FGFR1 Amplification Is Often Homogeneous and Strongly Linked to the Squamous Cell Carcinoma Subtype in Esophageal Carcinoma

Katharina von Loga*, Jule Kohlhaussen*, Lia Burkhardt, Ronald Simon, Stefan Steurer, Susanne Burdak-Rothkamm, Frank Jacobsen, Guido Sauter, Till Krech

Department of Pathology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

These authors contributed equally to this work.

kvonloga@uke.de

Abstract

Background and Aims
Amplification of the fibroblast growth factor receptor 1 (FGFR1) is believed to predict response to multi-kinase inhibitors targeting FGFR1. Esophageal cancer is an aggressive disease, for which novel targeted therapies are highly warranted.

Methods
This study was designed to investigate the prevalence and clinical significance of FGFR1 amplification in a tissue microarray containing 346 adenocarcinomas and 254 squamous cell carcinomas of the esophagus, using dual-labeling fluorescence in situ hybridization (FISH) analysis.

Results
FGFR1 amplification, defined as a ratio of FGFR1:centromere 8 copy numbers ≥ 2.0, was more frequently seen in squamous cell carcinoma (8.9% of 202 interpretable cases) than in adenocarcinoma (1.6% of 308; p<0.0001). There was no association between FGFR1 amplification and tumor phenotype or clinical outcome. To study potential heterogeneity of FGFR1 amplification, all available tumor blocks from 23 FGFR1 amplified tumors were analyzed on conventional large sections. This analysis revealed complete homogeneity of FGFR1 amplification in 20 (86.9%) primary tumors and in all available lymph node metastases. Remarkably, FGFR1 amplification was also seen in dysplasia adjacent to tumor in 6 of 9 patients with FGFR1 amplified primary cancers.

Conclusions
In conclusion, FGFR1 amplification occurs in a relevant subgroup of carcinomas of the esophagus and may play a particular role for development of squamous cell cancers. The
high homogeneity of FGFR1 amplification suggests that patients with FGFR1 amplified esophageal cancers may particularly benefit from anti-FGFR1 therapies and prompt for clinical studies in this tumor type.

Introduction

Esophageal cancer is an aggressive disease presenting with two histologically and genetically distinct subtypes, i.e. adenocarcinomas (EADC and ESCC). Patients with esophageal neoplasias are usually diagnosed at advanced stages [1,2] and, thus, have a generally poor prognosis with 5-year survival rates typically not extending 10–25% (URL: http://www.cancer.org) [1,3]. Because curative therapy options in patients with advanced disease are lacking, there is an urgent need for novel and effective drugs.

Targeted cancer therapies have successfully entered clinical routine in several tumor types. Particularly growth factor receptors, such as HER2, EGFR, VEGFR or c-KIT, which are strongly up regulated in many cancers, have proven to represent efficient anti-cancer therapy targets [4–10]. There is growing evidence that targeting of the fibroblast growth factor receptor 1 (FGFR1) holds promising clinical potential [11,12]. FGFR1 plays an important role in cell differentiation and growth by downstream signaling to the nucleus involving either the Ras/MAPK- or PI3/Akt-pathways [13,14]. An important mechanism of oncogenic FGFR1 activation is amplification of its gene locus at chromosome 8p11, which is found in 10–20% of squamous cell carcinomas of the lung [15–18], in about 10% of hormone receptor positive breast carcinoma [19–21], 10–17% head and neck squamous cell carcinomas [22] and 6% of small cell carcinomas of the lung [23]. Little is known about the clinical significance of FGFR1 amplification in esophageal cancer or about possible differences between histological subtypes. Reported FGFR1 amplification frequencies in studies on 32–189 esophageal cancers range between 6–21% in squamous cell cancers [24,25] and 9% in adenocarcinomas [25], but the impact on patient prognosis is largely unknown. Only one study on Asian ESCC patients suggested that FGFR1 amplification might be linked to poor outcome [26].

To better understand the prognostic role of FGFR1 amplification in Caucasian patients, we employed fluorescence in-situ hybridization (FISH) analysis for precise determination of the FGFR1 amplification rate in a large tissue microarray made from 254 ESCC and 346 EADC patients with histopathological and clinical follow-up data of Caucasian origin.

Material and Methods

Esophageal cancer TMA

The esophageal cancer TMA utilized for this study consists of 600 formalin-fixed paraffin-embedded tissue samples including 346 esophageal adenocarcinomas and 254 esophageal squamous cell carcinomas, and was extended based on an earlier TMA containing 292 cancers [27]. All patients had undergone surgery between 1992–2011 at the surgical department of the University Medical Center Hamburg-Eppendorf. The female to male ratio in our cancers was 117 to 483, which corresponds to the observed incidence of these tumor types [28,29]. Two pathologists (KVL, TK) reviewed all tumor slides. All work has been carried out in compliance with the Helsinki Declaration. The general usage of archived diagnostic left-over tissues for manufacturing of tissue microarrays (TMAs) and their analysis for research purposes as well as patient data analysis has been approved by the local ethics committee (Ethics commission Hamburg, WF-049/09 and PV3652). The authors KVL and FJ acted as the treating physicians/
pathologists and had access to identifying patient information at the time point when the tissues were collected but not at the time point when the study was conducted. The tissues were collected during routine cancer surgery. All tissues had been collected and used for TMA manufacturing prior to this study. The ethics committee reviewed and approved the lack of consent procedure.

The TMA manufacturing process was described earlier in detail [30]. In short, one 0.6 mm core was taken from a representative tissue block from each patient. Tissue sample were distributed on two TMA blocks, containing 346 and 254 cancer cores, respectively. In addition, both blocks comprise tissue controls of normal esophageal epithelium. Tumor grade and stage were defined according to the International Union Against Cancer (UICC) and the WHO [3,31]. Clinical data of patients were retrospectively evaluated. The medium follow-up period was 27.7 months (range 0–215 months). An overview of all histological and clinical data is given in Table 1.

**Fluorescence in situ hybridization (FISH)**

A dual color FISH probe set was used for FGFR1 amplification analysis. The probe set combined a home-made spectrum green labeled FGFR1 probe (chromosome 8 locus 8p 11.22–23, made from bacterial artificial chromosome (BAC) clone RP11-350N15) and a commercial probe.

**Table 1. Esophageal Carcinoma—Array.**

|                | ESCC (n = 254) | EADC (n = 346) |
|----------------|---------------|---------------|
| Gender         |               |               |
| female         | 69            | 48            |
| male           | 185           | 298           |
| Tumor          |               |               |
| pT1a           | 16            | 33            |
| pT1b           | 34            | 49            |
| pT2            | 49            | 36            |
| pT3            | 139           | 206           |
| pT4a           | 6             | 18            |
| pT4b           | 10            | 4             |
| Nodal          |               |               |
| pNX            | 9             | 8             |
| pN0            | 116           | 107           |
| pN1            | 56            | 63            |
| pN2            | 48            | 80            |
| pN3            | 25            | 88            |
| Metastasis     |               |               |
| pM0            | 206           | 307           |
| pM1            | 48            | 39            |
| Grade          |               |               |
| G1             | 4             | 26            |
| G2             | 164           | 125           |
| G3             | 86            | 189           |
| G4             | 0             | 6             |
| UICC           |               |               |
| IA             | 40            | 71            |
| IB             | 24            | 12            |
| II A           | 46            | 29            |
| IIB            | 13            | 14            |
| III A          | 35            | 57            |
| III B          | 26            | 54            |
| III C          | 22            | 72            |
| IV             | 48            | 37            |

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spectrum red labeled probe for the centromeric region of chromosome 8 (Zytovision, Bremerhaven, Germany). Freshly cut TMA sections (4 micrometer thick) were deparaffinized and proteolytically pretreated using a commercial kit (Zytolight FISH-Tissue Implementation Kit, Zytovision, Bremerhaven, Germany), followed by dehydration in 70%, 90% and 99% ethanol, air drying and codenaturation in a Thermobrite hybridization oven (Abbott, Chicago, USA) for 10 minutes at 75°Celsius. Hybridization was overnight at 37°Celsius. Slides were then washed and counterstained with 0, 2 micromol/l of DAPI.

Scoring of FISH signals

Presence of tumor cells was verified in each spot by comparison of a hematoxilin and eosin (H&E) stained adjacent reference section of the TMA. Two experienced technicians (SS, SE) estimated the predominant gene and centromere copy numbers in at least 20 non-overlapping tumor cells in each tissue spot. Data from our laboratory have previously shown that diagnosis of amplification based on signal number estimation is highly reliable [32,33].

High-level FGFR1 amplification was defined as presence of \( \geq 10 \) FGFR1 gene signals or an \( FGFR1/\text{centromere 8} \) ratio of \( \geq 3.0 \). Tumors with a ratio of \( \geq 2.0 \) but \( < 3.0 \) were considered low-level amplification. All other cancers were considered non-amplified. These included cancers with normal (two) copies of \( FGFR1 \) and centromere 8, cancers with polyploidy of chromosome 8 (ratio \( > 0.8 \) but \( < 1.2 \) and more than two \( FGFR1 \) copies) as well as cancers with an \( FGFR1 \) copy number gain not reaching the threshold for amplification (ratio \( \geq 1.2 \) but \( < 2.0 \)). Examples of tumor spots with and without \( FGFR1 \) amplification are shown in Fig 1 (Fig 1).

Large section validation

To estimate the degree of intratumoral heterogeneity of \( FGFR1 \) amplification, all available primary and metastasis tumor blocks of all cancers showing \( FGFR1 \) amplification according to the TMA analysis, including 18 ESCC and 5 EADC, were analyzed for amplification on conventional large sections (4 \( \mu \)m thickness). The number of \( FGFR1 \) and centromere 8 FISH signals were counted in at least 20 non-overlapping cell nuclei, and the average \( FGFR1 \) and centromere 8 copy numbers were calculated per sample. The \( FGFR1/\text{centromere 8} \) ratio was calculated from these values. High-level and low-level \( FGFR1 \) amplification was defined as described above. Heterogeneity was defined as presence of \( FGFR1 \) non-amplified and amplified tumor areas within the same cancer (Fig 1B). If present, adjacent dysplasia was also evaluated.

For comparison of \( FGFR1 \) expression levels in tumors with and without \( FGFR1 \) amplification, tissue blocks containing 70% or more tumor cells were selected that had been used for TMA manufacturing before. For RNA isolation, one 0.6 mm tissue core was taken from each tumor block. The deparaffinized and air-dried cores were grinded in liquid nitrogen before total RNA was isolated using a commercial kit (RNeasy FFPE kit #744044, QIAGEN) following the manufacturers instructions except for prolonged (overnight) proteinase digestion. cDNA was synthesized from 0.5 to 1 mg total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems #4368814). Quantitative reverse transcriptase PCR (qRT-PCR) was carried out in duplicate using combinations of primer pairs and TaqMan probes targeting mRNA sequences of \( FGFR1 \) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers were obtained from Applied Biosystems (Darmstadt). The \( GAPDH \) gene served as an internal control for the normalization of \( FGFR1 \) RT-PCR products. The PCR program included a 10 minute denaturation at 95°C followed by 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C. Relative quantification results were calculated according to the \( \Delta \Delta CT \) method [34].
Statistical analysis

Statistical calculations were performed using SAS’ JMP (version 9.0) statistical software. To compare categorical variables such as grade, stage and molecular features, contingency tables were calculated applying chi²-test and Fisher’s exact tests. Survival curves were calculated according to the Kaplan-Meier method and compared with the Logrank test. Cox regression was used to assess independency of molecular, morphological and clinical parameters to predict patient survival.

Results

Technical aspects

FISH analysis was successful in 510 of 600 (85%) arrayed tumors including 202 squamous cell carcinomas and 308 adenocarcinomas. Analysis failures were either due to insufficient
hybridization efficiency or issues connected to the TMA technology, such as missing samples or absence of unequivocal cancer cells in a tissue spot.

Prevalence of **FGFR1** amplifications and their association to esophageal cancer phenotype and patient prognosis

Low- and high-level **FGFR1** amplifications were significantly more frequent in ESCC (18 of 202, 8.9%) than in EADC (5 of 308, 1.6%, \( p < 0.0001 \)). **FGFR1** amplification was typically high-level according to our predefined criteria: 67% (12 of 18) **FGFR1**-amplified ESCC, and 80% (4 of 5) **FGFR1**-amplified EADC showed high-level amplification. Due to these strong differences in amplification frequencies between squamous cell- and adenocarcinomas, associations to phenotype and clinical outcome were calculated separately in each subgroup. All results are summarized in Table 2. These analyses did not reveal significant associations between **FGFR1** amplification and tumor phenotype or clinical outcome, neither in the subset of 202 ESCC, nor in the subset of 308 EADC (Fig 2). A total of 21 squamous cell carcinomas and 4 adenocarcinomas harbored **FGFR1** copy number increases that did not reach the predefined threshold for amplification, including 15 cancers with polyploidy of chromosome 8 (15 ESCC) and 10 cancers with **FGFR1** gains (6 ESCC and 4 EADC). The results of these cases are shown in S1 Table.

Heterogeneity analysis

All 23 amplified cancers were further analyzed in order to assess the level of homogeneity/heterogeneity of **FGFR1** amplifications. Data are summarized in Table 3. Overall, **FGFR1** amplification was homogenous in 20 (86.9%) and heterogeneous in 3 (13.0%) amplified cancers. All available lymph node metastasis (n = 7) showed a homogeneous amplification pattern, even in one case with heterogeneous amplification of the primary tumor. Remarkably, **FGFR1** amplification was also observed in 6 of 8 patients (75%) where areas of dysplastic squamous epithelium were found adjacent to invasive cancer.

Association between **FGFR1** gene amplification and mRNA expression

mRNA expression results were retained from 8 of 10 **FGFR1** amplified and **FGFR1** non-amplified each. The average mRNA expression level was 14919.4 in **FGFR1** amplified as compared to 5485.8 in **FGFR1** non-amplified (\( p = 0.1869 \)). Of note, most **FGFR1** amplified cancers had expression levels that were in the range of **FGFR1** non-amplified samples. Highest expression levels were found in two amplified samples harboring 9.35 and 35.55 **FGFR1** gene copies according to FISH analysis (Fig 3).

Discussion

Our data demonstrate marked differences in the prevalence of **FGFR1** gene amplification between squamous cell carcinomas and adenocarcinomas of esophageal carcinomas.

In this study, we employed FISH analysis for **FGFR1** gene copy analysis. FISH is regarded as the most precise means for gene copy number measurement in histological sections, because it is not disturbed by the presence of non-cancerous cells in the tissue samples. Previous studies on Caucasian ESCC employing the less quantitative CGH analysis reported 6–21% **FGFR1** amplifications in cohorts of 32 and 70 ESCC [24,25]. However, our finding of 8.9% **FGFR1** amplification in ESCC is almost identical to a recent FISH study on Asian ESCC, reporting 8.6% amplification using the same threshold (ratio ≥ 2.0) for amplification [26]. Data from The Cancer Genome Atlas (TCGA https://tcga-data.nci.nih.gov/tcga) on esophageal carcinomas (September 2015) indicate **FGFR1** amplification in 11.1% of 45 squamous cell carcinomas...
with data on copy number alterations, which is also well in line with 8.9% in our study. That no FGFR1 amplification was reported in 25 adenocarcinomas further supports the concept of marked differences in the FGFR1 amplification frequencies between these two histological subtypes. TCGA data, moreover, suggest that FGFR1 mutations are rare events (<2%) in this tumor type. It is, therefore, likely that the putative oncogenic function of amplified FGFR1 is typically mediated by the wild type gene.

Comparison of FGFR1 mRNA expression levels in a small set of FGFR1 amplified and non-amplified cancers revealed a wide range of expression levels in both subgroups. An overall high FGFR1 expression level in amplified cancers was mainly driven by two FGFR1 amplified cancer with particularly high mRNA expression levels. These findings suggest that gene amplification is one important mechanism for FGFR1 overexpression but also indicates that other mechanisms can lead to a significant up-regulation of FGFR1 expression.

We found a striking predominance of FGFR1 amplification in squamous cell cancers (8.9%) as compared to adenocarcinomas (1.6%) in our study on 510 esophageal cancers. A higher prevalence of FGFR1 amplification in ESCC as compared to EADC had also been suggested in

Table 2. FGFR1 amplification in ESCC and EADC.

|          | ESCC  |              |              |              |         |         |         |         |         |         |         |         |
|----------|-------|--------------|--------------|--------------|---------|---------|---------|---------|---------|---------|---------|---------|
|          | n     | all ampl. (%)| low ampl. (%)| high ampl. (%)| p-value | n       | all ampl. (%)| low ampl. (%)| high ampl. (%)| p-value |
| all cancers | 202   | 18 (8.9)     | 6 (3.0)      | 12 (5.9)     |         | 308     | 5 (1.6)      | 1 (0.3)      | 4 (1.3)      |         |
| Gender    | female | 53          | 4 (7.5)     | 1 (1.9)      | 3 (5.7) | 41       | 1 (2.4)     | 0         | 1 (2.4)     |         |
|           | male   | 149         | 14 (9.4)    | 5 (3.4)     | 9 (6.0) | 267      | 4 (1.5)     | 1 (0.4)     | 3 (1.1)     | 0.733   |
| Tumor     | pT1a   | 9           | 1 (11.1)    | 0           | 1 (11.1) | 29       | 0         | 0         | 0         |         |
|           | pT1b   | 27          | 3 (11.1)    | 0           | 3 (11.1) | 44       | 1 (2.3)    | 0         | 1 (2.3)     |         |
|           | pT2    | 38          | 4 (10.5)    | 2 (5.3)     | 2 (5.3) | 27       | 1 (3.7)    | 0         | 1 (3.7)     |         |
|           | pT3    | 116         | 9 (7.8)     | 4 (3.4)     | 5 (4.3) | 188      | 3 (1.6)    | 1 (0.5)    | 2 (1.1)     |         |
|           | pT4a   | 5           | 1 (20.0)    | 0           | 1 (20.0) | 16       | 0         | 0         | 0         |         |
|           | pT4b   | 7           | 0           | 0           | 0       | 0.6832   | 4         | 0         | 0         | 0.9748   |
| Nodal     | pNX    | 4           | 0           | 0           | 0       | 8        | 0         | 0         | 0         |         |
|           | pN0    | 95          | 8 (8.4)     | 3 (3.2)     | 5 (5.3) | 96       | 3 (3.1)    | 0         | 3 (3.1)     |         |
|           | pN1    | 45          | 4 (8.9)     | 1 (2.2)     | 3 (6.7) | 54       | 0         | 0         | 0         |         |
|           | pN2    | 41          | 5 (12.2)    | 1 (2.4)     | 4 (9.8) | 71       | 1 (1.4)    | 0         | 1 (1.4)     |         |
|           | pN3    | 17          | 1 (5.9)     | 1 (5.9)     | 0       | 0.7451   | 79        | 1 (1.3)    | 1 (1.3)     | 0       | 0.2219   |
| Metastasis| pM0    | 165         | 12 (7.3)    | 4 (2.4)     | 8 (4.8) | 273      | 5 (1.8)    | 1 (0.4)    | 4 (1.5)     |         |
|           | pM1    | 37          | 6 (16.2)    | 2 (5.4)     | 4 (10.8) | 0.2525  | 35        | 0         | 0         | 0       | 0.5543   |
| Grade     | G1     | 3           | 0           | 0           | 0       | 23       | 0         | 0         | 0         |         |
|           | G2     | 136         | 13 (9.6)    | 4 (2.9)     | 9 (6.6) | 111      | 0         | 0         | 0         |         |
|           | G3     | 63          | 5 (7.9)     | 2 (3.2)     | 3 (4.8) | 0.9308  | 174       | 5 (2.9)    | 1 (0.6)     | 4 (2.3) | 0.2528   |
| UICC      | IA     | 28          | 2 (7.1)     | 0           | 2 (7.1) | 63       | 1 (1.6)    | 0         | 1 (1.6)     |         |
|           | IB     | 22          | 1 (4.5)     | 0           | 1 (4.5) | 11       | 1 (9.1)    | 0         | 1 (9.1)     |         |
|           | IIA    | 40          | 4 (10.0)    | 2 (5.0)     | 2 (5.0) | 28       | 1 (3.6)    | 0         | 1 (3.6)     |         |
|           | IIB    | 9           | 2 (22.2)    | 1 (11.1)    | 1 (11.1) | 10       | 0         | 0         | 0         |         |
|           | IIA    | 27          | 0           | 0           | 0       | 50       | 0         | 0         | 0         |         |
|           | IIB    | 25          | 2 (8.0)     | 1 (4.0)     | 1 (4.0) | 49       | 1 (2.0)    | 0         | 1 (2.0)     |         |
|           | IIC    | 14          | 1 (7.1)     | 0           | 1 (7.1) | 63       | 1 (1.6)    | 1 (1.6)    | 0         |         |
|           | IV     | 37          | 6 (16.2)    | 2 (5.4)     | 4 (10.8) | 0.4709  | 34        | 0         | 0         | 0       | 0.6935   |
a previous study comparing 70 ESCC and 189 EADC [25]. In addition, differences in the \textit{FGFR1} amplification rate between squamous cell carcinoma and adenocarcinomas has also been reported from cancers of the lungs [35,36]. These findings suggest a particular role of \textit{FGFR1} activation for the development of a squamous cell phenotype. It is possible, that this finding is linked to specific mutagenic agents such as cigarette smoke. It is well known that squamous cell cancers of the esophagus and lungs are linked to smoking [37–39]. Differences in the amplification frequency between ESCC and EADC have also been reported from other genes, including SOX2, PIK3CA, MYC, CCND1, which had a higher amplification frequency in ESCC, and GATA4 as well as GATA6, which had a higher amplification frequency in EADC [25]. Of note, many of these genes are transcription factors. It is, thus, tempting to speculate that amplification and overexpression of these genes results in activation of specific genetic programs that favor development of the one ore the other histological subtype of esophageal carcinomas. In fact, amplification of GATA4 and GATA6 is often found in adenocarcinomas from other origins [25,40].

An early role of \textit{FGFR1} activation for squamous cell phenotype development is supported by our analysis of ESCC precursor lesions. It can be expected that molecular events arising before or during malignant transformation should be present in all cancer cells of the resulting tumor bulk. We found \textit{FGFR1} amplification in six of eight samples of squamous cell dysplasia adjacent to \textit{FGFR1} amplified cancers, and 15 of 18 \textit{FGFR1} amplified ESCC showed homogeneous amplification. These findings, despite the low number of cases, might suggest that \textit{FGFR1} amplification is an early event in ESCC. A tumor-initiating role of \textit{FGFR1} is also supported by studies from other cancer types. For example, \textit{FGFR1} amplification was found in in-situ carcinomas and low-grade ER positive breast cancers [41,42] and in early stage lung cancers [35].

Only recently, FGFR1 has gained considerable interest as a target for gene specific therapies. A multitude of selective and non-selective small molecule inhibitors targeting FGFR1 and

![Fig 2. Raw Survival of ESCC patients. Red line: no \textit{FGFR1} amplified tumor patients. Blue line: \textit{FGFR1} amplified tumor patients.](https://doi.org/10.1371/journal.pone.0141867.g002)
Table 3. Homogeneity/Heterogeneity analysis of FGFR1 amplified tumors.

| N° | Sub-type | Age | Gender | pT | pN | pM | G | UICC | FGFR1 in the PT | Cen 8* | FGFR1 Ratio | Homo/ Hetero* | FGFR1 in the LN | Cen 8* | FGFR1 Ratio | PT | LN | Dys |
|----|----------|-----|--------|----|----|----|---|------|----------------|--------|-------------|-------------|----------------|--------|-------------|----|----|-----|
| 1  | ESCC     | 76  | m      | 1b | 1  | 1  | 1 | IV   | 35.55          | 3.35   | 10.6        | homogeneous  | Ampl, high    | Ampl | 3.05        | 6.97 | Ampl | no  |
| 2  | ESCC     | 62  | m      | 1b | 2  | 1  | 1 | IV   | 35.5            | 4.1    | 8.55       | homogeneous  | Ampl, high    | Ampl | 3.05        | 6.97 | Ampl | no  |
| 3  | ESCC     | 71  | m      | 2  | 1  | 0  | 2 | IIB  | 17.25          | 2.8    | 6.16        | homogeneous  | Ampl, high    | Ampl | 2.15        | 9.07 | Ampl | no  |
| 4  | ESCC     | 53  | m      | 3  | 2  | 1  | 3 | IV   | 15.2            | 2.3    | 6.61        | homogeneous  | Ampl, high    | Ampl | 2.35        | 3.94 | Ampl | no  |
| 5  | ESCC     | 71  | m      | 3  | 1  | 1  | 2 | IV   | 14.3            | 2.75   | 5.20        | homogeneous  | Ampl, high    | Ampl | 2.15        | 9.07 | Ampl | no  |
| 6  | ESCC     | 53  | m      | 3  | 0  | 0  | 2 | IIA  | 14.0            | 2.35   | 5.96        | homogeneous  | Ampl, high    | Ampl | 2.15        | 9.07 | Ampl | no  |
| 7  | ESCC     | 56  | m      | 3  | 0  | 0  | 3 | IIA  | 10.85           | 4.25   | 2.55        | homogeneous  | Ampl, high    | Ampl | 2.15        | 9.07 | Ampl | no  |
| 8  | ESCC     | 53  | f      | 4a | 2  | 0  | 3 | IIIIC | 10.45          | 3.2    | 3.27        | homogeneous  | Ampl, high    | Ampl | 3.2         | 2.83 | Ampl | no  |
| 9  | ESCC     | 52  | m      | 2  | 0  | 0  | 2 | IIB  | 10.3            | 3.1    | 3.32        | homogeneous  | Ampl, high    | Ampl | 2.15        | 9.07 | Ampl | no  |
| 10 | ESCC     | 61  | f      | 3  | 2  | 0  | 2 | IIIIB | 9.5             | 2.5    | 3.80        | homogeneous  | Ampl, high    | Ampl | 2.1         | 2.74 | Ampl | no  |
| 11 | ESCC     | 60  | f      | 1b | 0  | 0  | 2 | IA   | 8.4             | 2.45   | 3.43        | homogeneous  | Ampl, high    | Ampl | 2.15        | 9.07 | Ampl | no  |
| 12 | ESCC     | 70  | m      | 1a | 0  | 0  | 2 | IIA  | 6.7             | 2.05   | 3.27        | homogeneous  | Ampl, high    | Ampl | 2.15        | 9.07 | Ampl | no  |
| 13 | ESCC     | 47  | m      | 2  | 0  | 1  | 2 | IV   | 7.6 + 3.4       | 2.7    | 2.76        | heterogeneous | Ampl, low      | Ampl | 2.1         | 2.74 | Ampl | no  |
| 14 | ESCC     | 66  | m      | 3  | 0  | 0  | 2 | IIA  | 6.3 + 2.55      | 2.3    | 2.74        | heterogeneous | Ampl, low      | Ampl | 2.1         | 2.74 | Ampl | no  |
| 15 | ESCC     | 71  | m      | 3  | 2  | 0  | 3 | IIIIB | 9.5             | 4.0    | 2.38        | homogeneous  | 12.25         | 2.4   | 5.10        | Ampl | Ampl | no  |
| 16 | ESCC     | 42  | m      | 2  | 1  | 0  | 3 | IIIIB | 9.35            | 3.5    | 2.67        | homogeneous  | 11.05         | 4.5   | 2.46        | Ampl | Ampl | no  |
| 17 | ESCC     | 62  | m      | 3  | 3  | 1  | 2 | IV   | 5.15            | 2.3    | 2.24        | homogeneous  | Ampl, low      | Ampl | 2.2         | 2.46 | Ampl | no  |
| 18 | ESCC     | 67  | f      | 3  | 0  | 0  | 2 | IIA  | 5.05            | 2.0    | 2.53        | homogeneous  | Ampl, low      | Ampl | 2.15        | 9.07 | Ampl | no  |
| 19 | EADC     | 62  | f      | 3  | 0  | 0  | 3 | IIA  | 30.75           | 5.05   | 6.09        | homogeneous  | Ampl, high    | Ampl | 2.15        | 9.07 | Ampl | no  |
| 20 | EADC     | 60  | m      | 3  | 2  | 0  | 3 | IIIIB | 18.5            | 5.35   | 3.46        | homogeneous  | Ampl, high    | Ampl | 2.15        | 9.07 | Ampl | no  |
| 21 | EADC     | 56  | m      | 2  | 0  | 0  | 3 | IIB  | 9.85            | 3.0    | 3.28        | homogeneous  | Ampl, high    | Ampl | 2.15        | 9.07 | Ampl | no  |
| 22 | EADC     | 80  | m      | 1b | 0  | 0  | 3 | IA   | 8.63            | 2.38   | 3.63        | homogeneous  | Ampl, high    | Ampl | 2.15        | 9.07 | Ampl | no  |
| 23 | EADC     | 73  | m      | 3  | 3  | 0  | 3 | IIIIC | 6.25            | 2.85   | 2.19        | homogeneous  | Ampl, low      | Ampl | 2.15        | 9.07 | Ampl | no  |

*average copy number counted in 20 cell nuclei,
**Homogeneity/Heterogeneity, PT: primary tumor, LN: lymph node, Dys: dysplasia

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related tyrosine kinases are currently under investigation in preclinical and clinical trials, including the non-selective inhibitors dovitinib, Ki23057, and ponatinib, and the highly selective inhibitors AZD4547 and BGJ398. Preclinical studies have demonstrated the efficacy of AZD4547 and BGJ398 on FGFR gene—amplified cancers both in cell line and mouse models [43–47]. In a phase II clinical trial (NCT01795768), AZD4547 showed therapeutic efficacy as a second-line treatment in patients with FGFR1- and FGFR2-amplified breast cancer, squamous cell carcinoma of the lung, or gastro-esophageal adenocarcinoma. In addition, one pre-clinical study suggests that such treatment may also hold promise for esophageal cancer [48]. The lack of relevant intratumoral heterogeneity of FGFR1 amplifications in our study suggests that anti-FGFR1 therapies may be effective in esophageal cancers harboring this alteration, and encourages future clinical trials in FGFR1 amplified ESCC.

FGFR1 amplification was unrelated to tumor stage, grade, lymph node metastasis and clinical outcome in the 202 esophage squamous cell carcinomas analyzed in this study. Additional data on the clinical relevance of FGFR1 alterations in ESCC are only available from Asian patients. Two studies on 526 Korean and 79 Japanese patients report associations between FGFR1 amplification [26] and immunohistochemical overexpression [49] and shorted overall survival. It is possible that ethnic differences between Caucasian and Asian patients might account for the discrepant findings. Such ethnic differences have been described for various relevant molecular cancer features, including HER2 amplification in breast cancer [50], TMPRSS2-ERG gene fusion in prostate cancer [51], and MET mutation in lung cancer [52].

In this study, a tissue microarray composed from a single 0.6 mm punch per tissue sample was used. We have previously shown that using multiple cores (e.g. 3 cores per tissue spot) does not necessarily increase the ability to identify associations of biomarkers with tumor phenotype and prognosis but has always the disadvantage of additional work and tissue requirements [53]. Using multiple cores can be useful to increase the number of analyzable cancers but can lead to statistical problems if unequal amounts of tissue are analyzed per tumor. In fact, there is a large number of studies using TMAs with one 0.6 mm cores that confirm the known prognostic relevance of virtually all previously established clinically useful biomarkers,
for instance, between alterations of HER2 [54] or p53 [55] and survival in breast cancer, between vimentin expression and prognosis in kidney cancer [56], and even between heterogeneous markers such as Ki67 labeling index and prognosis in urinary bladder cancer [57], breast cancer [58] and prostate cancer [53].

Data from The Cancer Genome Atlas (TCGA) on 70 esophageal carcinomas (45 ESCC and 25 EADC) suggest that FGFR1 mutations are rare events (< 2%) in this tumor type. It is, therefore, likely that the putative oncogenic function of FGFR1 is typically mediated by the wild type gene.

In summary, the results of our study provide strong evidence that FGFR1 amplification is an early molecular event linked to the squamous cell subtype of esophageal cancers. The high homogeneity and high level of FGFR1 amplification argues for FGFR1 representing a promising drug target in ESCC.

Supporting Information

S1 Table. FGFR1 gene copy number alterations. Legend S1. Polyploidy: ratio >0.8 but <1.2 and more than two FGFR1 copies, Gain: ratio >1.2 but < 2.0.

Author Contributions

Conceived and designed the experiments: KVL JK RS GS TK. Performed the experiments: KVL LB JK RS TK. Analyzed the data: KVL JK RS LB SS FJ SBR TK. Contributed reagents/materials/analysis tools: KVL JK RS TK. Wrote the paper: KVL JK RS TK.

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