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EGFR, HER2, survivin, and loss of pSTAT3 characterize high-grade malignancy in salivary gland cancer with impact on prognosis☆

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1. Introduction

Salivary gland carcinomas are rare tumors characterized by an enormous morphological diversity between different subtypes going along with diverse clinical courses [1].

Functional activation of epidermal growth factor receptors (EGFR) plays a central role in tumorigenesis of different types of epithelial cancers [2]. In breast cancer, gene amplification and protein overexpression of HER2 are associated with poor prognosis [3] and represent a precondition for trastuzumab therapy [4]. Increased gene copy number of *EGFR* correlates with poor prognosis in head and neck squamous cell carcinomas (SQCCs) [5]. In colorectal cancer, increased EGFR gene copy number determined by fluorescence in situ hybridization (FISH) was associated with higher response rates to EGFR inhibitors [6,7]. In salivary gland cancer, EGFR and HER2 are reported to be expressed in different subtypes with impact on prognosis. Especially for salivary duct carcinomas (SDCs), amplification of HER2 has already been described [8-10]. To date, however, results of initial phase II studies for anti-EGFR or anti-HER2 therapy are rather disappointing [11,12].

Survivin shows antiapoptotic, pro-proliferative, and proangiogenic activity and is up-regulated in most malignancies leading to chemoresistence and poor survival [13,14].

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway has been found to be activated in a number of solid tumors such as head and neck SQCC or non–small lung cancer with clinical implications [15]. Survivin and pSTAT3 are currently attracting attention as novel therapeutic targets (eg, YM155, JAK inhibitors) [13,15].

The aim of this study was to analyze the protein and gene status of EGFR and HER2 in a cohort of 286 salivary gland carcinomas by immunohistochemistry and FISH analysis with respect to clinicopathologic parameters and prognosis. In the same context, the expression of survivin and pSTAT3 was evaluated and correlated to EGFR and HER2.

2. Materials and methods

2.1. Patients and tumor samples

Two hundred eighty-six 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 were reviewed. The medical records were obtained from the clinical tumor 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.

At diagnosis patients were staged according to the TNM system of the American Joint Committee on Cancer 2010 [16]. Two hundred eighty-four patients underwent conservative or radical primary surgery (151 total parotidectomies, 35 partial parotidectomies, 42 submandibulectomies, and 56 local resections). Lymph node dissection (mostly conservative) was performed in 205 patients. Postoperative radio- or radiochemotherapy was applied in 167 cases with high-grade malignancy, positive margins, lymph node metastases, or distant metastasis.

The mean follow-up of all patients was 4.86 (0.4-24.5) years. The cohort comprised 136 male and 150 female patients with a mean age at diagnosis of 60.6 (11-99) years. Two hundred (69.9%) parotid, 42 (14.7%) submandibular, 1 (0.3%) sublingual, and 43 (15.0%) minor gland carcinomas were recorded. 54.7% of the patients presented with high (III, IV) UICC tumor stages. Cervical lymph node metastases were obvious in 30.7%, and distant metastases occurred in 11.1% of the patients. In 222 cases (78.4%), close resection margins (R0) were achieved; 47 (16.6%) patients ended up with microscopic (R1) and 14 (4.9%) patients with macroscopic (R2) residual tumor after surgery. Recurrence was observed in 68 (25.0%) patients.

2.2. Histology and classification

The hematoxylin-eosin slides from paraffin wax–embedded tumors of all patients were independently reviewed by 2 experienced pathologists without knowledge of the initial diagnosis. All tumors were classified according the contemporary World Health Organization's classification of salivary gland tumors [1]. Grading was based on a 3-tiered grading system as recently described (Table 1) [17,18]. The 33 cases of carcinoma ex pleomorphic adenoma were classified and graded according to the malignant component of the tumor. All cases of SQCC were classified as primitive of the salivary glands after intensive staging procedures (computed tomography (CT) or magnetic resonance imaging (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.

2.3. Immunohistochemistry

A tissue microarray (TMA) with 2.0-mm-diameter punch cores was constructed from formalin-fixed paraffin-

Table 1 Histology and grading

Histology	Grade			Total
	1	2	3	
Acinic cell carcinoma (ACCC)	34	0	6 [a]	40
Adenoid cystic carcinoma (ADCC)	0	39	11	50
Mucoepidermoid carcinoma (MEC)	28	4	11	43
Salivary duct carcinoma (SDC)	0	2	31	33
Adenocarcinoma NOS (ACNOS)	1	5	29	35
Squamous cell carcinoma (SQCC)	0	12	13	25
Myoepithelial carcinoma (MYEC)	4	6	8	18
Basal cell adenocarcinoma (BCAC)	5	0	2 [a]	7
Epithelial myoepithelial carcinoma (EMC)	2	0	2 [a]	4
Polymorphous low grade adenocarcinoma (PLGAC)	11	0	0	11
Oncocytic carcinoma	0	0	8	8
Malignant mixed tumor	0	0	5	5
Undifferentiated carcinoma	0	0	4	4
Cystadenocarcinoma	2	0	0	2
Sebaceous carcinoma	1	0	0	1
Total	88	68	130	286

[a] Dedifferentiated carcinoma.

embedded tissue blocks of all patients as previously described [19]. Hematoxylin-eosin–stained TMA sections were used for reference histology.

Immunostaining of EGFR, HER2, survivin, and pSTAT3 was performed on 5-μm sections of the TMAs and applied according to manufacturer's instructions. In brief, after dewaxing, washing, and rehydration of the slides through xylene and graded alcohols, microwave heating in citrate buffer was used for antigen retrieval. In the case of EGFR, proteinase K was applied for epitope retrieval. Endogenous peroxidase was blocked in Chem-Mate peroxidase-blocking solution (Dako Cytomation, Glostrup, Denmark). Antibodies were used as follows: HER2—Dako, A0485, polyclonal rabbit, dilution 1:250, pH7,1; detection iVIEW DAB (Ventana, Medical Systems, Tucson, AZ, USA); EGFR—EGFR pharmDx, clone 2-18C9, monoclonal mouse, dilution 1:400, detection En Vision (Dako); Survivin—Abcam, ab469, polyclonal rabbit, dilution 1:500, detection EnVision (Dako); *pSTAT3* (Tyr705) (D3A7)—Cell Signaling, monoclonal rabbit, dilution 1:50, detection EnVision (Dako).

The immunostaining for EGFR and HER2 was semiquantitatively evaluated based on intensity of membrane reactivity following the original DAKO Herceptest criteria with a threshold of 10% immunopositive cells: 0, negative (no reactivity or reactivity in <10% of cells); 1+, weak reactivity in >10% of cells, 2+ moderate reactivity in >10% cells; 3+ strong reactivity in >10% cells. Nuclear and cytoplasmic staining intensity (0, 1+, 2+, 3+) of pSTAT3 was evaluated with a threshold of 10% immunopositive cells. For survivin, nuclear and cytoplasmic staining intensity (0, 1+, 2+, 3+ with a threshold of 10% immunopositive cells) was multiplied with the percentage of the staining area generating a immunoreactive score (IRS) ranging from 0 to 300 points. The IRS of survivin was classified into 4 subgroups (0-29, 30-99, 100-199, 200-300). For EGFR, HER2, and pSTAT3, cases with 3+ staining patterns (overexpression) were considered positive, whereas cases with 0, 1+, and 2+ staining were considered negative for further dichotomized analysis. For survivin, an IRS ≥ 100 was assessed as positive expression. Immunostaining patterns of survivin (n = 64) and pSTAT3 (n = 66) were also documented in normal salivary gland tissues as controls.

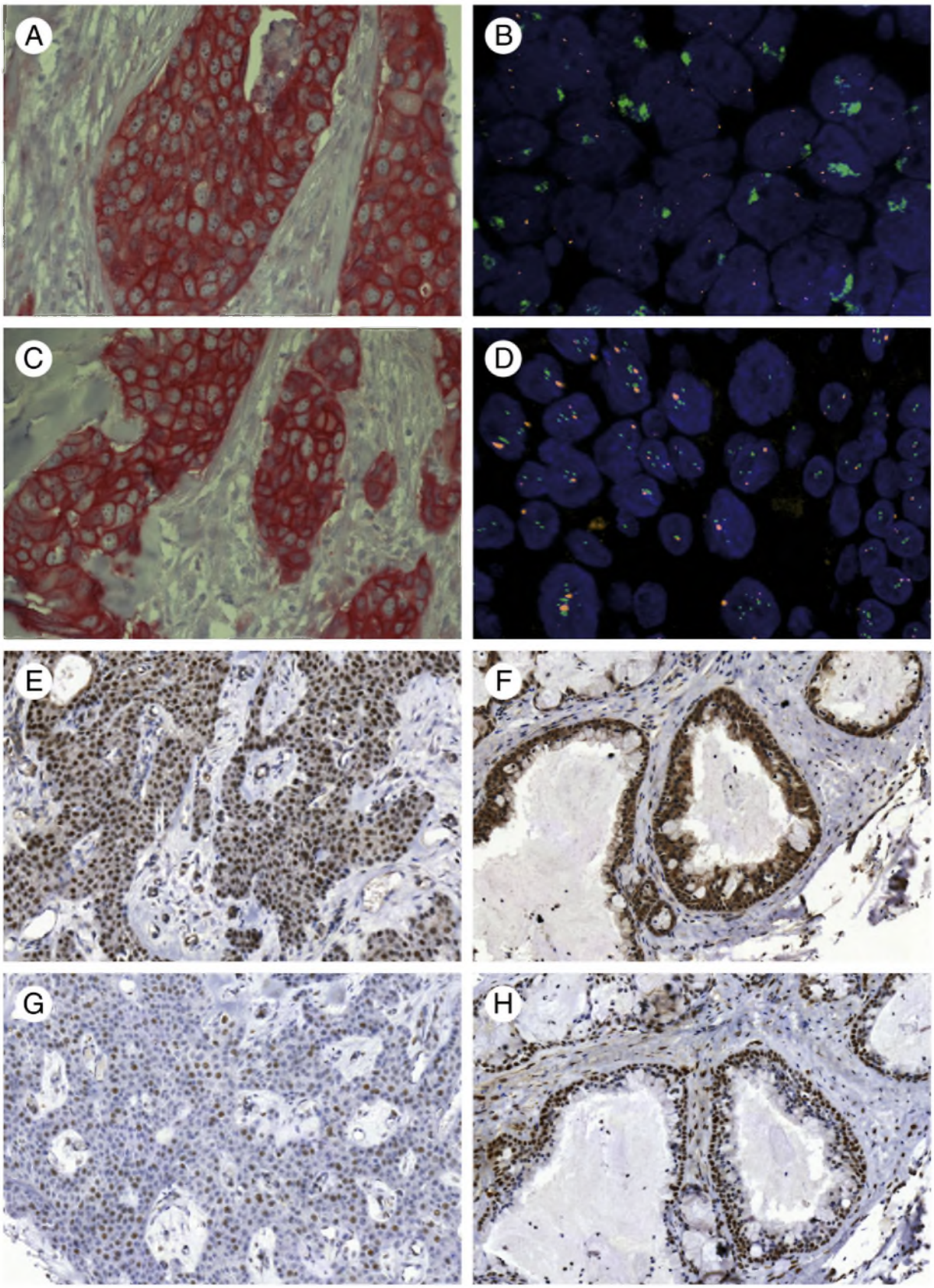
2.4. Fluorescence in situ hybridization

As described in detail elsewhere [20], TMA sections were mounted on charged slides (SuperFrost Plus; Menzel GmbH, Braunschweig, Germany). Hematoxylin/eosin-stained TMA sections were used for reference histology. FISH was performed with the use of directly labeled Zyto*Light* SPEC EGFR/CEN7 and SPEC HER2/CEN17 dual color probe (ZytoVision Ltd, Bremerhaven, Germany). After probe hybridization, nuclei were counterstained with antifading DAPI (4′,6-diamidino-2-phenylindole) Vectashield (Vector Laboratories, Burlingame, CA) and were analyzed by epifluorescence microscopy using the AxioImager-Z1 (Zeiss, Göttingen, Germany). Hybridization signals of 50 nuclei were manually counted on single cell basis by 2 independent observers.

The FISH ratio was assessed as the number of genes proportional to the number of centromeres as published by Cappuzzo et al [21]. Samples were grouped as normal disomy, ≤2 centromere signals in ≥50% of cells; low polysomy/trisomy, ≥3 centromere signals in ≥40% of cells, excluding cases with high polysomy or gene amplification; high polysomy, ≥4 centromere signals in ≥40% of cells, excluding cases with gene amplification; and gene amplification, ratio of gene/chromosome ≥2 or clusters of probes (>10 copies/tumor cell) in ≥40% of cells. Disomy and trisomy/low polysomy were grouped as FISH negative, whereas high polysomy and amplification were classified as FISH positive (HP/A) or high gene copy number in dichotomization [5,22].

2.5. Statistical analysis

Data were analyzed with SPSS for Windows, version 15.0 (SPSS, Erkrath, Germany). Relationships between dichotomized parameters were examined using Fisher exact probability test ($P < .05$). 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.


A
B
C
D
E
F
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H

Table 2 EGFR, HER2, survivin, pSTAT3 and clinicopathologic parameters

Parameter	EGFR				HER2				nSurvivin		npSTAT3	
	IH		FISH		IH		FISH		IH		IH	
	0, 1+, 2+	3+	D/LP	HP/A	0, 1+, 2+	3+	D/LP	HP/A	0-99	100-300	0, 1+, 2+	3+
Histology												
ACCC	38	1	38	0	35	2	38	1	29	5	16	19
ADCC	41	6	39	0	46	0	44	1	39	1	20	23
MEC	26	12	38	3	37	1	38	4	29	7	30	6
SDC	23	8	20	12	15	16	17	14	14	6	20	2
ACNOS	26	7	22	6	25	7	23	6	23	3	23	3
SQCC	12	10	16	8	21	1	18	7	14	8	19	2
MYEC	16	2	15	1	17	1	16	1	14	3	12	5
PLGAC	10	1	8	0	11	0	9	0	8	2	8	2
Others	24	6	18	12	31	0	20	8	19	8	23	6
Age												
<70	**150**	**22**	141	21	154	16	149	22	**129**	**21** *	**100**	**51**
>70	**66**	**31** ***	74	21	84	12	75	20	**60**	**23**	**72**	**17** *
Grade												
G1-G2	**129**	**18**	**134**	**7**	**140**	**5**	**141**	**6**	**120**	**12**	**85**	**51**
G3	**87**	**35** ***	**80**	**35** ***	**98**	**23** ***	**82**	**36** ***	**69**	**31** ***	**86**	**17** ***
T stage												
T1-T2	129	28	**140**	**17**	142	13	**142**	**16**	**121**	**20**	103	43
T3-T4	82	25	**70**	**25** **	91	15	**77**	**26** **	**65**	**23** *	65	25
N stage												
N0	148	31	**155**	**21**	**166**	**9**	**163**	**19**	**138**	**24**	**109**	**57**
N1-3	60	22	**52**	**21** **	**64**	**19** ***	**53**	**23** ***	**46**	**19** *	**58**	**9** ***
M stage												
M0	190	45	190	40	210	23	200	37	170	35	**145**	**66**
M1	24	7	22	2	25	5	21	5	18	7	**24**	**2** *
Recurrence												
No	159	33	160	27	171	18	**167**	**23**	137	30	119	51
Yes	47	16	46	12	56	7	**48**	**15** *	47	10	45	15
Total	216	53	214	42	238	28	223	42	189	43	171	68

Abbreviations: D, disomy; LP, low polysomy; HP, high polysomy; A, amplification; HP/A, high gene copy number; IH, immunohistochemistry; n, nuclear; ACCC, acinic cell carcinoma; ADCC, adenoid cystic carcinoma; MEC, mucoepidermoid carcinoma; SDC, salivary duct carcinoma; ACNOS, adenocarcinoma not otherwise specified; SQCC, squamous cell carcinoma; MYEC, myoepithelial carcinoma; PLGAC, polymorphous low-grade adenocarcinoma.

Statistically significant associations are given in bold.

* $P \leq .05$.

** $P \leq .01$.

*** $P \leq .001$ (Fisher exact test).

3. Results

3.1. Clinicopathologic characteristics

The histologic diagnoses are shown with grading in Table 1. The 5-and 10-year disease-specific overall survival (DSS) rates of all carcinomas were 75.3% and 69.7%, respectively. The 5-and 10-year DSS of the most frequent subtypes were as follows: acinic cell carcinoma (ACCC) 96.4% and 89.5%, adenoid cystic carcinoma (ADCC) 77.1% and 71.2%, mucoepidermoid carcinoma (MEC) 80.7% and 76.3%, SDC 59.0% each, adenocarcinoma not otherwise specified (ACNOS) 57.7% each, primary SQCC 59.1% each, myoepithelial carcinoma (MYEC) 68.5% each, polymorphous low-grade adenocarcinoma (PLGAC) 87.5% each. Results of univariate and multivariate survival analysis for dichotomized clinicopathologic parameters are shown in Table 3. In multivariate analysis, high-grade malignancy was the strongest negative parameter for survival.

Fig. 1 A, EGFR IH (SDC, 3+ membranous staining, original magnification ×200); B, EGFR FISH (SDC, cluster amplification); C, HER2 IH (SDC, 3+ membranous staining, original magnification ×200); D, HER2 FISH (MEC, amplification). E, Survivin IH (MEC, 3+ nuclear, 1+ cytoplasmic staining, nuclear IRS 240 pts, original magnification ×100); F, survivin IH (MEC, 3+ nuclear, 2+ cytoplasmic staining, nuclear IRS 210 pts, original magnification ×100); G, PSTAT3 IH (MEC, 1+ nuclear staining, original magnification ×100); H, PSTAT3 IH (MEC, 3+ nuclear staining, original magnification ×100).

3.2. EGFR

3.2.1. Immunohistochemistry

Of 269 salivary gland carcinomas, 66 (24.5%) showed weak (1+), 125 (46.5%) moderate (2+), and 53 (19.7%) strong (3+) membranous staining for EGFR. Twenty-five (9.3%) carcinomas were completely negative for EGFR expression. Overexpression of EGFR (3+, Fig. 1A) was most frequent in MEC (12/38, 31.6%), SDC (8/31, 25.8%), and SQCC (10/22, 45.5%, Table 2). EGFR overexpression was associated with age >70 ($P < .001$) and high-grade malignancy ($P = .001$) for the totality of tumors. No significant correlations were found with T stage, N stage, recurrence, and distant metastases ($P > .05$). 54.5% (6/11) of high-grade MECs presented overexpression of EGFR in contrast to only 22.2% (6/27) of low- and intermediate-grade MECs ($P = .068$). Strong membranous EGFR staining was significantly associated with worse disease-specific survival in univariate analysis in the totality of tumors ($P < .001$, Fig. 2) and in MECs separately ($P < .001$).

3.2.2. Gene analysis

Categorial classification showed 176 (68.5%) of 256 tumors with disomy for chromosome 7, 39 (15.2%) with trisomy/low polysomy, 39 (15.2%) with high polysomy, and 3 (1.2%) tumors (1 MEC, 1 SDC and 1 ACNOS) with amplification of the *EGFR* gene (Fig. 1B). Increased gene copy number (high polysomy or amplification) of *EGFR* was most frequently found in SDC (12/32, 37.5%), SQCC (8/24, 33.3%), and ACNOS (6/28, 21.4%, Table 2). 53.8% (21/39) of the carcinomas with increased *EGFR* gene copy number also showed overexpression of the protein, whereas EGFR overexpression was rare (28/201, 13.9%) in tumors with disomy of the *EGFR* gene ($P = .001$). Two of the three amplified tumors (SDC and MEC) presented overexpression (3+) of the EGFR protein, whereas for the third carcinoma (ACNOS) with *EGFR* amplification, moderate protein staining (2+) was observed. FISH positivity was associated with high-grade malignancy ($P < .001$), high T stage ($P = .003$), and high N stage ($P = .003$), whereas no significant correlations were observed with age, recurrence, and distant metastases ($P > .05$). High-grade MECs also correlated with *EGFR* FISH positivity in contrast to low and intermediate MECs ($P = .011$). Increased *EGFR* gene copy number was clearly associated with unfavorable disease-specific survival in the whole sample of tumors ($P < .001$, Fig. 2), in MECs ($P = .026$), and in SQCCs ($P = .020$).

3.3. HER2

3.3.1. Immunohistochemistry

Of 266 tumors, 66 (24.8%) showed weak (1+), 106 (39.5%) moderate (2+), and 28 (10.5%) strong (3+)

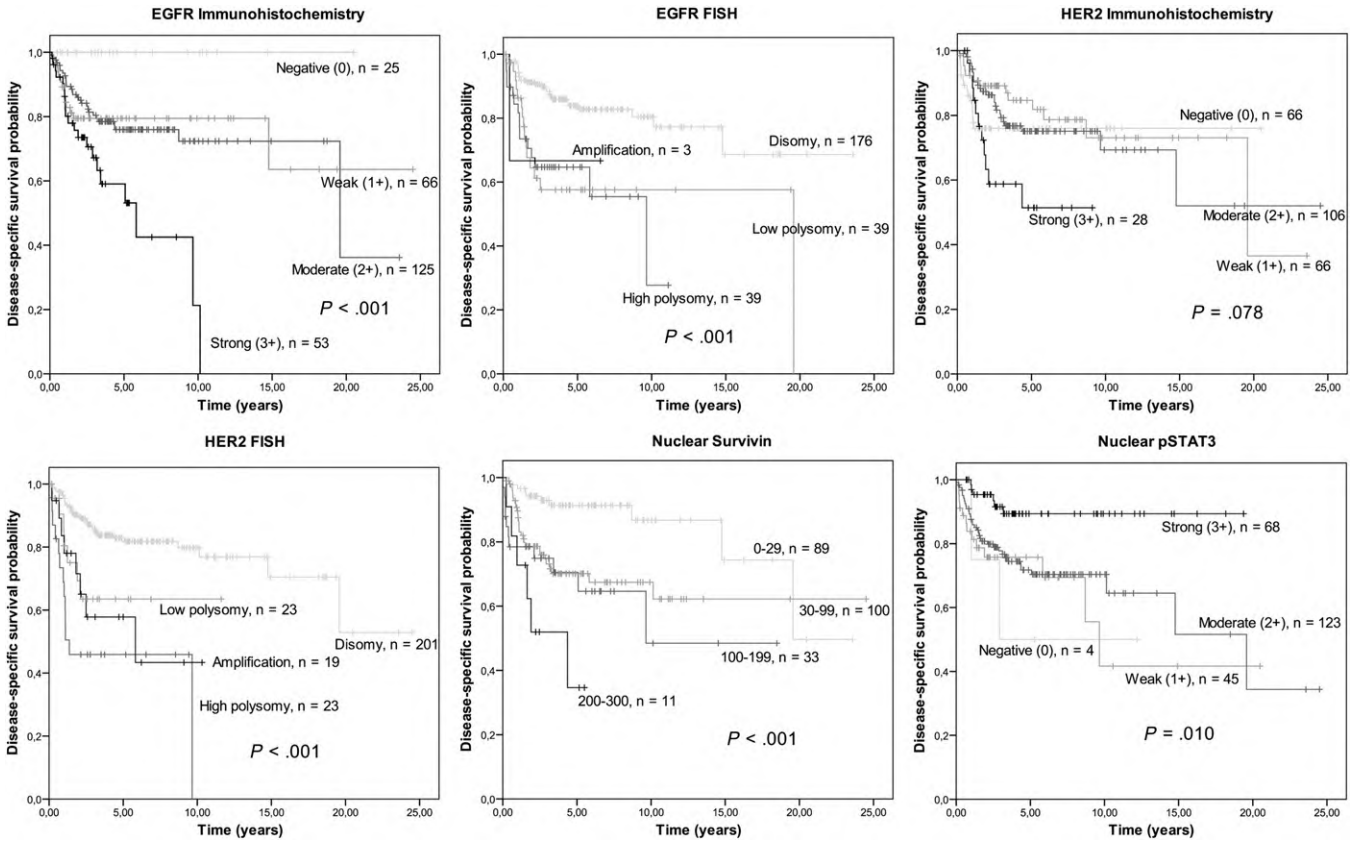

Fig. 2 Kaplan-Meier survival analysis for EGFR, HER2, survivin, and pSTAT3.

Table 3 Univariate (Kaplan-Meier—log-rank) and multivariate (Cox regression—Enter) analysis

Variable	Coding	Univariate	Multivariate	
		Log-rank	Significance	HR (95% CI)
Age	<70 y *vs* ≥ 70y	**<0.001**	**0.016**	2.30 (1.17-4.52)
Grade	G1/G2 *vs* G3	**<0.001**	**<0.001**	5.58 (2.39-13.03)
T stage	1,2 *vs* 3,4	**<0.001**	**0.008**	2.52 (1.27-5.00)
N stage	0 *vs* 1,2,3	**<0.001**	**0.020**	2.16 (1.13-4.11)
R stage	R0 *vs* R1/R2	**<0.001**	**<0.001**	4.31 (2.11-8.78)
EGFR FISH	D/LP *vs* HP/A	**0.001**	0.260	0.60 (0.25-1.46)
HER2 FISH	D/LP *vs* HP/A	**<0.001**	**0.026**	2.62 (1.12-6.12)
nsurvivin	0-99 *vs* 100-300	**0.002**	0.456	1.31 (0.64-2.68)
npSTAT3	3 *vs* 0,1+,2+	**0.001**	**0.045**	3.59 (1.03-12.49)

Abbreviations: n, nuclear; HR, hazard ratio; CI, confidence interval; R, residual tumor.

membranous staining of HER2. Sixty-six (24.8%) carcinomas were completely negative of HER2 expression. SDC (16/31, 51.6%) and ACNOS (7/32, 21.9%) showed the highest proportions of HER2 overexpression (3+) (Table 2). HER2 overexpression (Fig. 1C) was associated with high-grade malignancy ($P < .001$) and positive nodal status ($P < .001$). No significant associations were found between HER2 overexpression and age, T stage, M stage, or recurrence ($P > .05$). Membranous HER2 staining did not reach statistical significance in univariate survival analysis ($P = .078$, Fig. 2). When dichotomized (0, 1+, 2+ versus 3+), however, overexpression of HER2 showed significantly worse survival ($P = .016$, Table 3).

3.3.2. Gene analysis

Of 265 tumors, 201 (75.6%) showed disomy for chromosome 17, 23 (8.6%) cases with trisomy/low polysomy, and 23 (8.6%) tumors with high polysomy. *HER2* amplification (Fig. 1D) was found in 19 (7.1%) tumors (1 ADCC, 2 MEC, 11 SDC, 2 ACNOS, 2 SQCC). 48.1% of the carcinomas with overexpression of the HER2 protein showed high polysomy or amplification (HP/A) of the gene ($P < .001$). High gene copy number of *HER2* was frequent in SDC (45.2%), SQCC (28.0%), and ACNOS (20.7%, Table 2). 31% (9/29) of SDCs presented concomitant HER2 amplification and protein overexpression. As shown in Table 2, *HER2* FISH positivity was associated with high-grade malignancy ($P < .001$), advanced T stage ($P = .002$), positive N stage ($P < .001$), and tumor recurrence ($P = .04$) in the totality of tumors. With respect to MECs, all 4 cases of increased *HER2* gene status were observed in high-grade variants ($P = .002$). Increased *HER2* gene status strongly correlated with unfavorable survival in all tumors together ($P < .001$, Fig. 2) as well as in ADCCs ($P = .004$), SQCCs ($P = .006$), and MYECs ($P < .001$) separately. Increased gene copy number of *HER2* was also a significant ($P = .026$) negative prognostic parameter in multivariate analysis (Table 3).

3.4. Survivin

3.4.1. Immunohistochemistry

Survivin was evaluated for nuclear and cytoplasmic staining patterns (Fig. 1E-G). Nuclear survivin was positive (IRS ≥ 100 points) in 44 (18.9%) of 232 tumors, whereas positive cytoplasmic reactivity of survivin was found in 55 (23.6%) of 232 cases. Nuclear survivin was completely absent (0 points) in 43 (67.2%) of 64 normal salivary gland tissues resulting in a mean IRS of 8.3 points. In contrast, the IRS of nuclear survivin was 58.4 points for the carcinomas. Positive nuclear survivin expression (IRS ≥ 100) was frequently found in SDC (6/20, 30.0%) and SQCC (8/22, 36.4%) while almost absent in ADCC (1/40, 2.5%, Table 2). Positive cytoplasmic survivin expression (IRS ≥ 100) accumulated in ACCC (10/34, 29.4%) and MEC (11/36, 30.6%), whereas it was rarely found in SQCC (1/22, 4.5%). Although cytoplasmic survivin staining did not show any correlations to other clinicopathologic parameters, positive nuclear survivin was associated with age >70 ($P = .014$), high-grade malignancy ($P < .001$), advanced T stage ($P = .036$), and N stage ($P = .015$) in the totality of tumors. In contrast, no significant associations with recurrence and distant metastases were found ($P > .05$). Moreover, nuclear survivin expression was not associated with tumor grade within the different subtypes including the group of MECs ($P > .05$). In univariate analysis strong nuclear survivin staining significantly correlated with worse survival ($P < .001$, Table 2) in all tumors together as well as in MECs ($P = .030$) and MYECs ($P = .013$) separately. Interestingly, positive cytoplasmic survivin expression (≥100 points) was inversely associated with better survival rates in MECs ($P = .054$) and ADCCs ($P = .217$) without reaching statistical significance.

3.5. pSTAT3

3.5.1. Immunohistochemistry

PSTAT3 was exclusively expressed in the cell nuclei of the carcinomas (Fig. 1H-I). Four (1.7%) of 239 tumors were

negative for pSTAT3 staining, 45 (18.8%) showed weak (1+), 123 (51.3%) moderate (2+), and 68 (28.3%) strong (3+) nuclear staining of pSTAT3. Normal salivary glands (n = 66) expressed strong (3+) nuclear pSTAT3 in 81.8% (n = 54) resulting in a mean staining score of 2.8, whereas the mean staining score for the carcinomas was 2.1. Highest proportions of strong pSTAT3 staining were found in ACCC (54.3%) and ADCC (53.5%, Table 2). Overexpresssion of pSTAT3 was rarest in SDC (9.1%), ACNOS (11.5%), and SQCC (9.5%). pSTAT3 positivity (3+) was associated with age <70 (P = .018), low and intermediate grade (P < .001), absence of lymph node metastases (P = .001), and absence of distant metastases (P = .011). Five (82.3%) of 6 ADCCs with distant metastases showed negative expression of pSTAT3 in comparison to only 38.9% (14/36) of ADCCs without distant metastases (P = .075). No correlations were observed between pSTAT3 expression and T stage or recurrence (P > .05). With respect to the separate subtypes, pSTAT3 expression showed no association with tumor grade neither in the group of MECs nor in other entities (P > .05). Considering all tumors together, pSTAT3 overexpression was significantly correlated with higher disease-specific survival rates in univariate (P = .010, Fig. 2) and multivariate analyses (P = .045, Table 3).

3.6. Correlations between EGFR, HER2, survivin and pSTAT3

Correlations between EGFR, HER2, nuclear survivin, and nuclear pSTAT3 are shown in Table 4. Parameters were dichotomized as shown in Table 2. For both EGFR and HER2, increased gene copy number (high polysomy or gene amplification) was strongly associated with protein overexpression ($P \leq .001$). Of 39 tumors, 25 (64.1%) with high copy number of HER2 also showed an increased copy number of EGFR ($P < .001$). Overexpression of nuclear survivin correlated with *EGFR* (P = .017) and *HER2* (P = .051) FISH positivity, whereas cytoplasmic overexpression of survivin was associated with *EGFR* (P = .004) and *HER2* (P = .044) FISH negativity. Moreover, overexpression of nuclear survivin was correlated to loss of pSTAT3 (P < .001). Only 1 of 68 tumors strongly expressing pSTAT3 was positive for nuclear survivin. Increased gene copy number of *EGFR* and *HER2* was highly associated with loss of pSTAT3 expression (P = .001 and P = .002, respectively).

4. Discussion

4.1. EGFR and HER2

This study revealed that high-grade salivary gland carcinomas harbor an increased protein expression and gene copy number of EGFR and HER2. FISH positivity of *EGFR* and *HER2* was most frequent in SDCs, SQCCs, and adenocarcinomas not otherwise specified. Amplification of *HER2* was found in 35% (11/31) of SDCs exceeding the 12.1% (8/66) reported by Williams et al [9]. Protein overexpression of EGFR was predominant in MECs, SQCC, and SDCs, whereas HER2 expression was most frequently found in SDCs and adenocarcinomas not otherwise specified according to previous studies [8,9,23-26]. In the present investigation, expression of EGFR and HER2 was strongly associated with increased gene copy number, confirming the results of Lujan et al [23] for EGFR in MECs and the findings of Williams et al [9] for HER2 in SDCs. Although overexpression of EGFR was found in nearly 20% of the carcinomas, gene amplification was a rather rare event (1%). This phenomenon has already been described before [9,23,27] and is supposed to be the result of increased activity of the EGFR promoter or of deregulations at translational and posttranslational levels [28]. In this investigation of a cohort of different types of salivary gland carcinomas, both EGFR- and HER2- protein and gene status correlated with tumor stage, tumor grade, and disease-specific survival of patients. With respect to MECs, EGFR and HER2 positivity characterizes high-grade variants and may therefore be useful to differentiate grade 3 from grade 1 and 2 malignancy in daily practice.

Although immunhistochemical EGFR expression does not characterize patients who may respond to anti-EGFR drugs [2,7], high polysomy or amplification of the *EGFR* gene detected by FISH may present a promising predictor of therapy response as reported for non–small cell lung cancer and colorectal cancer [29,30]. In the present study,

Table 4 Correlations between EGFR, HER2, survivin, and pSTAT3 (Fisher Exact test)

	EGFR IH	EGFR FISH	HER2 IH	HER2 FISH	nsurvivin	npSTAT3
EGFR IH	—	<0.001	0.449	0.014	0.037	0.019*
EGFR FISH		—	0.038	<0.001	0.017	0.001*
HER2 IH			—	<0.001	0.060	0.028*
HER2 FISH				—	0.051	0.002*
nsurvivin					—	<0.001*
npSTAT3						—

NOTE. Dichotomization of parameters as shown in Table 2.
Abbreviations: IH, immunohistochemistry; n, nuclear, * inversely associated.

only 3 tumors showed an *EGFR* gene amplification. However, 15.2% of the carcinomas had an increased *EGFR* copy number based on *CEN-7* polysomy. In breast cancer, combined protein overexpression and gene amplification of *HER2* pose the prerequisite for trastuzumab therapy [4,31]. As there is a certain proportion of salivary gland carcinomas, especially SDCs (31%) in our own study meeting these conditions, anti-HER2 therapy may be applicable to these tumors. Objective response to trastuzumab has been reported for SDCs in the first clinical studies [25,32].

4.2. Survivin

The present investigation elucidates the prognostic impact of the apoptosis inhibitor survivin in salivary gland cancer. Although largely absent in normal salivary gland tissue, high expression of nuclear survivin has been found in high-grade carcinomas with accumulation in SDCs and SQCCs. Moreover, increased nuclear survivin expression correlated with negative prognostic parameters such as advanced T and N stage and was significantly associated with lower disease-specific survival rates. Recently, Stenner et al observed a correlation of cytoplasmic survivin expression with worse survival in a cohort of salivary adenocarcinomas (n = 27) and MECs (n = 21) [33]. This group evaluated the percentage of cytoplasmic positive tumor cells without regarding the staining intensity and had a mean follow-up time of only 30 months. Nuclear staining of survivin was only found in a very few cases in this investigation. For the 21 MECs, univariate survival analysis revealed a statistically nonsignificant trend for worse survival of cytoplasmic survivin expression, whereas in our own study on 36 MECs, only nuclear survivin correlated with unfavorable survival (P = .030). Interestingly, positive cytoplasmic survivin expression was inversely associated with better survival in our MECs.

Nuclear survivin expression has been reported to show prognostic significance in 37 ADCCs [34], which could not be confirmed by our investigation, as only 1 of 40 ADCCs presented high nuclear survivin expression. In the mentioned study, staining intensity and percentage were evaluated in the same manner as in our own investigation; however, a lower cut-off (22 points, score 4) for positivity in semiquantitative immunohistochemical analysis was chosen, which may explain the different results. Nuclear survivin is reported as a negative prognostic parameter in different cancer types such as oropharyngeal SQCCs, non-small cell lung carcinomas, hepatocellular carcinomas, or breast cancer [14,35-38]. As mentioned above, however, there are also immunohistochemical studies describing cytoplasmic survivin staining as an unfavorable parameter of survival for pancreatic cancer or oral SQCC [39,40], which represents the current inconsistency about the clinical implications for subcellular survivin localization. One important aspect seems to be a survivin shuttle between the nucleus and the cytoplasm depending on certain survivin splice variants (*survivin* ΔEx3 and *survivin* 2β) and mediated by the nuclear export receptor Crm 1 [13,41]. Nuclear survivin is suspected to control cell division with a proliferative aggressive phenotype in cancer expression, whereas cytoplasmic/mitochondrial survivin is considered to be cytoprotective [42]. In breast cancer, a high cytoplasmic-to-nuclear ratio of survivin was associated with improved overall survival, whereas nuclear expression was associated with reduced survival [43]. We observed nuclear survivin expression positively correlated to increased *EGFR* and *HER2* gene copy number, whereas cytoplasmic survivin presented with *EGFR*- and *HER2*- disomy or low polysomy status. In this context, Papanikolaou et al [44] found up-regulated survivin in breast cancer cells in the presence of HER2, whereas HER2 knockdown led to down-regulation of survivin. Survivin expression seems also to be dependent on the EGFR-PI3K-AKT-pathway because inhibition with the EGFR-TKI inhibitor gefitinib caused reduced survivin expression in *EGFR* mutation–positive non–small cell lung cancer [45]. Likewise, lapatinib treatment of vestibular schwannoma cells lead to survivin down-regulaton [46].

4.3. pSTAT3

Constitutive activation of STAT3 is involved in deregulation of the cell cycle, increased proliferation, and inhibition of apoptosis, and depends on transforming growth factor–induced activation of EGFR [47]. The present investigation revealed a nearly exclusive nuclear expression pattern of pSTAT3 in salivary gland cancer. Compared with that in normal salivary gland tissues, the staining intensity of pSTAT3 is reduced in the carcinomas. We found that weak expression or complete loss of nuclear pSTAT3 is associated with negative clinical prognostic parameters such as high-grade malignancy, lymph node metastases, and distant metastases. In contrast, strong expression of nuclear pSTAT3 is correlated with favorable survival in univariate und multivariate analysis. These findings are unexpected because activated STAT3 is described as a negative risk factor in different types of cancer such as cervical, prostate, laryngeal, gastric, or breast cancer [48]. However, the results are in accordance with previous reports on head and neck SQCCs [49], rectal cancer [48], prostate cancer [50], or breast cancer [51]. The reason for these contradictory observations is rather unclear. Dolled-Fillhart et al evaluated the immunohistochemical staining intensity of pSTAT3 in 346 node-negative breast carcinomas analogous to our methods and reported a better prognosis for nuclear pSTAT3 expression, concluding that tumors activating the STAT3 pathway are less aggressive than tumors that progress in the absence of STAT3 activation [51]. In the present study, tumors with weak or loss of nuclear pSTAT3 expression were strongly associated with high gene copy number of *EGFR* and *HER2*. Moreover, all tumors with strong pSTAT3 staining showed low survivin expression levels. It may be concluded that tumors activating the nuclear STAT3 pathway are less aggressive than tumors activating

the EGFR, HER2, or survivin pathway. A tumor-suppressing activity of STAT3 has been supposed, too. In an $Apc^{Min/+}$ mouse model, STAT3 expression promoted early intestine adenoma formation. However, at later stages of tumorigenesis, loss of STAT3 enhanced progression toward invasive carcinomas [52]. In glioblastomas, STAT3 was functioning suppressively in PTEN-deficient tumors, while promoting tumorigenesis in EGFRvIII expressing tumors [53].

In conclusion, this investigation shows that overexpression and increased gene copy number of EGFR and HER2 is found in different types of salivary gland cancer with impact on survival. Amplification is extremely rare, except for *HER2* in SDCs. High nuclear expression of the apoptosis inhibitor survivin, and weak expression or loss of nuclear pSTAT3 characterizes high-grade salivary gland carcinomas with unfavorable prognosis. Nuclear pSTAT3 seems to function as a tumor suppressor in the absence of EGFR, HER2, and survivin. Besides new molecular parameters, histologic grade remains the strongest predictor of survival.

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