Heinzle et al. *Differential Effects of the Fibroblast Growth Factor Receptor-4 Polymorphic Alleles (G388R) on Colon Cancer Growth and Metastasis.*

Supplemental Material

1. Detailed information on Materials and Methods

Allele-specific expression of FGFR4

A calibration curve was constructed from the ct-values obtained from defined mixtures or $FGFR4^{arg}$ and $FGFR4^{gly}$ expression vectors using the Taqmen Genotyping assay. $\Delta ct^{(arg\text{-}gly)}$ was calculated and plotted on the y-axis with log2 of the mixing ratios on the x-axis. It was then used for semi-quantitative assessment of allele-specific FGFR4 expression was determined from the same cDNA that was also used for qRT-PCR (compare §3, Figure 2s c).

Table 1s: Antibodies used for Western blotting

Antibody recognizing	Catalog #	Company	Dilution
ERK1/2	06-182	Upstate, Lake Placid, NY	1:5000
phospho-ERK1/2 (Thr202/204)	9910	Cell Signalling, Boston, MA	1:1000
S6 Ribosomal Protein	2212		1:2000
phospho- S6 (Ser240/244)	2215		1:1000
GSK3ß	9315		1:1000
phospho-GSK3ß	9323		1:1000
FRS2α	10818-Mo2	Abnova, Taipee, Taiwan	1:500
phospho-FRS2α	3861	Cell Signalling	1:250
PLCγ	5690		1:1000
phospho-PLCγ	2821		1:1000
c-src	Y011091	Applied Biological Materials Inc. Richmond, BC	1:1000
phospho(418)-c-src	Y021168		1:1000
ß-actin	AC-15	SIGMA, Saint Louis, MO	1:1000

2. FGFR4 genotyping of colorectal tumor patients

Population-based study population

In an ongoing molecular epidemiology CRC study of Austria (CORSA) 3471 caucasian participants were recruited since May 2002 within a large province-wide screening project in the province Burgenland, Austria and participants with a positive fecal occult blood testing receive further diagnostic workup such as colonoscopies. These persons were asked to participate in our "Molecular epidemiology study of CRC". Results of colonoscopies were collected in a central database and standardized documentation guidelines were followed. Demographic and anthropometric factors as well as dietary and smoking habits were assessed by a short questionnaire. All subjects gave written informed consent. The study was approved by the institutional ethic review board "Ethikkommission Burgenland".

Cases were newly diagnosed within this screening project, previously untreated, and histological confirmed CRC patients. The polyp group consisted of 1330 patients and was classified as high risk and low risk polyps according to histological appearance. Specifically, adenomatous villous, adenomatous tubulovillous, and co-occurrence of adenomatous tubular with tubulovillous polyps were classified as high risk polyps while hyperplastic polyps which have no malignant potential and adenomatous tubular polyps were assigned to the low risk group.

The control group (n=1794) consists of participants that were free of polyps and CRC at the time of colonoscopy. Based on these criteria the participants were assigned to four groups: CRC patients (n=178), high risk polyp group (n=328), low risk polyp group (n=1059), and controls. In the control group 46.6% of the probands were male and 53.4% female. In the carcinoma group the percentage of males was 63.2%; in the polyp groups it was 66.8% and 65.1% for high risk and low risk respectively. The pattern of confounding variables (age, body mass index, meat consumption and smoking) is summarised in table 2s.

Genotype analysis

DNA was extracted by QIAamp DNA Blood Mini Spin protocol (Qiagen) from peripheral blood. Genotyping was performed with 7500 Fast Real-Time PCR System (Applied Biosystems) using TaqMan predesigned SNP genotyping assay. The reaction contained 20ng genomic DNA, TaqMan Genotyping Master Mix (Applied Biosystems) and 40x TaqMan Genotyping Assay (Applied Biosystems) containing the wild-type and the mutant allele in a total volume of 10µl. $FGFR4^{arg}$ specific probes were labelled with FAM and $FGFR4^{gly}$ probes with VIC. Universal reaction conditions were 2min at 50°C, 10 min at 95°C, 40 cycles with

15s at 95°C, and annealing/ extension at 60°C for 1min. Allelic discrimination was carried out by measurement of fluorescence yields of the two different dyes at 60°C. Genotyping was done blinded to case–control status, and 10% of samples were randomly repeated for quality control, with complete congruence.

Genotypic counts of controls were tested for Hardy–Weinberg equilibrium using a v2 test. Linkage disequilibrium (LD) statistics were computed using Haploview 4.0. Multiple logistic regressions were applied to compare individuals of the control group against three different risk groups defined in table 1s. Age, sex, and body mass index were used as confounders. Odds ratios (ORs) and 95% confidence intervals (CI) were estimated for each polymorphism and haplotype; reference categories were wild type and the most frequent haplotype, respectively.

Analysis of the data was performed using the software R Ver 2.6.2. All p-values are two-sided; p-values<0.05 were considered to be statistically significant.

Hospital-based study population

A second hospital-based study population of 81 cancer patients was recruited to obtain tissue specimen from both tumor tissue and normal mucosa. These patients were not diagnosed due to a screening program but after displaying CRC related symptoms. Complete clinical and pathological information was available permitting the analysis of histopathology parameters with FGFR4 expression and genotype. The patients had given their informed consent and the study was approved by the ethics committee of the Vienna communal hospitals.

Due to the different setting of diagnosis the tumor staging was shifted to higher stages when compared to the population diagnosed in the context of the screening campaign (Table 2s).

Table 2s: Characteristics of the CORSA study population:

	CRC patients n = 178 (5.3%)	**High risk adenomas** n = 328 (9.8%)	**Low risk adenomas** n = 1,059 (31.5%)	**Controls** n = 1,794 (53.4%)
Age (years)				
< 50	8 (9.2)	44 (15.1)	136 (14.0)	353 (20.3)
50 – 60	18 (20.7)	60 (20.6)	227 (23.4)	426 (24.5)
60 – 70	24 (27.6)	109 (37.3)	349 (36.0)	550 (31.7)
70 – 80	31 (35.6)	77 (26.4)	245 (25.3)	391 (22.5)
> 80	6 (6.9)	2 (0.7)	13 (1.34)	18 (1.0)
Body mass index (kg/m2)				
< 19	1 (1.2)	1 (0.3)	8 (0.8)	7 (0.4)
19–B25	17 (19.5)	49 (16.8)	182 (18.8)	377 (21.7)
25–B30	35 (40.2)	141 (48.3)	439 (45.3)	729 (41.9)
> 30	28 (32.2)	95 (32.5)	326 (33.6)	564 (32.5)
Missing	6 (6.9)	6 (2.1)	15 (1.6)	61 (3.5)
Meat consumption				
High	348 (20.0)	12 (13.8)	78 (26.7)	233 (24.0)
Rather high	700 (40.3)	39 (44.8)	120 (41.1)	411 (42.4)
Low	608 (35.0)	33 (37.9)	77 (26.4)	296 (30.5)
None	21 (1.2)	0 (0)	5 (1.7)	5 (0.5)
Missing	61 (3.5)	3 (3.5)	12 4.1)	25 (0.5)
Smoking				
Current	13 (14.9)	52 (17.8)	189 (19.5)	239 (13.8)
Former	34 (39.1)	88 (30.1)	296 (30.5)	464 (26.7)
Never	40 (46.0)	144 (49.3)	469 (48.4)	993 (57.1)

Table 3s: Comparison of staging in the CORSA screening and hospital populations of CRC patients

	Screening study Burgenland		Hospital	
Stage	Patients		Patients	
	Number	%	Number	%
I	15	27.3	5	6.6
II	21	38.3	37	48.7
III	12	28.6	23	30.3
IV	7	12.7	11	14.5

3. FGFR4 over-expressing cell lines

Choice of cell lines

FGFR4 expression of available cell lines was determined from RNA isolated from semi-confluent cultures by qRT-PCR using a Taqman expression kit as described in materials and methods. Genotype and allelic expression was determined from DNA and RNA respectively using a Taqman SNP assay. Expression was found to be lowest in SW480 and T84 cells that have a gly/gly genotype. Cell lines that have a arg/gly (HT29, Colo201, LT97) or arg/arg (Caco2) genotype but also some gly/gly cell lines (e.g. HCT116, SW620) have a higher RNA expression (Figure 1s). Based on these results and the suitability of their general growth characteristics SW480, HCT116 and HT29 cells were chosen for over-expression experiments. For these cells lines also FGFR4 protein expression on the cell surface was determined by FACS-analysis using a PE-coupled anti-FGFR4 antibody.

Proof of over-expression

RNA was isolated from stable transfectants and both the amount and the allelic identity of FGFR4 were determined by qRT-PCR. The highest over-expression was observed in SW480 cells that had the lowest endogenous expression of the receptor. For this cell line clone pools with a 3-4-fold over-expression were chosen for further analysis. The transfected genes were the predominant FGFR4 alleles expressed. In HCT116 that have higher endogenous FGFR4 expression over-expression was only 1.23-fold for $FGFR4^{gly}$ and 1.7-fold for $FGFR4^{arg}$ resulting in a shift of gene expression to the arg-allele at a ratio of 1:1. In HT29 cells over-expression was 2.5-fold for $FGFR4^{arg}$ and 1.5-fold for $FGFR4^{gly}$ causing a shift to the gly-allele at a ratio of 1:3 (figure 2s a).

FGFR4 on the cell surface was determined by FACS using an anti-FGFR4-antiboy coupled to PE. It was increased 4-12-fold by transfection of an arg-allele, while the gly allele had much less impact on the amount of FGFR4 detectable on the cell membrane (1.6-4-fold; figure 2s b).

To analyze the ratio of arg/gly alleles in the expressed RNA we used the reagents from the SNP assay and constructed a standard curve from defined mixtures of pure $FGFR4^{arg}$ and $FGFR^{gly}$ and the resulting Δct between the two alleles (figure 2s c, d).

4. FGFR4 knock-down

Efficiency of FGFR4 knock down was assessed at the RNA level by qRT-PCR and at the protein level by FACS analysis. Efficiency was very high in HT29 cells repressing FGFR4 RNA level to 10% of control and protein to 20% of control. HCT116 was less efficient with 20 and 35% respectively (figure 3s, a,b).

Legends to supplemental figures

Figure 1s: FGFR4 expression and genotype of colorectal tumor cell lines

RNA was isolated from cultures at 50% confluency and FGFR4 expression determined by qRT-PCR using GAPDH as house keeping gene for normalization. FGFR4 genotype was determined from the genomic DNA of the cell lines using real time SNP assays.

Figure 2s: FGFR4 over-expression in stable transfectants

(a) RNA was isolated from clone pools of cells transfected with $FGFR4^{gly}$ or $FGFR4^{arg}$. Cells transfected with the empty vector were used as controls. FGFR4 expression was determined by qRT-PCR and normalized first to GAPDH expression and then to expression in vector controls.

(b) Cells were harvested from semi-confluent cultures and stained with a PE-coupled anti-FGFR4 antibody. FGFR4 protein level was determined by FACS analysis.

(c) A calibration curve was constructed from the ct-values obtained from defined mixtures or $FGFR4^{arg}$ and $FGFR4^{gly}$ expression vectors using the TaqMan Genotyping assay. $\Delta ct^{(arg\text{-}gly)}$ was calculated and plotted on the y-axis with log2 of the mixing ratios on the x-axis.

(d) $FGFR4^{gly}$ and $FGFR4^{arg}$ allele ratios were determined from the cDNAs described in (a) using SNP assay reagents and the arg/gly ratio in the cDNA calculated from the Δct of the 2 allelic forms and the standard curve shown in figure 2s a

Figure 3s: Efficiency of FGFR4 knock-down

HT29 and HCT116 cells were transfected with siRNA targeting FGFR4 or a scrambled control siRNA. 24h later cells were harvested for isolation of RNA and determination of FGFR4 mRNA by qRT-PCR (a). At the same time cells were trypsinized and FGFR4 on the cell surface measured by FACS analysis (b).

Figure 1s

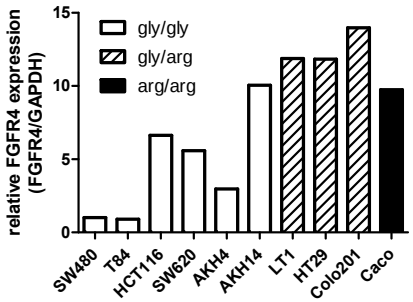
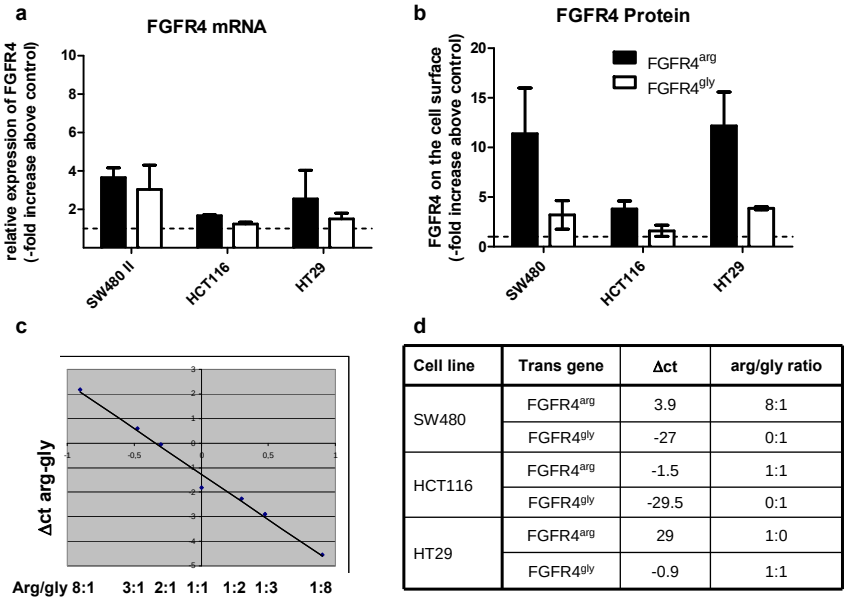


Figure 2s



Cell line	Trans gene	Δct	arg/gly ratio
SW480	FGFR4^{arg}	3.9	8:1
	FGFR4^{gly}	-27	0:1
HCT116	FGFR4^{arg}	-1.5	1:1
	FGFR4^{gly}	-29.5	0:1
HT29	FGFR4^{arg}	29	1:0
	FGFR4^{gly}	-0.9	1:1

Figure 3s: Knock down: gene expression