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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 *FGFR4^{gly}* or *FGFR4^{arg}* alleles. The biologic analyses revealed an oncogenic importance for both polymorphic alleles, but *FGFR4^{gly}* was the stronger inducer of tumor growth, whereas *FGFR4^{arg}* was the stronger inducer of migration. An evaluation of clinical specimens revealed that FGFR4 was upregulated in 20/71 patients independent of gly³⁸⁸arg status. There was no correlation between the presence of an *FGFR4^{arg}* allele and CRC or polyp risk in 3,471

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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participants of the CORSA study. However, among 182 patients with CRC, *FGFR4^{arg}*-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 *FGFR4^{arg}*-carriers are at a higher risk for more aggressive tumors and therefore may profit from early detection measures.

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 (gly³⁸⁸arg; rs351855) has been described and has pathophysiologic impact in tumor development (1). Specifically, the *FGFR4^{arg}* 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 *FGFR4^{arg}* 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 *FGFR4^{arg}* allele. For this purpose, the biologic impact of *FGFR4^{gly}* and *FGFR4^{arg}* 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 Trifast reagent according to the manufacturer's instructions (PeqLab). cDNA 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 x -fold increase 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 C_t method.

Reagents from the TaqMan Genotyping assay and a standard curve constructed with defined mixtures of pure *FGFR4^{gly}* and *FGFR4^{arg}* DNA were used on cDNA to assess the ratio of expressed alleles in heterozygous cells from the C_t between *FGFR4^{arg}* and *FGFR4^{gly}* (for details, see Supplementary Material).

Overexpression of *FGFR4* in CRC cell lines

pcDNA3 plasmids expressing VSV-tagged forms of *FGFR4^{gly}* or *FGFR4^{arg}* 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 μ 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, 0.6 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 γ , FRS2 α , c-src, ERK, GSK3 β , and S6. A detailed list of the antibodies used can be found in Supplemental Materials (Supplementary Table S1). Bands were detected using second antibodies coupled to horseradish peroxidase and chemoluminescence 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&6D3; 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 1×10^3 cells per well into 96-well plates for growth curves and 3×10^3 for knockdown experiments. Viability was determined by MTT assay (Easy4U; Biomedica).

DNA synthesis was determined by incubation with ³H-thymidine (1 μ 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- μ m pore-size polyester tracketched membrane filters (BD-Falcon) in 24-well plates at a density of 0.5×10^5 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^{gly}* or *FGFR4^{arg}* as well as control transfectants were suspended in serum-free medium at a density of 1×10^6 cells/50 μ 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 Supplementary 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 χ^2 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 $\alpha = 5\%$ 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.

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 I using contingency tables and Fisher exact or χ^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/argheterozygous and expresses high levels of mainly *FGFR4^{arg}* (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^{arg}* and 3-fold for *FGFR4^{gly}*. In HCT116 that expressed higher levels of *FGFR4^{gly}*, overexpression was only 60% and 25% for *FGFR4^{arg}* and *FGFR4^{gly}*, respectively, but *FGFR4^{arg}* transfection shifted the arg/gly-ratio from 0:1 to 1:1. *FGFR4* protein was increased 3-fold (*FGFR4^{arg}*) and 1.6-fold (*FGFR4^{gly}*). In the *FGFR4^{arg}* expressing HT29 cells, RNA was upregulated 2.5- and 1.5-fold with *FGFR4^{gly}* transfection shifting the arg/gly ratio to 1:1. Protein overexpression was 12-fold and 3.6-fold for *FGFR4^{arg}* and *FGFR4^{gly}* (details are shown in Supplementary Fig. S2). Impact of *FGFR4* overexpression on logarithmic growth under standard culture conditions was only seen in SW480 cells (Supplementary Fig. S3).

However, overexpression of *FGFR4^{gly}* but not *FGFR4^{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^{gly}* was the stronger activator compared with *FGFR4^{arg}* (gly vs. arg significantly different at $P = 0.03$). In HCT116, agar growth was not further increased by *FGFR4^{gly}* and even decreased by transduction of *FGFR4^{arg}* creating a highly significant difference between the 2 allelic forms. In contrast, in the *FGFR4^{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^{arg}* was the more potent gene compared with *FGFR4^{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^{arg}* and *FGFR4^{gly}*, and no significant difference between the 2 allelic forms). In HT29 cells that endogenously express high levels of *FGFR4^{arg}*, no additional effect on cell migration was induced by *FGFR4^{arg}*. *FGFR4^{gly}* caused a 25% reduction of migration capacity ($P=0.0002$ compared with control as well as with *FGFR4^{arg}*; 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 α was increased in *FGFR4* overexpressing SW480 cells compared with the control (SW480^{co}) even though the amount of FRS2 α found in the particulate fraction was reduced. PLC γ phosphorylation was decreased in *FGFR4* transfectants. The amount of FRS2 α and PLC γ protein recruited to the membrane was distinctly higher in SW480^{gly} than SW480^{arg}, whereas specific phosphorylation of the signaling molecules was similar in SW480^{arg} and SW480^{gly} cells (Fig. 3A and B). In addition, c-src protein was upregulated in SW480^{arg} but not SW480^{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 β 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^{gly}, SW480^{arg}, and SW480^{co} 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 cytokeratin 20 expressing, poorly differentiated cells. The

FGFR4^{gly} gene significantly enhanced tumor growth as compared with the SW480^{co} ($P=0.0002$), whereas the *FGFR4^{arg}* variant had no impact. The fraction of proliferating Ki67-positive cells was similar in SW480^{co} and SW480^{arg} tumors ($29.0 \pm 5.7\%$ and $25.1 \pm 3.1\%$, respectively), but increased in the SW480^{gly} tumors ($39.1 \pm 3.0\%$; $P=0.05$; Fig. 4A 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 was found in the lung of an animal from the *FGFR4^{arg}* group. The other lungs obtained from the SW480^{arg} group contained large clusters of tumor cells (>10 cells), whereas only single tumor cells or small clusters were found in the lungs of SW480^{gly} and control animals. The number and size of metastatic colonies for each mouse were scored showing a higher average score for the SW480^{arg} than for control or SW480^{gly} 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 CORSAs 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 CORSAs 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 CORSAs screening study; Supplementary Table S2). Additional details can be found in Supplementary Materials.

On the basis of the strong promigratory impact of *FGFR4^{arg}* even in an *FGFR4^{gly}* 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 CORSAs 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, 1.10–359.00 by Fisher exact test) for tumors stage II or higher. The main difference in the CORSA population was the presence of 33% *FGFR4^{arg}* hetero- and homozygous individuals in the stage I group, whereas the fraction of *FGFR4^{arg}* 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 background—low expression, high *FGFR4^{gly}* expression, and high *FGFR4^{arg}* 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 *FGFR4^{gly}* was the stronger stimulator of malignant cell growth, whereas *FGFR4^{arg}* was the stronger activator of cell migration. The differential impact was more obvious in the cell lines with high endogenous *FGFR4* expression. In the *FGFR4^{gly}* background of HCT116 cells *FGFR4^{gly}* had no additional effect on malignant growth but still stimulated cell migration, whereas *FGFR4^{arg}* stimulated cell migration, but inhibited anchorage-independent growth. In contrast, in the *FGFR4^{arg}* expressing HT29 cells both alleles increased malignant growth, whereas no additional effect of *FGFR4^{arg}* and inhibition by *FGFR4^{gly}* was observed for cell migration. This indicates that both polymorphic alleles are capable of counteracting the main activity of the respective other form. This agrees with reports on inhibition of cell migration by *FGFR4^{gly}* in breast cancer cells by Bange and colleagues (1). However, this only occurred in a high *FGFR4^{arg}* background, whereas

FGFR4^{gly} was fully capable of stimulating cell migration in a low *FGFR4* background in CRC cells.

The most consistent difference between the *FGFR4^{gly}* and *FGFR4^{arg}* alleles was observed in colony formation assays where *FGFR4^{arg}* 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 *FGFR4^{arg}* 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, SW480^{gly} cells grew to larger tumors locally, whereas SW480^{arg} 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 FRS2 α and survival signaling downstream of PI3K as indicated by increased phosphorylation of the Akt-substrate GSK3 β and of S6. Differential activity was seen with the primary receptor substrates PLC γ and FRS2 α that were better recruited into the signaling complex by *FGFR4^{gly}*, and for c-src that was increased by *FGFR4^{arg}*. 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 mRNA 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^{gly}* and *FGFR4^{arg}* were still clearer: *FGFR4* protein on the cell membrane was increased 4- to 12-fold in *FGFR4^{arg}* transfectants, but only 1.6- to 4-fold in *FGFR4^{gly}* cells. This strongly suggests higher stability of the *FGFR4^{arg}* protein as compared with *FGFR4^{gly}* similar to observations in prostate cancer where increased stability and sustained phosphorylation of *FGFR4^{arg}* 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 a survival and migration factor in CRC cells (17, 20) and a strong ligand for *FGFR4* (34). Stronger membrane recruitment and activation of FRS2 α by *FGFR4^{gly}* explains the stronger growth impact of the receptor on tumor cell growth by a higher activity of canonical survival signaling (35, 36). Activation of downstream survival

signaling is similar in both transfectants suggesting a different pathway is used that still needs to be elucidated.

The CORSA 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 CORSA 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 patients. 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^{arg}* 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^{gly}* homozygous. In the CORSA population, the number of stage I tumors was much higher because of the diagnosis in the context of a screening program and also included 33% *FGFR4^{arg}* carriers. This difference and the small number of patients in the CORSA population prevented observation of a significant effect of *FGFR4^{arg}* on tumor stage. However, a similar trend toward increased frequency of *FGFR4^{arg}* 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^{arg}* 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^{arg}* allele may serve as a prognostic marker for more aggressive tumors and that *FGFR4^{arg}* carriers may profit from CRC screening and early detection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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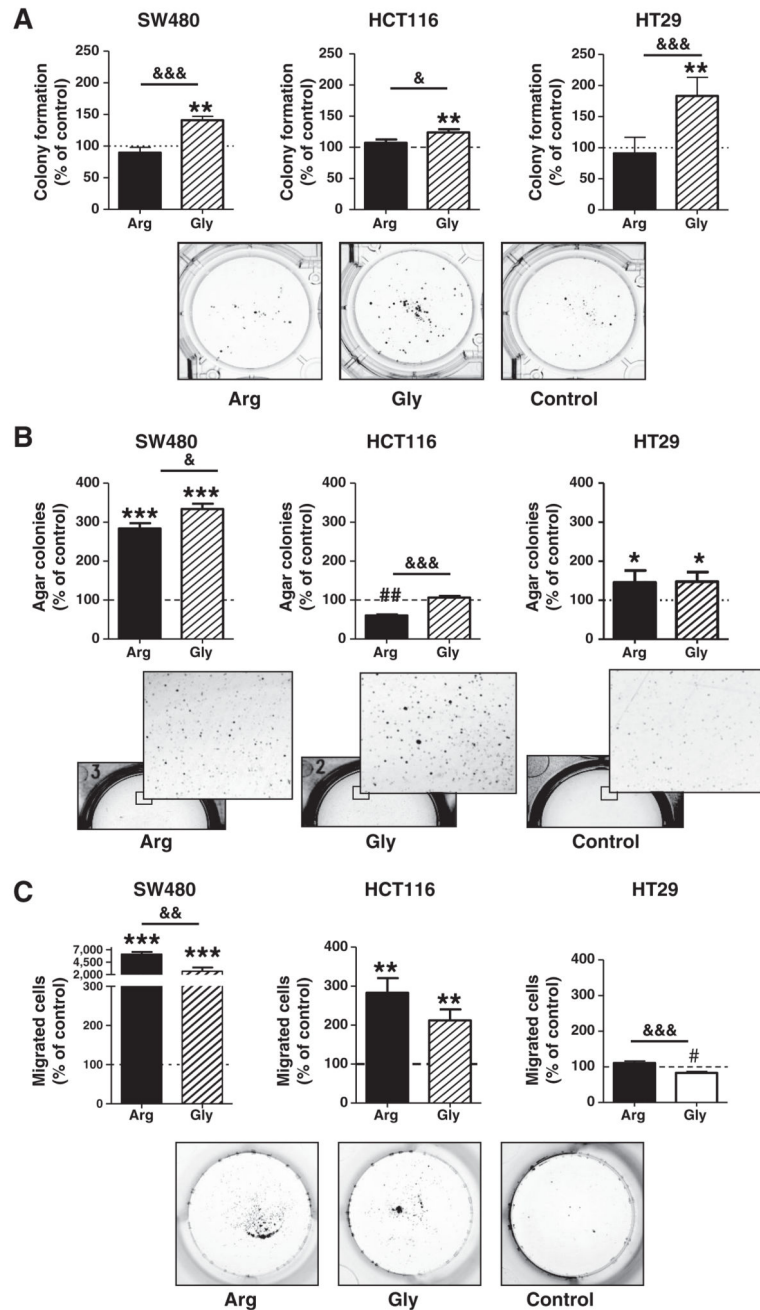


Figure 1.

Impact of *FGFR4* overexpression on tumor cell growth and migration *in vitro*. Stable transfectants overexpressing *FGFR4^{arg}* or *FGFR4^{gly}* 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 2×10^4 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 \pm 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^{gly}* and *FGFR4^{arg}* groups at $P < 0.05$, 0.01, and 0.001, respectively.

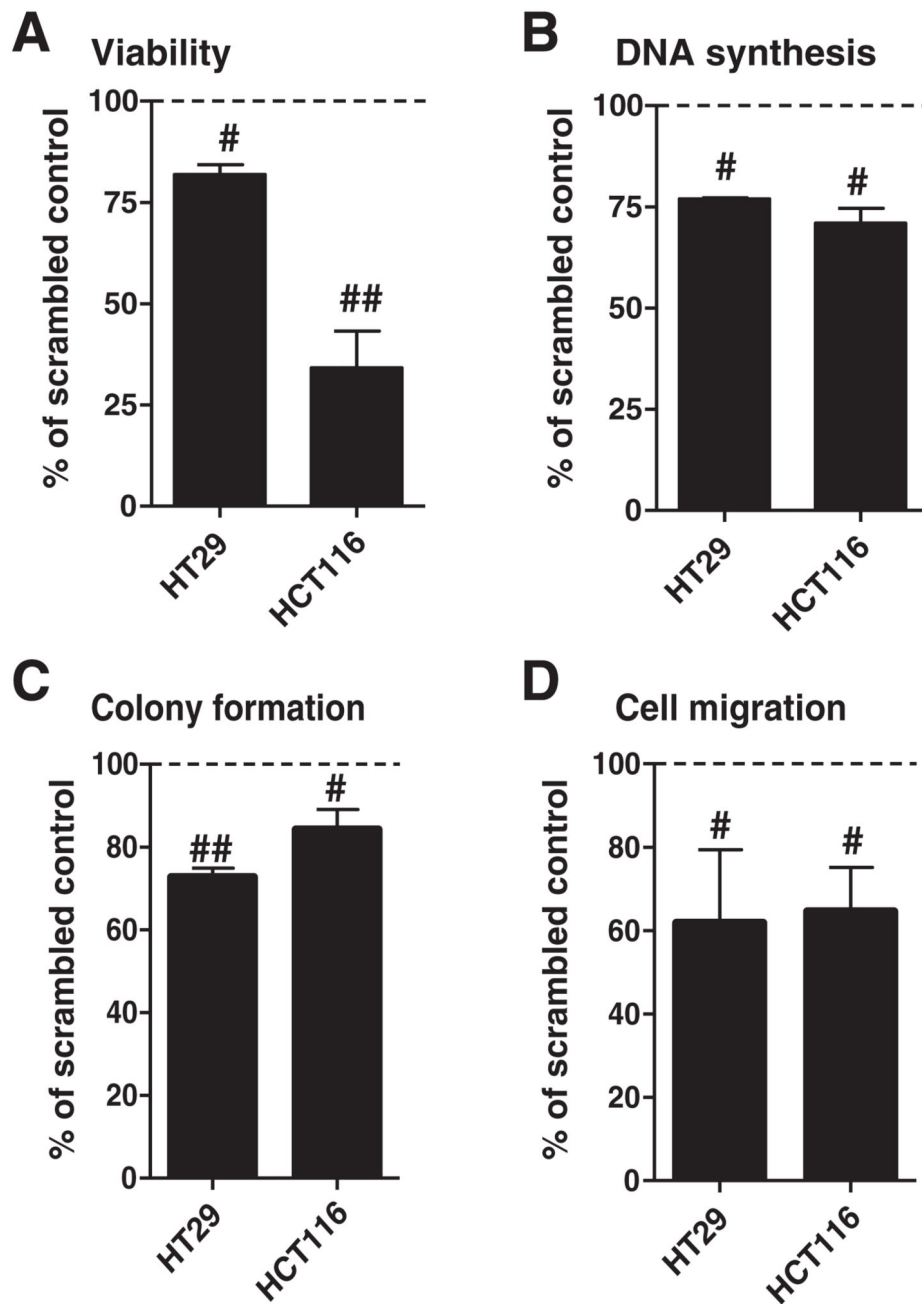
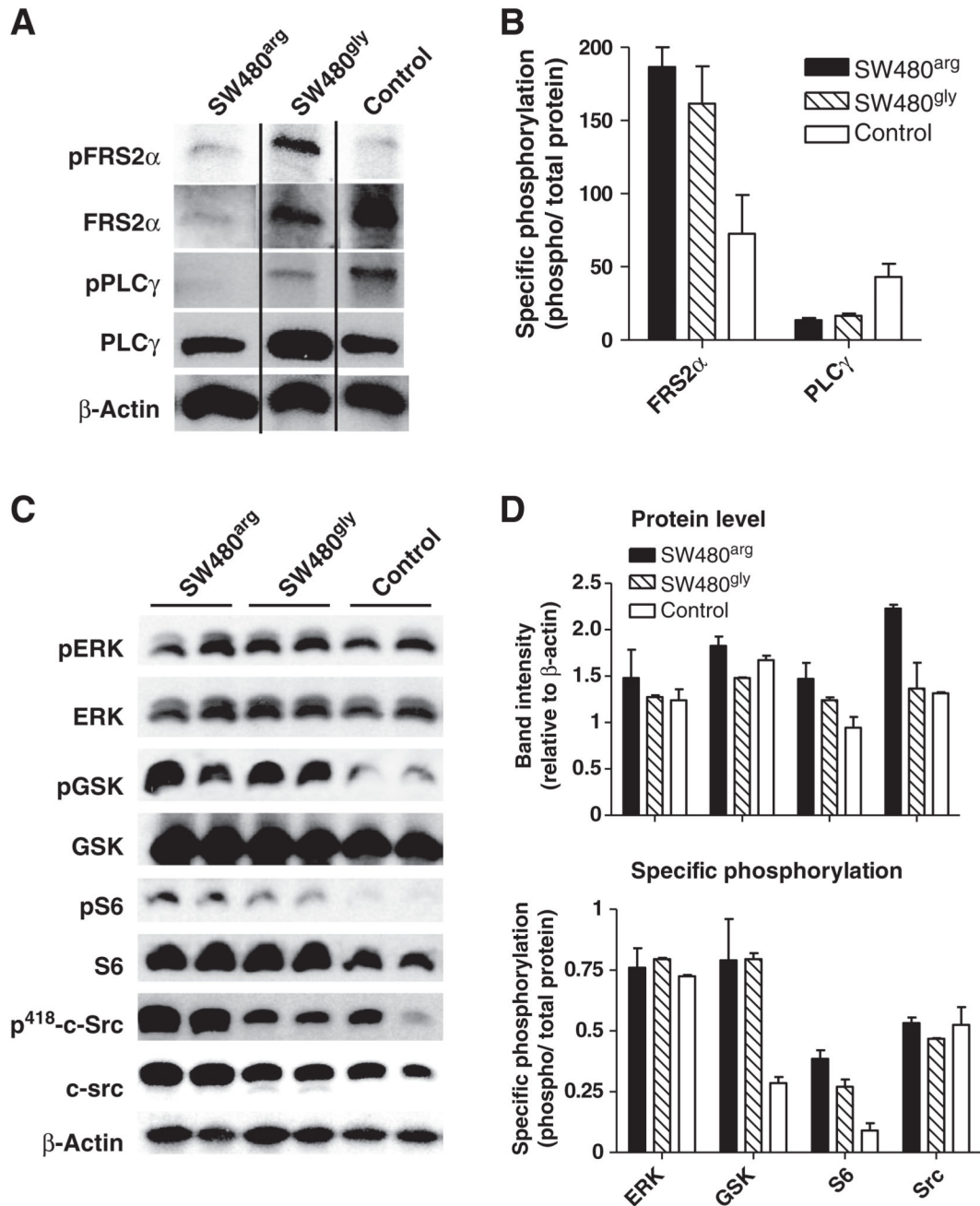


Figure 2.

Impact of *FGFR4* knockdown on clonogenicity and migration *in vitro*. siRNA oligonucleotides were introduced by lipofection into HCT116 and HT29 cells. Controls were transfected with scrambled control siRNA. A, five days after knockdown, cell viability was measured by MTT assay. B, 24 hours after knockdown, proliferation was determined by ^3H -thymidine uptake. From parallel cultures, cells were harvested 24 hours after transfection and plated at 100 and 200 cells/6-well for colony formation assays (C) and 2×10^4 cells/filter for migration assays (D). Results were pooled from at least 3 independent

experiments and presented as mean \pm SD. # and ## indicate a decrease as compared with control at $P < 0.05$ and 0.01 , respectively.

**Figure 3.**

FGFR4-dependent downstream signaling. A and B, semiconfluent cultures of SW480 transfectants were starved and cell membranes were prepared and extracted. Protein amount and phosphorylation of PLC γ and FRS2 α was analyzed by Western blot analysis. C and D, from parallel cultures, protein lysates were prepared after 24 hours for analysis of signaling molecules using phospho-specific antibodies. Photographs of Western blot analysis show results of representative gel runs. Quantification was conducted from 2 independent experiments using duplicate samples.

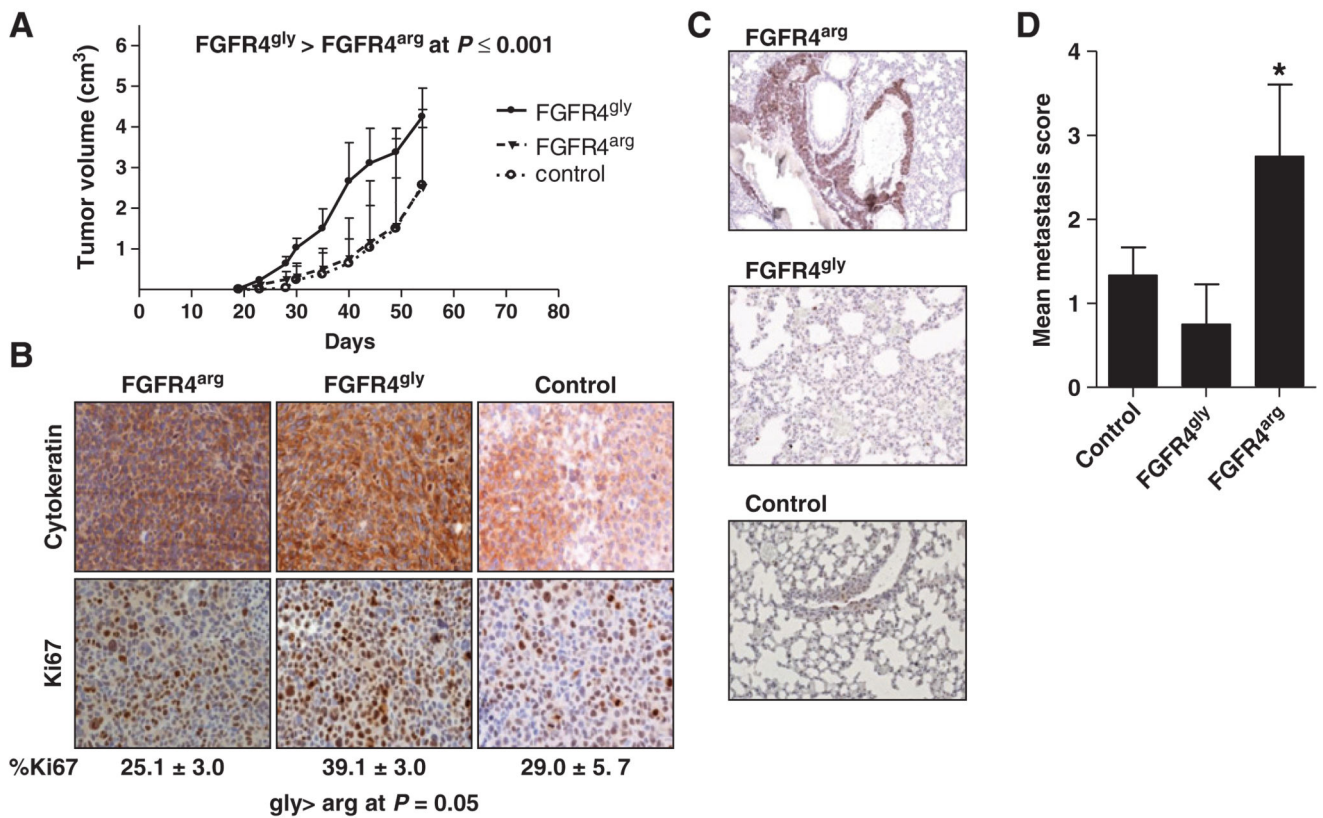


Figure 4.

Impact of *FGFR4* overexpression on tumor growth and metastasis *in vivo*. 10^6 cells each of $SW480^{gly}$, $SW480^{arg}$, and $SW480^{co}$ cells were injected s.c. into SCID mice. A and B, tumor growth was monitored and the mice sacrificed when tumor size reached 5 cm^3 or after 9 weeks, whichever came first. The tumors were analyzed by immunohistochemistry using antibodies recognizing cytokeratin 20 and Ki67. C and D, lungs were isolated from tumor-bearing mice and fixed with formalin. Serial sections were stained using antibodies recognizing Ki67 to detect tumor cells that were scored according to the criteria given in Supplementary Materials.

Table 1
Characteristics of the CORSA study population

	CRC patients <i>n</i> = 178 (5.3%)	High-risk adenoma patients <i>n</i> = 292 (9.0%)	Controls <i>n</i> = 1,794 (53.4%)
Sex			
Male	55 (63.2)	195 (66.8)	810 (46.6)
Female	32 (37.8)	97 (33.2)	928 (53.4)
Age, y			
<50	8 (9.2)	44 (15.1)	353 (20.3)
50–60	18 (20.7)	60 (20.6)	426 (24.5)
60–70	24 (27.6)	109 (37.3)	550 (31.7)
70–80	31 (35.6)	77 (26.4)	391 (22.5)
>80	6 (6.9)	2 (0.7)	18 (1.0)
Body mass index, kg/m ²			
<19	1 (1.2)	1 (0.3)	7 (0.4)
19–25	17 (19.5)	49 (16.8)	377 (21.7)
25–30	35 (40.2)	141 (48.3)	729 (41.9)
>30	28 (32.2)	95 (32.5)	564 (32.5)
Missing	6 (6.9)	6 (2.1)	61 (3.5)

Table 2
FGFR4 (rs351855) genotype distribution and CRC risk

Genotype	Control	CRC			CRC + high-risk adenomas		
		Cases	OR (95% CI)	Significance level	Cases	OR (95% CI)	Significance level
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.

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

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

NOTE: Genotype information was obtained from blood samples 57 of patients with CRC in the CORSА population and from tissue 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.