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Fibroblast Growth Factor Receptor 1 as a Putative Therapy Target in Colorectal Cancer

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Abstract

Background/Aims—Resembling a potential therapeutic drug target, fibroblast growth factor receptor 1 (FGFR1) amplification and expression was assessed in 515 human colorectal cancer (CRC) tissue samples, lymph node metastases and CRC cell lines.

Methods—FGFR1 amplification status was determined using fluorescence in situ hybridization. Additionally, we assessed protein levels employing Western blots and immunohistochemistry. The FGFR1 mRNA localization was analyzed using mRNA in situ hybridization. Functional studies employed the FGFR inhibitor NVP-BGJ398.

Results—Of 454 primary CRCs, 24 displayed FGFR1 amplification. 92/94 lymph node metastases presented the same amplification status as the primary tumor. Of 99 investigated tumors, 18 revealed membranous activated pFGFR1 protein. FGFR1 mRNA levels were independent of the amplification status or pFGFR1 protein occurrence. In vitro, a strong antiproliferative effect of NVP-BGJ398 could be detected in cell lines exhibiting high FGFR1 protein.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

Conclusion—FGFR1 is a potential therapeutic target in a subset of CRC. FGFR1 protein is likely to represent a central factor limiting the efficacy of FGFR inhibitors. The lack of correlation between its evaluation at genetic/mRNA level and its protein occurrence indicates that the assessment of the receptor at an immunohistochemical level most likely represents a suitable way to assess FGFR1 as a predictive biomarker for patient selection in future clinical trials.

Keywords

Fibroblast growth factor receptor 1; Colorectal cancer; NVP-BGJ398

Introduction

Fibroblast growth factor receptors (FGFRs) activating mutations and overexpression trigger the development of various cancers, e.g. bladder, breast, ovarian, renal cell and squamous cell lung cancer [1–3]. FGFRs are encoded by 4 genes (FGFR1–4) and activated after ligand binding. They regulate downstream signaling pathways via two substrates, FRS2 and phospholipase-C γ . Among others, FRS2 activates mitogen-activated protein kinase and the phosphoinositide-3-kinase pathway [4]. Distribution and function of FGFRs are highly cell type specific [5]. *FGFR1* amplification is a putative biomarker of a subset of FGFR1-driven cancers of different origin and histology [2, 3]. So far, patients with colorectal cancer (CRC) are not included in clinical trials utilizing anti-FGFR therapy. However, Nakao et al. [6] and others [7] have described *FGFR1* amplification (~ 5%) in CRC.

Targeted therapies are being tested in functional and clinical trials [3, 8] utilizing specific small molecule inhibitors exemplified by NVP-BGJ398, inhibiting auto-phosphorylation and catalytic activity [3]. To date, patient recruitment for targeted anti-FGFR therapy is solely based upon FGFR1 gene copy number status. However, other factors such as epigenetic control of transcription and post-translational modification of the *FGFR1* gene clearly influence mRNA and also protein levels. The *FGFR1* copy numbers were studied here in a relevant cohort of primary and metastasized CRC, resembling a putative target for inhibitor therapy. Furthermore, we determined FGFR1 mRNA and protein levels evaluating the molecular consequences of gene amplification in tissues and cell lines.

Materials and Methods

Patient Cohort

Human tissue samples were collected at the University Hospitals of the Charité Berlin (n = 416, between 1995 and 2009), and Bonn (n = 99, between 2005 and 2010). The Bonn cohort was available for immunostaining as well as mRNA and protein measurements. Representative samples of all relevant tumor localizations and histological subtypes of primary CRCs were taken into account [colon ascendens (n = 79), colon transversum (n = 35), colon descendens (n = 35), sigma (n = 127), rectosigmoid (n = 20), rectum (n = 95), flexura dextra (n = 14), flexura sinistra (n = 16), cecum (n = 55), multifocal (n = 8), and unknown site (n = 31)]. The predominant histological subtype was adenocarcinoma (81.8%). The median age of the patients at diagnosis was 68 years in 279 male and 236 female patients.

59.5% (292/491) patients presented with lymph node metastases (LNM). In 99 cases, corresponding LNM were further assessed. Clinicopathological data was available for all 515 patients (table 1).

Tissue Microarray Construction

Tissue microarray (TMA) construction was performed as described earlier [9]. In short, 3 representative cores measuring 0.6 mm in diameter from each formalin-fixed paraffin-embedded primary tumor and its corresponding LNM were assembled into TMA blocks and stained with hematoxylin and eosin.

FGFR1 Fluorescence in situ Hybridization

FISH was performed for the detection of *FGFR1* amplification status on genomic level in TMAs [1]. In brief, we selected the *FGFR1* target probe (red fluorescent signal) for hybridization spanning the 8p11.22–23 locus (RP11-148d12) and a commercially available reference probe (green fluorescent signal) located on the stable centromeric region of chromosome 8 (MetaSystems, Altussheim, Germany). Only nuclei displaying green reference signals were included for the determination of the *FGFR1* copy number status. The evaluation was performed independently by two evaluators with a fluorescence microscope (Zeiss, Jena, Germany) and the FISH software Metafer 4 (MetaSystems). We evaluated at least 100 nuclei per sample. Tumors were classified as high-level amplified (HLA) if the number of red target signals was at least nine times higher than the number of green reference signals in 20% of nuclei. An accumulation of many inseparable target gene signals (cluster formation) was also assorted to HLA. Samples with fewer than nine but more than two excess red target signals relative to the number of green reference signals in 20% of cells were classified as low-level amplified (LLA). We observed high intratumoral uniformity in tumors exhibiting FGFR1 amplification (80% of cell nuclei) [10].

Cell Lines and Reagents

All media contained 10% fetal bovine serum and 40 µg/ml streptomycin. All cell lines originated from human colorectal adenocarcinoma: HCT116 [obtained from ATCC, McCoy's 5A Medium (Gibco)], SNU-C1 [obtained from ATCC, RPMI 1640 medium (Gibco)], and HDC9 [German Cancer Research Center (DKFZ), RPMI 1640 medium (Gibco)].

Cell Line Screening

Cell lines were preselected by screening for a characteristic peak in 8p11.23–8p11.22 containing *FGFR1* within the single nucleotide polymorphism data using the online platform of the Broad Institute (www.Broadinstitute.org/tumorscape). Several cell lines (LS123, HCT116, HDC9, SNU-C1, HT29, SW837, SW620, DLD1, Isreco1, SW480) were screened for an amplification of the *FGFR1* gene by performing metaphase FISH [11].

Inhibition Assay

NVP-BGJ398 was from Active Biochem (Maplewood, N.J., USA), dissolved in dimethyl sulfoxide (DMSO). Aliquots were stored at –20 °C. Triplets of 5,000 cells of SNU-C1/

HDC9 and 1,200 cells of HCT116 were incubated for 4 h in 96-well plates. Subsequently, different amounts ranging from 0.5 to 5 μ M of NVP-BGJ398 were added. Analysis of viable cells was performed with MTT (Roche Diagnostics, Mannheim, Germany) after 48 and 72 h. Controls received 5 μ l DMSO. After solubilization, the formazan dye was quantified by scanning with a multi-well spectrophotometer (ELISA reader).

Real-Time Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from cell lines using the RNeasy Mini Kit 50 (Qiagen). cDNA conversion was performed using a Super-Script[®] VILO[™] cDNA Synthesis Kit (Life Technologies). The cDNA was preamplified utilizing a Taq PCR Master Mix (Light-Cycler 480 Probes Master; Roche Diagnostics). Sequences for the primers were: β -actin: (forward) gcacccagcacaatgaaga, (reverse) cgatccacacggagtacttg; fgfr1: (forward) ggcagcatcaaccacacata, (reverse) taccaggccactgttt.

Quantitative RT-PCR was performed employing a LightCycler 480 system (Roche Diagnostics) using TaqMan[®] analysis.

High-Resolution Melting Analysis

High-resolution melting (HRM) analyses were performed on a LightCycler 480 (Roche Diagnostics) [12]. The amplicon length for exon 2 is 142 bp flanking the hot spot mutation of codon 12 and 13 KRAS, and the amplicon length for exon 3 is 186 bp flanking codon 59, 61 and 63 of KRAS.

Sanger Sequencing

Sanger sequencing analyses were performed determining the KRAS mutation status and the HRM results. The HRM PCR products were directly used for sequencing using a Big Dye Terminator v1.1 Cycle Sequencing Kit. Sequences were run on an ABI Prism 3130 automated sequencer. Data was edited utilizing Sequencer analysis software (all Applied Biosystems, Darmstadt, Germany).

Western Blots

Western blots were performed as described earlier [13]. Antibodies used: FGF receptor 1 (Epitomics, 1: 500), pFGFR1 (phos-pho-Y766, Abnova, 1: 250), ERK (Cell Signaling (Boston, Mass., USA), 1: 500), pERK (Cell Signaling, 1: 500), pAkt (Cell Signaling, 1: 500), Akt (Cell Signaling, 1: 500), and β -actin (Sigma-Aldrich, 1: 5,000). The concentrations of the inhibitor were 0, 2, 4, and 8 μ M.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on prepared cytopins of the treated cell lines and in a subset (99 patients) of our cohort by Ventana Discovery automated immunostaining system (Ventana Medical Systems, Tucson, Ariz., USA) according to the manufacturer's protocol. Antibodies used: primary polyclonal phospho-FGFR1 antibody (pFGFR1 Y766, Abnova, 1: 250) and primary cleaved caspase-3 antibody (DCS Innovative Diagnostic Systems, 1: 100). For detection we utilized ChromoMap DAB kit (UI-traMap DAB anti-Rb detection system; Ventana Medical Systems).

Evaluation of the Immunohistological Stainings

After digitalization (Mirax scanner), all TMA slides were scored by two independent investigators. Immunostaining was quantified by assessing the staining intensity according to a semi-quantitative score ranging from 0 to 3, for negative (0), weak (1+), medium (2+), and strong (3+) immunoreactivity for nuclear, cytoplasmatic and membranous localization, respectively. In a subset of cases, tissue staining presented in a heterogeneous fashion which were then counted to the prevailing intensity level (>60% of the tumor tissue).

FGFR1 mRNA in CRC

The FGFR1 mRNA status was determined in 99 primary CRC tumors using mRNA in situ hybridization (ISH) with the RNA-scope technology (Advanced Cell Diagnostics, Hayward, Calif., USA, www.acdbio.com/). TMA slides were digitized using a Mirax scanner. The evaluation of the staining was graded as follows: no staining (0), a tumor exhibiting 1–3 dots per cell in >50% of the cells (2+), tumors exhibiting 1–3 dots/cell in >1%, but <50% of cells are scored as 1+, while clusters of dots in <50% of cells or 3–5 dots in >50% of cells or >5 dots in >10% of cells is scored as 3+. A tumor exhibiting clusters of dots in >50% of the cells was scored as 4+.

Statistical Analysis

All statistical analyses were done with R version 2.13.0 on a Mac OS X 10.6.8 system. The Kruskal-Wallis test was used for ordinally scaled non-parametric data. The Fisher test was used for comparison of nominal data. Comparison of mean values was done by t test or analysis of variance in case of more than two groups. p values < 0.05 were considered to be statistically significant. Correlation tests were performed with the Spearman method for non-parametric data.

Results

FGFR1 Amplification Frequency in CRC

Of the 515 primary CRCs, 454 were assessable. Of these, 5.3% (24/454) displayed *FGFR1* amplification (4 HLA and 20 LLA) (table 2).

In the subgroup of cases with available LNM, 4.3% (4/94) displayed *FGFR1* amplification in the primary tumor and its corresponding LNM (1 HLA and 3 LLA). In patients with *FGFR1* amplification in LNM, the *FGFR1* status always reflected the changes seen in the primary tumor. 2.1% (2/94) of the cases displayed *FGFR1* amplification in only the primary tumor (2 LLA) but not in the metastases (fig. 1a–f; table 2).

IHC of pFGFR1

We assessed activated phosphorylated FGFR1 receptor (pFGFR1) in a subcohort of cases (n = 99). 18 cases revealed a membranous staining of pFGFR1, sometimes heterogeneously (fig. 2a–c). 15 of these cases displayed a weak membranous pFGFR1 localization (1+). Three cases showed medium membranous pFGFR1 localization (2+). The nuclear localization of the cases presented as follows: 40 cases without pFGFR1 (0), 17 with weak pFGFR1 (1+), 33 with medium pFGFR1 (2+), and 9 with strong pFGFR1 (3+). When

scoring the cytoplasmatic pFGFR1, we found 65 cases without pFGFR1 (0), 27 with weak pFGFR1 (1+), 3 with medium pFGFR1 (2+) and 4 with strong pFGFR1 (3+) (table 2). Taken together, pFGFR1 was localized as follows: 28 cases displayed no pFGFR1 in any cell compartment (0), 25 cases showed a weak pFGFR1 (1+), 35 cases exerted a medium pFGFR1 (2+) and 10 cases revealed a strong pFGFR1 (3+) in at least one localization.

In 24 cases, pFGFR1 was distributed over two or three cell compartments (membrane, cytoplasm and nucleus). In 34 cases, pFGFR1 was localized in only one cell compartment. Interestingly, nuclear occurrence of pFGFR1 proved to be the most frequent localization among these cases (28/34).

FGFR1 mRNA in CRC

We determined the FGFR1 mRNA status of the patients of the Bonn cohort (n = 99), employing mRNA ISH. 17 cases showed no (0), 29 cases low (1+), 29 cases medium (2+), 17 cases high (3+) and 7 cases very high (4+) FGFR1 mRNA levels (table 2; fig. 2d–f).

Statistical Evaluation of FGFR1 Amplification/Protein Levels/mRNA Status

There was no significant association of gene copy number status with either FGFR1 mRNA or pFGFR1 protein status of all three cell compartments.

Interestingly, patients harboring a *FGFR1* amplification mostly presented with a medium nuclear pattern (2+) of pFGFR1 (5/6 cases). Also, patients with *FGFR1* amplification always had at least a baseline mRNA level (1+). Association of pFGFR1 protein of all cell compartments with clinicopathological data revealed a significant correlation of nuclear localization with a lymphatic invasion ($p < 0.01$) and angioinvasion ($p = 0.02$). Additionally, membranous ($p < 0.01$) and cytoplasmatic ($p < 0.03$) pFGFR localization occurred significantly more often in male patients (table 1).

FGFR Inhibitor NVP-BGJ398 Reduces Cell Growth Dependence on FGFR1 mRNA/Protein Levels

We employed three representative CRC cell lines for functional studies, taking *FGFR1* gene expression, mRNA levels and protein levels into account. SNU-C1 displayed low-level amplification in the *FGFR1* gene locus, but interestingly showed the lowest occurrence of FGFR1 on the mRNA and protein level as compared to all other screened cell lines (fig. 3a–c). The cell lines HDC9 and HCT116 showed higher protein and mRNA levels of FGFR1 compared to SNU-C1 and were not amplified in the FGFR1 gene locus. HDC9 and SNU-C1 proved to be KRAS wild-type, whereas HCT116 displayed a KRAS mutation (GGC to GAC mutation in codon 13).

Inhibition of HDC9 and HCT116 cell lines by NVP-BGJ398 showed a decrease in cell viability, whereas SNU-C1 cell viability was unaffected by small molecule inhibition (fig. 4). HDC9 was the most sensitive cell line followed by HCT116, only responding to high inhibitor concentrations (fig. 4). Sensitivity to inhibition with NVP-BGJ398 was more related to FGFR1 mRNA and protein levels as well as the KRAS mutation status than to the *FGFR1* amplification status.

Decrease of Phosphorylation of FGFR1 in Cell Lines with High pFGFR1 Levels (HDC9 and HCT116) after Treatment with NVP-BGJ398

We further determined the effects of NVP-BGJ398 on the responsive cell lines HCT116 and HDC9. Both cell lines showed a clear downregulation of the activated pFGFR1, whereas total FGFR1 showed no decrease upon treatment.

The KRAS-mutated cell line HCT116 showed no change in the activation of the two main downstream pathways of FGFR1 (ERK1/2 and Akt). In contrast, HDC9 cells showed a decrease of phosphorylation of the downstream molecule ERK. The levels of total ERK1/2 and total Akt remained unaltered after inhibition (fig. 5).

NVP-BGJ398 Induces Apoptosis in HDC9 Cells

In HDC9, but not in SNU-C1 and HCT116 (data not shown), the IHC staining with cleaved caspase-3 revealed an increase of the protein in a dose-dependent manner, indicating increased apoptosis (fig. 6a–c).

Discussion

Certain gene alterations lead to a new definition of tumor subgroups enabling individualized therapy options based upon genetic screening [14]. Among these, altered FGFR1 in various cancers including CRC could be indicative for a specific subgroup benefiting from special treatment strategies [3, 5].

So far, patients are selected by determination of FGFR1 amplification status for targeted anti-FGFR therapies. We aimed (1) to probe for *FGFR1* amplification status in primary and metastatic CRC, and (2) to redefine patient selection criteria for therapy recruitment by determining the *FGFR1* copy number status, mRNA and protein quantity in tissue and in CRC cell lines.

Previously, in a small cohort using aCGH, a genomic gain was preliminarily reported in the 8p region between 41 and 48 Mb (containing *FGFR1* among several other genes) [6]. We confirm here the *FGFR1* amplification frequency of about 5% using FISH in a larger cohort. Moreover, we report the same *FGFR1* copy number status in metastatic disease as in the corresponding primary tumor, indicating *FGFR1* amplification as a clonal event during tumor progression. This is supported by data from lung cancer [10].

Interestingly, mRNA and protein levels of the non-amplified cell lines (HDC-9 and HCT116) were higher than in the LLA cell line (SNU-C1). Accordingly, no significant correlation was obvious between *FGFR1* amplification and high levels of mRNA and pFGFR1. Therefore, we suggest that other regulatory epigenetic mechanisms play a crucial role causing alterations of transcriptional and translational processes in the levels of FGFR1 mRNA and protein. The interaction between genetic and epigenetic alterations in CRC is increasingly under discussion [15–17]. SNU-C1 was not sensitive to NVP-BGJ398 treatment supporting recent data [18]. HDC9 cells showed reduced cell growth and increased apoptosis under treatment. Despite elevated FGFR1 protein levels and a reduction of pFGFR1 level on inhibition, HCT116 was only affected by NVP-BGJ398 in higher

concentrations. Reduced sensitivity might be due to an activating KRAS mutation since KRAS is downstream of FGFR1. It is important to take downstream targets into account predicting responsiveness to targeted therapies with compounds like NVP-BGJ398 [18, 19]. pFGFR1 proved to be not only localized in the cell membrane, but also in cytoplasm and nucleus in our cohort. The signaling cascade of the nuclear FGFR1 receptors differs from the membranous receptor signaling cascade. It serves as a KRAS-independent transcriptional regulator for genes controlling proliferation and cell cycle in other tumors [20]. This has an intriguing impact on our study since the compound can cross cellular membranes. Therefore, NVP-BGJ398 may impact on FGFR receptors regardless of their subcellular localization [3, 21]. In higher concentrations, NVP-BGJ398 may interact with intracellular receptor proteins, impairing viability at higher concentrations, as shown in the example of HCT116. In this cell line, we demonstrate slightly reduced viability despite KRAS mutation pointing to inhibited intracellular FGFR1 receptors. The differing localizations of receptors may affect prognosis and pathological features of patients with CRC [22]. Activated FGFR exists in all subcellular localizations [20, 23, 24]. Our data indicate a significant correlation of strong nuclear pFGFR1 levels with a significantly higher risk of lymphovascular invasion, thus indicating a negative prognostic feature.

FGFR1 is a potential therapeutic target in a subset of CRC. FGFR1 is likely to represent a central factor limiting the efficacy of FGFR inhibitors. The lack of correlation between its evaluation at genetic/mRNA level and its protein levels indicates that the assessment of the receptor at an immunohistochemical level most likely represents a suitable way to assess FGFR1 as a predictive biomarker for patient selection in future clinical trials.

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Fig. 1. FISH images of CRC cases with different *FGFR1* copy number status: diploid *FGFR1* copy number status in CRC sample (a) and corresponding lymph node (b), low-level *FGFR1* amplification in CRC sample (c) with corresponding lymph node (d), and high-level *FGFR1* amplification in CRC sample (e) with corresponding lymph node (f).

Fig. 2. Immunohistological stainings. **a–c** Membranous localization of pFGFR1 in CRC: no pFGFR1 (**a**), heterogeneous localization (**b**), and high pFGFR1 levels (**c**). **d–f** FGFR1 mRNA status: score 0 (**d**), score 2+ (**e**), and score 4+ (**f**).

Fig. 3.

Amplification, mRNA, and protein status of FGFR1 in HCD9, HCT116, and SNU-C1 cells.

a Relative mRNA levels of FGFR1 in HDC9, SNU-C1 and HCT116 employing qRT-PCR (normalized to β -actin). HDC9 displays the highest levels, followed by HCT116 and SNU-C1. **b** FISH images of (1) inter- and metaphase nucleus of HDC9 cells harboring no *FGFR1* amplification, (2) inter- and metaphase nucleus of the *FGFR1*-amplified SNU-C1 cells, and (3) inter- and metaphase nucleus of the HCT116 cells with no *FGFR1* amplification. **c** FGFR1 protein levels in HDC9, SNU-C1, and HCT116 cells as assessed by immunoblotting. HDC9 displays the highest amounts, followed by HCT116 and SNU-C1.

Fig. 4.

FGFR inhibitor NVP-BGJ398 reduces cell growth correlating with FGFR1 mRNA/protein. Inhibition of HDC9 and HCT116 cell lines using NVP-BGJ398 (48 and 72 h) showed a decrease in cell viability, whereas SNU-C1 cell viability was not affected. HDC9 was the most sensitive cell line followed by HCT116 showing only a response at high concentrations of the inhibitor. Viability (NVP-BGJ398 treatment as compared to DMSO control) was determined via MTT assay as described. Viability of 1.0 means 100% cell survival. The number of viable cells is relative to the untreated control cells at the same time points (48 and 72 h).

Fig. 5.

Western blots of pFGFR1/FGFR1 and downstream molecules. HCT116 and HDC9 cells showed a clear downregulation of the activated pFGFR1, whereas total FGFR1 showed no decrease in the treated cell lines. HDC9 cells showed a decrease in the phosphorylation of the downstream molecule pERK. HCT116 showed no change in the activation of pERK and pAkt. The levels of total ERK1/2 and total Akt remained the same after inhibition.

Fig. 6. Immunohistological staining of HDC9. Increase of cleaved caspase-3 in HDC9 cells after inhibition for 48 h with rising amounts of NVP-BGJ398: 0 µM NVP-BGJ398 (a), 0.5 µM NVP-BGJ398 (b), and 1 µM NVP-BGJ398 (c).

Table 1

Clinicopathological data available for 515 patients

	Total cohort (n = 515), n (median age = 68 years)	Bonn cohort (n = 99), n (median age = 65 years)	FGFR1 membranous (p value)	FGFR1 nuclear (p value)	FGFR1 cytoplasmatic (p value)	FGFR1 mRNA expression (p value)	FGFR1 genetic expression (p value)
Age, years							
68	258						
68	257						
65		50					
65		49					
Sex			<0.01	0.26	<0.03	0.2	0.88
Male	279	54					
Female	236	45					
pT stage			0.24	0.73	0.62	0.16	0.21
1	16	4					
2	68	4					
3	312	55					
4	95	33					
Data unknown	24	3					
pN stage			0.51	0.18	0.63	0.44	0.6
0	199	0					
1	148	50					
2	142	44					
3	2	2					
Data unknown	24	3					
M stage			0.49	0.8	0.17	0.46	0.93
M0	417	70					
M1	77	29					
Data unknown	21	0					

	Total cohort (n = 515), n (median age = 68 years)	Bonn cohort (n = 99), n (median age = 65 years)	FGFR1 membranous (p value)	FGFR1 nuclear (p value)	FGFR1 cytoplasmatic (p value)	FGFR1 mRNA expression (p value)	FGFR1 genetic expression (p value)
Grading			0.14	0.19	0.51	0.51	0.79
G1	11	1					
G2	293	49					
G3	185	44					
Data unknown	26	5					
Lymphovascular invasion							
L0	196	12	0.38	<0.01	0.44	0.59	0.59
L1	270	60					
Data unknown	49	27					
V0	349	29					
V1	87	13	0.9	0.02	0.29	0.4	0.83
Data unknown	79	57					

Table 2

Overview of amplification frequencies in our cohort

	Primary CRC (n = 454)	Primary CRC + LNM (n = 94)		pFGFR1 (protein) (n = 99)			FGFR1 (mRNA) (n = 99)
		primary CRC + LNM	primary CRC (LNM not amplified)	membrane	cytoplasm	nucleus	
No amplification	430	88	0				
LLA	20	3	2				
HLA	4	1	0				
0				81	65	40	17
1+				15	27	17	29
2+				3	3	33	29
3+				0	4	9	17
4+							7