Differential Effects of Polymorphic Alleles of FGF Receptor 4 on Colon Cancer Growth and Metastasis

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Abstract

A gly388arg polymorphism (rs351855) in the transmembrane domain of the fibroblast growth factor receptor (FGFR4) is associated with increased risk, staging, and metastasis in several different types of cancer. To specifically assess the impact of the polymorphic FGFR4 in colorectal cancer (CRC), we engineered CRC cell lines with distinct endogenous expression patterns to overexpress either the FGFR4gly or FGFR4arg alleles. The biologic analyses revealed an oncogenic importance for both polymorphic alleles, but FGFR4arg was the stronger inducer of tumor growth, whereas FGFR4gly was the stronger inducer of migration. An evaluation of clinical specimens revealed that FGFR4 was upregulated in 20/71 patients independent of gly388 arg status. There was no correlation between the presence of an FGFR4arg allele and CRC or polyp risk in 3,471 participants of the CORSA study. However, among 182 patients with CRC, FGFR4arg-carriers had a fivefold higher risk of tumors that were stage II or greater. Together, our results established that both allelic forms of FGFR4 exert an oncogenic impact and may serve equally well as therapeutic targets in CRC. One important implication of our findings is that FGFR4arg-carriers are at a higher risk for more aggressive tumors and therefore may profit from early detection measures.

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Introduction

For the fibroblast growth factor receptor 4 (FGFR4), a polymorphism that causes a substitution of an arginine instead of a glycine in the transmembrane domain of the receptor (gly388 arg; rs351855) has been described and has pathophysiologic impact in tumor development (1). Specifically, the FGFR4arg allele was found to be associated with increased cancer risk in the prostate (2) and with more aggressive tumors, metastasis, therapy resistance, and poor outcome in lung carcinomas (3), head and neck cancer (4, 5), breast cancer (1, 6), and melanomas (7). In the colon, 2 studies report an association of the FGFR4arg allele with tumor size and nodal status (1, 8) and with recurrence (8). Another study did not find a significant impact of the polymorphic alleles on colorectal cancer (CRC) pathophysiology (9). To date, no mechanistic studies that investigate the cell biologic impact of FGFR4 variants on malignant cell characteristics in CRC are available.

The cellular mechanism underlying FGFR4-effects on carcinogenesis in general is still poorly understood. FGF signaling plays essential roles in embryogenesis, development, and wound healing through activation of cell growth, survival, and cell migration (10–13). These effects have also been observed in malignant cells for several members of the FGF family (14–17). Consistent with this cell biologic pattern of FGF effects, therapy resistance conferred by FGFR4 in breast cancer cells was reported to involve expression of antiapoptotic genes (18). In addition, differential promigratory activity of the gly388 or arg388 forms of FGFR4 has been implicated in the formation of more aggressive breast tumors (19).

It is, therefore, the aim of this study to address the hypothesis that the gly388 and arg388 alleles of FGFR4 have differential impact on the malignant characteristics of CRC cells, thus causing an increased risk of CRC tumor progression and metastasis in individuals carrying an FGFR4arg allele. For this purpose, the biologic impact of FGFR4gly and FGFR4arg has been assessed using cell line models and the association of SNP rs351855 and expression with CRC risk and prognostic parameters has been analyzed in individuals participating in the CORSA study and CRC patients of hospitals in Vienna and Budapest.

Materials and Methods

Cell lines

SW480, SW620, HCT116, HT29, Colo201, T84, and Caco2 CRC cell lines were obtained from the American Type Culture...
Collection and kept under standard tissue culture conditions using Minimal Essential Medium containing 10% fetal calf serum (FCS). Cell lines were authenticated by analysis at the Genetic Resources Core Facility of the Johns Hopkins University.

Isolation of DNA and genotyping

DNA was extracted by standard protocols (Qiagen) and genotyping was conducted with ABI Prism 7500 Sequence Detection System (Applied Biosystems) using a TaqMan SNP-assay (Applied Biosystems 4351379). A detailed description can be found in Supplementary Materials.

Isolation of RNA and quantitative RT-PCR

Total RNA was isolated from subconfluent cultures or frozen colon tissue specimens using Trifact reagent according to the manufacturer’s instructions (PeqLab). DNA was synthesized using RevertAid MMuLV reverse transcriptase (Fermentas) and random hexamer primers (GE Healthcare).

TaqMan assays from Applied Biosystems were used to determine expression of FGFR4 (Hs00242558_m1) and GAPDH (Hs99999905_m1) mRNAs by quantitative RT-PCR (qRT-PCR). Expression in cell lines was calculated as an \( \Delta \Delta C_t \) above the respective controls; expression in tumors was calculated as an \( x \)-fold change compared with the corresponding normal mucosa using GAPDH as control gene and the \( \Delta \Delta C_t \) method.

Reagents from the TaqMan Genotyping assay and a standard curve constructed with defined mixtures of pure \( FGFR4^{\beta} \) and \( FGFR4^{\gamma} \) DNA were used on cDNA to assess the ratio of expressed alleles in heterozygous cells from the density of 0.5 \( /C_2 \) 105 cells/cm2. After a migration period of 24 hours, filters were removed and cells in the lower chamber were counted.

Overexpression of FGFR4 in CRC cell lines

pcDNA3 plasmids expressing VSV-tagged forms of \( FGFR4^{\beta} \) or \( FGFR4^{\gamma} \) that were kindly provided by A. Ullrich (Martinsried, Germany) were introduced into SW480, HCT116, and HT29 cells by lipofection with Transfectin (BioRad). Controls were transfected with GFP or the pcDNA3 vector and stable transfectants were selected in the presence of geneticin (G418).

Knockdown of gene expression

siRNAs specifically targeting \( FGFR4 \) were purchased from Ambion (Applied Biosystems) and transfected into 70% confluent cultures kept in medium containing 10% FCS using 3 \( \mu \)L siLentFect (BioRad) and 20 pmol of the siRNA per well in culture medium without serum. A scrambled siRNA without sequence homology to known human genes served as negative control. After 24 and 48 hours, RNA and protein were isolated to verify knockdown efficiency. Functional and growth assays were initiated 24 hours after transfection.

Protein isolation and Western blotting

To determine the impact of FGFR4 on intracellular signaling activity semiconfluent cultures of SW480 transfectants were starved and lysed for protein analysis 24 hours later. Cell membranes were prepared by cell lysis in Dounce buffer (10 mmol/L Tris HCl, 0.5 mmol/L MgCl2 protease and phosphatase inhibitors, pH7.6), followed by homogenization in tonicity restoration buffer (10 mmol/L Tris HCl, 0.5 mmol/L MgCl2, 0.06 mol/L NaCl and protease and phosphatase inhibitors, pH7.6) and ultracentrifugation (100,000 g, 90 minutes). Total protein extraction and Western blotting was conducted as described (17) using phosphospecific antibodies recognizing PLC, FRS2α, c-src, ERK, GSK3β, and S6. A detailed list of the antibodies used can be found in Supplemental Materials (Supplementary Table S1). Bands were detected using secondary antibodies coupled to horseradish peroxidase and chemiluminescence staining reagents (GE Healthcare). Band intensity was quantified from the x-ray films using ImageQuant software (GE Healthcare).

FGFR4 on the cell surface

Trypsinized cells were stained with a PE-coupled monoclonal antibody recognizing the N-terminus of FGFR4 (clone 4F&6D3s; Biolegend). Control stains were done using a PE-coupled antimouse control antibody. Fluorescence-activated cell sorting (FACS) analysis was conducted on a FACS Calibur (Becton Dickinson).

Cell viability and growth assays

Cells were seeded at a density of 1 \( \times 10^5 \) cells per well into 96-well plates for growth curves and 3 \( \times 10^5 \) for knockdown experiments. Viability was determined by MTT assay (Easy4U; Biomedica).

DNA synthesis was determined by incubation with \( ^3 \)H-thymidine (1 \( \mu \)Ci/mL) as described previously (14, 17).

For assessment of anchorage-independent growth, 5,000 cells/well were suspended in 0.25% agar prepared in RPMI medium containing 20% FCS and incubated for 2 to 3 weeks before counting the number of colonies microscopically.

Clonogenicity was determined from cells plated at a density of 100 or 200 cells/well onto 6-well plates in medium containing 10% FCS by staining with 0.01% of crystal violet solution to assess colony formation (14, 17).

Cell migration assay

Cells were seeded into 8-μm pore-size polyester track-etched membrane filters (BD-Falcon) in 24-well plates at a density of 0.5 \( \times 10^5 \) cells/cm2. After a migration period of 24 and 48 hours for SW480 and HCT116 and 96 hours for HT29 cells, filters were removed and cells in the lower chamber were stained with crystal violet and colony number evaluated using Lucia software. Alternatively, migration was determined by scratch assay as described in ref. 20 with identical results.

Tumor growth and metastasis

SW480 cells selectively overexpressing \( FGFR4^{\beta} \) or \( FGFR4^{\gamma} \) as well as control transfectants were suspended in serum-free medium at a density of 1 \( \times 10^6 \) cells/50 \( \mu \)L and subcutaneously injected into the rear flanks of immunodeficient severe combined immunodeficient mice (SCID)/Balb/c recipient mice (female, aged 4 weeks, Harlan Winkelmann). Tumor formation was monitored by palpation and tumor size was determined...
using a Vernier caliper. Tumor volume was calculated using the formula (smaller diameter² / larger diameter) / 2. All experiments were conducted in quadruplicates and carried out according to the Austrian and FELASA guidelines for animal care and protection. Tissue sections of experimental tumors were analyzed by immunohistochemistry using antibodies directed against cytokeratin 20 and Ki67 as described previously (21).

Mouse lungs were prepared for immunohistochemistry and metastasizing tumor cells in lung sections were identified by their expression of Ki67. Metastasis was scored according to the number and size of metastatic foci as described in Supplemental Materials.

Hospital study population and tissue specimens

Tissue specimens were collected from patients undergoing surgery for CRC in hospitals in Vienna and Budapest ("hospital population"). Informed consent was obtained from all patients. Immediately after surgery, tissue specimens were frozen in liquid N2 until extraction of nucleic acids. All diagnostic information on tumor location, staging, and grading is available and the pattern of staging is given in Supplemental Table S2. Tumor tissue had a tumor cell content of at least 70% as judged from the histology of immediately adjacent tissue. The study had prior approval of the local ethics committees.

FGFR4 expression was analyzed from both the tumor and the adjacent normal mucosa by qRT-PCR. The rs351855 polymorphism was determined from the tumor tissue using the TaqMan Genotyping assay.

Population-based study population

Within a province-wide screening project in eastern Austria, Caucasian participants were recruited for the molecular epidemiology CRC study of Austria (CORSA). Participants with a positive fecal occult blood testing underwent colonoscopies and were asked to participate in the molecular epidemiology study. All subjects gave written informed consent. The study was approved by the Institutional Ethic Review Board. Details of the study population are described in refs. 22–24.

The control group (n = 1,794) consisted of participants that were free of polyps and CRC shown by colonoscopy. The adenoma group consisted of 1,330 and the CRC group of 178 patients who were newly diagnosed and previously untreated. CRC and polyp diagnosis was histologically confirmed and the adenoma group was classified in a high-risk (n = 292) and a low-risk (n = 1,038) subgroup based on the histology report. Sex, age, and nutrition have been shown to impact on CRC risk independent of most genetic variants (25, 26). The pattern of these confounding variables is summarized in Table 1.

Statistical evaluation of data

The statistical analysis of CRC risk in relation to patient genotype is described in detail in refs. 22 and 23. Genotypic counts of controls were tested for Hardy–Weinberg equilibrium using a v2 test. Linkage disequilibrium statistics were computed using Haploview 4.0. Multiple logistic regressions were applied to compare individuals of the control group against the CRC group and the CRC + high-risk adenoma group. ORs and 95% confidence intervals (CI) were estimated using the software R Ver 2.6.2. All P values are 2-sided; P values less than 0.05 were considered to be statistically significant.

Power calculations were carried out with a calculator available from (27). Given the observed genotype distribution [50% homozygous wild type (gly/gly), 40% heterozygous (arg/gly), and 10% homozygous mutant (arg/arg)] ORs of 2.36 can be recognized with a power of 80% and a significance level α = 5% for carcinoma versus controls, arg/arg versus gly/gly. For carcinoma + high risk adenoma versus controls, arg/arg versus gly/gly the OR was 1.71.

Table 1. Characteristics of the CORSA study population

<table>
<thead>
<tr>
<th></th>
<th>CRC patients n = 178 (5.3%)</th>
<th>High-risk adenoma patients n = 292 (9.0%)</th>
<th>Controls n = 1,794 (53.4%)</th>
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<tr>
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<td></td>
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<tr>
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<td>55 (63.2)</td>
<td>195 (66.8)</td>
<td>810 (46.6)</td>
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<td>97 (33.2)</td>
<td>928 (53.4)</td>
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<td>&lt;50</td>
<td>8 (9.2)</td>
<td>44 (15.1)</td>
<td>353 (20.3)</td>
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<td>18 (20.7)</td>
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<td>426 (24.5)</td>
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<td>24 (27.6)</td>
<td>109 (37.3)</td>
<td>550 (31.7)</td>
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<td>31 (35.6)</td>
<td>77 (26.4)</td>
<td>391 (22.5)</td>
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<td>&gt;80</td>
<td>6 (6.9)</td>
<td>2 (0.7)</td>
<td>18 (1.0)</td>
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<td>Body mass index, kg/m²</td>
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<td>7 (0.4)</td>
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<td>6 (2.1)</td>
<td>61 (3.5)</td>
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</table>
Tissue expression data were analyzed by paired sample \( t \) test after obtaining a Gaussian distribution by transforming to log values. The relationship between SNP rs351855 and tumor stage was determined in comparison to stage 1 using contingency tables and Fisher exact or \( \chi^2 \) test using Graphpad Prism. Tumor growth was analyzed by 2-way ANOVA and cell biologic results were analyzed by Student \( t \) test or Kruskal–Wallis test depending on the results of normality testing.

Results

Impact of FGFR4 overexpression on malignant characteristics in vitro

SW480 and HCT116 cells are gly-homozygous cells expressing low and high levels of FGFR4, respectively, HT29 is gly/arg-heterozygous and expresses high levels of mainly FGFR4\textsuperscript{gly}, (for details, see Supplementary Fig. S1). These cells were transfected with FGFR4 expression vectors and clone pools were selected that stably overexpress a specific FGFR4 allele. In SW480 transfectants, overexpression was 3- to 4-fold on the RNA level and because of their low endogenous expression, the cells mainly expressed the transfected allele. FGFR4 at the cell membrane was increased 11-fold for FGFR4\textsuperscript{gly} and 3-fold for FGFR4\textsuperscript{arg}. In HCT116 that expressed higher levels of FGFR4\textsuperscript{gly}, overexpression was only 60% and 25% for FGFR4\textsuperscript{arg} and FGFR4\textsuperscript{gly}, respectively, but FGFR4\textsuperscript{arg} transfection shifted the arg/gly-ratio from 0.1 to 1.1. FGFR4 protein was increased 3-fold (FGFR4\textsuperscript{arg}) and 1.6-fold (FGFR4\textsuperscript{gly}). In the FGFR4\textsuperscript{arg} expressing HT29 cells, RNA was upregulated 2.5- and 1.5-fold with FGFR4\textsuperscript{gly} transfection shifting the arg/gly-ratio to 1:1. Protein overexpression was 12-fold and 3.6-fold for FGFR4\textsuperscript{arg} and FGFR4\textsuperscript{gly} (details are shown in Supplementary Fig. S2). Impact of FGFR4 overexpression on logarthmic growth under standard culture conditions was only seen in SW480 cells (Supplementary Fig. S3).

However, overexpression of FGFR4\textsuperscript{gly} but not FGFR4\textsuperscript{arg} enhanced the capacity to attach and form colonies in very low-density cultures by 20% to 60% in all 3 cell lines (Fig. 1A). Differential impact on anchorage-independent growth in soft agar was also observed depending on the endogenous FGFR4 expression level: in SW480 cells, growth stimulation was strong with both alleles and FGFR4\textsuperscript{arg} was the stronger activator compared with FGFR4\textsuperscript{gly} (gly vs. arg significantly different at \( P = 0.003 \)). In HCT116, growth was not further increased by FGFR4\textsuperscript{arg} and even decreased by transduction of FGFR4\textsuperscript{gly} creating a highly significant difference between the 2 allelic forms. In contrast, in the FGFR4\textsuperscript{arg} expressing HT29 cells agar growth was similarly stimulated by either FGFR4 allele (Fig. 1B).

Both FGFR4 alleles had strong impact on tumor cell migration in SW480 cells. With a 60-fold increase FGFR4\textsuperscript{arg} was the more potent gene compared with FGFR4\textsuperscript{gly} (25-fold; difference significant at \( P = 0.003 \)). The impact was much weaker in HCT116 cells (2.8- and 2-fold, respectively, for FGFR4\textsuperscript{gly} and FGFR4\textsuperscript{arg}, and no significant difference between the 2 allelic forms). In HT29 cells that endogenously express high levels of FGFR4\textsuperscript{arg}, no additional effect on cell migration was induced by FGFR4\textsuperscript{arg}. FGFR4\textsuperscript{arg} caused a 25% reduction of migration capacity (\( P = 0.0002 \) compared with control as well as with FGFR4\textsuperscript{gly}, Fig. 1C).

Knockdown of FGFR4 expression

Knockdown of FGFR4 expression in the cell lines HCT116 and HT29 by lipofection with siRNA-oligonucleotides caused suppression of FGFR4 mRNA to 10% to 20% of control level (Supplementary Fig. S4). Shifts in the gly/arg ratio of HT29 cells were not observed. On the protein level for both cell lines, the cell population expressing FGFR4 on their cell surface was reduced to 20% to 40% of the controls. Suppression of FGFR4 expression reduced viability to 80% (\( P = 0.0005 \)) of control in HT29 cells and to 35% (\( P = 0.029 \)) in HCT116 (Fig. 2A). DNA synthesis was inhibited by 25% in both cell lines (Fig. 2B). Colony formation in low-density cultures was inhibited by 28% in HT29 (\( P = 0.005 \)) and 16% in HCT116 cells (\( P = 0.029 \); Fig. 2C). Impact on cell migration was more pronounced in both cell lines with an inhibition of about 40% (\( P = 0.005 \) for HT29 and \( P = 0.031 \) for HCT116; Fig. 2D). An impact on anchorage-independent growth could not be detected in either cell line (data not shown).

Impact on downstream signaling

Phosphorylation of the primary FGFR-target FRS2\(\alpha\) was increased in FGFR4 overexpressing SW480 cells compared with the control (SW480\textsuperscript{gly}) even though the amount of FRS2\(\alpha\) found in the particulate fraction was reduced. PLC\(\gamma\) phosphorylation was decreased in FGFR4 transfectants. The amount of FRS2\(\alpha\) and PLC\(\gamma\) protein recruited to the membrane was distinctly higher in SW480\textsuperscript{gly} than SW480\textsuperscript{arg}, whereas specific phosphorylation of the signaling molecules was similar in SW480\textsuperscript{arg} and SW480\textsuperscript{gly} cells (Fig. 3A and B). In addition, c-src protein was upregulated in SW480\textsuperscript{arg} but not SW480\textsuperscript{gly} cells. The src protein was phosphorylated at tyrosine 418 indicating activation of kinase activity at a similar level in all transfectants (Fig. 3C and D). FGFR4-dependent phosphorylation of GSK3\(\beta\) and S6 was observed—showing activation of survival pathways downstream of the phosphatidylinositol-3-kinase. Phosphorylation of ERK was not affected (Fig. 3C and D).

Tumor growth in vivo

As the results of the in vitro growth, assays strongly indicated a differential protumorigenic impact of FGFR4 overexpression, SW480\textsuperscript{arg}, SW480\textsuperscript{arg}, and SW480\textsuperscript{gly} cells were injected s.c. into SCID mice to assess local tumor growth and metastasis. All 3 cell lines grew to local tumors consisting mainly of cytokeakin 20 expressing, poorly differentiated cells. The FGFR4\textsuperscript{arg} gene significantly enhanced tumor growth as compared with the SW480\textsuperscript{gly} (\( P = 0.0002 \)), whereas the FGFR4\textsuperscript{arg} variant had no impact. The fraction of proliferating Ki67-positive cells was similar in SW480\textsuperscript{arg} and SW480\textsuperscript{arg} tumors (29.0 ± 5.7% and 25.1 ± 3.1%, respectively), but increased in the SW480\textsuperscript{arg} tumors (39.1 ± 3.0%; \( P = 0.05 \); Fig. 3A and B).

To assess metastasis, lungs from tumor-bearing mice were analyzed in serial sections stained with an antibody detecting Ki67 that is not expressed in normal lung tissue. The only metastatic lesion large enough to be observed macroscopically

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FGFR4 in Colorectal Cancer

Figure 1. Impact of FGFR4 overexpression on tumor cell growth and migration in vitro. Stable transfectants overexpressing FGFR4<sup>Arg</sup> or FGFR4<sup>Gly</sup> in SW480, HCT116, or HT29 cells were used to assess growth and malignant characteristics in vitro. Stable transfectants with the pcDNA3 vector were used as controls. A, 100 and 200 cells were seeded/6-well, medium was changed after 24 hours, and the number of colonies counted after 10 days of growth. B, five thousand cells each were suspended in soft agar medium and plated in 6-well plates. The number of colonies was counted at a magnification of 10-fold after 3 weeks of growth. C, cell migration was determined by filter migration assay from 2 × 10⁶ cells/24-well. Photographs of cultures show representative results from SW480 transfectants. Quantitative results from all cell lines were calculated as % of control, pooled from at least 3 independent experiments, and presented as mean ± SD.

*, **, *** indicate an increase as compared with the control at P < 0.05, 0.01, and 0.001, respectively. ## indicates a decrease as compared with control at P < 0.01. &, &&, and &&& indicate a difference between the FGFR4<sup>Arg</sup> and FGFR4<sup>Gly</sup> groups at P < 0.05, 0.01, and 0.001, respectively.

was found in the lung of an animal from the FGFR4<sup>Arg</sup> group. The other lungs obtained from the SW480<sup>Gly</sup> group contained large clusters of tumor cells (>10 cells), whereas only single tumor cells or small clusters were found in the lungs of SW480<sup>Arg</sup> and control animals. The number and size of metastatic colonies for each mouse were scored showing a higher average score for the SW480<sup>Arg</sup> than for control or SW480<sup>Gly</sup> tumors (Fig. 4C and D; P = 0.05).
FGFR4 expression and SNP rs351855 in human CRC

FGFR4 gene expression was determined from paired tissue specimen obtained from patients undergoing surgery for CRC (hospital population). FGFR4 was found overexpressed 2-fold or more as compared with normal mucosa in a subgroup of 20/71 (28%) of these specimens. The range of relative expression levels varied from 0.04 to 33.76 resulting in a mean of 2.73 ± 0.69 (increased above control at P = 0.015). However, expression level did not correlate with either histopathological parameters or the patients’ SNP rs351855.

FGFR4 allele distribution was analyzed from the genomic DNA of 3,471 participants of the CORSA study. The genotype distribution in controls was in Hardy-Weinberg equilibrium. Multiple logistic regression was applied to compare individuals of the control group against 2 different case groups: the CRC group and the combined CRC + high-risk adenoma group (Table 1). The prevalence of homozygous arg/arg genotype in the control population was 8.1%, whereas it was 11.8% and 8.5% in the CRC and CRC + high-risk adenoma group, respectively, which was not statistically different from the control population. This resulted in a relative CRC risk of 1.42 (95% CI, 0.68–2.93) for developing CRC and 1.03 (95% CI, 0.77–1.36) for developing CRC or a high-risk adenomatous polyp conferred by SNP rs351855 (Table 2). Both were not statistically different from risk of the homozygous gly/gly population.

Histopathologic parameters were available for 55 of the CRC patients in the CORSA study population. As this was not sufficient for a meaningful analysis, further genotype information was obtained from 122 tissue specimens of the hospital population. Because of the different settings for the diagnosis, the tumor stage distribution was different for the 2 CRC groups. Specifically, the percentage of stage I tumors was lower in the hospital population (3.9% vs. 26.8% in the CORSA screening study; Supplementary Table S2). Additional details can be found in Supplementary Materials.

On the basis of the strong promigratory impact of FGFR4 gly388arg even in an FGFR4 homozygous background, the study populations were grouped into patients with a gly/gly genotype and arg carriers consisting of both heterozygous and arg-homozygous patients. To determine whether the different stage distribution of the CORSA and hospital populations affects, the association of SNP rs351855 with tumor stage, the data for both populations were analyzed separately with similar results (Table 3). In the hospital population, all stage I tumors were obtained from gly-homozygous patients and the fraction of arg carriers increased with tumor stage, resulting in a 19.4-fold risk (95% CI, 1.10–339.00 by Fisher exact test) for tumors stage II or higher. The main difference in the CORSA population was the presence of 33% FGFR4 gly/homozygous individuals in the stage I group, whereas the fraction of FGFR4 hetero- and homozygous genotypes in stage II was lower compared with the hospital population (52% vs. 62%). No differences in allele distribution between the 2 populations were seen for stages III and IV. Because of the small number of cases in the CORSA population, no significant results could be obtained. However, the trend of increasing fraction of arg carriers with higher tumor stage was similar to the hospital population. In the combined hospital and CORSA population, patients carrying the arg allele had a 5-fold increased risk of tumor stage II or higher already at diagnosis (95% CI, 1.75–14.60 by Fisher exact test). SNP rs351855 did not correlate with any other parameter including tumor size and grade (data not shown).

Discussion

The gly388arg polymorphism of FGFR4 has been described as a predisposing and/or prognostic factor for malignancies of the lung (28, 29), the prostate (30), the head and neck (4), the breast (6), the liver (31), and for melanomas (7). For CRC, there are 3 prior reports: Bange and colleagues have shown a correlation of the FGFR4 arg allele with tumor size and metastasis (1) and Gordon and colleagues described a correlation with recurrence after chemoradiation (8). In contrast, Spinola and colleagues did not observe any association of the SNP rs351855 with either risk or prognostic parameters (9).

In this study, we have constructed moderately FGFR4 over-expressing cell lines for analysis of the cellular mechanisms...
induced by the 2 polymorphic forms of FGFR4. We have used 3
different cell lines SW480, HCT116, and HT29 that represent 3
possible variations of endogenous FGFR4 expression back-
ground—low expression, high FGFR4gly expression, and high
FGFR4arg expression—resulting in 3 sets of isogenic human cell
lines. Using an assay panel informative about malignant
growth and cell migration, we observed that both
FGFR4 alleles stimulated anchorage-independent growth as well as cell
migration in SW480 cells that have low endogenous
FGFR4 expression. Comparison of the 2 alleles revealed that
FGFR4gly was the stronger stimulator of malignant cell growth, whereas
FGFR4arg was the stronger activator of cell migration. The
differential impact was more obvious in the cell lines with high
endogenous FGFR4 expression. In the FGFR4gly background of
HCT116 cells FGFR4arg had no additional effect on malignant
growth but still stimulated cell migration, whereas FGFR4arg stimulated cell migration, but inhibited anchorage-independent
growth. In contrast, in the FGFR4gly expressing HT29 cells
both alleles increased malignant growth, whereas no addi-
tional effect of FGFR4arg and inhibition by FGFR4gly was
observed for cell migration. This indicates that both polymor-
phic alleles are capable of counteracting the main activity of
the respective other form. This agrees with reports on inhi-
bition of cell migration by FGFR4gly in breast cancer cells by
Bange and colleagues (1). However, this only occurred in a high
FGFR4arg background, whereas FGFR4gly was fully capable of
stimulating cell migration in a low FGFR4 background in CRC
cells.

The most consistent difference between the FGFR4gly and
FGFR4arg alleles was observed in colony formation assays
where FGFR4arg did not stimulate any of the cell lines used.
The assay determines the combined effect of cell attachment and growth potential so that both the weaker impact of
FGFR4arg on growth and viability and the reduction in cell
attachment inherent in the ability to better migrate should
contribute to this effect.
SW480 transfectants were used for tumorigenicity studies in vivo, because they mainly express the transfected FGFR4 allele. After xenotransplantation under the skin of SCID mice, SW480gly cells grew to larger tumors locally, whereas SW480arg cells had a higher tendency to metastasize confirming the differential oncogenic effect of FGFR4 polymorphic alleles in vivo.

Analysis of human tissue specimen supports the same conclusions: overall expression of FGFR4 in human tumor specimens was elevated about 2-fold because of very strong upregulation (up to 30-fold) in a 28% subgroup of patients. Expression level was not related to histopathologic parameters or FGFR4 genotype, however. Both polymorphic alleles were affected in a similar way indicating that both alleles have a similar potential of tumorigenic impact in CRC.

With regard to downstream signaling activity, both polymorphic forms of FGFR4 activated FRS2α and survival signaling downstream of PI3K as indicated by increased phosphorylation of the Akt-substrate GSK3β and of S6. Differential activity was seen with the primary receptor substrates PLCγ and FRS2α that were better recruited into the signaling complex by FGFR4gly, and for c-src that was increased by

| Table 2. FGFR4 (rs351855) genotype distribution and CRC risk |
|-----------------|-----------|----------|-----------------|-----------------|-----------|
| Genotype        | CRC Case | OR (95% CI) | Significance level | CRC + high-risk adenomas |
| gly/gly         | 802      | 42       | 1.0              | n.s.             | 190       | 1.00 | n.s. |
| gly/arg         | 723      | 33       | 0.87 (0.54–1.40) | n.s.             | 148       | 0.86 (0.67–1.09) | n.s. |
| arg/arg         | 135      | 10       | 1.41 (0.68–2.93) | n.s.             | 25        | 0.81 (0.51–1.28) | n.s. |

NOTE: Genotype was analyzed from blood samples of participants of the CORSA study and analyzed in relation to CRC and high-risk polyp diagnosis.

Abbreviation: n.s., not significant.
FGFR4<sup>gly</sup>. The latter effect has been correlated with FGFR-dependent migration signaling in previous studies. Association of c-src activation with higher receptor stability and extended signaling activity with induction of cell migration has been described for N-CAM-mediated induction of cell migration in HeLa cells (32).

We actually did observe higher miRNA levels in CRC cell lines carrying an arg allele than in gly/gly homozygous cell lines, similar to observations in breast cancer cell lines (1). On the protein level, the differences of expression between FGFR4<sup>gly</sup> and FGFR4<sup>arg</sup> were still clearer: FGFR4 protein on the cell membrane was increased 4- to 12-fold in FGFR4<sup>arg</sup> transfectants, but only 1.6- to 4-fold in FGFR4<sup>gly</sup> homozygous cell lines, in accordance with previous studies (35, 36). Activation of downstream survival signaling has been discussed as an essential contribution to the metastatic phenotype (33).

In the absence of exogenously added ligands, activation of FGFR4-dependent signaling has to come from autocrine growth factors in the culture supernatant. For the SW480 cells, this will mainly be FGF18 that is still needs to be elucidated.

The CORSRA study population consists of 3,471 participants. As it was conducted within the context of a screening program, it provided a very well defined control population consisting of those participants that were found free of both polyps and cancer by colonoscopy. A disadvantage of the early-detection setting is the low incidence of CRC. Only 2.8% of the participants had already developed cancer and the staging was generally lower than in the hospital-based CRC group. Incidence of high-risk adenomas among the CORSRA participants was 14% and overall adenoma incidence was 42%. On the basis of the power analysis for the cohort, an OR of 2.36 for the arg/arg group could have been determined with a significance level of \( P = 0.05 \). On the basis of the multistep nature of CRC, adenoma incidence can be regarded as representative of tumor initiation (37, 38). Therefore, we extended analysis to the larger group of CRC + high-risk adenomas that allowed recognition of an OR = 1.71 for the arg/arg group. Actual differences in ORs observed in our study were too small to be considered significant.

In contrast, significant correlation was observed within the CRC group between the presence of an FGFR4<sup>arg</sup> allele in the patients’ genome and higher tumor stage but not with tumor size or grade. In the hospital population for patients carrying the arg allele, the relative risk of their tumor being stage II or higher was 19-fold. All CRC patients with stage I tumors in the hospital population were FGFR4<sup>arg</sup>. Therefore, we extended analysis to the larger group of CRC + high-risk adenomas that allowed recognition of an OR = 1.71 for the arg/arg patients. Actual differences in ORs observed in our study were too small to be considered significant.

### Table 3. FGFR4 (rs351855) genotype distribution and CRC tumor stage

<table>
<thead>
<tr>
<th>Stage</th>
<th>gly/gly-</th>
<th>arg/arg</th>
<th>gly/arg</th>
<th>OR (95%CI)</th>
<th>Significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>15</td>
<td>5</td>
<td>25</td>
<td>4.5</td>
<td>1.48–13.44</td>
</tr>
<tr>
<td>II</td>
<td>33</td>
<td>49</td>
<td>59</td>
<td>6.7</td>
<td>2.10–21.16</td>
</tr>
<tr>
<td>III</td>
<td>19</td>
<td>40</td>
<td>67</td>
<td>4.0</td>
<td>1.06–15.14</td>
</tr>
<tr>
<td>IV</td>
<td>9</td>
<td>12</td>
<td>57</td>
<td>5.1</td>
<td>1.75–14.60</td>
</tr>
<tr>
<td>≥ II</td>
<td>61</td>
<td>101</td>
<td>62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hospital patients only</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>23</td>
<td>38</td>
<td>62</td>
<td>18.0</td>
<td>0.95–341.20</td>
</tr>
<tr>
<td>III</td>
<td>15</td>
<td>32</td>
<td>68</td>
<td>23.1</td>
<td>1.20–444.40</td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>8</td>
<td>57</td>
<td>14.4</td>
<td>0.67–310.10</td>
</tr>
<tr>
<td>≥ II</td>
<td>44</td>
<td>78</td>
<td>64</td>
<td>19.4</td>
<td>1.05–359.40</td>
</tr>
<tr>
<td>CORSRA patients only</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>10</td>
<td>5</td>
<td>33</td>
<td></td>
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<tr>
<td>II</td>
<td>10</td>
<td>11</td>
<td>52</td>
<td>2.2</td>
<td>0.56–8.69</td>
</tr>
<tr>
<td>III</td>
<td>4</td>
<td>8</td>
<td>66</td>
<td>4.0</td>
<td>0.80–20.02</td>
</tr>
<tr>
<td>IV</td>
<td>3</td>
<td>4</td>
<td>57</td>
<td>2.7</td>
<td>0.42–16.83</td>
</tr>
<tr>
<td>≥ II</td>
<td>17</td>
<td>23</td>
<td>57</td>
<td>2.1</td>
<td>0.81–5.28</td>
</tr>
</tbody>
</table>

NOTE: Genotype information was obtained from blood samples 57 of patients with CRC in the CORSRA population and from tumor of 127 patients at hospitals in Vienna and Budapest. *, **, and *** represent differences to stage I at \( P ≤ 0.05 \), \( P ≤ 0.01 \), and \( P ≤ 0.001 \), respectively. Abbreviation: n.s., not significant.
the small number of patients in the CORSA population prevented observation of a significant effect of FGFR4<sup>arg</sup> on tumor stage. However, a similar trend toward increased frequency of FGFR4<sup>arg</sup> carriers in higher tumor stages was observed as in the hospital population. Addition of the CORSA patients in the overall analysis lowered the extent of the impact FGFR4<sup>arg</sup> had on the risk of higher stage tumors but did not abolish it. This further supports a role for the polymorphic allele in tumor progression and an invasive phenotype. These results are in agreement with the studies of Bange and colleagues (1) and Gordon (8) who reported aspects of more aggressive tumor behavior. It contradicts Spinola’s report of no impact of FGFR4 on CRC (9). Like ours, all previous studies report on patient cohorts smaller than 200.

In summary, the results obtained from the functional characterization of our cell line models show oncogenic effects in CRC for both polymorphic forms of FGFR4. Consequently, both FGFR4 forms are suitable candidate therapeutic targets in CRC. In addition, results on allele distribution in CRC patients that need to be tested in a larger population indicate that the FGFR4<sup>arg</sup> allele may serve as a prognostic marker for more aggressive tumors and that FGFR4<sup>arg</sup> carriers may profit from CRC screening and early detection.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**


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