**FGFR1, 2 and 3 protein overexpression and molecular aberrations of FGFR3 in early stage non-small cell lung cancer**

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**Abstract**

This study aimed to determine protein expression levels of fibroblast growth factor receptors (FGFR) 1, 2 and 3 in early stage non-small cell lung cancer (NSCLC). Additionally, a screen to define the frequency of FGFR3-TACC3 translocation and FGFR3 amplification was performed. Archived tissues from 653 NSCLC samples (adenocarcinoma (AC), squamous cell carcinoma (SCC) and large cell carcinoma (LCC)) were analysed with immunohistochemistry (IHC) for expression of FGFR1, 2 and 3. Expression levels of FGFR1, 2 and 3 were correlated with clinicopathological features. The presence of FGFR3-TACC3 translocation was detected by RT-PCR and FGFR3 amplification was detected by fluorescence in situ hybridization. FGFR1, 2 and 3 proteins were highly expressed in 64 (10.6%), 76 (12.9%) and 20 (3.3%) NSCLC tumour samples, respectively. Protein expression of FGFR1 was significantly related to worse overall survival in NSCLC. Furthermore, FGFR1 protein expression was associated with light smoking and histological subtype (AC), FGFR2 protein expression with female gender, younger age, histological subtype (AC) and lower tumour stage, and FGFR3 protein was significantly over-expressed in tumours of older patients and SCC histology. The FGFR3-TACC3 fusion was detected in 3.0% (6/200) of NSCLC samples and the FGFR3 gene was amplified in 4.7% of IHC positive NSCLC samples (2/43). FGFR1, 2 and 3 proteins are expressed in a high number of early stage NSCLC and FGFR1 protein expression may serve as a prognostic biomarker. Recurrent translocations and amplifications in FGFR3 can be found in NSCLC. This study shows that FGFR family members are frequently aberrant in NSCLC and could be interesting therapeutic targets for the treatment of NSCLC.

**Keywords:** non-small cell lung cancer (NSCLC); fibroblast growth factor receptor (FGFR); immunohistochemistry (IHC); FGFR3-TACC3 translocation; FGFR3 amplification

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All authors declare that they have no conflicts of interest.

**Introduction**

Lung cancer is the leading cause of cancer-related deaths in the western world. Non-small cell lung cancer (NSCLC) accounts for over 75% of all lung cancer cases and consists of adenocarcinoma (AC), squamous cell carcinoma (SCC) and large cell carcinoma (LCC). Approximately half of the patients presenting with NSCLC have curable disease, but around 30% of them will eventually develop an
incurable metastatic stage [1]. Insights into genomic aberrations in cancer cells and the development of targeted therapies have led to significant improvements in treatment and survival. For patients with advanced NSCLC with tumours harbouring an EGFR mutation or ALK rearrangement, a tyrosine kinase inhibitor has replaced chemotherapy as the first choice of treatment ie, erlotinib/gefitinib/afatinib and crizotinib respectively [2,3]. However, particularly in the western population the incidence of these aberrations is rare and they occur mainly in the AC histological subtype, so only a minority of patients have benefited so far. Thus, there is still a great need for new targets to augment treatment options and improve prognosis for advanced NSCLC, especially in squamous cell lung carcinoma.

Recently, a novel potential therapeutic target has been identified in cancer: the fibroblast growth factor receptor (FGFR) family. The FGFR family members play a physiological role in stimulating cell proliferation and migration as well as in promoting survival of various types of cells [4]. In the FGFR pathway, 18 ligands (fibroblast growth factors, FGFs) can bind to four homologous receptor tyrosine kinases FGFR1-4. Binding of FGF ligand activates FGFR1-4, which subsequently leads to a highly complex signalling response through multiple pathways – the MAPK signalling cascade and the PI3K/AKT pathway, among others [5,6]. In cancer, different FGFR aberrations have been identified that constitutively activate these downstream pathways and contribute to tumour development. These include receptor overexpression through gene amplification or post-transcriptional regulation, FGFR mutations, FGFR translocations, alternative splicing of FGFR, isoform switching and paracrine/autocrine activation through overexpression of FGFs in cancer or stromal cells [7]. Apart from NSCLC, aberrant FGFR signalling has been observed in many cancers, such as breast, ovarian, bladder, gastric, prostate, endometrial, head and neck, colorectal cancer and glioblastoma [7–9]. In tumours with FGFR1-3 aberrations, inhibition with several small molecules known to target the FGF/FGFR-pathway has shown anti-tumour activity in vitro [10–16]. Clinical trials with FGFR inhibitors in advanced NSCLC are ongoing, whether combined with chemotherapy or not, and partial responses have been observed [17,18].

Recently we developed a high-throughput platform for systematically profiling kinase fusions, which enabled the identification of a recurrent FGFR3-TACC3 translocation in a cohort of 95 early stage NSCLC samples. Both samples carrying this fusion were of squamous histology and showed FGFR3 protein overexpression. We also identified two SCC samples that carried an activating mutation in FGFR3 [19]. The mutation causes a serine-to-cysteine substitution at position 249 and is the most common FGFR3 activating mutation identified in bladder cancer. FGFR3-TACC3 translocations and FGFR3 mutations may become an interesting target for therapy in SCC, but many aspects regarding the FGFR-signalling pathway in NSCLC remain unknown. Insights into the frequency of protein (over)expression of FGFR1, 2 and 3 may assist in a better understanding of the role of this pathway in NSCLC and help to identify patients who may benefit from targeted therapy.

Here, we assessed a large cohort of 653 early stage NSCLC samples for FGFR1, 2 and 3 protein expression using immunohistochemistry (IHC). In light of our recent finding of a recurrent FGFR3-TACC3 translocation in the squamous subgroup of NSCLC and because of the lack of targeted therapy options for SCC of the lung, we subsequently screened a subset of FGFR3 IHC positive samples for additional FGFR3 molecular aberrations.

Material and methods

Sample collection and patient cohort

Inclusion criteria for this cohort were patients who had undergone a lung resection between 1990 and 2013 at one of four Dutch medical centres. Exclusion criteria were non-NSCLC histology eg, SCLC, metastatic non-NSCLC, a synchronous primary tumour, unavailability of tumour tissue and unavailability of patient follow-up data. The cohort included 768 samples with adequate patient and tumour characteristics. For all these patients, formalin-fixed, paraffin-embedded (FFPE) tumour samples were collected. After a second pathology review where samples without sufficient tumour material or histological subtype other than AC, SCC and LCC were excluded, 635 samples were eligible for further analysis. The cohort consisted of 329 AC, 278 SCC and 28 LCC samples. Median follow-up time was 96.0 months (95% CI: 86–103). All tumours were classified according to the 2004 WHO classification system. TNM classification was redefined for resections that were done before 2010 according to the 7th lung cancer TNM classification and staging system. Light smokers were defined as having fewer than 10 pack years (PY) including never smokers. The Translational Research Board of the Netherlands Cancer Institute-Antoni van
Leeuwenhoek hospital (NKI-AVL) approved the use of patient material in this study.

All FFPE tissues were constructed into tissue microarrays (TMAs) using a TMA Grand Master instrument (3D HISTECH, Budapest, Hungary). From each FFPE tissue block, three cores (0.6 mm) were punched from tumour areas that had been marked by a pathologist on HE slides, and arrayed into a recipient TMA donor block.

A subset of 200 samples for which RNA was available was screened for the FGFR3-TACC3 translocation. Of these 200 samples, 44 were kinome capture sequenced and analysed for fusion detection together with 15 matched Fresh Frozen (FF) samples previously reported by Majewski and colleagues [19]. The FGFR3 IHC positive samples of this 200-sample-subset were selected for FGFR3-amplification screening by fluorescence in situ hybridization (FISH). In addition, we screened for the S249C FGFR3 activating mutation previously found by Majewski and colleagues in the SCC subgroup.

Immunohistochemical staining for FGFR1, 2 and 3

Immunohistochemical staining was performed using the Ventana Benchmark Ultra stainer (Ventana Medical Systems, Tucson, AZ). In short, for FGFR1 and FGFR2 IHC staining 3 µm TMA slides were deparaffinized using EZ prep buffer (Ventana Medical Systems) and pretreated for 24 min at 95°C. Slides were then counterstained with Haematoxylin II for 8 min, and Bluing Reagent for 4 min (Ventana Medical Systems), after which slides were dehydrated and cover slipped. Slides were scored by two observers (S.W. and W.T.). Protein expression of FGFR1 and FGFR2 was quantified as a percentage (range 0–100%) of positive cells present among all tumour cells present in the TMA cores. Stain intensity was not scored because it was homogenous among all tumours. A mean score was calculated over one to three available measurements for each patient. Previous studies with FGFR IHC all used different arbitrary cutoffs [13,20–24], so we used FGFR1 and 2 as continuous variables. When assessing the prognostic value of FGFR expression for survival, FGFR1 and 2 were used both as a continuous and ordinal four-level variable, where <10% was considered negative (N), 10–50% low (L) expression, 50–80% median (M) and 80% and above was considered high (H). Expression of FGFR3 was semi-quantitatively scored based on the intensity: 0 (negative, N), 1+ (low, L), 2+ (high, H). The percentage of cells was not scored because it was homogenous among all tumours, in all of them it was 100%. The highest score among all three measurements of the same tumour was used for further analysis. FGFR1, FGFR2 and FGFR3 IHC scores were unavailable for 34 (5.4%), 45 (7.1%) and 23 (3.6%) of the samples, respectively. This was due to the lack of viable tumour in any of the three cores or the loss of all three cores on the TMA after staining. Samples were left out of the analysis only when all three FGFRs scores were unavailable. To verify the extent to which tumour heterogeneity would influence the FGFR IHC scoring we also scored some whole slides (n = 22), but in none of these cases did this lead to a different score (ordinal four-level variable for FGFR1 and 2 or intensity score for FGFR3).

Fusion detection and validation

Patient material was available from FFPE tissues. Sequencing libraries were constructed with a TruSeq mRNA library preparation kit using Ribo-ZeroTM (Epicentre). Capture enrichment was performed with the human kinome DNA capture baits (Agilent Technologies, Santa Clara, CA). Captured libraries were sequenced on an Illumina HiSeq2000 platform with a paired-end 51 base protocol. Sequences were aligned to the human genome (Hg19) with TopHat [25]. Two pipelines were used to identify and rank candidate fusion genes: TopHat-fusion [26] and de novo transcript assembly with Trinity [27]. We selected candidate fusion transcripts with at least one spanning read and one spanning pair. Detailed selection criteria were previously described [19]. We excluded fusions also identified in an unrelated normal sample.

Each fusion transcript was assessed to determine if the transcript would produce an in-frame fusion. The Maxima First Strand cDNA Synthesis Kit was used to produce input cDNA for RT-PCR (Thermo Scientific, Newington, NH). PCR primers were designed to amplify across the fusion breakpoints and products were confirmed by capillary sequencing using the Big Dye
 Terminator V3.1 Sequencing Kit (Applied Biosystems, Foster City, CA). A complete list of the validated fusion transcripts and the primers used for the validation is reported in supplementary material, Table S1. The FGFR3-TACC3 fusion transcript was screened with two different primer pairs previously reported [19,28].

**Mutation screening**

The activating mutation in FGFR3 was verified by PCR amplification from cDNA (F: 5′-CATTGGAGGCTAAGGCT; R: 5′-AGCACGTAACGTAGGTTG) and capillary sequencing using the Big Dye Terminator V3.1 Sequencing Kit (Applied Biosystems, Foster City, CA).

**Fluorescence in situ hybridization**

Fresh cut 4 μm sections of formalin-fixed paraffin embedded tissue were submitted to dual-colour FISH analysis using an IGH/FGFR3 translocation dual fusion FISH probe developed at Cytocell (Cambridge, UK). One probe set was designed in the short arm of chromosome 4 (red signal), where lies the FGFR3 gene and the other one in the long arm of chromosome 14 (green signal), where lies the IGH gene. FISH was performed according the standard method with some minor modifications. The slides were counterstained in phosphate-buffered saline containing 4′,6 diamidino-2 phenylindole dihydrochloride (DAPI) and mounted with Vectashield. Since there are no standard criteria to evaluate FGFR3 FISH assays in NSCLC yet, we decided to follow the strategy suggested by Schildhaus HU et al for scoring FGFR1 amplifications. We counted 20 tumour cells from three areas, resulting in a total of 60 nuclei. We called high level amplification if the FGFR3/IGH ratio was equal to or above 2 or the average gene copy per nucleus was equal to or above 6. When a sample showed a FGFR3/IGH ratio of 1.5, so not sufficient for a high-level amplification status but different from an unamplified status, the sample was defined as having a low level amplification or FGFR3 gain, and below 1.5 was defined as a normal FGFR3 copy-number [29]. We performed the FISH on a whole slide (in addition to the TMA) if samples were of low quality on the TMA or they were scored as positive for FGFR3 amplification on the TMA.

**Statistical analysis**

All analyses were carried out using IBM SPSS statistics 22 and R version 3.2. The data were summarized using standard descriptive statistics and frequency tabulations. FGFR1 and 2 expression were treated as continuous variables whereas FGFR3 was treated as an ordinal variable with three levels: negative, low, high.

Univariate associations between the FGFR protein expression and the patients’ clinical demographic variables age, gender, smoking history, histological type and pathological stage were assessed using a linear by linear association test and Spearman rank correlation for ordinal variables, Fisher’s exact test for categorical variables and Wilcoxon-Mann-Whitney or Kruskal-Wallis test for continuous variables. Multivariate associations between FGFR protein expression and clinical variables were assessed using linear regression for FGFR1 and 2 and logistic regression for FGFR3 after dichotomizing the latter into negative versus positive (low and high).

For survival analysis, overall survival (OS) was estimated from time of surgery until death or end of follow-up. Survival curves were estimated using the Kaplan-Meier method and compared between patients groups by four-level (FGFR1 and 2) or three-level (FGFR3) IHC scores by Logrank tests. The association between OS and FGFR expression was further analysed with univariable and multivariable Cox proportional hazard models. To correct for confounding variables, expression of all three FGFRs and patient characteristics were included in the multivariable

| Table 1. Patient and tumour characteristics of the non-small cell lung cancer cohort. PY = pack years |
|---------------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Gender                         | Male              | Female            | Median age at surgery (years, range) | 64 (30–84) |
| Neo-adjuvant therapy           | Chemotherapy      | Concurrent chemo radiotherapy | Sequential chemo radiotherapy | Erlotinib [52] | Radiotherapy | No adjuvant therapy | 532 (84.8%) |
| Smoking                        | Light smokers <10 PY | Heavy smokers ≥10 PY | Unknown          | 90 (14.2%) |
| Histology                      | Adenocarcinoma    | Squamous cell carcinoma | Large cell carcinoma | 38 (0–150) |
| Tumour stage at resection      | Tis               | Stage I           | Stage II          | Stage III       | Stage IV       | Unknown          | 2 (0.3%) |
| Median overall survival (months, range) | 49.0 (0–289) |
model as cofounders and the model was stratified for tumour stage. Median follow-up time was estimated by a reversed Kaplan-Meier method. Also, in the multivariate models FGFR3 was entered as a two-level factor (negative versus positive). Two-sided p-values below 0.05 were considered significant throughout all statistical computations.

Results

FGFR1, FGFR2 and FGFR3 protein are highly expressed in a subset of NSCLC

Patient characteristics and adequate tumour material were available for 635 samples. The median age at surgery was 64 years and 84.1% were early stage (≤ stage II). Of the 48 light smokers 33 were never

| FGFR1* | FGFR2* | FGFR3† |
|--------|--------|--------|
| N      | 601    | 590    | 612    |
| Negative | 117 (19.5%) | 269 (45.6%) | 505 (82.5%) |
| Low     | 267 (44.4%) | 166 (28.1%) | 87 (14.2%)  |
| Medium  | 153 (25.5%) | 79 (13.4%)  | NA       |
| High    | 64 (10.6%)  | 76 (12.9%)  | 20 (3.3%)  |

*Negative <10%, low 10 <50%, median 50 <80%, high ≥80%.
†Negative 0, low 1+, high 2+.

Figure 1. FGFR1, 2 and 3 protein expression by immunohistochemistry and FGFR3 copy numbers by FISH in non-small cell lung cancer. (A, B, C) Representative immunohistochemical staining of FGFR1, 2 and 3 negative samples versus FGFR1, 2 and 3 positive (high expression) samples, respectively. FGFR1: ab10646, abcam; FGFR2: ab10648, abcam; FGFR3: Clone B-9, sc-13121, Santa Cruz Biotechnology. (D, E) FGFR3 amplification and FGFR3 gain, respectively, assessed by dual-colour FISH analysis using an IGH/FGFR3 translocation dual fusion FISH probe (Cytocell).
smokers. Median OS was 148 months (95% CI: >103 months). Table 1 summarizes the clinicopathological characteristics of our patient cohort.

High protein expression of FGFR1, FGFR2 and FGFR3 was found in 64 (10.6%), 76 (12.9%) and 20 (3.3%) of NSCLC tumour samples, respectively (Table 2, Figure 1). Of all samples, 21.3% showed high-level expression of at least one of the three FGFRs. No samples showed high expression of all three FGFRs. There was a positive correlation between FGFR1 and FGFR2 expression ($R = 0.20$ (95% CI: 0.13–0.28)). No association was seen between expression of FGFR1 or 2 with expression of FGFR3. Nor could mutual exclusivity be determined, because there were samples showing overlap of high FGFR1 or 2 expression and high FGFR3 expression.

FGFR expression differs significantly between histological subtypes

We correlated gender, age, smoking status, histology and tumour stage with FGFR expression scores. Interestingly, FGFR expression differed significantly between histological subtypes: FGFR1 and FGFR2 were significantly associated with AC histological subtype ($p < 10^{-6}$ and $p < 10^{-15}$ respectively), whereas FGFR3 was significantly associated with SCC ($p < 10^{-12}$) (Figure 2, supplementary material, Table S2).

There was a significant difference in FGFR2 expression between genders, with higher FGFR2 expression in females (mean 34.1% versus 23.2%, p-value <0.0001). FGFR2 correlated negatively with age for the whole cohort ($R = −0.16$ (95% CI: −0.24 to −0.08)) and for the female patients only ($R = −0.16$ (95% CI: −0.28 to −0.04)). There was a negative correlation between tumour stage and FGFR2 expression (rho = −0.12, $p = 0.002$). FGFR3 correlated positively with age (rho = 0.16, $p < 0.001$). FGFR1 expression was associated with light smoking ($p = 0.02$) and FGFR3 expression was associated with heavy smoking (higher PY, $p < 0.0001$). All univariate associations are summarized in supplementary material, Table S3.
In the multivariate models, the associations of FGFR expression with their distinct histological subtype remained statistically significant. FGFR1 was still associated with FGFR2 expression. FGFR2 remained associated with gender and FGFR3 with a rising number of pack years (Table 3).

FGFR1 protein expression is related to poor overall survival in NSCLC

Higher expression of FGFR1 was associated with worse OS: HR 1.06 per 10%-point increase in FGFR1 level (95% CI 1.02–1.11, \( p = 0.01 \)) (Figure 3). When including gender, age, smoking status, histology as cofounders into the cox model and stratifying for tumour stage, the prognostic effect of FGFR1 remains statistically significant (\( p = 0.006 \)) (supplementary material, Table S4). Expression of FGFR2 (\( p = 0.51 \)) and FGFR3 (\( p = 0.13 \)) were not associated with a difference in OS (Figure 3).

FGFR3-TACC3 translocations and FGFR3 gene amplification occur in a small subset of NSCLC

RNA was available for 200 out of 635 samples in the cohort at the time of the study (111 (55.5%) AC, 76 (38.0%) SCC and 13 (6.5%) LCC). These samples were screened by RT-PCR for the presence of the FGFR3-TACC3 fusion. We detected six samples (3.0%) in which FGFR3 was fused with TACC3. FGFR3-TACC3 fusions occurred in 4/76 (5.3%) SCC and in 2/111 (1.8%) AC. Further details of IHC, histology and expression of FGFR1 and FGFR2 are summarized in Table 4 for these six samples. All patients had over 25 pack-years, three were male, four had stage I and two had stage II disease and all six patients were still alive at the end of follow-up.

Table 3. Multi-variate analysis: linear regression analysis for FGFR1 and 2 protein expression and logistic regression for FGFR3 protein expression with clinicopathological variables in non-small cell lung cancer

| Variable          | FGFR1* | FGFR2* | FGFR3† |
|-------------------|--------|--------|--------|
| FGFR1*            | NA     | pos, \( p = 0.0005 \) | pos, \( p = 0.24 \) |
| FGFR2*            | pos, \( p = 0.0005 \) | NA     | neg, \( p = 0.83 \) |
| FGFR3†            | pos, \( p = 0.21 \) | neg, \( p = 0.61 \) | NA     |
| Gender†           | pos, \( p = 0.46 \) | pos, \( p = 0.01 \) | neg, \( p = 0.69 \) |
| Age at diagnosis§ | pos, \( p = 0.50 \) | neg, \( p = 0.26 \) | pos, \( p = 0.17 \) |
| Smoking status¶   | neg, \( p = 0.17 \) | pos, \( p = 0.58 \) | pos, \( p = 0.008 \) |
| SCC versus AC     | neg, \( p = 0.002 \) | neg, \( p < 10^{-7} \) | pos, \( p < 10^{-6} \) |
| LCC versus AC     | neg, \( p = 0.47 \) | neg, \( p = 0.10 \) | pos, \( p = 0.15 \) |
| Tumour stage      | pos, \( p = 0.31 \) | neg, \( p = 0.30 \) | neg, \( p = 0.98 \) |

Direction of association and adjusted \( p \)-values are given.

*Per 10%-point increase.
†Dichotomized into negative (0) and positive (1+2+).
‡Female versus male.
§Per year.
¶FGFR1 and 2 as heavy versus light smoking; FGFR3 as PY.

SCC, squamous cell carcinoma; AC, adenocarcinoma; LCC, large cell carcinoma.

In the multivariate models, the associations of FGFR expression with their distinct histological subtype remained statistically significant. FGFR1 was still associated with FGFR2 expression. FGFR2 remained associated with gender and FGFR3 with a rising number of pack years (Table 3).

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Figure 3. FGFR1 protein expression is related to poor overall in non-small cell lung cancer. Survival curves by Kaplan–Meier method. (A, B) FGFR1 and 2 compared as an ordinal four-level variable by Log-rank test. (C) FGFR3 compared as an ordinal three-level variable by Logrank test. FGFR3 high protein expression is a rare event (\( n = 20 \)), which might have influenced the statistical significance of this test.
In order to understand whether high FGFR3 levels by IHC could be explained by FGFR3 amplification we screened 43 samples (36 SCCs, 6 ACs, 1 LCC) with FGFR3 IHC or 2 using FISH. FGFR3 was amplified in two samples (4.7%) (FGFR3/IGH ratio ≥ 2) (Figure 1D) and two samples showed an intermediate pattern that we could define as low level amplification or gain (Figure 1E). Interestingly, both samples with the FGFR3 gain were positive for the FGFR3-TACC3 translocation by RT-PCR. Also, we screened the 141 FFPE samples of the 200 samples for which RNA was available that were not previously screened for the S249C FGFR3 activating mutation in the Majewski paper, but we did not find any new cases.

**Discussion**

This study shows that high protein expression of FGFR1, 2 and 3 occurs frequently in NSCLC and that recurrent FGFR3-TACC3 fusions and FGFR3 amplifications are found. Also, FGFR1 protein expression may serve as a prognostic biomarker. These results suggest that the FGFR pathways play an important role in growth and malignant progression of NSCLC.

Histological subtype seems to be an important determinant of which FGFR protein is expressed. FGFR1 expression is significantly associated with AC, as was previously reported by Seo et al [30]. The same association was seen for FGFR2 protein expression, similarly to Behrens et al [22]. In contrast, FGFR3 protein expression was predominantly seen in SCC samples. To our knowledge, no data have been published concerning FGFR3 IHC and histology. Higher FGFR1 protein expression was associated with worse OS, which is in concordance with other studies highlighting the possible prognostic effect of FGFR1 protein expression [22,31]. We did not detect an association of FGFR2 and FGFR3 with OS.

It is an interesting finding that FGFR2 is associated with female gender in our cohort. We did not have data about the menopausal state of the female patients, but age and FGFR2 expression correlated negatively in the female cohort alone. Also, FGFR2 expression in the youngest quartile of women (age 31–53 year) was significantly higher compared to the upper three quartiles (44.5% versus 30.6%). These findings suggest an effect of hormonal status on FGFR2 expression or a synergy between oestrogen receptor and FGFR signalling in lung carcinogenesis. In NSCLC, there is indeed evidence of cross-talk between oestrogen and other growth factor pathways [32]. FGFR2 mutations have been found in approximately 10% of endometrial cancers [33] and FGFR2-amplification in 4% of triple-negative breast cancer [34]. Remarkably, there was no statistically significant association between FGFR1 expression and gender, although FGFR2 expression was significantly associated with both. However, the association between FGFR1 expression and gender does exist when comparing dichotomized (above versus below median) FGFR1 scores between genders instead of using FGFR1 as a continuous variable, but the latter method was deemed more appropriate for there is no consensus about cut-offs and this way less information is lost. Also, the proportion of FGFR2 positive FGFR1 negative samples in females was slightly higher than in males (cut-off by median score) and the association between FGFR1 and gender is much weaker and indeed disappears when compensating for FGFR2 in a bivariate model.

The strong association of histological subtype and FGFR protein expression suggests that FGFR3 positive tumours are a distinct subgroup within NSCLC. Because of the lack of targeted therapy options in the squamous sub-group of NSCLC and because of our recent finding regarding FGFR3 molecular aberrations [19], we screened a subset of our cohort for additional FGFR3 molecular aberrations depending on DNA/RNA availability. We detected a frequency of 5.3% of FGFR3-TACC3 fusions in our SCC sample group, which is higher than previously reported (1.8–3.0%) [16,35]. However, it has to be taken into account that our sample size was small and that we did not have data about the menopausal state of the female patients.
account that this subset was enriched for FGFR3 IHC positive samples. We also found two AC samples carrying the FGFR3-TACC3 fusion (1.8% in the AC group only). The translocation has been described in AC previously [35,36]. Only four out of six samples carrying the FGFR3-TACC3 fusion showed positive FGFR3 IHC staining. This discrepancy may show that the FGFR3-TACC3 fusion does not always lead to protein (over)expression. The expression levels of the FGFR3-TACC3 transcript by RT-qPCR confirmed that samples with negative FGFR3 IHC staining but carrying the FGFR3-TACC3 fusion (Table 4, samples #5 and #6) showed very low FGFR3-TACC3 mRNA expression (data not shown). In addition, diagnostic and technical issues regarding IHC staining must also be taken into consideration: tumour heterogeneity, unclear standards on FGFR IHC scoring, and the quality of the FGFR3 antibody [13,20–24,35]. This will need further investigation.

Because FGFR3 protein overexpression may also be explained by increased FGFR3 copy number (GCN, Gain Copy Number), we assessed the amplification status of FGFR3 using FISH. We found two samples with FGFR3 amplification (4.7%). FGFR3 amplifications are rare events in bladder cancer (3.4% of cases) [37], but no studies have so far investigated the amplification status of FGFR3 in NSCLC specifically. The Cancer Genome Atlas (TCGA) reported a lower frequency of FGFR3 amplification (1% in SCC and AC populations together), but our selection of FGFR3 IHC+ samples for the FISH screening could explain this difference. Furthermore, our FISH assay was specifically designed to detect FGFR3 amplification, which makes it a more sensitive assay than the CGH technology used by the TCGA. This finding shows that FGFR3 amplification is a rare event in our sample set, similar to bladder cancer. We found two different patterns of high level amplification: a cluster-pattern which consists of a tight accumulation of more than six FGFR3 signals in the nuclei (IHC 2+, Figure 1D); and FGFR3 signals spread in the nuclei. The low level amplification (gain) found in two of the samples positive for the FGFR3-TACC3 translocation (Figure 1E) might be explained by the formation of this fusion via tandem duplication [9,38].

Still, we could not detect an underlying molecular aberration in the majority of the screened samples with high FGFR3 protein expression. On the one hand, diagnostic issues may play a role. FFPE archival methods lead to chemical modifications (methylene dimerization or monomethylolation) and partial degradation of the RNA, which makes RNA extraction, reverse transcription and quantitation a difficult process. Therefore, fusion-transcript detection in such poor quality material can be very challenging and lead to false negative results. Unfortunately, FF material was available for only a very limited number of samples in our cohort (16%, 33/210) to confirm the absence/presence of the event by screening both FF/FFPE samples. In addition, the result that the FGFR3-TACC3 frequency in our cohort is even higher than previously reported highlights the reliability of our screening procedure. On the other hand, aberrant pathway activation needs to be considered. Marek and colleagues described coexpression of FGFR2 and FGFRs (mRNA and IHC) in NSCLC cell lines without underlying molecular aberrations [39]. These findings, which suggest a FGFR autocrine and/or paracrine loop, were seen not only in NSCLC but also in other solid tumours [5,6,20,22,40–47].

There is evidence that FGFR3-TACC3 is an oncogenic driver in several cancer types, such as bladder cancer, glioblastoma and nasopharyngeal carcinoma. In these cancers, responses to FGFR inhibition have been observed in FGFR3-TACC3 translocated cell lines [16,28,38,48,49]. Similarly, Ba/F3 cells expressing the FGFR3-TACC3 fusion were sensitive to FGFR3 inhibition [36]. Direct correlation of FGFR3-TACC3 translocation and increased sensitivity to FGFR inhibition in NSCLC specifically is still under investigation, but response rates to FGFR inhibition as shown by recent phase II clinical trials with the FGFR inhibitors AZD4547 and BGJ398 in FGFR1 amplifed lung cancers have been unsatisfactory [50,51]. It is not clear at present whether this lack of clinical response is due to issues with detection of those tumours in which FGFR is a true driver of the oncogenic phenotype for the reasons mentioned above, or whether feedback regulation of signalling limits the effects of FGFR inhibition in lung cancer. We are currently studying the latter possibility using functional genetic approaches.

In conclusion, FGFR1, 2 and 3 proteins are often overexpressed in early stage NSCLC with distinctive expression according to histological subtype. Also, recurrent molecular aberrations such as FGFR3 amplification and FGFR3-TACC3 translocation are present, making these pathways an interesting target for the treatment of NSCLC. Our results contribute to the refinement of selection of patients who may benefit from FGFR inhibition.

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Author contributions

WT involved in writing the protocol synopsis, conceived and carried out experiments, data collection, analysed the data, generated the figures and tables; LM conceived and carried out experiments, data collection and analysed the data; SW involved in writing the protocol synopsis, sample collection, conceived and carried out experiments; AJ conceived and carried out experiments; DP sample collection, conceived and carried out experiments; VvdN analysed the data, generated the figures and tables; EJ data and sample collection; KK conceived and carried out experiments; TP conceived and carried out experiments; TS conceived and carried out experiments; JB data and sample collection; CvN sample collection; RB involved in writing the protocol synopsis; MvdH involved in writing the protocol synopsis; All authors were involved in writing the paper and had final approval of the submitted and published versions.

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**SUPPLEMENTARY MATERIAL ONLINE**

**Table S1.** List of the validated fusion transcripts and the primers used

**Table S2.** FGFR1, 2 and 3 immunohistochemical expression in non-small cell lung cancer by histological subtype

**Table S3.** The association between FGFR1, 2 and 3 protein expression with clinicopathological features

**Table S4.** Cox-regression analysis of overall survival on clinicopathological variables stratified for tumour stage