] Expanding data on microscopic features and an improved understanding of the molecular and genetic events involved brought forth the most recent WHO (World Health Organisation) classification of salivary gland tumours (2005)

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which contains 24 different entities.[1] Surgery and – in patients with adverse risk factors – adjuvant radiation remain the therapeutic standard in the primary treatment of salivary gland cancer.[5] Beyond that, there is no evidence that systemic chemotherapy prolongs or improves survival.[6,7] The same holds true for molecular targeted therapy; until now, no biological has been established for the treatment of salivary gland cancer. The overall survival rate of salivary gland cancer at 5 years is reported to be 76%, the 5-year disease-free survival rate to be 69%.[8] The TNM status, the age and the treatment modality still remain the only reliable clinicopathological prognostic factors so far.[8] Besides many other investigated markers, only the Ki67 proliferative index is a widely accepted histopathological marker for the prognosis of salivary gland cancer. A high Ki67 index has been found to correlate with poor overall survival.[9] The enormous effort to identify, characterise, and validate meaningful biomarkers will represent a step forward in the individualisation of salivary gland cancer classification, therapy, and prognosis.

Apoptosis has an essential role in the normal development of tissues and is frequently impaired in human malignancy. The inhibitor of apoptosis protein survivin (BIRC5/baculoviral inhibitor of apoptosis repeat-containing protein 5), primarily expressed in embryonic cells, is both an anti-apoptosis and a pro-survival factor, and studies have demonstrated its importance in apoptosis during embryonic salivary gland development.[10] Survivin is expressed only to a very low extent in differentiated normal adult cells of any organ[11] but is highly expressed in a wide range of cancer tissues[12] and thus may represent one of a few 'universal' tumour antigens. Qi et al. were the first to show a significant difference in the expression of survivin mRNA between benign and malignant salivary gland tumours.[13] Because survivin is a potent caspase-inhibitor, its over expression in cancer cells is implicated in their resistance to different apoptotic stimuli including chemotherapy. Survivin also seems to be a promising cancer gene because of the potential utilisation of the survivin pathway as an ideal molecular target for cancer therapy.[14–16] Recently, Nikitakis et al. could show in two adenocarcinoma cell lines that inhibition of the oncogenic signal transducer and activator of transcription 3 (STAT3)-survivin pathway in these cells can be achieved by selective targeting techniques or treatment with the non-steroidal anti-inflammatory drug (NSAID) sulindac.[17] However, Nikitakis et al. found no statistically significant differences in the immunohistochemical survivin expression between benign and malignant salivary gland tumours, or amongst the various examined histopathological subtypes of salivary gland cancer.[18]

The aim of this study was to assess the long-term prognostic value of survivin in a large group of histological different parotid gland cancers (PGC).

2. Materials and methods

2.1. Patient data

The study was conducted according to the Declaration of Helsinki. In a retrospective design, we analysed salivary gland tumour samples obtained at the time of surgical resection from 143 consecutive patients newly diagnosed with a PGC (Table 1). All patients were treated at the Department of Otorhinolaryngology, Head and Neck Surgery at the University Hospital of Cologne between 1986 and 2006. The treatment modality employed was primary definitive surgery, and none of the patients had been previously treated with chemotherapeutic agents or radiotherapy. All patients were free of distant metastasis (cM0) as evaluated by means of a computed tomography scan of the thorax, an ultrasound examination of the abdomen and a bone scintigraphy. The type of surgery and a post-operative radiation were applied in accordance with the patients' cancer stage.

The patients' age ranged from 7 to 89 years with a male to female ratio of 1 to 1.07. The classification of the primary tumours was determined following WHO 2005 criteria.[1] Besides the seven predominant histological subtypes (Table 1), other subtypes were epithelial-myoepithelial carcinoma ($n = 4$), lymphoepithelial carcinoma ($n = 2$), myoepithelial carcinoma ($n = 5$), salivary duct carcinoma ($n = 3$) and basal cell adenocarcinoma ($n = 4$). As classified retrospectively by the pathologists according to Barnes et al.,[1] mucoepidermoid carcinomas and acinic cell carcinomas were graded into low grade and high grade tumours whereas all adenocarcinomas, adenoid cystic carcinomas, squamous cell carcinomas, carcinomas ex pleomorphic adenoma and undifferentiated carcinomas were poorly differentiated. Follow-up data were collected at periodic visits in intervals of 3–6 months at our outpatients department. The mean follow-up time was 54.8 months (range 0.3 to 269.3).

In addition, samples of healthy parotid gland tissue were included in the study to serve as negative controls. Sections from colorectal adenocarcinomas served as positive controls.

2.2. Immunohistochemistry

Tissue specimens were fixed in 10% buffered formaldehyde and embedded in paraffin according to routine methods. Slides from all tumour blocks were reviewed by a single pathologist to mark representative areas of the tumour for further processing by tissue micro array (TMA). To account for tumour heterogeneity, for each patient two 2.0 mm diameter cores were taken randomly from within these areas of the tumour (resulting in more than 6 mm^2 of tumour tissue per patient). Multiple TMA containing up to 28 crores were constructed using a manual tissue arrayer (Beecher Instruments Inc., Sun Prairie, WI, USA). Briefly, cores of tumour tissue were removed and inserted into a pre-cored hole in a recipient paraffin block. Four micron TMA sections were then cut, mounted onto *Histobond* glass slides (Marienfeld, Lauda-Königshofen, Germany), deparaffinised and rehydrated using graded ethanols.

Immunohistochemistry was conducted as described previously.[19] Antigen retrieval was done by heating the slides in citrate buffer at 60 °C in an incubator overnight. For staining of survivin, slides were incubated with 2 $\mu g\ ml^{-1}$ anti-survivin rabbit polyclonal antibody (Novus Biologicals Inc., Littleton, CO, USA) at 4 °C over night. After subsequent staining with chromogen AEC (DAKO, Glostrup, Denmark) for 30 minutes, sections were counterstained with haematoxylin (DAKO) and mounted using *Aquamount* (Lerner Laboratories,

Table 1 – Clinicopathological data of 143 patients diagnosed with parotid gland cancer.

Clinicopathological feature	*n*	%	5-year disease-free survival	
			%	*p*
Mean age	58.8 ± 1.4			
Gender				
Male	69	48.3		
Female	74	51.7		
Parotidectomy				
Lateral	17	12.1		
Subtotal	4	2.8		
Total	66	46.8		
Radical	54	38.3		
Neck dissection				
None	20	14.1		
Selective	62	43.6		
Radical	60	42.3		
Radiotherapy postoperatively	82	59.0		
T classification				
pT1	26	18.4	76.9	0.012
pT2	24	17.0	81.1	
pT3	27	19.1	66.3	
pT4	64	45.5	48.5	
N classification				
pN0	83	58.9	72.6	0.029
pN1	15	10.6	71.6	
pN2	41	29.1	42.5	
pN3	2	1.4	*	
Grading				
low grade	51	37.5	75.2	<0.0001
high grade	82	60.3	58.0	
R classification[#]				
R0	95	71.4	69.9	0.007
R1	33	24.8	47.4	
R2	5	3.8	25.0	
Relapse	51	37.5		
Death	42	30.0		
Histology				
Adenocarcinoma	29	20.3	64.2	
Adenoid cystic carcinoma	27	18.9	59.0	
Mucoepidermoid carcinoma	22	15.4	87.5	
Acinic cell carcinoma	17	11.9	71.6	
Squamous cell carcinoma	12	8.4	34.1	
Undifferentiated carcinoma	10	7.0	35.7	
Carcinoma ex pleomorphic adenoma	8	5.6	50.0	
Others	18	12.5	62.9	

Percentage values are rounded; valid percentages in case of missing data.

* All cases are censored.

\# R0, no residual tumour; R1, microscopic residual tumour; R2, macroscopic residual tumour.

Pittsburgh, PA, USA) as a mounting medium. For quality control of staining, negative control slides without primary antibody were included for each tumour section.

All sections were examined independently by at least two observers who were blinded to the patients' clinical information. The survivin staining pattern was granular and localised in the cytoplasm. It was evaluated semi quantitatively by comparing the immunostaining within the tumour with those of the control specimens. Intensity of staining was not considered for evaluation. For each section of every single TMA core at least 500 carcinoma cells were analysed in six randomly selected fields using high-power (40× objective, 10× ocular) magnification. According to Lo et al.,[20] a mean percentage of cytoplasmic positive tumour cells was determined and a level of immunoreaction <5% was graded as negative. There were only very few cases with discrepancies of immunostaining between a patients' two cores. In tumours showing heterogeneity of staining, the immunostaining was

judged according to the prominent pattern. Whenever there were discrepancies in the evaluation between observers, a consensus was reached using a multi-headed microscope. The Cohen's kappa coefficient for the inter-observer agreement was 0.741.

2.3. Statistical analysis

All statistical analyses were performed using *PASW 18.0* (SPSS Inc., Chicago, IL, USA). The association between experimental findings and clinicopathological variables was analysed using Student's *t*-test for quantitative and χ^2-test for categorical data. An ROC curve analysis was done to evaluate the performance of our diagnostic test in relation to its sensitivity and specificity. This ROC curve was linear and did not show a characteristic cut-off point. Thus, for practical and statistical purposes as based on the results, a grouping was done, defining a percentage of immunoreaction between 0–50% as low and >50% as high. A COX regression for continuous data was performed as well. Disease-free survival and overall survival rates were estimated for a time period of 5 years using the Kaplan–Meier algorithm for incomplete observations. The overall survival time was defined as the interval between the date of diagnosis and the last date when the patient was known to be alive (censored) or date of death for any reason (uncensored). The disease-free survival rate was measured as the period of time between the date of diagnosis and the date of the last follow-up examination in which the patient was disease-free (censored), or the date of first recurrence (uncensored). Univariate analysis of the various variables was performed with the Log-Rank test. A Cox proportional hazards ratio model was used to determine independent predictors of disease-free survival using factors being significant ($p \leqslant 0.05$) on univariate analysis as covariates. The hazard ratio (HR) and the respective 95% confidence interval (95% CI) were named. A p-value $\leqslant 0.05$ was considered to be statistically significant.

3. Results

3.1. Expression of survivin

In healthy control parotid gland tissue, no survivin expression could be detected. Cytoplasm of carcinoma cells showed variable survivin staining intensities in all histological subtypes with a range of immunopositive tumour cells from 0% to 100% (Fig. 1, Table 2). A positive cytoplasmic expression of survivin was found in 61.5%, a high expression in 25.9% of all specimens. We only found sporadic cases of nuclear survivin staining, regardless of the cytoplasmic staining pattern. There were no discrepancies between the observers in the blinded analysis of the slides.

3.2. Clinicopathological characteristics

There was no significant association between cytoplasmic survivin expression (high versus low) and the patients' age or gender, the clinical or the pathological staging variables T, N, M or the grading (all $p > 0.05$). There was a significant difference of cytoplasmic survivin expression in relation to the histological subtypes of PGC ($p = 0.011$). Low survivin was predominantly found in acinic cell carcinoma (94.1% of tumours), carcinomas ex pleomorphic adenoma (87.5%), adenoid cystic carcinoma (81.5%), adenocarcinoma (75.9%), mucoepidermoid carcinoma (68.2%) and undifferentiated carcinoma (60.0%) whereas in squamous cell carcinoma predominantly high survivin (66.7%) was found (Table 2). There were significant differences of the tumour stage in relation to the histological subtypes of PGC ($p = 0.013$). The tumour stage in most patients with a mucoepidermoid carcinoma was lower than in the patients with other tumour entities.

3.3. Survival analysis

In our patients, the estimated 5-year overall survival rate was 67.9%. The 5-year disease-free survival rate of all patients was 62.6% with mucoepidermoid carcinomas having the best (87.5%) and squamous cell carcinomas having the worst (34.1%) prognosis (Table 1). The type of surgery and a postoperative radiation had no significant influence on the 5-year disease-free and overall survival rate.

For the whole group, the estimated 5-year overall survival rate for patients with a high cytoplasmic expression of survivin was significantly worse than for patients with a low cytoplasmic expression of survivin (44.2% versus 74.6%; HR = 2.703; 95% CI = 1.373-5.322; $p = 0.003$; test power 93.4%) (Table 2). Furthermore, a high cytoplasmic survivin expression significantly indicated a poor 5-year disease-free survival rate compared to patients with a low cytoplasmic survivin expression (31.3% versus 71.5%; HR = 3.076; 95% CI = 1.638–5.775; $p < 0.0001$, test power 99.7%) (Table 2). A COX regression on continuous data revealed a worse overall survival (risk for 1% elevation of survivin: HR 1.012, $p = 0.011$; risk for 10% elevation of survivin: HR 1.127, $p = 0.011$) and disease-free survival (risk for 1% elevation of survivin: HR 1.009, $p = 0.040$; risk for 10% elevation of survivin: HR 1.094, $p = 0.040$). Regarding the different histological subtypes examined, significant differences of the survival parameters with a poor prognosis in case of high survivin expression were found for adenocarcinomas, adenoid cystic carcinomas and undifferentiated carcinomas whereas in mucoepidermoid carcinomas an analogical trend could be observed. In acinic cell carcinomas, squamous cell carcinomas and carcinomas ex pleomorphic adenoma no such trend could be assessed. A high cytoplasmic survivin expression significantly indicated a poor 5-year disease-free ($p = 0.002$) as well as 5-year overall survival ($p = 0.002$) in high grade but not in low grade tumours (Table 2).

Besides cytoplasmic survivin expression and histological subtype, the only other significant clinicopathological parameters for a poor 5-year disease-free survival rate were the pT and pN classification, the tumour grading and the residual tumour (*R*) classification (Table 1). In patients, additionally treated by radiation, a high cytoplasmic survivin expression significantly indicated a poor 5-year disease-free survival rate (72.1% versus 27.5%; $p = 0.002$).

3.4. Multivariate analysis of prognostic factors

Using the Cox proportional hazards model, we performed a multivariate analysis to assess the independent predictive value of the cytoplasmic survivin expression for the disease-free

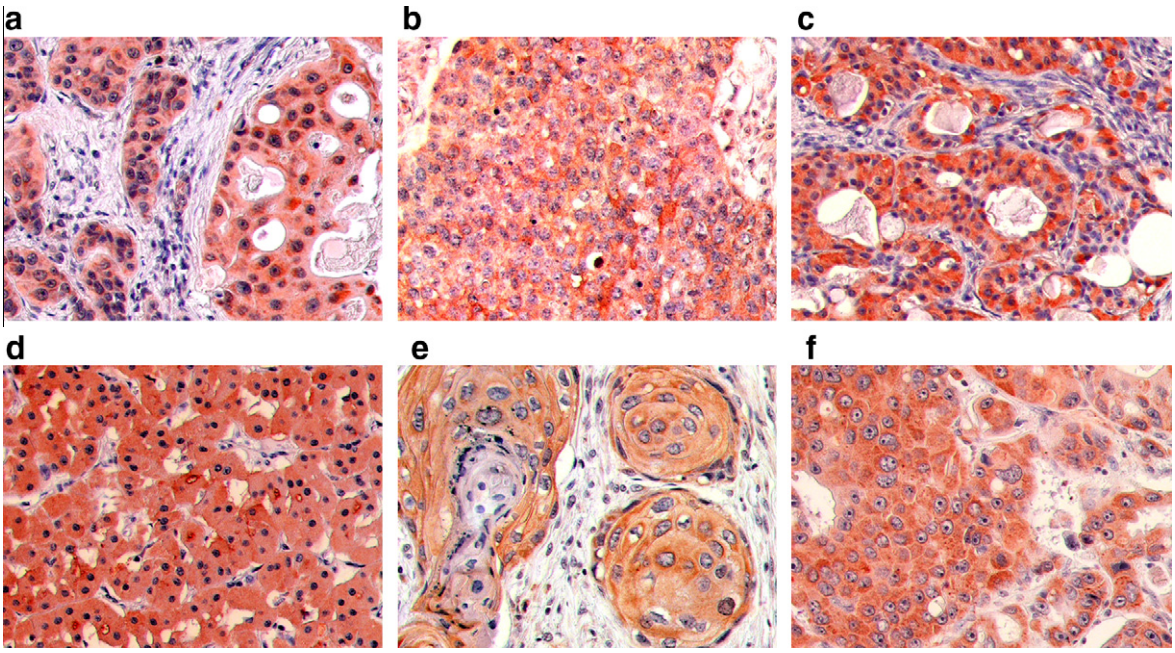

Fig. 1 – Immunohistochemical staining for survivin in parotid gland cancer. High cytoplasmic survivin expression as seen in adenocarcinoma (a), adenoid cystic carcinoma (b), mucoepidermoid carcinoma (c), acinic cell carcinoma (d), squamous cell carcinoma (e) and undifferentiated carcinoma (f). Original magnification 400×.

survival. The following prognostic variables were included in the model: cytoplasmic survivin expression, histological subtype, pT and pN classification, tumour grading and resection status. Concerning the 5-year disease-free survival, the multivariate analysis revealed that the cytoplasmic survivin expression was the only significant negative prognostic factor (HR = 2.543; 95% CI = 1.035–6.249; $p = 0.042$).

4. Discussion

Our data of 143 patients with PGC demonstrates that compared to healthy tissue, cytoplasmic survivin is expressed in different amounts in various histological subtypes. This expression is independent of the patients' clinicopathological characteristics. Moreover, a high cytoplasmic expression of survivin is a significant prognostic indicator for a poor overall as well as a poor disease-free survival. This applies not only to all PGC but also to most of the various histological subtypes examined.

In accordance with previous studies[18] we did not find any survivin expression in normal parotid gland tissue since survivin is physiologically only expressed during embryonic salivary gland development.[10] For benign and malignant salivary gland tumours a cytoplasmic survivin expression has been described by means of immunohistochemistry,[18] and Giefing et al. showed amplifications of 13q22.1–22.2 in salivary gland tumours by using comparative genomic hybridisation analysis. This region harbours KLF5, a regulator of survivin expression.[21] On RNA-level, Qi et al. could show a significant higher expression of survivin mRNA in malignant than in benign salivary gland tumours.[13] Even though morphologically heterogenous, we could detect cytoplasmic expression of survivin in all examined histological subtypes of PGC. More than half of the individual tumour samples showed a positive expression and about one fourth showed a high expression. Even without categorising the tumours into a low and a high expressing group, we made some essential observations. The highest cytoplasmic expressions of survivin were found in squamous cell and undifferentiated carcinomas. These were the subtypes having the poorest 5-year disease-free survival in our study. Primary squamous cell[22] and undifferentiated carcinomas[23–25] of the parotid gland are known to have the poorest prognosis. Thus, it can be speculated, that this is at least in part a direct result of an inhibition of apoptosis in the tumour cell cycle by an over expression of survivin. In other head and neck squamous cell carcinomas similar observations were made with cytoplasmic survivin expression levels up to 83% having a poor prognosis.[19,26] Mucoepidermoid carcinomas on the other hand are known to have the best prognosis.[27,28] Supporting our observation, we could show that these tumours were of lower tumour stages, had a lower survivin expression and a better 5-year disease-free survival.

We found only very sporadically nuclear survivin staining; even though in other head and neck carcinomas, survivin is described to exist in the nucleus as well.[19,29] As the nuclear pool of survivin is likely to be involved in promoting cell proliferation, whereas the cytoplasmic pool of survivin may participate in controlling cell survival[15,16] we suggest, that survivin is mainly involved in cell survival in PGC. Categorising the tumours into a low and a high expressing group, we could furthermore demonstrate that cytoplasmic survivin was a highly significant predictor of a poorer overall as well as disease-free survival. With respect to the different histological subtypes, we found significant differences in the expression of survivin in adeno-, adenoid cystic, mucoepidermoid, squamous cell and undifferentiated carcinomas. Significant differences in the survival parameters were found in adeno-, adenoid cystic and undifferentiated carcinomas. In

Table 2 – Survivin expression and survival parameters of different histological subtypes of parotid gland cancer.

Histological subtype	*n*	Mean survivin expression in %	Low survivin		High survivin		*p*	5-year disease-free survival			5-year overall survival		
			n	(%)	*n*	(%)		Low survivin (%)	High survivin (%)	*p*	Low survivin (%)	High survivin (%)	*p*
All PGC	143	24.9 ± 2.7	106	(74.1)	37	(25.9)	<0.0001	71.5	31.3	<0.0001	74.6	44.2	0.003
Adenocarcinoma	29	17.1 ± 5.6	22	(75.9)	7	(24.1)	0.006	75.5	0.0	0.001	64.2	0.0	0.017
Adenoid cystic carcinoma	27	19.1 ± 5.3	22	(81.5)	5	(18.5)	0.022	69.8	0.0	0.008	88.4	60.0	0.086
Mucoepidermoid carcinoma	22	34.0 ± 5.9	15	(68.2)	7	(31.8)	0.005	100.0	60.0	0.106	93.3	80.0	0.543
Acinic cell carcinoma	17	11.8 ± 6.4	16	(94.1)	1	(5.9)	0.332	71.6	*		84.0	*	
Squamous cell carcinoma	12	57.6 ± 9.7	4	(33.3)	8	(66.7)	0.001	37.5	38.1	0.520	0.0	62.5	0.214
Undifferentiated carcinoma	10	36.9 ± 12.6	6	(60.0)	4	(40.0)	0.037	50.0	0.0	0.008	66.7	0.0	0.025
Carcinoma ex pleomorphic adenoma	8	11.3 ± 5.3	7	(87.5)	1	(12.5)	0.351	50.0	*		50.0	*	
low grade tumours	51	25.5 ± 4.3	39	(76.5)	12	(23.5)	<0.0001	81.2	51.9	0.081	89.4	75.0	0.393
high grade tumours	82	22.7 ± 3.5	62	(75.6)	20	(24.4)	<0.0001	67.7	20.5	0.002	67.2	40.7	0.002

Percentage values are rounded; valid percentages in case of missing data.

* All cases are censored.

all other subtypes either a similar, non-significant trend could be observed or the group numbers were too small with only censored cases. These results once more suggest that the inhibition of apoptosis by survivin might play an important role during tumour progression in PGC independent of the histology, confirming survivin as a universal tumour antigen. Survivin might serve as a prognostic indicator not only in PGC in general but also within the different histological subtypes meaning that besides the histologic type, the behaviour of PGC is considerably predicted by the presence or absence of survivin. Looking at more definitive tumour grades in histologic subtypes where grading is known to affect prognosis, we could furthermore show that survivin serves as a significant prognostic indicator in high grade tumours rather than in low grade tumours. As survivin has a variety of functions[30] different pathways might be involved. Survivin over expression is mediated besides other mechanisms by the proteosome-mediated degradation of p53, which inhibits p53-mediated down regulation of survivin promoter constructs.[31] Biallelic loss of the gene or transcriptional silencing of p53 has been reported in oropharyngeal squamous cell carcinoma cell lines, resulting in a complete loss of transcript in tumour cells.[32] This might be an essential effect in PGC as well. Different to Qi et al. in a study about survivin mRNA,[13] where the difference in the expression of survivin mRNA in malignant salivary gland tumour was significant in the pathological grade, lymph node status and relapse of the tumour, we did not find any correlation with these clinicopathological parameters. This makes survivin even more interesting as a survival prognosticator.

It has lately been shown in a study on 311 surgically resected gastric cancer specimens, that survivin plays a prognostic role as a downstream molecule of STAT3 activation in adenocarcinomas of that organ system.[33] In this context, inhibition of the STAT3-survivin pathway seems to be a hopeful way in growth suppression with many substances on the way to be tested in different cancers.[34–36] Identification of new targets, by means of immunohistochemistry, through expression microarray and other global methods of molecular analysis, and improvements in delivery and targeting may further improve efficacy. Targeting survivin may provide a novel perspective in cancer therapy by simultaneously disabling multiple signalling circuitries. Currently, several clinical trials targeting survivin with various approaches ranging from immunotherapy to antagonists and molecular manipulation of survivin expression are under way in other malignancies and might be broadly applicable to PGC as well, enhancing chemo and radiation therapy.[37,38] Further analyses of the STAT3-survivin pathway on a molecular level in larger group numbers are needed in order to confirm our results and to find suitable targets in anticancer therapy of PGC. Survivin or its serum antibody may also be used as a tumour marker for PGC by means of an enzyme-linked immunosorbent assay as described in other head and neck cancers.[39]

Conflict of interest statement

None declared.

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