Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

A Functional Germline Variant in *GLI1* Implicates Hedgehog Signaling in Clinical Outcome of Stage II and III Colon Carcinoma Patients

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Abstract

Purpose: Cumulating evidence indicates that germline variants in the Wnt, Notch, and Hedgehog pathways are involved in colon carcinoma progression and metastasis. We investigated germline polymorphisms in a comprehensive panel of Wnt, Notch, and Hedgehog pathway genes to predict time to recurrence (TTR) and overall survival in patients with stage II and III colon carcinoma.

Experimental Design: A total of 742 consecutively collected patients with stage II and III colon carcinoma were included in this retrospective study. Genomic DNA was analyzed for 18 germline polymorphisms in Wnt, Notch, and Hedgehog pathway genes (*SFRP*, *DKK 2* and *3*, *AXIN2*, *APC*, *MYC*, *TCF7L2*, *NOTCH2*, and *GLI1*) by TaqMan 5′-exonuclease assays.

Results: In univariate analysis, the homozygous mutant variant of *GLI1* rs2228226 G>C was significantly associated with decreased TTR in a recessive genetic model after adjustment for multiple testing [HR = 2.35; confidence interval (95% CI), 1.48–3.74; $P < 0.001$] and remained significant in multivariate analysis including clinical stage, lymphovascular-, vascular-, and perineural-invasion (HR = 2.43; CI 95%, 1.52–3.87; $P < 0.001$). In subanalyses, the association was limited to patients with surgery alone (HR = 3.21; CI 95%, 1.59–6.49; $P = 0.001$), in contrast with patients with adjuvant chemotherapy (HR = 0.82; CI 95%, 0.35–1.95; $P = 0.657$). When the subgroup of patients with "high-risk" *GLI1* rs2228226 C/C genotype was analyzed, no benefit of adjuvant 5-fluorouracil–based chemotherapy could be found.

Conclusion: This is the first study identifying *GLI1* rs2228226 G>C as an independent prognostic marker in patients with stage II and III colon carcinoma. Prospective studies are warranted to validate our findings. *Clin Cancer Res; 20(6); 1687–97. ©2014 AACR.*

Introduction

Colorectal carcinoma is the third cause of cancer-related deaths in the United States and the second cause of cancer mortality in Europe (1, 2). Across all stages, approximately 30% of patients with colon carcinoma develop synchronous or metachronous metastases (3). The 5-year survival rate of patients with colon carcinoma with metastatic disease is less than 10% (4).

In the absence of adjuvant chemotherapy, approximately 50% of patients with colon carcinoma with resectable disease are cured by surgery alone, whereas 50% relapse. Using adjuvant chemotherapy following surgery rescues approximately 15% of patients from the relapsing group. In current practice, the majority of these patients with colon carcinoma receive adjuvant treatment unnecessarily, either because they were cured by surgery alone or because they will relapse despite adjuvant treatment. It is therefore essential to identify patients who will benefit from adjuvant therapy, sparing other needless toxicity and the financial burden of chemotherapy that will not work (5–7). Tumor recurrence after curative surgery remains a major obstacle for improving overall cancer survival, which may be, in part, due to the existence of cancer stem cells (CSC). Current therapies target populations of rapidly growing and differentiated tumor cells, but have shown to lack activity against CSCs (8, 9). CSCs therefore may have an important role in tumor recurrence despite adjuvant chemotherapy (9, 10). There is strong evidence that the embryonic signaling pathways Wnt, Notch, and Hedgehog operate in CSCs and drive tumor progression, metastasis, and chemoresistance (11–18).

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doi: 10.1158/1078-0432.CCR-13-1517

Translational Relevance

Germline variants in cancer stem cells (CSC) may have an important role in tumor recurrence despite adjuvant chemotherapy. In the present study, we investigated germline polymorphisms in a comprehensive panel of genes in the Wnt, Notch, and Hedgehog pathways that have been previously investigated for their biologic function and/or associated with CSCs and cancer risk or clinical outcome to predict tumor recurrence in patients with stage II and III colon carcinoma. These common DNA-sequence variations may alter the gene function and/or activity, including transcription, translation, or splicing, thereby causing interindividual differences in relation to tumor recurrence capacity. Our study provides the first evidence that *GLI1* rs2228226 G>C may predict early tumor recurrence in patients with stage II and III colon carcinoma.

There is substantial germline genetic variability within the genes of the Wnt, Notch, and Hedgehog pathways, including multiple single-nucleotide polymorphisms (SNPs). These common DNA-sequence variations may alter the gene function and/or activity, including transcription, translation, or splicing, thereby causing interindividual differences in relation to tumor recurrence capacity and chemoresistance (19–29). Furthermore, common gene variants may also predict chemoresistance and toxicity to 5-fluorouracil (5-FU) and/or oxaliplatin as recently shown for the *thymidylate synthetase, 5 methyltetrahydrofolate-homocysteine methyltransferase reductase, multidrug resistance protein 2, dihydropyrimidine dehydrogenase*, and the *X-ray repair cross-complementing protein 1 genes* (30–35).

In the present study, we investigated 18 germline polymorphisms in a comprehensive panel of genes in the Wnt, Notch, and Hedgehog pathways that have been previously investigated for their biologic function and/or associated with cancer risk or clinical outcome to predict tumor recurrence in patients with stage II and III colon carcinoma. This study was conducted adhering to the reporting recommendations for prognostic tumor marker studies (36, 37).

Materials and Methods

Eligible patients

Between 1995 and 2011, 742 patients with histopathologically confirmed stage II and III colon carcinoma were consecutively recruited at the Division of Clinical Oncology, Department of Medicine, Medical University of Graz (Graz, Austria). Tissue samples from 522 patients were available for current genetic analyses. Tissue samples were provided by the Biobank of the Medical University of Graz, the Department of Pathology of the General Hospital Graz West and the Department of Pathology of the General Hospital Leoben (Leoben, Austria). Patients treated with adjuvant chemotherapy received 5-FU–based regimens. All patients were included in the colon carcinoma surveillance program of the Division of Clinical Oncology of the Medical University of Graz, providing history and physical examination and carcinoembryonic antigen determination every 3 months for 3 years and every 6 months at years 4 and 5 after surgery, colonoscopy at year 1 and thereafter every 3 to 5 years, and chest X-ray and abdominal ultrasound or CT scans of chest and abdomen every 6 months for the first 5 years and in 12 months interval in years 6 to 10 after diagnosis. Patient data were collected retrospectively through chart review. This study has been approved by the Institutional Review Board of the Medical University of Graz. All participants were Caucasians.

Candidate gene polymorphisms

Common and putatively functional Wnt, Notch, and Hedgehog gene polymorphisms were selected using stringent and predefined selection criteria: (i) minor allele frequency (MAF) $\geq$10% in Caucasians (based on the population genetics section in the Ensembl Genome Browser), (ii) polymorphism that could alter the function of the gene in a biologically relevant manner [either published data or predicted function using Functional-Single-Nucleotide-Polymorphism (F-SNP) database; refs. 38, 39], and (iii) published clinical associations (e.g., cancer risk and/or clinical outcome or chemoresistance). As it was not possible to select all Wnt, Notch, and Hedgehog pathway gene variants matching these criteria for study power reasons, we focused on the most promising genes and polymorphisms. Table 1 summarizes the genes and polymorphisms investigated in our study cohort, including location and function/clinical association.

Isolation of genomic DNA and determination of single-nucleotide polymorphisms

Genomic DNA was extracted from paraffin-embedded normal tissue adjacent to the tumor samples to obtain germline DNA. DNA isolation was performed by use of the QIAamp DNA Mini Kit (Qiagen) and according to the manufacturer's instructions. Genotypes were centrally determined by 5′-exonuclease assay (TaqMan) at the Medical University of Graz. Primer and probe sets were designed and manufactured using Applied Biosystems "Assay-by-Design" custom service (Applera). General TaqMan reaction conditions were according to the manufacturer of the assays. As a control for consistency of genotyping methods, determination of genotypes was repeated in at least 96 samples. The rules of good laboratory and clinical practice were observed. The investigator analyzing the germline polymorphisms was blinded to the clinical dataset.

Immunohistochemistry

Immunohistochemistry was performed on a Ventana XT immunostainer using UltraView DAB as the detection kit and CC1 32 minutes as heat-induced epitope retrieval. The primary antibody was incubated for 32 minutes each: anti-interleukin (IL)-17 antibody ab 9565/Abcam in a dilution of 1:40, anti-IL23 antibody ab115759/Abcam in a dilution of 1:50, and anti-GLI1 (H-300) sc-20687 Santa Cruz Biotechnology, Inc. in a dilution of 1:30. The tumor center of

Table 1. Analyzed Wnt/Notch/Hedgehog pathway genes and polymorphisms

Pathway	Gene	Function	rs-number	Base exchange	Location	Association
WNT	*SFRP*	Soluble Wnt receptor	rs1802073	C>A	Nonsynonymous	Rectal cancer risk (26)
WNT	*SFRP*	Soluble Wnt receptor	rs288326	G>A	Nonsynonymous	CRC risk (21, 22)
WNT	*SFRP*	Soluble Wnt receptor	rs7775	C>G	Nonsynonymous	CRC risk (21, 22)
WNT	*DKK2*	Inhibits Wnt by binding to LRP5/6	rs17037102	G>A	Nonsynonymous	RCC outcome (20)
WNT	*DKK3*	Inhibits Wnt by binding to LRP5/6	rs3206824	A>G	Nonsynonymous	RCC risk (20)
WNT	*DKK3*	Inhibits Wnt by binding to LRP5/6	rs1472189	C>T	3 UTR	RCC outcome (20)
WNT	*DKK3*	Inhibits Wnt by binding to LRP5/6	rs7396187	C>G	Intron	RCC risk (20)
WNT	*AXIN2*	Suppressor	rs11079571	A>G	Intron	BC risk (23)
WNT	*AXIN2*	Suppressor	rs4791171	A>G	Intron	BC risk (23)
WNT	*AXIN2*	Suppressor	rs3923086	T>G	Intron	BC risk (23)
WNT	*AXIN2*	Suppressor	rs3923087	A>G	Intron	BC risk (23)
WNT	*APC*	Suppressor	rs454886	T>C	Intron	BC risk (23)
WNT	*AXIN2*	Suppressor	rs2240308	G>A	Nonsynonymous	NSCLC risk (24)
WNT	*MYC*	Wnt enhancer	rs6983267	G>T	8q24, noncoding, near MYC	CRC risk (27)
WNT	*TCF7L2*	Transcription factor activator	rs12255372	G>T	Intron	CRC risk (25)
WNT	*TCF7L2*	Transcription factor activator	rs7903146	C>T	Intron	CRC risk (28)
NOTCH	*NOTCH2*	NOTCH receptor	rs11249433	T>C	1p11.2, within noncoding gene	BC (29)
HEDGEHOG	*GLI1*	Transcriptional activator	rs2228226	G>C	Nonsynonymous	IBD risk (47)

Abbreviations: CRC, colorectal cancer; RCC, renal cell cancer; BC, breast cancer; NSCLC, non–small cell lung cancer; IBD, inflammatory bowel disease; NA, not available.

the stained slides was captured with a ×20 objective on an Eclipse 80i microscope with Digital sight DS-Fi1 digital camera and NIS-Elements D Version 3.21.04 software, Nikon with same correction for brightness and white balance for all images. On the basis of the images, staining intensity in the tumor cells was visually semiquantified and classified by low, moderate, and high expression.

Statistical analysis

The endpoint of the study was time to recurrence (TTR). TTR was calculated from the date of diagnosis of colon cancer to the date of the first observation of tumor recurrence. TTR was censored at the time of death or at the last follow-up if the patient remained tumor recurrence free at that time. The statistical power to detect or exclude effects for the SNPs we investigated depended on MAF and SNP effect size. For the variant with the lowest MAF, *SFRP* rs7775, the present study had a power of 0.98, 0.89, or 0.70 to detect or exclude a HR of 2.0, 1.7, or 1.5 for recurrence. The statistical power increased with higher MAF and/or higher HRs. The secondary endpoint was overall survival (OS). OS was defined as the time from date of diagnosis of colon cancer to death from any cause. Allelic distribution of the polymorphisms was tested for deviation from Hardy–Weinberg equilibrium using HW Diagnostics-Version 1.beta (Fox Chase Cancer Center, Philadelphia, PA). The distribution of polymorphisms across baseline demographic, clinical, and pathologic characteristics was examined using Fisher exact test. The association of clinicopathological features and polymorphisms with TTR and OS was analyzed using Kaplan–Meier curves and log-rank test. In the multivariate Cox regression analyses, the models were adjusted for significant clinicopathological features from univariate analysis of TTR and OS. The true mode of inheritance of all polymorphisms tested has not been established yet and we evaluated a codominant, dominant, or recessive genetic model where appropriate. The significance threshold for an overall type I error rate of 0.05 was set at $P < 0.003$ based on a conservative Bonferroni correction for multiple comparison. The interactions between polymorphisms and adjuvant chemotherapy on TTR were tested by comparing likelihood ratio statistics between the

Table 2. Clinicopathological characteristics and TTR and OS in univariate analysis

			TTR		OS	
Parameter	***N***	**%**	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Gender						
Male	416	56.1	1 (reference)	0.750	1 (reference)	0.401
Female	326	43.9	0.96 (0.74–1.24)	—	0.89 (0.67–1.17)	—
Tumor location						
Left	274	36.9	1 (reference)	0.168	1 (reference)	0.025
Right	468	63.1	0.83 (0.64–1.08)	—	0.73 (0.55–0.96)	—
Tumor size						
T1	12	1.6	1 (reference)	—	1 (reference)	—
T2	32	4.3	0.97 (0.10–9.35)	<0.001	1.33 (0.16–11.43)	<0.001
T3	536	72.2	3.27 (0.46–23.36)	—	2.26 (0.32–16.15)	—
T4	162	21.8	6.58 (0.91–47.35)	—	5.56 (0.77–40.05)	—
Lymph node involvement						
N0	298	40.2	1 (reference)	—	1 (reference)	—
N1	276	37.2	1.44 (1.03–2.02)	<0.001	1.33 (0.93–1.88)	<0.001
N2	167	22.5	3.86 (2.80–5.32)	—	3.12 (2.23–4.36)	—
Unknown	1	0.1	—	—	—	—
Tumor grade						
G1	37	5	1 (reference)	—	1 (reference)	—
G2	480	64.7	1.16 (0.57–2.37)	0.337	0.77 (0.37–1.57)	0.032
G3	224	30.2	1.40 (0.68–2.91)	—	1.12 (0.54–2.33)	—
Unknown	1	0.1	—	—	—	—
Lymphovascular invasion						
No	532	71.7	1 (reference)	0.001	1 (reference)	0.009
Yes	210	28.3	1.58 (1.21–2.06)	—	1.47 (1.10–1.96)	—
Vascular invasion						
No	665	89.6	1 (reference)	<0.001	1 (reference)	0.001
Yes	77	10.4	2.40 (1.73–3.32)	—	2.06 (1.43–2.96)	—
Perineural invasion						
No	721	97.2	1 (reference)	<0.001	1 (reference)	0.006
Yes	21	2.8	3.64 (2.12–6.26)	—	2.45 (1.29–4.62)	—
Clinical stage						
II	295	39.8	1 (reference)	<0.001	1 (reference)	<0.001
III	446	60.1	2.26 (1.69–3.03)	—	1.92 (1.42–2.58)	—
Unknown	1	0.1	—	—	—	—
Adjuvant chemotherapy						
No	256	34.5	1 (reference)	0.239	1 (reference)	0.300
Yes	483	65.1	1.18 (0.90–1.56)	—	0.86 (0.65–1.14)	—
Unknown	3	0.4	—	—	—	—

baseline and nested Cox proportional hazards models that include the multiplicative product term. Case-wise deletion for missing polymorphisms was used in univariate and multivariate analyses. The association between the *GLI1* rs2228226 genotypes and *GLI1*, IL-17, and IL-21 expression in tumor was examined using χ^2 test. All analyses have been performed using the SPSS 21.0 statistical software package (SPSS Inc.).

Results

The baseline characteristics of the 742 patients included in this analysis are summarized in Table 2. A total of 231 patients received infusional 5-FU monotherapy (bolus of 5-FU (450 mg/m2)-leucovorin (20 mg/m2) day (d)1-d5 or bolus of 5-FU (500 mg/m2)-leucovorin (500 mg/m2) weekly for 6 consecutive weeks), 110 patients capecitabine monotherapy (capecitabine (2500 mg/m2) d1-d14), 108 patients FOLFOX (oxaliplatin (85 mg/m2) d1, leucovorin (200 mg/m2) d1 and d2, bolus of 5-FU (400 mg/m2) d1 and d2 and 5-FU (600 mg) d1 and d2), 16 patients XELOX [(oxaliplatin (130 mg/m2) d1 and capecitabine (2000 mg/m2) d1-d14], and the treatment regimen of 18 patients was unknown. The median age at time of diagnosis was 64 years (range 27–95 years), with a median follow-up time of 64.8 months (range 1–199 months). The median

TTR was 54.5 months (range 1–199 months) and the median OS was 64.8 months (range 1–199 months). The genotyping quality control provided a genotype concordance of 100%. Genotyping was successful in at least 91% of patients for each polymorphism analyzed, with the exception of *DKK3* rs7396187 (86.8%). In failed cases, genotyping was not successful because of limited quantity and/or quality of extracted genomic DNA. The genotype frequencies for all polymorphisms were within the probability limits of Hardy–Weinberg equilibrium.

In our study cohort, we found a significant association between tumor size, lymph node involvement, lymphovascular-, vascular-, and perineural-invasion, and clinical stage with TTR and OS. In addition, tumor location and histopathological grade were significantly associated with OS (Table 2). When the polymorphisms were correlated with the clinicopathological features, we found a significant association between *APC* rs454886 G>A and tumor size ($P = 0.001$) and vascular invasion ($P = 0.001$), observing larger tumors and increased vascular invasion in patients carrying the wild-type. Furthermore, patients with colon cancer with *NOTCH2* rs11249433 T>C wild-type showed significantly increased lymphovascular invasion ($P = 0.001$). No association was found between the other tested polymorphisms and clinicopathological features (data not shown).

The associations between all polymorphisms tested and TTR and OS are provided in Table 3. *GLI1* rs2228226 G>C, *AXIN2* rs3923086 T>G, and *AXIN2* rs4791171 A>G showed an association with TTR in a codominant model ($P < 0.05$; Table 3). In multiple testing, only *GLI1* rs2228226 G>C using a recessive genetic model remained significant for TTR in univariate analysis [HR = 2.35; 95% confidence interval (CI), 1.48–3.74; $P < 0.001$]. Patients harboring the homozygous mutant variant (C/C) had a median TTR of 52.2 months, in contrast with patients carrying the G/G or G/C genotype with a median TTR of 121.8 months (Fig. 1). In OS analyses, no statistically significant association between the polymorphisms and OS could be found (Table 3). In the multivariate analysis including clinical stage (because clinical stage derives from tumor size and lymph node involvement, which all have been significant in univariate analysis, only clinical stage was incorporated in the multivariate model), lymphovascular-, vascular-, and perineural-invasion, the homozygous mutant variant of *GLI1* rs2228226 G>C remained significantly associated with decreased TTR (HR = 2.43; 95% CI, 1.52–3.87; $P < 0.001$).

In interaction analysis, there was a significant association between *GLI1* rs2228226 G>C and adjuvant chemotherapy with TTR ($P < 0.05$). When only patients with surgery alone were analyzed, we found a highly significant association between *GLI1* rs2228226 G>C and TTR (HR = 3.21; 95% CI, 1.59–6.49; $P < 0.001$). Patients harboring the homozygous mutant variant showed a median TTR of 49.9 months, whereas patients harboring the G/G or G/C genotype had a median TTR of 123.6 months (Fig. 2). In multivariate analysis including clinical stage, lymphovascular-, vascular-, and perineural-invasion, we observed a statistical trend toward decreased TTR in patients carrying the homozygous mutant variant (HR = 2.35; 95% CI, 1.13–4.850; $P = 0.022$). In patients with adjuvant chemotherapy, we found no significant association between *GLI1* rs2228226 G>C and TTR (HR = 1.99; 95% CI, 1.06–3.72; $P = 0.031$). In this subgroup, patients harboring the C/C genotype had a median TTR of 52.5 months, in contrast with patients carrying the G/G or G/C genotype with a median TTR of 119.1 months (Fig. 3).

To evaluate whether "high-risk" patients based on the *GLI1* rs2228226 G>C polymorphism (40 patients) benefit from adjuvant chemotherapy compared with surgery alone, we performed a Kaplan–Meier analysis and log-rank test for this subgroup. According to the treatment regimen [surgery alone (19 patients) vs. surgery plus adjuvant chemotherapy (21 patients; 8 patients received 5-FU monotherapy, 7 patient capecitabine, and 6 patients FOLFOX], no significant difference in TTR was identified in this high-risk subgroup (HR = 0.82; 95% CI, 0.35–1.95; $P = 0.657$; Fig. 4).

When we correlated the *GLI1* rs2228226 genotypes using the recessive genetic model with *GLI1*, IL-17, and IL-21 expression in tumor in a subset of patients ($n = 27$ for wild-type and heterozygous mutant and $n = 12$ for homozygous mutant), we found no significant association ($P = 0.697$, $P = 0.338$, $P = 0.596$, respectively).

Discussion

It is becoming increasingly apparent that disease progression and chemoresistance are driven by a multitude of signaling networks and the analysis of a single marker may fail to predict clinical outcome and treatment efficacy with a high degree of accuracy and reproducibility. Therefore, it is critical to adopt and implement a pathway-based approach. In the present study, we investigated germline polymorphisms in a comprehensive panel of the Wnt, Notch, and Hedgehog pathway genes to predict tumor recurrence in patients with stage II and III colon carcinoma. Our results indicate that *GLI1* rs2228226 G>C may be an independent prognostic marker. Our findings further suggest that patients harboring the homozygous mutant variant do not benefit from adjuvant 5-FU–based chemotherapy.

The exact molecular mechanisms involved in how the *GLI1* rs2228226 G>C polymorphism exerts effect on colon carcinoma outcome are not clarified yet. Nonsynonymous polymorphisms result in amino acid changes and thus may affect the protein function (40). We used the F-SNP database to predict the functional effects of the analyzed polymorphisms. F-SNP gathers computationally predicted functional information about polymorphisms, particularly aiming to facilitate identification of disease-related polymorphisms in association studies (38, 39). When used for *GLI1* rs2228226 G>C, F-SNP predicted changes in splicing regulation and posttranslation, thus supporting the effects seen in our study. In a recent study, however, Páez and colleagues investigated the association of *GLI1* rs2228226 with TTR in 234 patients with stage III and high-risk stage II patients, all treated with adjuvant 5-FU–based chemotherapy, but

Table 3. Association between the polymorphisms and TTR and OS in univariate analysis

		TTR		OS	
Polymorphism	***N***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
SFRP rs1802073					
C/C	217	—	—	—	—
C/A	231	0.98 (0.78–1.23)	0.858	0.93 (0.72–1.19)	0.568
A/A	63	—	—	—	—
SFRP rs288326					
G/G	7	—	—	—	—
G/A	103	1.04 (0.74–1.48)	0.809	0.78 (0.55–1.10)	0.156
A/A	363	—	—	—	—
SFRP rs7775					
C/C	6	—	—	—	—
C/G	64	1.01 (0.66–1.54)	0.967	1.04 (0.65–1.68)	0.866
G/G	436	—	—	—	—
DKK2 rs17037102					
G/G	408	—	—	—	—
G/A	88	0.83 (0.56–1.23)	0.351	0.89 (0.58–1.37)	0.586
A/A	4	—	—	—	—
DKK2 rs3206824					
A/A	288	—	—	—	—
A/G	188	1.01 (0.79–1.30)	0.922	1.05 (0.80–1.37)	0.745
G/G	34	—	—	—	—
DKK2 rs1472189					
C/C	241	—	—	—	—
C/T	220	0.90 (0.71–1.15)	0.405	1.11 (0.85–1.44)	0.438
T/T	50	—	—	—	—
DKK2 rs7396187					
C/C	14	—	—	—	—
C/G	156	1.19 (0.87–1.63)	0.285	0.91 (0.66–1.26)	0.575
G/G	283	—	—	—	—
AXIN2 rs11079571					
A/A	22	—	0.171	—	0.645
A/G	189	1.23 (0.92–1.64)	—	1.08 (0.79–1.48)	—
G/G	283	—	—	—	—
AXIN2 rs4791171					
A/A	205	—	—	—	—
A/G	251	0.72 (0.56–0.93)	0.012	0.80 (0.60–1.05)	0.108
G/G	52	—	—	—	—
AXIN2 rs3923086					
T/T	107	—	—	—	—
T/G	261	1.31 (1.05–1.65)	0.019	1.19 (0.93–1.52)	0.160
G/G	142	—	—	—	—
AXIN2 rs3923087					
A/A	255	—	—	—	—
A/G	210	0.78 (0.60–1.02)	0.073	0.85 (0.63–1.14)	0.270
G/G	32	—	—	—	—
APC rs454886					
T/T	267	—	—	—	—
T/C	201	0.76 (0.60–1.01)	0.054	0.79 (0.58–1.07)	0.121
C/C	30	—	—	—	—
AXIN2 rs2240308					
G/G	114	—	—	—	—
G/A	267	0.80 (0.64–1.00)	0.054	0.90 (0.71–1.15)	0.407
A/A	129	—	—	—	—

(Continued on the following page)

Table 3. Association between the polymorphisms and TTR and OS in univariate analysis (Cont'd)

		TTR		OS	
Polymorphism	***N***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Near *MYC* rs6983267					
G/G	112	—	—	—	—
G/T	252	0.85 (0.66–1.08)	0.189	0.95 (0.73–1.24)	0.719
T/T	112	—	—	—	—
TCF7L2 rs12255372					
G/G	252	—	—	—	—
G/T	199	1.09 (0.85–1.40)	0.485	0.10 (0.76–1.31)	0.974
T/T	40	—	—	—	—
TCF7L2 rs7903146					
C/C	248	—	—	—	—
C/T	205	1.06 (0.83–1.36)	0.649	0.93 (0.71–1.22)	0.601
T/T	43	—	—	—	—
NOTCH2 rs11249433					
T/T	176	—	—	—	—
T/C	244	0.96 (0.77–1.19)	0.682	1.05 (0.83–1.33)	0.696
C/C	85	—	—	—	—
GLI1 rs2228226					
G/G	249	—	—	—	—
G/C	209	1.36 (1.06–1.74)	0.015	1.22 (0.93–1.61)	0.156
C/C	40	—	—	—	—

found no clinical effect (41). Moreover, in genome-wide association studies, *GLI1* rs2228226 has not been identified as a prognostic or predictive marker in colorectal cancer (42–44).

The Hedgehog signaling pathway induces expression of the gene *SNAIL1*, a transcription repressor of E-cadherin. Its transcriptional upregulation is directly mediated by the transcription factor *GLI1* (17). Patched 1

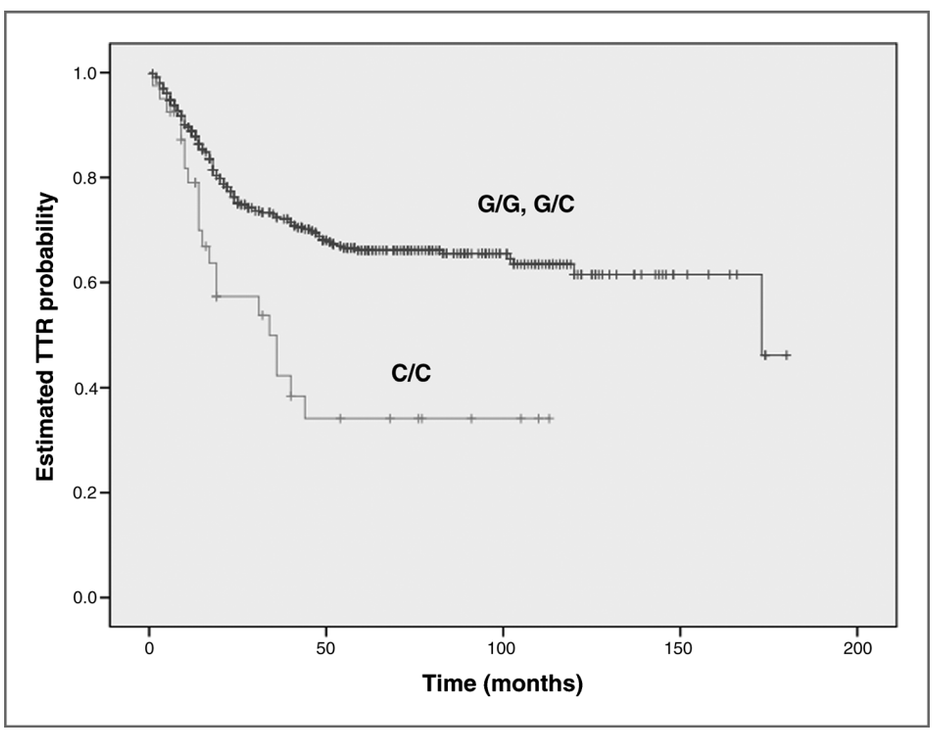

Figure 1. Association between *GLI1* rs2228226 G>C and TTR in all patients with colon carcinoma.

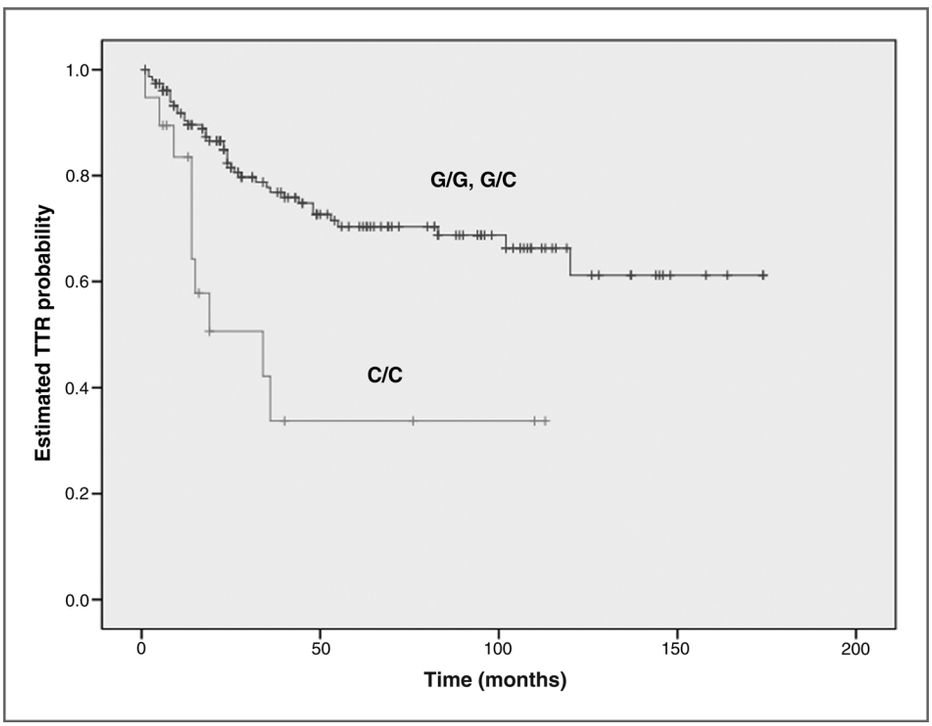

Figure 2. Association between *GLI1* rs2228226 G>C and TTR in patients with colon carcinoma with surgery alone.

(PTCH1), a membrane protein, functions as a tumor suppressor and normally inhibits the membrane protein Smoothened (SMO) from activating *GLI1*. The binding of one of the three Hedgehog ligands (Sonic, Indian, or Desert) to PTCH1 abrogates its repressive effects on SMO allowing the translocation of *GLI1* to the nucleus where it induces the expression of multiple target genes (45, 46). The Hedgehog signal transduction pathway regulates many processes of development and tissue homeostasis and is dysregulated in malignancies

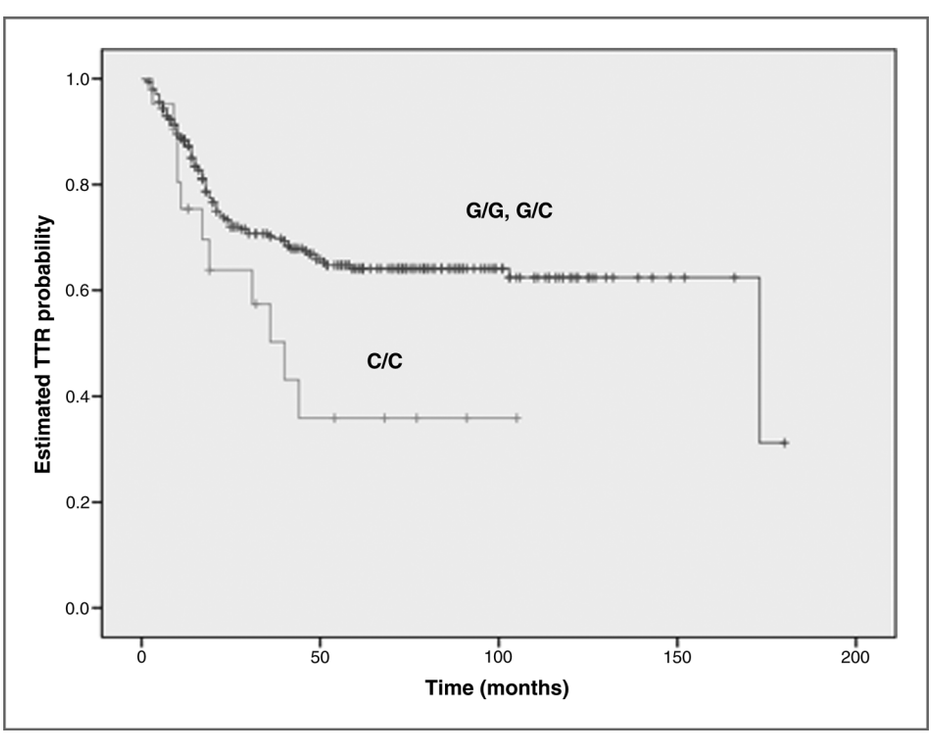

Figure 3. Association between *GLI1* rs2228226 G>C and TTR in patients with colon carcinoma with adjuvant chemotherapy.

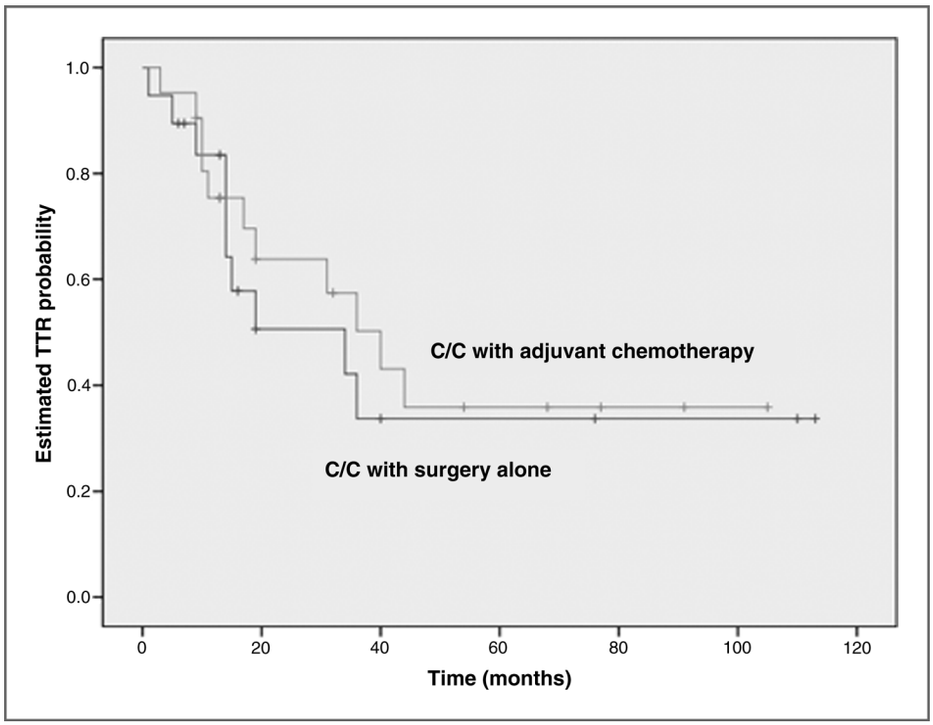

Figure 4. Association in patients with colon carcinoma homozygous mutant (C/C) for *GLI1* rs2228226 G>C (40 patients) between adjuvant chemotherapy (21 patients) or surgery alone (19 patients) and TTR.

and inflammatory diseases of the gastrointestinal tract (47–50).

Increasing evidence supports the involvement of inflammation in cancer progression and metastasis (51, 52). Hedgehog signaling plays a crucial role in the inflammatory response because Sonic Hedgehog is critical for T-lymphocyte development, adult human $CD4^+$ T-cell activation, and myeloid cell maturation (53–56). Recently, Lees and colleagues demonstrated an overall downregulation of Hedgehog signaling pathway activity, including *GLI1* and PTCH, in colonic inflammation in humans. Furthermore, they identified the *GLI* rs2228226 G>C polymorphism as functionally deficient in activating *GLI*-responsive transcription *in vitro*, showing a 50% less efficient transcriptional activity compared with the wild-type (57). *GLI1* rs2228226 G>C encodes a change from glutamine to glutamic acid, causing a significant charge change in a conserved region adjacent to the known transactivation region of *GLI1*, that may directly modify transactivation activity and/or affect protein stabilization (58). In addition, Lees and colleagues showed in an established mouse model of colitis that animals carrying the mutant allele of *GLI1* rs2228226 G>C develop severe intestinal inflammation, indicating that tolerance to inflammatory stimuli requires a fully functional Hedgehog signal transduction network (51). The most highly expressed cytokine in mice harboring the mutant allele of *GLI1* rs2228226 G>C in their study was IL-23, a molecule that promotes the differentiation of T-helper IL-17–producing (TH17) cells that are involved in inflammation processes, including inflammatory bowel disease (57). IL-23 is also known as a procarcinogenic cytokine, which is mainly produced by tumor-associated macrophages in the tumor microenvironment, via direct transcriptional activation of the *IL-23/p19* gene (59). In the study by Lees and colleagues, also IL-17, a cytokine closely associated with IL-23, was markedly upregulated in animals harboring the *GLI1* rs2228226 G>C mutant variant (57). Grivennikov and colleagues investigated mechanisms responsible for tumor-elicited inflammation in a mouse model of colorectal carcinogenesis, which, like human colorectal carcinoma, also exhibited upregulation of IL-23 and IL-17 (59, 60). They found that IL-23 signaling promotes tumor growth and progression, and the development of tumoral IL-17 response, resulting in an additional aggravation of disease progression (60). Efforts to target pathogenic Hedgehog signaling have steadily progressed from the laboratory to the clinic, and the recent approval of vismodegib for patients with advanced basal cell carcinoma represents an important milestone (61–66). However, in a recent phase II study, vismodegib did not add to the efficacy of standard first-line treatment for metastatic colorectal cancer (67).

In our study cohort, we found a statistically significant association between *GLI1* rs2228226 G>C and TTR, showing a decreased TTR in patients carrying the homozygous mutant genotype. Hence, we hypothesize that patients with colon carcinoma harboring the functionally deficient homozygous mutant variant, which is associated with upregulation of IL-23 and IL-17, are more likely to develop a recurrent disease, due to a supportive inflammatory microenvironment for tumor growth. However, we could not experimentally underline this biologic function because the *GLI1* rs2228226 genotypes were not significantly associated with *GLI1*, IL-17, and IL-21 expression in tumor in a subset of our patient cohort. Our results further suggest that patients harboring the

homozygous mutant variant of *GLI1* rs2228226 G>C do not benefit from adjuvant 5-FU–based chemotherapy. We also found a significant association between *APC* rs454886 G/G and larger tumor size and increased vascular invasion, furthermore, patients with *NOTCH2* rs11249433 TT showed an increased lymphovascular invasion. F-SNP predicted changes in the transcriptional regulation for the intronic *APC* rs454886 G>A. No prediction could be provided for the intergenic located *NOTCH2* rs11249433 T>C by the software (38, 39). Because the biologic function of these SNPs is unknown, these associations remain to be elucidated.

The strength of the present study is the large sample size and the long follow-up period. However, because of the retrospective study design, a selection bias cannot be fully excluded. The subgroup of "high-risk" *GLI1* rs2228226 C/C included overall only 40 patients and only 21 patients treated with various chemotherapy regimens. Therefore, it is currently unknown whether this association is truly significant and/or whether only patients with monotherapy or both, mono- and combination therapy do not benefit in this "high-risk" subgroup. Another limitation is the lack of the microsatellite instability (MSI) status in our study cohort; hence the evaluation of *GLI1* rs2228226 G>C in comparison with MSI was not feasible. Finally, the method of preservation of the tissue samples was performed by three different institutions over a number of years, undermining the consistency of sample preparation.

In conclusion, this study provides the first evidence that *GLI1* rs2228226 G>C may predict early tumor recurrence in patients with stage II and III colon carcinoma. Prospective studies are warranted to validate our findings.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Szkandera, M. Pichler, G. Absenger, M. Stotz, H. Samonigg, M. Asslaber, T. Winder, W. Renner

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Grant Support

This work was supported by funds of the Oesterreichische Nationalbank (Anniversary Fund, project number: 14258).

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Received June 2, 2013; revised December 22, 2013; accepted December 27, 2013; published OnlineFirst January 27, 2014.

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