

Fibroblast growth factor receptor 1 amplification is a common event in squamous cell carcinoma of the head and neck

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Recently, we characterized *fibroblast growth factor receptor 1* amplification as a target for a rational therapy in lung squamous cell carcinoma. Patients harboring this genetic event are currently eligible for treatment with antifibroblast growth factor receptor small-molecule inhibitors in phase I clinical trials. This has the potential to significantly improve standard therapy for lung squamous cell carcinoma patients. The aim of this study was to elucidate whether *fibroblast growth factor receptor 1* amplification is also a common genetic event in head and neck squamous cell carcinoma. For this purpose, we assembled a cohort of 555 patients, including 264 with metastatic disease and 147 with recurrent disease. Formalin-fixed, paraffin-embedded material of primary tumors, metastases and recurrences were assessed for *fibroblast growth factor receptor 1* copy number status using fluorescence *in situ* hybridization. Human papilloma virus status was detected by p16 immunohistochemistry staining and PCR-ELISA. Molecular parameters were correlated with each other and with clinicopathological data. We found 15% of primary head and neck squamous cell carcinoma to display a *fibroblast growth factor receptor 1* amplification. In nearly all cases, metastatic and recurrent tumor samples shared the same amplification status as the corresponding primary tumor. *Fibroblast growth factor receptor 1* amplification was associated with nicotine and alcohol consumption, but was mutually exclusive with human papilloma virus infection. Amplification of the gene was associated with parameters of worse outcome. Our data identify *fibroblast growth factor receptor 1* amplification as a frequent event in primary and metastatic head and neck squamous cell carcinoma and represents a potential biomarker for more aggressive disease. *Fibroblast growth factor receptor 1*-amplified tumors could potentially comprise a subset of head and neck squamous cell carcinoma against which targeted small-molecule inhibitors hold therapeutic efficacy.

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Molecular profiling of human malignancies has become a major focus of research over the past

decade. Catalogs of genomic alterations for specific cancer types are leading to a better understanding of cancer biology and promoting the identification of molecular targets for rational therapies.^{1–3} In comparison to conventional chemotherapies, rational therapies have the advantage of a better toxicity profile through target selectivity.⁴ Moreover, they can be as effective as conventional therapies or induce synergistic effects.^{5–9}

Recently, we described fibroblast growth factor receptor 1 (FGFR1) as the first actionable target in

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squamous cell lung cancer. FGFR1 is a receptor tyrosine kinase located on chromosome 8p12. Receptor tyrosine kinases commonly contribute significantly to the development of cancer.¹⁰ When recognizing their fibroblast growth factor ligands, FGFRs undergo dimerization, leading to the phosphorylation of specific intracellular receptor domains, and ultimately leading to the activation of cytoplasmatic effector molecules. The effector pathways following FGFR activation include the Ras/MAPK and PI3/Akt pathways, which in turn transmit the activating signal into the nucleus, where cell differentiation and proliferation are regulated.¹¹

FGFR1 amplification was first described as a potential therapeutic target in breast adenocarcinomas.^{12,13} Soon after, Weiss *et al*¹⁴ identified *FGFR1* amplification as an actionable target in squamous cell carcinoma of the lung.¹⁴ Of note, FGFR inhibitors in use for clinical trials are not specific to a particular FGFR. The treatment of patients with lung cancer is currently the subject of clinical trials based on *FGFR1* amplification, and also based on the fact that both studies mentioned above and also others were able to demonstrate an oncogenic addiction to FGFR1 signaling of *FGFR1*-amplified cells, which are highly specific to inhibition with pan-FGFR inhibitors,^{14–16} as cells were sensitive to pan-FGFR inhibitors, although FGFR2-4 were not relevantly expressed.¹⁷ We recently described *FGFR1* amplifications in regional lymph node metastases of *FGFR1*-amplified squamous cell lung cancer, broadening the rationale for therapy with these small-molecule inhibitors to the setting of metastatic disease.¹⁸

Head and neck squamous cell carcinoma is the most common malignant tumors in the head and neck region. Although some head and neck squamous cell carcinoma can be cured by radical surgical resection, many patients are diagnosed at advanced stages of disease associated with local tumor invasion and metastatic disease. Therefore, the development of effective systemic therapies, including targeted therapies, remains an important area of intense research focus. As was recently the case for squamous cell lung cancer, currently there are no effective targeted therapies available for head and neck squamous cell carcinoma. Given the histomorphological and clinical similarities between head and neck squamous cell carcinoma and squamous cell lung cancer, we proposed that FGFR1 might be involved in the pathogenesis and aggressiveness of head and neck squamous cell carcinoma. Supporting this hypothesis, Freier *et al*¹⁹ recently reported *FGFR1* amplifications in 17% of oral squamous cell carcinomas in a limited number of patient samples. Furthermore, initial functional studies describe a potential role of FGFRs in head and neck squamous cell carcinoma. However, these studies have not examined amplification status of the *FGFR1* gene in relation to therapeutical sensitivity.¹⁷ In this study,

we set out to characterize the prevalence of *FGFR1* amplification in a comprehensive and clinically well-characterized cohort comprising primary, metastatic and recurrent head and neck squamous cell carcinoma of all localizations of the head and neck area.

Materials and methods

Patient Cohort

All patients were treated surgically with curative or palliative intent between 1997 and 2011 at the University Hospital of Bonn. Distribution of the clinicopathological data reflects a representative Caucasian cohort of surgically treated head and neck squamous cell carcinoma patients (Supplementary Material 1).

In our study, we included a total of 810 tumor samples derived from 555 patients with head and neck squamous cell carcinoma (488 cases primary tissue available, 23 cases only metastatic tissue available, 14 cases only recurrence tissue available, 5 cases only distant metastases available, 25 cases only clinicopathological data in statistical analysis) (Table 1). Sites of available primary tumor tissue origin were distributed as follows: hypopharynx ($n = 54$), oropharynx ($n = 156$), oral cavity ($n = 110$), larynx ($n = 161$) and unknown in seven patients (carcinoma of unknown primary).

Forty-eight percent (264/555) of all patients presented with lymph node metastasis. In 207/264 cases, we had primary tumor and corresponding lymph node metastasis available, while in 23 patients, we had only metastatic tissue without the corresponding primary tumor, and in 34 cases, lymph node metastases were clinically described, but no tissue was available for assessment. In patients with multiple positive lymph nodes, we assessed up to three lymph node metastases: in 217 cases there was 1 lymph node metastasis available, in 6 cases there was 2 lymph node metastases and in 4 cases there was 3 lymph node metastases, all of which were assessed. Overall, 241 lymph node metastases were analyzed.

Clinically, 24 patients displayed distant metastases (13/24 specimens available). Out of the cases

Table 1 Distribution of our cohort apportioned by tissue availability, assessability and *FGFR1* copy number status

Tissue	Tissue available	Tissue assessable		FGFR1 copy number status		
		Yes	No	No amplification	LLA	HLA
Primary tumors	488	452	36	384	52	16
Metastases	241	223	18	182	32	9
Distant metastases	13	13	0	9	3	1
Recurrences	68	64	4	55	7	2

with an available specimen, 5 cases derived from the lung, 5 cases from soft tissue, 2 cases out of the parotid gland and 1 case of bone metastasis. Out of the 13 available cases with distant metastases, we had corresponding primary head and neck squamous cell carcinoma available in 8 cases. Twenty-seven percent (147/555) patients experienced recurrence of disease, and recurrent tumor specimens were available in 68 cases. In 54 cases, the corresponding primary and recurrent tumors were both available for analysis. Therapy information, drinking habits and smoking status were available for almost all patients. The study was approved by the institutional ethics review board (no. 148/11).

Tissue Microarray Construction

Tissue microarray construction was applied as described earlier.²⁰ In summary, three representative cores measuring 0.6 mm in diameter from each formalin-fixed, paraffin-embedded primary tumor, its corresponding lymph node metastasis, distant metastasis, recurrence and benign tissue were assembled into tissue microarray blocks and stained with hematoxylin and eosin (H&E).

FGFR1 Fluorescence *In Situ* Hybridization

Overall, fluorescence *in situ* hybridization for the detection of *FGFR1* amplification status on genomic level was performed as described earlier.¹⁴ In brief, the *FGFR1* target probe (red fluorescent signal) spanning the 8p11.22–23 locus (RP11-148d12) and a commercially available reference probe (green fluorescent signal) located on the centromeric region of chromosome 8 (Metasystems, Altussheim, Germany) were selected for hybridization. Only nuclei displaying green reference signals were included for the determination of the *FGFR1* copy number status. All samples were independently analyzed by three evaluators (FG, AF, MB) under a $\times 63$ oil immersion objective with a fluorescence microscope (Zeiss, Jena, Germany). In each case, we assessed at least 100 tumor cell nuclei. A sample was considered amplified if at least 20% nuclei displayed the *FGFR1* amplification. A high-level amplification was defined as additional nine or more red target signals or clusters of target gene signals as compared with the green reference signals. Lower than nine but more than two red target signals as compared with the green reference signals were assigned to be low-level amplified.

P16 Immunohistochemistry

Serial sections of tissue microarrays were deparaffinized in xylene and stepwise incubated in 100–70% ethanol. Sections were pretreated in autoclave for

45 min (maximum temperature 125 °C for 3 min, cool down to 90 °) and in included pretreatment buffer. Primary monoclonal mouse anti-p16INK4A antibody (CINtec by MTM Laboratories AG, Heidelberg, Germany) was added to the slides for 60 min at room temperature, followed by incubation with a secondary HRP-conjugated goat anti-mouse antibody (MTM Laboratories) at room temperature for 1 h (for details see manufacturer's recommendations). After counterstaining of the peroxidase complex, sections were dehydrated and embedded with Eukitt (Fluka).

HPV Analysis

The HPV Type 3.5 LCD-Array Kit was used for the determination of HPV subtypes by hybridization to HPV-specific DNA probes (Chipron GmbH, Berlin, Germany). Amplification of HPV-specific DNA segments (L1 region) was achieved by using the primer sets HPV '125' and HPV MY09/MY11. Ten microliters of the amplification products were hybridized to HPV type-specific capture probes fixed to an LCD array chip. All steps were performed according to the manufacturer's recommendations.

Statistical Analysis

All statistical analysis and graphical output were carried out with R version 2.13.0.

For ordinally scaled nonparametric data, the Wilcoxon–Mann–Whitney *U*-test (W–M–W–*U*) was used. In case of more than two groups, we used the extended W–M–W–*U* test. Fisher's exact test was used for computing statistical significance. Mean value comparison were carried out by *t*-test or analysis of variance in case of more groups than two. In all tests, 0.05 was chosen as the level of significance. Two groups according to the median value were defined for age (62 years), recurrence-free survival (13 months) and overall survival (26 months). In Figure 2, values are displayed in logarithmic manner for better visualization. As the number 1 becomes 0, we added 1 count to every value to enable visualization of small numbers.

Results

FGFR1 Amplification Status in Primary Head and Neck Squamous Cell Carcinoma

Of the 488 analyzed primary tumor samples, 93% (452/488) cases were assessable. Of these, 23% (103/452) were from the oral cavity, 33% (148/452) from the oropharynx, 10% (47/452) from the hypopharynx and 33% (147/452) from the larynx, whereas in 7 (2%) cases the primary site was unknown. Of all assessable primary tumors, 15% (68/452) displayed an *FGFR1* amplification with 12% (52/452) low-level and 4% high-level amplification (16/452).

FGFR1 amplification was most common in primary squamous cell carcinomas of the hypopharynx (23%; 11/47) and larynx (18%; 26/147), and less common in squamous cell carcinomas of the oropharynx (14%; 21/148) and oral cavity (9%; 9/103), which are the areas commonly afflicted by HPV infection ($P < 0.05$). Control samples of benign squamous cell tissue were always negative for *FGFR1* amplification.

***FGFR1* Amplification Status in Metastatic Head and Neck Squamous Cell Carcinoma**

Of 264 patients with clinically determined metastatic diseases, we successfully assessed 223 lymph node metastases. Eighteen percent (41/223) displayed *FGFR1* amplification with 14% low-level amplification (32/223) and 4% high-level amplification (9/223).

Of the 207 cases with available corresponding primary tumor and lymph node metastasis, we had 26 non-comparable cases and 181 cases in which the primary and corresponding lymph node metastasis tissues were both assessable. Eighty-eight percent

(160/181) of cases displayed a concordant *FGFR1* copy number status in the primary and metastatic tissue. Of these, we found 4% (6/160) of the cases to be high-level amplified (Figure 1a and a1), 8% (13/160) cases low-level amplified and 88% (141/160) of the cases displayed no amplification (Figure 1c and c1). In 12% (21/181) cases, *FGFR1* copy number status was discordant (Figure 2a).

In the seven cases with more than one lymph node metastasis, all cases were assessable. Four cases had the same copy number status in all analyzed tissue and three cases had differing *FGFR1* copy numbers.

Furthermore, we had tumor tissue of 13 distant metastases. Of these, four displayed *FGFR1* amplification (2/4 lung, 2/4 others) and nine wild-type *FGFR1* (Figure 1c1) (3/9 lung, 6/9 others). Out of the eight corresponding cases of primary head and neck squamous cell carcinoma and distant metastases, five cases had identical *FGFR1* copy number status (3 × lung, 2 × others), all of which displayed a wild-type *FGFR1* copy number status. In three cases, we had differing *FGFR1* copy number status in primary tumor and distant metastases (2 × lung, 1 × others) (Figure 2b).

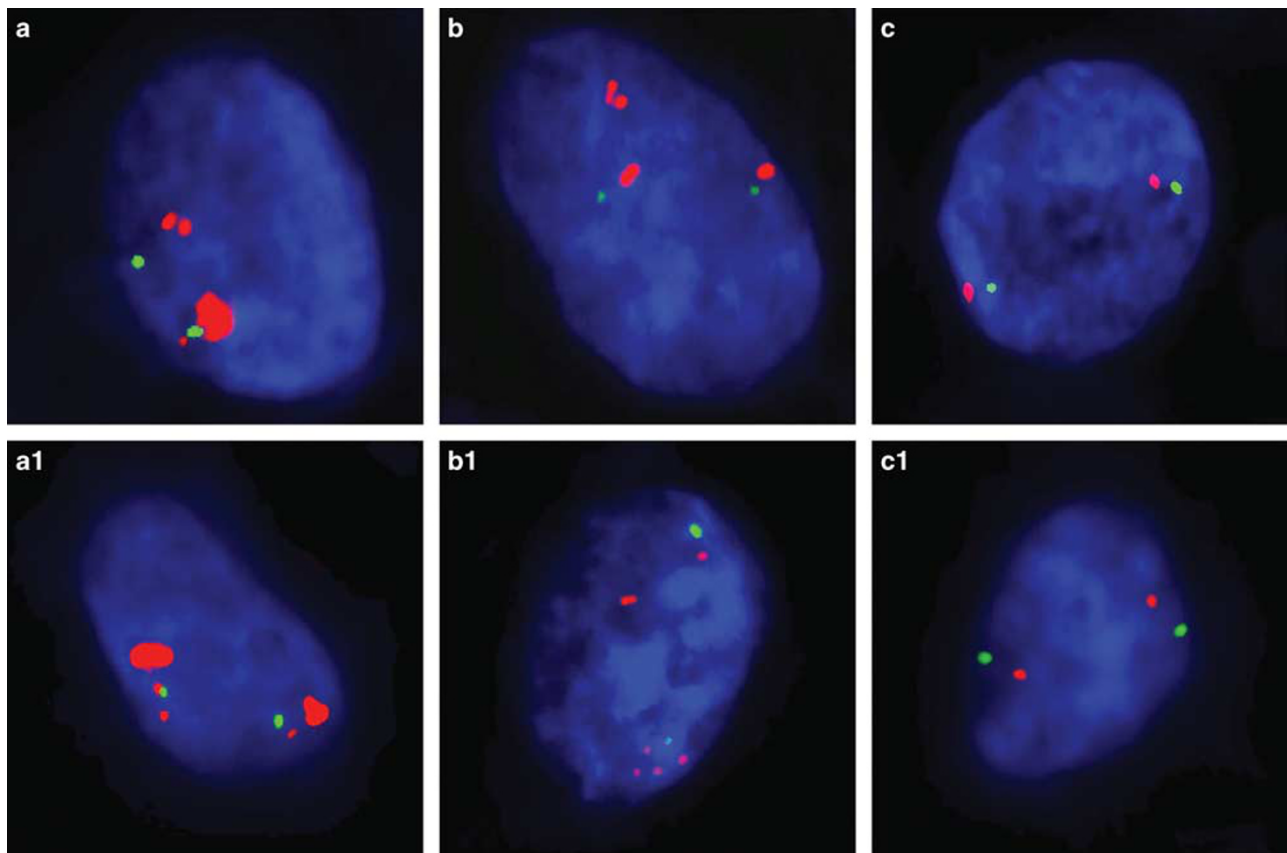


Figure 1 FISH images of primary tumors (a, b, c) (upper row) and their corresponding tumor tissues (lower row) (a1, b1, c1). A high level amplification (HLA) is represented by a primary tumor (a) and its corresponding lymph node metastasis (a1). (b) is an example of low level amplification (LLA) in a primary tumor and its corresponding recurrent tumor tissue (b1). An example of a wild type copy status is shown in a primary tumor (c) and its corresponding distant metastasis (c1).

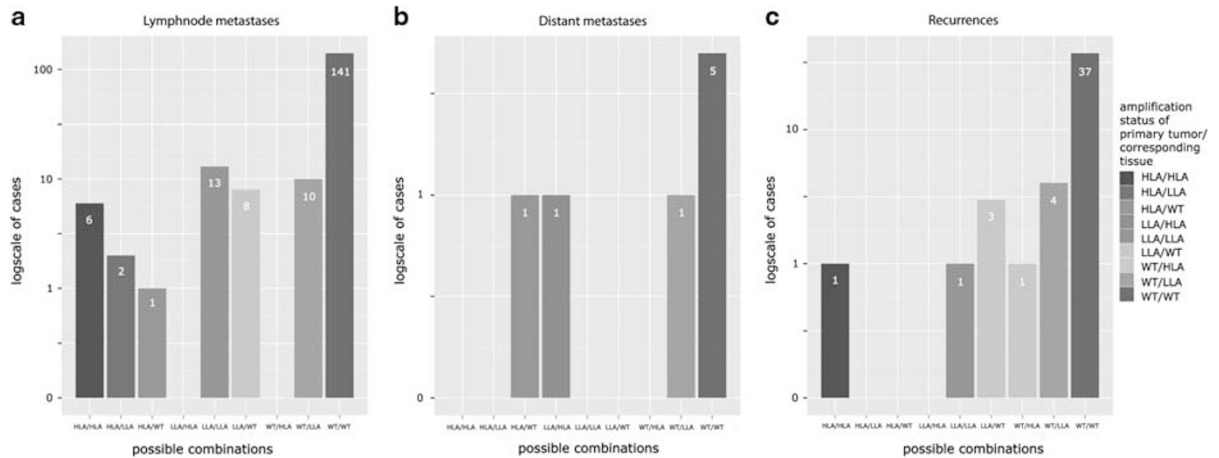


Figure 2 Histogram of *FGFR1* copy number status and corresponding tumor tissue. (a) Histogram of *FGFR1* amplification status in primary tumors and corresponding lymph-node metastases. The colors indicate the possible combinations of different amplification levels. (b) Histogram of the corresponding *FGFR1* amplification status in primary tumor and its corresponding distant metastasis. (c) Histogram of the corresponding *FGFR1* amplification status in primary tumor and its corresponding recurrent tumor. Data was logarithmically plotted. In order to include all detected cases, the value “1” was added to every count (for detailed information, see description statistical analysis in materials and methods).

FGFR1 Amplification in Recurrent Head and Neck Squamous Cell Carcinoma

A total of 68 cases of recurrent tumor tissue were available. Of these, 94% (64/68) were assessable. Primary and recurrent tumor tissue of the same patient were available in 54 cases, of which 47 (87%) were assessable: 83% (39/47) presented an identical copy number status in both primary tumor and recurrent tumor tissue (example of concordant wild-type *FGFR1*; Figure 1b and b1). In eight cases, the recurrent tumor displayed a different *FGFR1* copy number status as compared with the primary tumor (Figure 2c).

FGFR1 Amplification and Clinicopathological Features of Head and Neck Squamous Cell Carcinoma

All features of clinicopathological data in relation to *FGFR1* copy number status and statistical analysis are summarized in Table 2.

In detail, parameters of age at primary diagnosis and gender were available for all patients. *FGFR1* amplification was not significantly associated with age ($P = 0.30$), but occurred significantly more often in male patients ($P = 0.02$).

Smoking status was available for 434 patients. Of these, 352 had *FGFR1* assessable tumor tissue available. Eighty-eight percent (310/352) of assessable patients were smokers and 12% (42/352) were never-smokers. *FGFR1* amplification occurs significantly more often in the cohort of patients with a smoking history than in the group of patients who never smoked ($P = 0.04$). We also compared the number of pack years with the occurrence of *FGFR1* amplifications. Interestingly, *FGFR1* amplifications occur significantly more often with rising amount of pack years ($P = 0.02$). Status of alcohol abuse was

available for 411 patients, out of which 331 had *FGFR1* assessable tumor tissue available. Among the patients, 130 were never-drinkers, 73 were occasional drinkers and 128 medium to heavy drinkers. *FGFR1* amplification occurs significantly more often with rising alcohol consumption ($P < 0.05$).

Furthermore, we assessed the association between *FGFR1* amplification status and pathologic features of primary tumors. We grouped lower (pT1 and pT2) and higher T-stages (pT3 and pT4) and assessed if *FGFR1* amplification correlated with these subgroups. We found a significant association of *FGFR1* amplifications and higher T-stages ($P < 0.05$). Also, *FGFR1* amplification was significantly associated with the presence of lymphovascular invasion (L) ($P = 0.02$).

To assess whether *FGFR1* amplification status predicts occurrence of regional lymph node metastases, we correlated the *FGFR1* amplification status of primary tumors with clinical nodal status. Of the cases with *FGFR1* amplification ($n = 68$) in the primary tumor, 40% (27/68) had lymph node-negative disease and 60% (41/68) had lymph node-positive disease compared with 61% (209/384) lymph node-negative and 46% (175/384) lymph node-positive of cases without *FGFR1* amplification ($n = 384$), indicating that *FGFR1* amplification is significantly associated with the development of lymph node metastasis ($P = 0.02$).

Patients were screened for HPV infection by p16 immunohistochemistry staining of all patients' tissue, which revealed a positive immunohistochemistry status in 10% (47/488) of the cases. These cases were then investigated by LCD array, which further identified the occurrence and the subtypes of HPV. Seventy-seven percent (36/47) of p16-positive cases turned out to be truly HPV positive, resulting in an overall frequency of 7% (36/488) within our cohort. We found HPV16 in 32 cases, the combination

Table 2 Summary of clinico-pathological data in relation to *FGFR1* amplification

	No amplification	Low-level amplification	High-level amplification	Any amplification	P-value (Fisher's exact test)
<i>Age (years), median</i>					
< 62	185	22	6	28	0.295
≥ 62	199	30	10	40	
<i>Gender</i>					
Female	96	6	2	8	0.017*
Male	288	46	14	60	
<i>Site of origin</i>					
Oral, oropharynx	221	21	9	30	0.045*
Hypopharynx, larynx	157	30	7	37	
Unknown	6	1	0	1	
<i>Tobacco</i>					
Never-smoker	40	1	1	2	0.04153*
Smoker	255	43	12	55	
<i>Alcohol</i>					
Non-drinker	119	9	2	11	0.007795**
Occasional	60	10	3	13	
Medium-heavy	100	21	7	28	
<i>Pathological features</i>					
T1-T2	239	17	5	22	0.000***
T3-T4	123	31	10	41	
Lymphovascular invasion					
Negative	336	44	11	55	0.02*
Positive	29	7	4	11	
Lymph node metastases (at the time of presentation)					
Negative	182	18	4	22	0.02*
Positive	175	30	11	41	
Distant metastases (at the time of presentation)					
Negative	375	48	14	62	0.015*
Positive	9	4	2	6	
<i>HPV status</i>					
Negative	351	52	16	68	0.003127**
Positive	36	0	0	0	
<i>Survival</i>					
Recurrence-free survival, median 28 months					
< 28	107	14	8	22	0.6116
≥ 28	113	16	3	19	
Overall survival, median 26 months					
< 26	177	25	10	35	0.5959
≥ 26	190	27	5	32	

Features include age, gender, site of origin, tobacco, alcohol, pathologic features, HPV status treatment regimen and survival.

P-values indicate the significance of any *FGFR1* amplification (HLA and LLA vs. non-amplified) between the described subgroups and are calculated by Fisher's exact test.

Significance levels are 0.05 (*), 0.01 (**) and 0.001 (***).

of HPV16 and HPV35 one time, HPV35 one time and HPV33 two times. All cases displaying HPV infection had a wild-type *FGFR1* status. All *FGFR1*-amplified tumors lacked HPV infection. HPV-positive patients had a significantly better overall survival compared with HPV-negative patients ($P < 0.05$).

Treatment and Outcome

After primary surgical treatment of localized disease, 147 patients suffered from a recurrence, 87 local, 49 as lymph node metastases and 28 as distant

metastases. The median time until recurrence was 13 months, which did not differ among tumors on the basis of *FGFR1* amplification status ($P = 0.9$). Of these 147 patients, we had assessable primary and recurrent tumor tissue of 47 patients available. Seventeen patients underwent surgical treatment only, 15 patients were treated with postsurgical radiation, 10 patients with postsurgical radiochemotherapy and 3 patients were treated with radiochemotherapy only. In two cases, there was no therapy regimen determinable. Comparing *FGFR1* copy number status of primary tumors and recurrences, we found 39 patients with concordant and

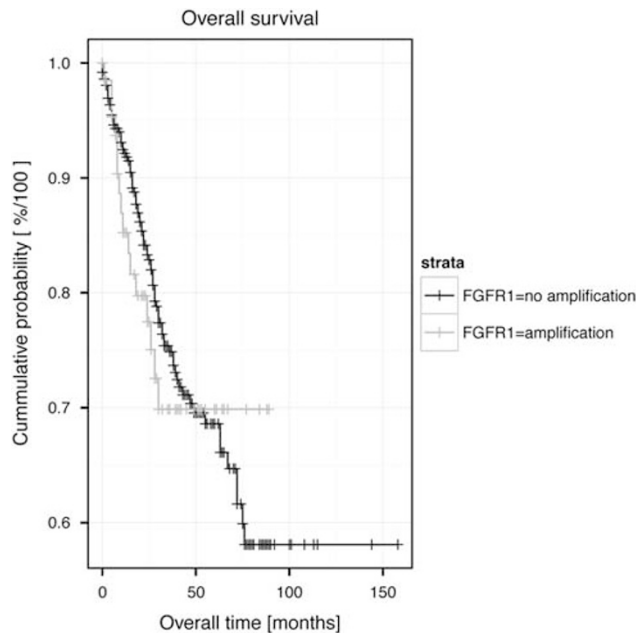


Figure 3 Kaplan Meier curve for overall survival related to *FGFR1* copy number status. *FGFR1* amplification is not significantly associated with better or worse overall survival.

8 patients with discordant gene copy number status in these tissues (for details see above). As discordance only occurred in eight patients, who underwent different therapy regimens (ie $4 \times$ surgery, $2 \times$ surgery and adjuvant radiochemotherapy, $1 \times$ surgery and radiation and $1 \times$ radio-chemotherapy), we do not observe a direct influence of a specific therapy regimen on the change of *FGFR1* copy number status from primary tumor to recurrent tumor tissue. Patient number of discordant *FGFR1* status is also underpowered for a reliable statistical analysis. There is no significant difference in overall survival between patients harboring a *FGFR1* amplification and those with *FGFR1* wild type ($P = 0.71$) (Figure 3).

Discussion

Frequent genetic alterations associated with tumorigenesis in cancers have become a major focus of research in hopes that such alterations will represent opportunities for the development of targeted therapies.¹ So far, patients with head and neck squamous cell carcinoma lack targeted therapy options and still have a rather limited functional and poor overall outcome in spite of improvements in conventional treatment modalities such as surgery and radiochemotherapy.

We and others previously discovered the highly smoking-related *FGFR1* amplification in approximately a fifth of all primary squamous cell lung cancer.^{14,15} This discovery resulted in the immediate initiation of a phase I clinical trial of small-molecule FGFR inhibitor therapy that is

enrolling patients diagnosed with stage IV disease suffering from the second recurrence with *FGFR1*-amplified primary squamous cell lung cancer.

Owing to known similarities in etiology, histology and risk factors between head and neck squamous cell carcinoma and squamous cell lung cancer, we hypothesized that *FGFR1* amplifications are also involved in the pathogenesis of head and neck squamous cell carcinoma. To test this hypothesis, we examined a large number of primary and metastatic head and neck squamous cell carcinoma to determine the prevalence of *FGFR1* amplification in these tumors. We found an overall *FGFR1* amplification rate of 15% in primary head and neck squamous cell carcinoma, which is close to the frequency described previously by Weiss *et al*¹⁴ for squamous cell lung cancer and also by Freier *et al*¹⁹ for oral squamous cell carcinomas. Mirroring our findings in lung cancers,¹⁴ acquisition of *FGFR1* amplification in head and neck squamous cell carcinoma showed a dose-dependent association with exposure to chemical carcinogens (eg smoking and, in the case of head and neck squamous cell carcinoma, alcohol abuse). In contrast, no *FGFR1* amplification was found in the prognostically more benign HPV-driven tumors, reinforcing the concept that this tumor subtype has its own specific pathogenesis and behavior. Confirming these observations, *FGFR1* amplification was detected more frequently in the squamous cell carcinoma of the hypopharynx and larynx, which are rather driven by chemical carcinogens than by HPV, as opposed to squamous cell carcinoma of the oropharynx and oral cavity, which are HPV positive in 30–65% of cases.^{21,22} Interestingly, positive HPV status and *FGFR1* amplification occurs mutually exclusively, implying completely independent pathways of carcinogenesis. This raises interesting questions regarding whether well-characterized downstream effectors of HPV infection that lead to head and neck squamous cell carcinoma are also involved in tumors characterized by *FGFR1* amplification. Smoking, on the other hand, was clearly associated with *FGFR1* amplification, raising interesting questions for future studies involving the mechanistic relationship between tobacco-associated cell damage and *FGFR1* amplification. Likewise, *FGFR1* amplification was seen more commonly among patients consuming alcohol, again providing fertile ground for new insights into the pathogenesis of head and neck squamous cell carcinoma. Damage occurring to the tissue because of radiation and/or chemotherapy does not seem to have an influence on the frequency of *FGFR1* amplification, as recurrence of tumors treated under these initially employed therapy regimens do not harbor more *FGFR1* amplifications than tumors treated purely with surgical resection.

As *FGFR1* is on its way to becoming an actionable target, it is important to consider patients with

multifocal disease, to assess whether separate tumor sites in a single patient should be tested individually for *FGFR1* amplification status. To that end, we assessed a large number of cases with corresponding lymph node metastases, and a number of cases with corresponding distant metastases, as well as corresponding recurrent tumors. We found that the *FGFR1* gene status is concordant in the vast majority of patients with metastatic or recurrent disease, although we did find exceptions to this tendency. Tissue microarrays were constructed by random selection of tissue cores from the whole tumor. Therefore, discordance could be due to molecular heterogeneity within the whole tumor. However, in fact, we assessed whole tumor sections in a subset of discordant cases and found that the distribution of *FGFR1* copy number status is the same as assessed on the tissue microarrays. Consequently, results derived from the tissue microarrays are reliable, although a theoretical limitation with regard to a sampling bias remains.

However, the clinical significance of this discordance is subject to further studies. In the meantime, it appears advisable to test all available tissue sites for *FGFR1* amplification status in patients being considered for treatment with FGFR inhibitors.

We assessed the utility of *FGFR1* amplification status as a prognostic biomarker, and although we did not find differences in survival time on the basis of *FGFR1* amplification, we did demonstrate an association between *FGFR1* amplification and various well-characterized parameters of poor prognosis, including advanced T-stage, lymphovascular invasion and regional and distant metastatic disease. As HPV infection is commonly linked with a favorable prognosis,²³ our data associating *FGFR1* amplification with a worse prognosis is consistent with mutual exclusion of HPV infection and *FGFR1* amplification. The lack of correlation between *FGFR1* amplification and overall survival could be due to the pronounced heterogeneity of our cohort with respect to treatment regimens.

In summary, *FGFR1* amplification is a frequent event in HPV-negative head and neck squamous cell carcinoma, and *FGFR1* copy number status was concordant between primary and metastatic tumors in the vast majority of cases. In the era of personalized cancer treatment, our findings emphasize the need to establish the prognostic and predictive significance of molecular alterations like *FGFR1* amplification for head and neck squamous cell carcinoma and to incorporate them into clinical algorithms when rational specific therapies exist. Further research will be necessary to elucidate the mechanistic relationships between *FGFR1* amplification and other well-established risk factors for head and neck squamous cell carcinoma, such as tobacco and alcohol, and molecular differences to HPV infection-driven head and neck squamous cell carcinoma.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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