Genomic aberrations of MDM2, MDM4, FGFR1 and FGFR3 are associated with poor outcome in patients with salivary gland cancer

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Fibroblast growth factor receptor 1 and 3 (FGFR1, FGFR3) impact on tissue homoeostasis, embryonic development and carcinogenesis. Murine double minute protein 4 (MDM4) and mouse double minute 2 homologue (MDM2) are regulators of p53-protein and may be the origin of an apoptosis overpowering cascade.

A collective of 266 carcinomas of salivary glands were investigated for MDM2, MDM4, FGFR1 and FGFR3 aberrations by fluorescence *in situ* hybridization (FISH). The results were matched with clinicopathological parameters and with expression of PTEN and p53.

MDM2 gene amplification ($n = 9$) and chromosomal aberrations (trisomy, $n = 47$; high polysomy, $n = 7$) are linked to high-grade malignancy ($P < 0.001$), lymph node metastasis ($P = 0.001$), advanced tumour size ($P = 0.013$) and stage ($P < 0.001$), gender ($P = 0.002$) and age ($P = 0.001$). MDM4 gene amplification ($n = 19$) and chromosomal aberrations (trisomy, $n = 34$; high polysomy, $n = 31$) are correlated to high-grade malignancy ($P < 0.001$), lymph node metastasis ($P = 0.008$), advanced tumour size ($P = 0.039$), stage ($P = 0.004$) and loss of PTEN ($P < 0.001$). Only, high-grade malignancy ($P < 0.001$), lymph node metastasis ($P = 0.036$) and advanced tumour stage ($P = 0.025$) are associated with FGFR3 amplification ($n = 1$) or chromosomal aberrations (low polysomy, $n = 61$; high polysomy, $n = 55$) but not with MDM4 alterations.

FGFR1 amplifications ($n = 5$) and chromosomal aberrations (trisomy, $n = 38$; high polysomy, $n = 30$) are associated with high-grade malignancy ($P < 0.001$), advanced tumour size ($P = 0.026$) and stage ($P = 0.004$), gender ($P = 0.016$) and age ($P = 0.023$).

Aberrations of MDM2, MDM4, FGFR1 and FGFR3 correlate with aggressive tumour growth and nodal metastasis. MDM2 ($P < 0.001$), MDM4 ($P = 0.005$) and FGFR3 ($P = 0.006$) alterations are associated with worse overall survival of patients with salivary gland cancer.

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Introduction

Salivary gland carcinomas are rare tumours and represent about 5% of all malignomas of the head and neck (1). Due to their histomorphological diversity, these tumours pose an enormous challenge regarding the histopathological diagnosis and the clinical management. As prognosis of these tumours is mainly determined by distant metastases investigators currently focus on possible targets for individual therapies. In this context, our research group found the receptor tyrosine kinases EGFR, HER2 and MET, which may function as therapy targets, to be overexpressed in different subtypes of salivary gland cancers (2–4). Fibroblast growth factor receptors (FGFR), consisting of FGFR1 – FGFR4, are transmembrane proteins with tyrosine kinase activity, which are currently also investigated as medical targets (e.g. Dovitinib) in different types of malignancies and also in salivary gland carcinomas (5, 6). FGFR influences embryonic development, tissue homoeostasis and cancer development via the RAS–MAPK or PI3K–AKT pathway in various cancer types (7–10). For example, FGFR3 is an activator of the p90 ribosomal S6 kinase (11) and therefore relevant for the initiation of oral cancer progression and an enhanced cell growth in oral cancer cells

(7, 12). Genomic aberrations of FGFR1 are found in breast cancer, lung cancer, oesophageal cancer and also in squamous cell carcinomas of head and neck (13). We could also recently show activity of the PI3K–AKT signalling in different subtypes of salivary gland carcinomas (14). The tumour suppressor gene PTEN (phosphatase and tensin homologue located on chromosome 10) is a major inhibitor of the PI3K–AKT cascade, and a strong association between loss of PTEN and high-grade salivary gland subtypes with worse prognosis was demonstrated (15).

The murine double minute protein 4 (MDM4) is a key regulator of the p53 protein by binding to the transactivation domain and inhibiting the transcriptional activity of p53 (16, 17). It consists of 11 exons and encodes a 490 amino acid protein (18). This protein may be the origin of a cascade, by which tumour cells can overpower apoptosis (19). In this context, amplifications or overexpression of MDM4 is documented in different tumour types including squamous cell carcinoma of the head and neck which are also correlated with a poor prognosis (20, 21). In addition, the mouse double minute 2 homologue (MDM2), which is structural similar to MDM4, shows oncogenic effects also by blocking the tumour suppressor p53 (22). The oncogenic capability is even more pronounced compared to the more selective MDM4 (23, 24). Thus, high levels of MDM2 are documented in different tumour types as well (22). The function of MDM2 and MDM4 in salivary gland cancer is rarely investigated to date (25, 26). Investigations of p53 brought ambivalent results with weak prognostic significance in some studies (27), whereas most others and also our own examinations could not find any significant impact of p53 (28–31).

To continue our previous work on salivary gland malignancies, this study explores the MDM2, MDM4, FGFR1 and FGFR3 expression in a representative cohort of salivary gland cancer by FISH analyses and correlates the results with the clinical outcome and with our former data on p53 and PTEN. To the best of our knowledge, this study is the first examination, which investigates the MDM4 expression in salivary gland tumours.

Patients and methods

Patients and therapeutic procedures

A total of 266 patients (135 female, 50.8%) with carcinomas of the major and minor salivary glands were examined. The mean age was 60.2 years (min 11, max 98). All patients were treated between 1984 and 2008 at the University Hospital Regensburg, the University Hospital Erlangen-Nuremberg and at the Hospital Clinic Nuremberg. In any case, a primary surgery was performed. Tumours were localized at the parotid gland in 188 (70.3%) cases, the submandibular gland in 36 (13.5%) cases and the sublingual gland in two (0.8%) cases. A total of 41 tumours (14.1%) were associated to the minor salivary glands.

A total of 70.7% (188 cases) underwent a conservative or radical neck dissection. A total of 156 patients (58.6%) were treated by adjuvant radio- or radiochemotherapy as a consequence of a N+ stage, perineural invasion, high-grade malignancy and positive resection margins. The pathological investigation of the neck dissection preparation showed cervical lymph node metastases in 31.7% (82 patients). A total of 29 patients (11.0%) were diagnosed with distant metastases. Overall, advanced tumour stages (UICC III, IV) were found in 55.3% of cases. The clinical tumour registries of Regensburg and Erlangen-Nuremberg recorded the follow-up data in consistence with the respective Research Ethic Guidelines of the medical faculties.

The follow-up time frame ranges from 0.1 to 21.2 years (mean 4.85). A recurrence was documented in 68 patients (26.6%). The disease-related death rate was 24.8% (66 patients) with a 5- and 10-year disease-specific survival rate of 74.6% and 69.3%, respectively.

Histological classification

All tumours were paraffin wax-embedded, stained by haematoxylin-eosin and examined by two independent experienced pathologists. On the basis of the current WHO classification of salivary gland tumours (32), the following tumour entities were integrated into the study: 40 acinic cell carcinomas (ACCC), 45 adenoid cystic carcinomas (ADCC), 45 mucoepidermoid carcinomas (MEC), 26 salivary duct carcinomas (SDC), 26 adenocarcinomas not otherwise specified (ACNOS), 26 squamous cell carcinomas (SQCC), 21 myoepithelial carcinomas (MYEC), eight polymorphous low-grade adenocarcinomas (PLGA), seven basal cell adenocarcinomas (BCAC), eight oncocytic carcinomas (OCC), five epithelial–myoepithelial carcinomas (EMC), three malignant mixed tumours (MMT), two undifferentiated carcinomas (UC), two large cell carcinomas (LCC) and two cystadenocarcinomas (CAC). PLGA, BCAC, OCC, EMC, MMT, UC, LCC and CAC were only found in a small number of patients and thus subsumed as 'others'. The grading was based on a three-tiered grading system (33, 34). ACCC, BCAC, EMC, CAC and PLGA were categorized as low grade (G1) as long as they were not dedifferentiated (G3). Otherwise, SDC, SQCC, MMT, OCC, UC and LCC were classified as high grade (G3). Mucoepidermoid carcinomas were graded analogous to the current three-tier WHO classification (32). In the rating of ACNOS, nuclear pleomorphism and mitotic activity were considered according to the Elston and Ellis grading of breast cancer (35). The malignant part of the tumour was crucial to the classification of the carcinomas ex pleomorphic adenoma. The mainly tubulo-cribriform adenoid cystic carcinomas were categorized as intermediate grade (G2), the mainly solid adenoid cystic carcinomas as high grade (G3). Myoepithelial carcinomas were classified as intermediate grade. In order to eliminate metastases to the salivary gland, intensive diagnostic procedures (CT/MRI of the head and neck, panendoscopy, X-ray/CT of the chest, ultrasonography of the abdomen) were performed in the group of SQCC. Moreover, squamoid variants of mucoepidermoid carcinoma were ruled out (36). By doing so, all cases of SQCC were classified as primary of the salivary gland.

Fluorescence in situ hybridization

First of all we prepared large-area slices of the tumour samples and performed a haematoxylin-eosin staining by which irrelevant areas like necrosis or haemorrhagic zones could be excluded from examination. By doing so, representing salivary gland tissue containing regions of the

tumour samples was identified for the fabrication of tissue microarray (TMA) with a core diameter of 2 mm. TMA slices were directly labelled after fixation on charged slides (SuperFrost Plus; Menzel GmbH, Braunschweig, Germany) and fluorescence *in situ* hybridization (FISH) was performed by ZytoLight SPEC FGFR1/CEN8, SPEC FGFR3/CEN4, SPEC MDM2/CEN12 and SPEC MDM4/1p12 dual colour probes (ZytoVision, Ltd, Bremerhaven, Germany). Afterwards, the counterstaining of the labelled hybridization nuclei was realized with antifading 4,6-diamidino-2-phenylindole Vectashield (Vector Laboratories, Burlingame, CA, USA). Complementary TMA sections were stained by haematoxylin–eosin for reference histology.

Twenty-five non-overlapped single cell nuclei were identified by two independent examiners by the help of epifluorescence microscopy (AxioImager-Z1; Zeiss, Göttingen, Germany). By doing so, the hybridization signals could be quantified. Samples of non-neoplastic salivary glands served as negative control.

The FISH ratio was assessed as the number of genes proportional to the number of centromeres, whereby the principles of Cappuzzo et al. (37, 38) were used to measure MDM2, MDM4, FGFR1 and FGFR3. Disomy: 2 gene/2 centromere signals in >50% of nuclei. Low polysomy/trisomy: 3 gene/3 centromere signals in >40%. High Polysomy: ≥4 gene/≥4 centromere signals in >40%. Amplification: ratio of gene/centromere ≥2, gene signals ≥10 or gene clusters. Deletion was defined as a ratio of gene/centromere <0.5 in more than 30% of nuclei. These principles were used for all analyses (Fig. 1).

Comparison to p53 and PTEN

The results of MDM2, MDM4, FGFR1 and FGFR3 were additionally correlated to p53 and PTEN. P53 was formerly evaluated in a smaller subset of salivary gland carcinomas by immunohistochemistry. In this study, p53 staining was assessed positive if more than 10% of tumour cells were stained (28). PTEN was investigated by immunohistochemistry and fluorescence *in situ* hybridization in 2012 in the same cohort of tumours as the current collective (15). Immunohistochemistry (IHC) for PTEN was semiquantitatively evaluated based on nuclear and cytoplasmic staining. 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 (39). Tumours were dichotomized into PTEN-negative (IRS 0–59) and PTEN-positive (IRS 60–300). In FISH analysis homozygous deletion of PTEN was defined by the simultaneous lack of both PTEN locus signals and by the presence of centromere signals in >20% of nuclei. Hemizygous deletion of PTEN was defined as >30% of tumour nuclei containing either one PTEN locus signal and ≥2 centromere signals or 2 PTEN locus signals and ≥4 centromere signals (relative deletions) (40). Both homo- and hemizygous deletion of PTEN were considered as PTEN loss.

Statistical analysis

Data were analysed with IBM SPSS Statistics 19 (Ehningen, Germany). Univariant survival curves were fabricated using the Kaplan–Meier method and distributions were compared by the log-rank test. The date of diagnosis and the disease-caused death respectively the end of follow-up were crucial for the determination of disease-specific overall survival. Clinicopathological features and the FISH results were compared using the chi-square test.

Results

One-hundred and eighty-four salivary gland carcinomas were examined by MDM2 FISH analysis. 121 cases (65.8%) showed physiological disomy. On the other hand, 47 (25.5%) patients featured trisomy, 7 cases (3.8%) showed high polysomy and 9 (4.9%) patients displayed an amplification. Disomy was most common in ACCC (96.2%), ADCC (91.7%) and MEC (79.3%). SQCC (38.9%), SDC (38.1%), MYEC (31.3%) and ACNOS (30.4%) showed trisomy frequently. High polysomy was evident most often in SQCC (11.1%), ACNOS (8.7%) and SDC (4.8%), amplifications were noticeable especially in ACNOS (13.0%) and MYEC (6.3%) (Table 1).

Aberrations of MDM2 (trisomy, high polysomy and amplification) were significantly linked to high-grade malignancy ($P < 0.001$), lymph node metastases ($P = 0.001$), advanced tumour size ($P = 0.013$), advanced tumour stage ($P < 0.001$), gender ($P = 0.002$) and advanced age ($P = 0.001$) (Table 2). Moreover, a significant impact of MDM2 on the disease-specific overall survival ($P < 0.001$) reinforces these data (Fig. 2).

MDM4 FISH analysis was performed on 234 salivary gland carcinomas. In 150 cases (64.2%) physiological disomy was detected, while 34 (14.5%) samples presented trisomy. A high polysomy could be revealed in 31 (13.2%) cases and an amplification in 19 (8.1%) cases. Disomy was most often found in epithelial–myoepithelial carcinomas (100%), polymorphous low-grade adenocarcinomas (83.3%), ACCC (75.0%), ADCC (85.0%) and MEC (74.4%). Basal cell adenocarcinomas (33.3%), MEC (21.1%) and SDC (20.0%) showed trisomy frequently. A high polysomy could be spotted most often in malignant mixed tumours (100%), SDC (36.0%) and ACNOS (19.2%), whereas amplifications were evident especially in oncocytic carcinomas (25.0%), ACNOS (19.2%) and SDC (12.0%) (Table 1).

Aberrations of MDM4 (trisomy, high polysomy and amplification) were significantly related to high-grade malignancy ($P < 0.001$), lymph node metastases ($P = 0.008$), advanced tumour size ($P = 0.039$) and tumour stage ($P = 0.004$) (Table 2). These findings are stressed by a significant impact of MDM4 aberrations on the disease-specific overall survival ($P = 0.017$) (Fig. 3).

The examination of 180 patients with carcinomas of the salivary glands by FGFR1 FISH analysis revealed 107 (59.4%) cases with disomy, 38 (21.1%) cases with low polysomy, 30 (16.7%) cases with high polysomy and 5 (2.8%) cases showed amplification. Disomy was primarily apparent in ACCC (96.3%), ADCC (86.4%) and MEC (79.3%). Low polysomy was commonest in ACNOS (52.2%), MYEC (40.0%), SQCC (35.3%) and SDC (28.6%). The most common entities with a high polysomy were SDC (28.6%) and SQCC (23.5%). Amplifications occurred especially in SQCC (5.9%), ADCC (4.5%) and ACNOS (4.3%) (Table 1).

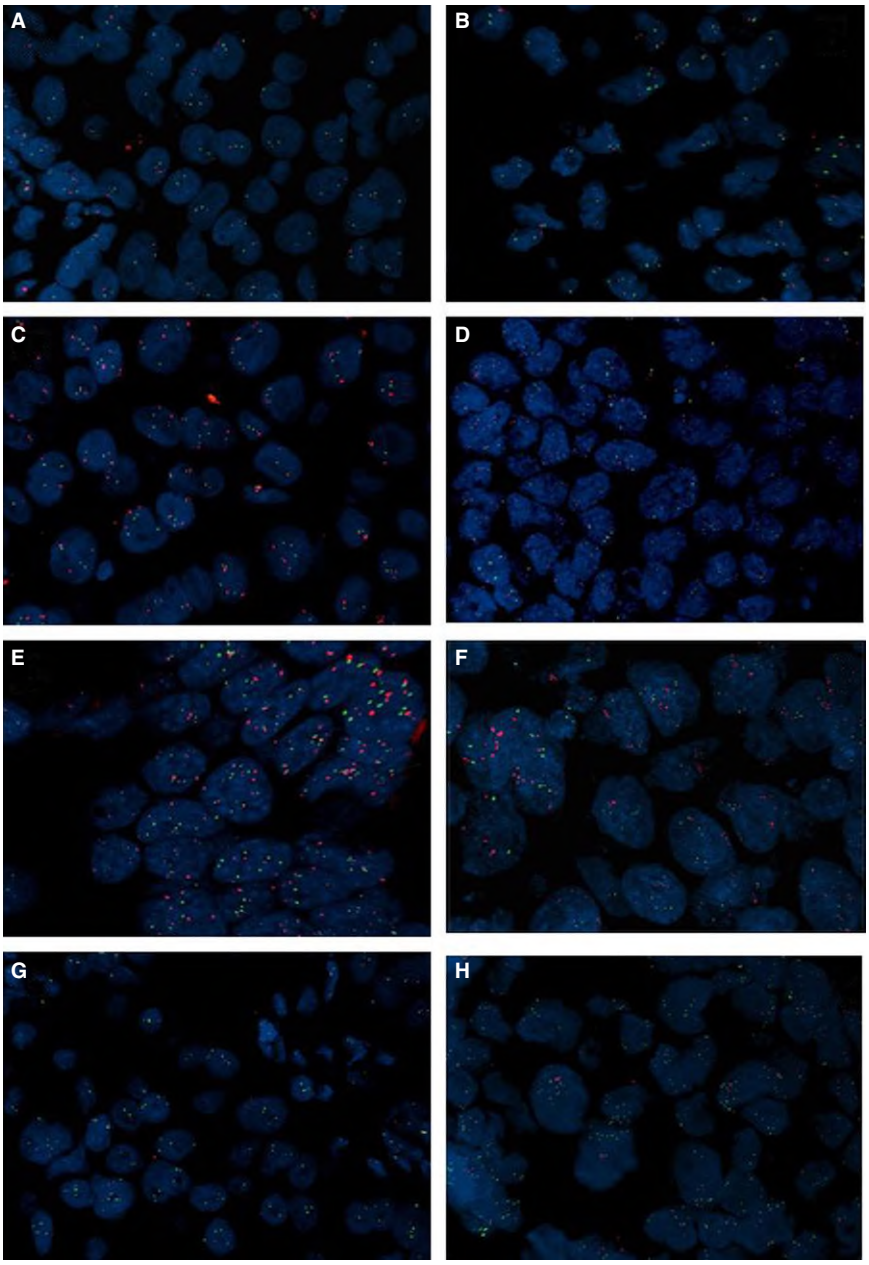


Figure 1 Samples of FISH analysis of FGFR3 and MDM4 in different subtypes of salivary gland carcinomas (red signal: centromere/green signal: gene locus): (A) Disomy of FGFR3 (×10) in carcinoma ex pleomorphic adenoma. (B) Disomy of MDM4 in an adenoid cystic carcinoma (×10). (C) Low polysomy of FGFR3 (×10) in carcinoma ex pleomorphic adenoma. (D) Low polysomy of MDM4 (×10) in adenocarcinoma NOS. (E) High polysomy of FGFR3 (×10) in mucoepidermoid carcinoma. (F) High polysomy of MDM4 (×10) in mucoepidermoid carcinoma. (G) Amplification of FGFR3 (×10) in an acinic cell carcinoma. (H) Amplification of MDM4 (×10) in myoepithelial carcinoma.

High-grade malignancy ($P < 0.001$), advanced tumour size ($P = 0.026$), advanced tumour stages ($P = 0.004$), gender ($P = 0.016$) and advanced age ($P = 0.023$) were clearly associated with FGFR1 aberrations (low polysomy, high polysomy and amplification). Lymph node metastasis ($P > 0.05$) was not significantly affected by FGFR1 (Table 2) as well as disease-specific overall survival ($P > 0.05$) (Fig. 4).

FGFR 3 FISH analysis was carried out in 211 salivary gland carcinomas. In doing so, 94 (44.5%) cases of disomy, 61 (28.9%) cases of low polysomy, 55 (26.1%) cases of high polysomy and 1 (0.5%) case of amplification were identified. Disomy was predominantly evident in ACCC (74.1%), MEC (54.1%) and ADCC (51.4%). Low polysomy was most often detected in MYEC (50.0%), ADCC (35.1%) and ACNOS (33.3%). SQCC (65.0%), ACNOS (41.7%) and SDC (30.4%) frequently presented high polysomy. The single case of an amplification was revealed in an ADCC (2.7%) (Table 1).

Compared to MDM4 only high-grade malignancy ($P < 0.001$), lymph node metastasis ($P = 0.036$) and advanced tumour stage ($P = 0.025$) were clearly associated

Table 1 Distribution of FGFR1, FGFR3, MDM2 and MDM4 gene status

Parameter	*Disomy (%)*	*Low polysomy (%)*	*High polysomy (%)*	*Amplification (%)*	*Total*
FGFR1-FISH					
ADCC	19 (86.4)	1 (4.5)	1 (4.5)	1 (4.5)	22
MEC	23 (79.3)	1 (3.4)	4 (13.8)	1 (3.4)	29
ACCC	26 (96.3)	1 (3.7)	0	0	27
ACNOS	7 (30.4)	12 (52.2)	3 (13.0)	1 (4.3)	23
SDC	9 (42.9)	6 (28.6)	6 (28.6)	0	21
SQCC	6 (35.3)	6 (35.3)	4 (23.5)	1 (5.9)	17
MYEC	9 (60.0)	6 (40.0)	0	0	15
Others	8 (30.8)	5 (19.2)	12 (46.2)	1 (3.8)	26
Total	107 (59.4)	38 (21.1)	30 (16.7)	5 (2.8)	180
FGFR3-FISH					
ADCC	19 (51.4)	13 (35.1)	4 (10.8)	1 (2.7)	37
MEC	20 (54.1)	11 (29.7)	6 (16.2)	0	37
ACCC	20 (74.1)	4 (14.8)	3 (11.1)	0	27
ACNOS	6 (25.0)	8 (33.3)	10 (41.7)	0	24
SDC	9 (39.2)	7 (30.4)	7 (30.4)	0	23
SQCC	5 (25.0)	2 (10.0)	13 (65.0)	0	20
MYEC	5 (27.8)	9 (50.0)	4 (22.2)	0	18
Others	10 (40.0)	7 (28.0)	8 (32.0)	0	25
Total	94 (44.5)	61 (28.9)	55 (26.1)	1 (0.5)	211
MDM2 FISH					
ADCC	22 (91.7)	2 (8.3)	0	0	24
MEC	23 (79.3)	5 (17.2)	1 (3.4)	0	29
ACCC	25 (96.2)	1 (3.8)	0	0	26
ACNOS	11 (47.8)	7 (30.4)	2 (8.7)	3 (13.0)	23
SDC	12 (57.1)	8 (38.1)	1 (4.8)	0	21
SQCC	9 (50.0)	7 (38.9)	2 (11.1)	0	18
MYEC	10 (62.5)	5 (31.3)	0	1 (6.3)	16
Others	9 (33.3)	12 (44.4)	1 (3.7)	5 (18.6)	27
Total	121 (65.8)	47 (25.5)	7 (3.8)	9 (4.9)	184
MDM4 FISH					
ADCC	34 (85.0)	2 (5.0)	2 (5.0)	2 (5.0)	40
MEC	29 (74.4)	5 (12.7)	4 (10.3)	1 (2.6)	39
ACCC	27 (75.0)	4 (11.1)	3 (8.3)	2 (5.6)	36
ACNOS	12 (46.2)	4 (15.4)	5 (19.2)	5 (19.2)	26
SDC	8 (32.0)	5 (20.0)	9 (36.0)	3 (12.0)	25
SQCC	15 (53.5)	5 (17.9)	5 (17.9)	3 (10.7)	28
MYEC	12 (63.2)	4 (21.1)	1 (5.2)	2 (10.5)	19
Others	13 (61.9)	5 (23.8)	2 (9.5)	1 (4.8)	21
Total	150 (64.2)	34 (14.5)	31 (13.2)	19 (8.1)	234

ACCC, acinic cell carcinoma; ACNOS, adenocarcinoma not otherwise specified; ADCC, adenoid cystic carcinoma; FGFR1, fibroblast growth factor receptor 1; FGFR3, fibroblast growth factor receptor 3; FISH, fluorescence in situ hybridization; MDM2, mouse double minute 2 homologue; MDM4, murine double minute protein 4; MEC, mucoepidermoid carcinoma; MYEC, myoepithelial carcinoma; SDC, salivary duct carcinoma; SQCC, squamous cell carcinoma.

with FGFR3 aberrations (low polysomy, high polysomy and amplification). Advanced tumour size ($P > 0.05$) was not affected by FGFR3 (Table 2). Nonetheless FGFR3 affected the disease-specific overall survival significantly ($P = 0.002$) (Fig. 5).

Regarding the connection between MDM4 and FGFR3 it was evident that MDM4 aberrations were adjuncted to FGFR3 aberrations explicitly ($P < 0.001$). Moreover aberrations of MDM4 were significantly correlated with aberrations of MDM2 ($P < 0.001$) and FGFR1 ($P = 0.004$) as well. Additionally high levels of MDM2 were associated with aberrations of FGFR1 ($P < 0.001$) and FGFR3 ($P < 0.001$). Beyond that a clear correlation between FGFR1 and FGFR3 aberrations was evident ($P = 0.002$).

The calculation of the hazard ratios revealed a higher risk for salivary gland tumours especially in MDM2 and FGFR3 aberrations (Table 3).

Comparison to p53 and PTEN

Comparison to p53 and PTEN is shown in Table 4. Positive immunostaining of p53 was higher in aberrant MDM2 (20.0% vs. 13.9%) and MDM4 (15.4% vs. 10.5%) without statistical significance ($P > 0.05$). However p53 positivity was associated with increased copy number of FGFR1 (38.5% vs. 9.8%, $P = 0.020$). P53 positivity was also higher in FGFR3 positive tumours than in negative ones (25.0% vs. 11.5%) but without statistical significance ($P > 0.05$).

Regarding PTEN we found both aberrant MDM2 ($P = 0.037$) and particularly MDM4 ($P > 0.001$) associated with genomic deletion of PTEN. 36.4% of tumours with aberrant MDM4 expression showed a deletion of PTEN compared to 16.1% PTEN deletions in non-aberrant MDM4 tumours. Immunohistochemical loss of PTEN was also significantly associated with MDM4 aberration ($P = 0.023$). With view to FGFR both FGFR1 (42.1% vs. 24.8%, $P = 0.161$) and FGFR3 (27.2 vs. 16.5, $P = 0.113$) copy number gain went along with higher percentages of PTEN deletions however not reaching statistical significance. Instead, increased copy number of FGFR1 was associated with immunohistochemical loss of PTEN expression ($P = 0.044$).

Discussion

In the present study, we explored the impact of MDM2, MDM4, FGFR1 and FGFR3 aberrations on the outcome of patients with salivary gland carcinomas.

Different groups could detect high levels of MDM4 in up to 50% in head and neck carcinomas (20). This is underlined by our study, where we found overexpression of MDM4 in more than 30% of the samples. Yu et al. mapped three single nucleotide polymorphisms (SNP) of MDM4. The combination of these SNP`s were responsible not only for higher risk of the development of oral and pharyngeal cancer, but also for advanced tumour stages and lymph node metastases (41). Hence, MDM4 overexpression seems to drive carcinogenesis and tumour progression and prognosticates poor outcome (42, 43). The study we present here corroborates previous findings as we revealed a significant association between MDM4 aberrations and poorly differentiated (high-grade) malignancies, lymph node metastasis and advanced tumour stage in salivary gland carcinomas.

Moreover a significant impact of MDM2, which features a similar biochemical structure as MDM4, on the occurrence of high-grade malignancy, lymph node metastasis, advanced tumour size, advanced tumour stage and disease-specific overall survival became obvious in this study. These findings are consistent with different other investigations where MDM2 overexpression was associated with the development of various human tumour types (44, 45). Mantesso et al. (46) could demonstrate the influence of a MDM2 overexpression on tumour progression in salivary gland carcinomas. Beyond that De Lima Mde et al. (47) presented the influence of MDM2 on tumorigenesis in adenoid cystic carcinomas, even though they could not connect high levels of MDM2 with tumour progression.

Table 2 FGFR1, FGFR3, MDM2 and MDM4 gene status and important clinicopathological parameters

	FGFR1 FISH		*FGFR3 FISH*		*MDM2 FISH*		*MDM4 FISH*	
	Disomy (%)	*Aberration (%)*	*Disomy (%)*	*Aberration (%)*	*Disomy (%)*	*Aberration (%)*	*Disomy (%)*	*Aberration (%)*
Age								
<70	**70 (66.0)**	**36 (34.0)**	59 (45.7)	70 (54.3)	**83 (75.5)**	**27 (24.5)**	100 (67.6)	48 (32.4)
>70	**37 (50.0)**	**37 (50.0)**	35 (42.7)	47 (57.3)	**38 (51.4)**	**36 (48.6)**	50 (58.1)	36 (41.9)
Gender								
Male	**40 (50.0)**	**40 (50.0)**	39 (38.6)	62 (61.4)	**43 (53.8)**	**37 (46.3)**	68 (57.7)	50 (42.3)
Female	**67 (67.0)**	**33 (33.0)**	55 (50.0)	55 (50.0)	**78 (75.0)**	**26 (25.0)**	82 (70.6)	34 (29.4)
No recurrence	**85 (64.4)**	**47 (35.6)**	72 (49.0)	75 (51.0)	**97 (71.3)**	**39 (28.7)**	111 (67.3)	54 (32.7)
Recurrence	**18 (42.9)**	**24 (57.1)**	20 (37.7)	33 (62.3)	**20 (48.8)**	**21 (51.2)**	35 (58.4)	25 (41.6)
T-stage								
T1–T2	**77 (64.7)**	**42 (35.3)**	59 (49.2)	61 (50.8)	**87 (71.3)**	**35 (28.7)**	**90 (69.8)**	39 (30.2)
T3–T4	**29 (48.3)**	**31 (51.7)**	34 (38.6)	54 (61.4)	**32 (53.3)**	**28 (46.7)**	**57 (56.4)**	44 (43.6)
Stadium								
I–II	**63 (69.2)**	**28 (30.8)**	**48 (53.3)**	**42 (46.7)**	**73 (79.3)**	**19 (20.7)**	**73 (74.5)**	**25 (25.5)**
III–IV	**43 (48.9)**	**45 (51.1)**	**45 (37.5)**	**75 (62.5)**	**47 (51.6)**	**44 (48.4)**	**75 (56.0)**	**59 (44.0)**
N-Stage								
N0	79 (63.2)	46 (36.8)	**65 (48.1)**	**70 (51.9)**	**92 (73.0)**	**34 (27.0)**	**106 (70.2)**	45 (29.8)
N1–N3	27 (50.0)	27 (50.0)	**26 (37.1)**	**44 (62.9)**	**27 (48.2)**	**29 (51.8)**	**39 (51.3)**	**37 (48.7)**
M-Stage								
M0	95 (57.9)	69 (42.1)	84 (44.7)	104 (55.3)	113 (67.3)	55 (32.7)	133 (63.9)	75 (36.1)
M1	10 (71.4)	4 (28.6)	8 (38.1)	13 (61.9)	6 (42.9)	8 (57.1)	15 (62.5)	9 (37.5)
Grading								
G1–G2	**66 (79.5)**	**17 (20.5)**	**62 (58.5)**	**44 (41.5)**	**69 (80.2)**	**17 (19.8)**	**84 (77.1)**	25 (22.9)
G3	**41 (42.3)**	**56 (57.7)**	**32 (30.5)**	**73 (69.5)**	**52 (53.1)**	**46 (46.9)**	**66 (52.8)**	59 (47.2)

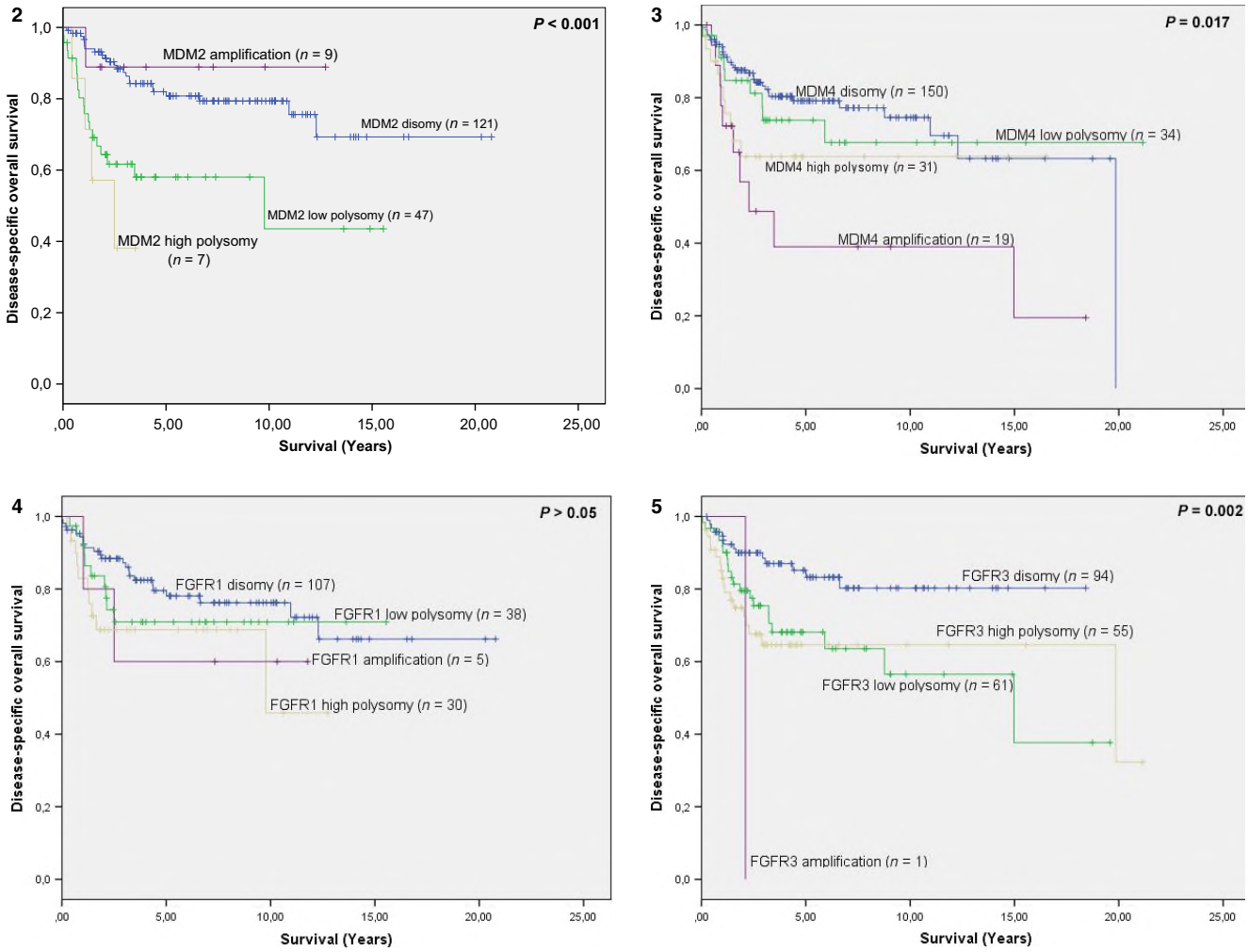
FGFR1, fibroblast growth factor receptor 1; FGFR3, fibroblast growth factor receptor 3; FISH, fluorescence *in situ* hybridization; MDM2, mouse double minute 2 homologue; MDM4, murine double minute protein 4.
Statistically significant associations are highlighted in bold.

Both MDM2 and MDM4 are substantial correcting variables of the tumour suppressor p53, which is turned off in 50% of tumours including squamous cell carcinomas of head and neck (48–52). Reduced levels of MDM2 and MDM4 create a p53-dependent apoptosis in different cells. On the other hand overexpression of MDM2 and MDM4 is a possible mechanism to operate against p53-related apoptosis, which is obvious in numerous cancer types (19, 51). In this context, MDM4 is able to stabilize the MDM2-p53 complex (53). We have already demonstrated impaired activity of p53 in certain subtypes of salivary gland cancer before (28, 54). However, in these investigations p53 did not show any significant impact on prognosis, which is in concordance with most other studies investigating p53 in salivary gland malignancies (29–31). In this study, now we additionally compared p53 expression to the current results of MDM2 and MDM4. In contrast to our expectation we did not find a significant correlation of MDM2 and MDM4 aberration with positive immunostaining of p53. One reason might be the lower case numbers (77 for MDM2 and 51 for MDM4) for this comparison. Another reason might be that mutation of p53 may not lead to protein over-expression, as non-sense and frameshift mutations may lead to the synthesis of a truncated or unstable protein not detectable by immunohistochemistry (55).

Interestingly, we found loss of PTEN strongly associated with aberrant and increased MDM2 and especially MDM4 expression. This is in line with findings in glioblastoma, where the PTEN tumour suppressor protein inhibits PI3K/AKT signalling, that promotes translocation of MDM2 into the nucleus. In addition, PTEN modulates MDM2 transcription and isoform selection by negatively regulating its promoter. In PTEN-null cells MDM2 promoter activity is upregulated, resulting in increased MDM2 expression (56). On the other hand transcription of PTEN is regulated by p53 (57).

Therapeutic inhibitors are developed, which are designed to block the MDM2-MDM4-p53-cascade. Gembarska et al. could exhibit the suppression of melanoma cells by inhibiting the MDM4-p53-pathway through application of SAH-p53-8. By doing so the effectivity of conventional chemotherapeutics was improved as well. Even the MDM2-p53-dependent effects were diminished although in a smaller extent (58). Secchiero et al. (59) presented the apoptotic effect of the MDM2-inhibitor Nutlin-3 on acute myeloid leukaemic blasts and therefore mentioned this as a potential therapeutic option. In particular, a combined inactivation of MDM4 and MDM2 by a stapled peptide (SAH-p53-8, RO-5963) could create apoptotic activity despite of MDM4 overexpression (60). Corresponding to our results high levels of MDM4 often correlate with high levels of MDM2. In view of this fact a double inhibition of MDM4 and MDM2 is possibly relevant. Although first phase I clinical studies present promising results, the possible toxic effects on normal tissue are uncertain and potential oncogenic adverse reactions with a long lag time are being debated (19, 20).

The activation of FGFR by FGF leads to an autophosphorylation and therefore to an activation of the tyrosine kinase domain. Via the RAS/MAPK- and the PI3K/AKT pathway cell proliferation is typically stimulated and cell survival enhanced (61). FGFR alterations as gene amplification, translocation and point mutations have been shown to contribute or even to trigger cancerogenesis for example



Figures 2-5 Kaplan–Meier survival rates of MDM2, MDM4, FGFR1 and FGFR3 FISH analyses.

in lung, breast, gastric, multiple myeloma, glioblastoma multiforme, uterine and bladder (61). Referring to salivary gland carcinomas recurrent mutations of the FGF/IGF/PI3K pathway were found in adenoid cystic carcinomas that may potentially offer new possibilities for therapy (62). Recently, copy number alterations of FGFR1 were detected by the use of FISH analysis in salivary duct carcinoma (26). In the current study FGFR aberrations were also investigated by FISH analysis.

Here we revealed FGFR1 aberrations predominantly in salivary duct carcinomas, adenocarcinomas NOS and squamous cell carcinomas. The aberrations are clearly associated with advanced tumour size, advanced tumour stage and high-grade malignancy (G3). This is in line with the fact that aberrations of FGFR1 are evident in different tumour types such as breast cancer, lung cancer, oesophageal cancer and also squamous cell carcinoma of the head and neck (13). The activation of the tyrosine kinase receptor FGFR1 by FGF2 enhances mitogenic activity, impairs cell differentiation and finally promotes advanced malignant characteristics (63–65). Concordantly, high expression of FGFR1 (and other receptors) in carcinomas ex pleomorphic adenoma (66), in adenoid cystic carcinomas and mucoepidermoid carcinomas (64) and in saliva and serum of patients with carcinomas of the salivary gland have been reported (67).

FGFR3 aberrations were mostly found in salivary duct carcinomas, adenocarcinomas NOS, squamous cell carcinomas and myoepithelial carcinomas. FGFR3 seems to play a pivotal role in carcinogenesis, for example of the bladder,

Table 3 FGFR1, FGFR3, MDM2 and MDM4 aberrations and the corresponding hazard ratio

Variable	*Coding*	*Univariant Log-rank*	*Multivariant*	
			Significance	*HR (95% CI)*
FGFR1 FISH	No CNG vs. CNG	>0.05	0.081	0.389 (0.135–1.123)
FGFR3 FISH	No CNG vs. CNG	0.002	0.016	3.600 (1.268–10.225)
MDM2 FISH	No CNG vs. CNG	<0.001	0.006	4.787 (1.581–14.499)
MDM4 FISH	No CNG vs. CNG	0.017	0.667	0.802 (0.294–2.191)

FGFR1, fibroblast growth factor receptor 1; FGFR3, fibroblast growth factor receptor 3; FISH, fluorescence *in situ* hybridization; MDM2, mouse double minute 2 homologue; MDM4, murine double minute protein 4; CNG, copy number gain.

Table 4 Comparison of MDM2, MDM4, FGFR1 and FGFR3 to p53 and PTEN

	MDM2		*MDM4*		*FGFR1*		*FGFR3*	
	Neg	*Pos*	*Neg*	*Pos*	*Neg*	*Pos*	*Neg*	*Pos*
P53								
Neg	62	4	34	11	**55**	**8**	23	15
Pos	10	1	4	2	**6**	**5***	3	5
PTEN (IH)								
>60	111	8	**120**	**54**	**101**	**17**	73	78
<60	32	5	**23**	**23***	**24**	**11***	14	25
PTEN (FISH)								
Disomy	**88**	**3**	**120**	**49**	79	11	71	75
Deletion	**30**	**5***	**23**	**28*****	26	8	14	28

FGFR1, fibroblast growth factor receptor 1; FGFR3, fibroblast growth factor receptor 3; FISH, fluorescence *in situ* hybridization; MDM2, mouse double minute 2 homologue; MDM4, murine double minute protein 4; PTEN, phosphatase and tensin homologue located on chromosome 10; IH, immunohistochemistry.
Statistically significant associations are highlighted in bold.
*$P < 0.05$.
***$P < 0.001$.

the cervix and multiple myeloma (12). High levels of FGFR3 lead to an enhanced cell proliferation, growth and survival (9). In this context, the PI3K–AKT and the MAPK pathway are discussed as possible mechanisms as outlined above (7, 9). For example, Vairaktaris et al. (7) examined oral mucosa cells with dysplasia or early tumour invasion and could reveal a significantly higher expression of FGFR3 in those cells. Moreover, Henson et al. described an FGFR3 overexpression in oral cancer cells compared to normal human oral keratinocytes. Beyond that they measured a 35% lower proliferation rate in oral cancer cells in the presence of reduced FGFR3 expression (12). These observations are in line with the results of our study. The genetic instability in our salivary gland cancer cells concerning FGFR3 was also obvious in oral squamous cancer cells. Zhang et al. (68) detected point mutations of FGFR3 in oral squamous cancer cells in 62% of the cases.

In this study, we additionally compared the expression of FGFR1 and FGFR3 to PTEN as a major inhibitor of the PI3K/AKT/mTOR-pathway. We found, that increased copy numbers of FGFR1 and FGFR3 went along with chromosomal PTEN deletion although not reaching statistical significance. Immunohistochemical loss of PTEN expression was significantly associated with genomic overexpression of FGFR1. So it seems, that loss of PTEN function facilitates FGFR-mediated PI3K–AKT signalling in certain types of high-grade salivary gland carcinomas. We also compared the FGF-receptors to p53 and found a significant correlation between FGFR1 and p53 immunostaining. However, as reported above, interpretation of immunohistochemical p53 staining should be performed with caution.

With respect to the clinical management of salivary gland cancer, elevated FGFR1/FGFR3 expression has been associated with resistance to endocrine therapy regimens (9, 62, 68, 69). For example, enhanced levels of FGFR3 in tamoxifen-resistant tumours were observed, that cause PI3K–AKT and PLCγ-activation (9). Furthermore FGFR3 seems to play a role in the development of a radioresistance of oral squamous carcinoma cells (70). As outlined above a reduced proliferation rate of oral cancer cells could be achieved by blocking the FGFR3 expression. This approach can potentially be enhanced by additional ionizing radiation (12). Moreover, the incubation of human cancer cells with FGFR inhibitors can boost the antiproliferational effect of conventional chemotherapeutics (5-fluorouracil, paclitaxel, etoposid) (71, 72).

Regarding FGFR the results of our study could contribute to an improvement of the patients' outcome by a supplementation of the medicamentous cancer therapies by tyrosine kinase inhibitors (TKI). The anticancer effects of the TKI Dovitinib is already evident in phase I, phase II and phase III clinical trials in the treatment of renal, breast, endometrial and urothelial cancer (10, 69). In particular Dovitinib is examined in phase II trials in the treatment of ACC with promising results (73). The origin of this approach is the observation, that dovitinib can suppress tumour growth in ACC xenograft model (74). Our results give hint that not only ACC but salivary gland carcinomas in general could be targeted with tyrosine kinase inhibitors as well. In this context, the identification of suitable patients with FGFR aberrations via FISH analysis might be helpful, as it was performed in our study.

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Conflict of interest

The authors declare that they have no conflict of interest.