

## GNAS1 T393C Polymorphism and Survival in Patients with Sporadic Colorectal Cancer

Ulrich H. Frey,<sup>1</sup> Hakan Alakus,<sup>2,4</sup> Jeremias Wohlschlaeger,<sup>2</sup> Klaus J. Schmitz,<sup>2</sup> Günther Winde,<sup>4</sup> Hans G. van Calker,<sup>5</sup> Karl-Heinz Jöckel,<sup>3</sup> Winfried Siffert,<sup>1</sup> and Kurt W. Schmid<sup>2</sup>

**Abstract Purpose:** Signaling via the G protein G $\alpha$ s pathway is linked to proapoptotic processes in cancer cell lines. We have recently shown an association between the *GNAS1* T393C polymorphism and disease progression in patients with bladder cancer with homozygous TT genotypes displaying increased transcription of G $\alpha$ s and a more favorable clinical course compared with C-allele carriers.

**Experimental Design:** In the present study, 151 patients with sporadic colorectal cancer were retrospectively genotyped to examine a potential association between T393C genotypes and survival. Moreover, two other single-nucleotide polymorphisms in common haplotype blocks within the gene *GNAS1* and their interaction with the T393C polymorphism were investigated.

**Results:** The allele frequency in the patients group was not significantly different from that of healthy blood donors. Kaplan-Meier curves for overall survival (mean follow-up, 43 months) showed that in International Union Against Cancer (UICC) stages I to II, the 5-year survival rate was significantly higher in TT genotypes (87.8%) compared with TC (71.0%) and CC genotypes (50.0%;  $P = 0.009$ ), whereas no genotype effect could be observed for UICC stages III to IV. In multivariate Cox proportional analysis the T393C polymorphism was an independent prognostic factor for survival. Homozygous CC patients were at highest risk for death (hazard ratio, 12.1;  $P = 0.006$ ) compared with TT genotypes. Heterozygous patients had an intermediate risk compatible with a gene-dose effect. The two haplotype blocks investigated were not associated with clinical outcome.

**Conclusions:** The results support the role of the T393C polymorphism as a marker for survival in patients with colorectal cancer stages I to II and in the identification of patients who may benefit from adjuvant chemotherapy.

Colorectal cancer is one of the leading causes of cancer-related death worldwide. In Germany, there has been a stable incidence of colorectal cancer since 1980, with 51,000 newly detected cases per year and an average 5-year survival rate after tumor resection in the range of 50% (1). Currently, the standard method for predicting outcome in patients is the tumor-node-metastasis system and the International Union Against Cancer (UICC) staging system (2). Patients with UICC stage III (any T, N<sub>1-2</sub>, M<sub>0</sub>, 5-year survival 30-55%) generally have a worse prognosis than patients with UICC stages I to II (T<sub>3-4</sub>, N<sub>0</sub>, M<sub>0</sub>, 5-year survival 60-95%). However,

patients with comparable pathologic disease stage exhibit significantly different survival, especially in stage II colon cancer (3).

The majority of patients with stage II disease is not treated with adjuvant chemotherapy. However, this treatment regimen is mandatory in patients presenting with stage III disease because decreases in relapse and mortality rates by 30% to 40% were observed compared with surgery alone (4). Up to 40% of patients with stage II colorectal cancer will develop recurrent disease during their lifetime and the role of adjuvant chemotherapy in this setting is still unclear (3, 5, 6). The identification of patients with high-risk colorectal cancer could help to set up novel treatment strategies in the lymph node-negative disease (UICC I-II) and may, in principle, improve outcome (7). Therefore, it is desirable to identify molecular markers offering to identify more aggressive colonic cancer phenotypes to individually tailor therapy. In fact, research in the past several years suggested that molecular markers could help to define subgroups of patients who, after surgery, may benefit from adjuvant chemotherapy (8-10). However, lack of standardization in the analysis of immunohistochemically detected biomarkers often prevents their application as prognostic factors. Most of this research has focused on properties of the tumor tissue itself (e.g., somatic mutations or differential expression of genes or proteins), whereas a

**Authors' Affiliations:** <sup>1</sup>Institute of Pharmacology, <sup>2</sup>Institute of Pathology, and <sup>3</sup>Institute of Medical Informatics, Biometry, and Epidemiology, University Hospital of Essen, Essen, Germany and <sup>4</sup>Department of Gastrointestinal Surgery and <sup>5</sup>Institute of Pathology, Klinikum Herford, Herford, Germany  
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**Note:** K-H. Jöckel, W. Siffert, and K.W. Schmid are members of the West German Cancer Centre Essen (WTZE), Universitätsklinikum Essen, Essen, Germany.

**Requests for reprints:** Ulrich H. Frey, Institut für Pharmakologie, Universitätsklinikum Essen, Hufelandstrasse 55, D-45122 Essen, Germany. Phone: 49-201-7233459; Fax: 49-201-7235968; E-mail: Ulrich.Frey@uni-essen.de.

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**Table 1.** Genotype distribution, demographic characteristics, grade, and stage of the tumor at primary diagnosis in 151 patients with colorectal cancer

	All	TT	TC	CC	P
n (%)	151	36 (23.8)	72 (47.7)	43 (28.5)	
Gender (M/F)	75/76	15/21	41/31	19/24	0.227
Mean age at diagnosis (y ± SD)	68.1 ± 10.1	68.8 ± 9.2	68.4 ± 9.6	66.9 ± 13.0	0.405
Tumor site					
Colon	80 (55.6)	23 (28.8)	36 (45.0)	21 (26.3)	
Rectum	64 (44.4)	13 (20.3)	32 (50.0)	19 (29.7)	0.509
Grade					
1	63 (43.4)	16 (25.4)	30 (47.6)	17 (27.0)	
2	67 (46.2)	15 (22.4)	32 (47.8)	20 (29.9)	
3	15 (10.3)	5 (33.3)	6 (40.0)	4 (26.7)	0.927
Tumor invasion					
pT <sub>1</sub>	12 (8.1)	7 (58.3)	3 (25.0)	2 (16.7)	
pT <sub>2</sub>	16 (10.7)	3 (18.8)	12 (75.0)	1 (6.3)	
pT <sub>3</sub>	83 (55.7)	18 (21.7)	37 (44.6)	28 (33.7)	
pT <sub>4</sub>	38 (25.5)	8 (21.1)	19 (50.0)	11 (28.9)	0.024
Lymph node status					
pN <sub>0</sub>	71 (48.6)	18 (25.4)	33 (46.5)	20 (28.2)	
pN <sub>+</sub>	75 (51.4)	18 (24.0)	35 (46.7)	22 (29.3)	0.978
Metastases					
pM <sub>0</sub>	115 (76.7)	28 (24.3)	53 (46.1)	34 (29.6)	
pM <sub>1</sub>	35 (23.3)	8 (22.9)	18 (51.4)	9 (25.7)	0.850
UICC stage					
I	20 (13.4)	8 (22.2)	10 (50.0)	2 (10.0)	
II	46 (30.9)	9 (19.6)	21 (45.7)	16 (34.8)	
III	48 (32.2)	11 (22.9)	22 (45.8)	15 (31.3)	
IV	35 (23.5)	8 (22.9)	18 (51.4)	9 (25.7)	0.420

NOTE: P values were calculated using  $\chi^2$  test for categorical variables and ANOVA for continuous variables.

potential role of genetic host factors has rarely been investigated (9–12).

We have recently shown that genotypes of the single-nucleotide polymorphism (SNP) T393C in the gene *GNAS1*, encoding the ubiquitously expressed G $\alpha$ s subunit of heterotrimeric G proteins, predict the clinical outcome of patients with urothelial carcinoma (13). Patients with TT genotypes showed a prolonged progression-free, metastasis-free, and overall survival compared with patients with TC and CC genotypes. We also showed that G $\alpha$ s mRNA expression is increased in TT genotypes, not only in bladder cancer but also in heart and fat cell specimens (13). These findings strongly suggest that increased G $\alpha$ s mRNA expression is a general phenomenon in individuals with the *GNAS1* 393TT genotype (13). Data from *in vitro* experiments indicate that increased expression of G $\alpha$ s enhances apoptosis (14–16). Hence, it is tempting to hypothesize that increased G $\alpha$ s expression with concomitantly enhanced apoptosis may be related to better survival in patients with TT genotype.

Thus, one aim of the present study was to accumulate further evidence that the T393C polymorphism-related altered expression of G $\alpha$ s is not only associated with the prediction of outcome in bladder cancer patients but may also represent a more general feature with the capacity to predict the clinical course in other tumors, too. To this end, we investigated a potential association

between genotypes of the T393C polymorphism and survival in a series of patients suffering from sporadic colorectal carcinoma. It was recently suggested that the T393C polymorphism lies within a recombination hotspot surrounded upstream and downstream within the same gene by two haplotype blocks (17). The second aim of the study was therefore to investigate possible associations of one SNP from the 5' block (T2291C, dbSNP rs6026584) and one SNP from the 3' block (T18830C, dbSNP rs234628) with outcome from colorectal cancer.

## Materials and Methods

**Patients.** All patients of this study were operated at a single Hospital (Department of General Surgery, Klinikum Herford, Germany) in the years 1996 to 1998 according to the recommendations of the German Society of Surgery. Mesorectal excision was not done at that time in the group of patients with rectal carcinoma of the middle and lower third of the rectum. Primarily, the files of all patients operated during this time period with cancer of the colon and rectum were retrieved and reviewed; finally, the study cohort (151 patients; for clinicopathologic data, see Table 1) consisted exclusively of patients with a meticulously complete follow-up record. The minimum follow-up period for patients still alive was 60 months.

Histopathologic diagnosis was made according to the WHO classification of tumors of the colon and rectum. Additionally, the tumors were staged and graded by standard histologic analysis according to the guidelines of International Union Against Cancer (2).

Patients with UICC stage III colorectal carcinoma received in the adjuvant setting a standardized chemotherapy with leucovorine 20 mg as an i.v. bolus on day 1 to 5 and fluorouracil 425 mg/m<sup>2</sup>/d as an i.v. bolus on day 1 to 5, repeated on day 28 for a total of 6 months [Mayo Clinic protocol (18)]. According to the recommendations of the German Surgical Oncology Working Group (CAO), patients with stage II colorectal carcinoma were not advised to any adjuvant treatment. The study was done according to the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital Essen.

**Blood donors.** The Caucasian control sample consisted of 820 healthy white individuals of either gender who were recruited at the local Department for Transfusion Medicine, University Hospital Essen. All samples were collected at random from subjects donating blood and the details of this sample have been published previously (19).

**Genotyping.** DNA was extracted from paraffin-embedded tumor tissue using a commercially available kit (QIAamp, Qiagen, Hilden, Germany). Genotypes for the T393C polymorphism were determined as described (13). The T2291C polymorphism was genotyped using primers 5'-AGGCAGAATTATGCTGTGGGA-3' and 5'-AGATCCGTGCCTCAGTTCCAC-3' to amplify a 508 bp fragment followed by restriction analysis with 1 unit *SspI* (New England Biolabs, Beverly, MA) resulting in CC = 508 bp, CT = 508, 318, 190 bp, and TT = 318, 190 bp. For the T18830C SNP, PCR was done using primers GNAS\_in11\_se 5' TTCAGGGGTTACGCTACC 3' and biotinylated primer GNAS\_in11\_as\_BT 5' GGCACAAACAAGGGGACT 3' resulting in a 200 bp fragment. Genotypes were determined using Pyrosequencing (20) with sequencing primer GNAS\_in11\_seq 5'-CTTTGCCCCCTC-3'.

**Statistical analysis and presentation of data.** The clinical outcome analyzed in this study was survival dependent on UICC stages and T393C genotypes. Kaplan-Meier plots and the log-rank test for trend were used to evaluate the relationship between UICC stages or T393C genotypes, and clinical outcome from the date of the primary diagnosis to the end of follow-up. Log-rank tests for T393C genotypes were done using all three genotypes unless stated otherwise. The effects of gender, UICC stages, grade, age, and T393C genotypes as prognostic factors for the clinical outcome were analyzed by stepwise multivariate Cox-regression analysis. Hazard ratios and 95% confidence intervals (95% CI) were calculated from the Cox-regression model including all factors for multivariate analysis and for the indicated factor for univariate analysis. Contingency tables and the Pearson's  $\chi^2$  test were used for categorical variables using T393C genotypes. Because the T393C polymorphism shows a gene-dose effect (13), linear ANOVA was used for comparison of continuous variables where appropriate. Haplotype analysis and control for deviation from the Hardy-Weinberg equilibrium were conducted with the public domain programs EH and Hardy-Weinberg equilibrium by J. Ott (<http://linkage.rockefeller.edu/ott/linkutil.htm>). The EH program analyzes linkage or absence of linkage between allelic markers by comparison of the observed and the predicted genotype distributions using contingency tables to test whether the observed distributions were compatible with a model of independent segregation (21). This allowed subsequent determination of estimated haplotype frequencies and estimated linkage disequilibrium coefficient,  $D$ , through the use of the 2LD program (22).  $D$  was expressed as  $D'$ , giving the value of  $D$  as a percentage of the maximum calculated value given the observed allele frequencies. Values of  $D'$  range between  $-1$  and  $+1$ . A  $|D'|$  value of 1 denotes complete linkage disequilibrium whereas a value of 0 denotes complete linkage equilibrium. Differences were regarded significant at  $P < 0.05$ . All statistical analyses were done using SPSS 11.0 (SPSS, Chicago, IL) or GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). Continuous variables are given as means  $\pm$  SD or  $\pm$  SE as indicated.

## Results

Demographic characteristics and tumor grade and stage in the whole case group and by genotype are displayed in Table 1.

The mean age was 68.1 years (range, 37-91 years) and mean follow-up time was 43 months (range, 1-92 months). Tumor site, grade, and UICC stage were compatible with those reported in the literature (23).

T393C genotype distribution is also displayed in Table 1. The frequency of the C allele (fC) in the patient group was 0.52 and the distribution was compatible with the Hardy-Weinberg equilibrium. To investigate whether the T393C polymorphism is predictive for colorectal cancer, we compared genotypes and allele frequencies with those from 820 healthy, white blood donors. Genotype distribution (TT,  $n = 182$ ; TC,  $n = 403$ ; CC,  $n = 235$ ) as well as C-allele frequency (fC = 0.53) was not significantly different from that of our patient group, which argues against an association of T393C genotypes with an increased risk for colorectal cancer. Gender, tumor site, grade, lymph node status, metastases, and UICC stage were not associated with genotypes (Table 1). However, the comparison of pathologic stages and genotype distribution showed a significant relationship between genotype and pathologic stage at the time of initial diagnosis. CC genotypes seem to be associated with higher pathologic stages compared with TT genotypes (Table 1;  $P = 0.024$ ).

To confirm that our sample displays the typical clinical course of patients with colorectal cancer, we calculated Kaplan-Meier curves for overall survival depending on the UICC stage. As shown in Fig. 1, overall survival was significantly dependent on pathologic stage ( $P < 0.0001$ ) and survival rates were comparable with published data (23).

**Clinical outcome by GNAS1 T393C genotypes.** Overall survival dependent on T393C genotypes was analyzed using Kaplan-Meier survival curves (Fig. 2). Survival was not significantly associated with T393C genotypes when all UICC stages were analyzed together (Fig. 2A). However, in UICC stages I to II, survival was significantly dependent on the T393C genotype with an apparent gene-dose effect (Fig. 2B;  $P = 0.009$ ). GNAS1 393C homozygous patients displayed a higher risk for death than T393 homozygous patients, with heterozygous patients being at intermediate risk [hazard ratios (HR): CC versus TT: 5.8; 95% CI, 1.4-15.9;  $P = 0.02$ ; CT versus TT: 2.8; 95% CI, 0.7-7.9;  $P = 0.19$ ]. Five-year survival rates were 87.8% for TT, 71.0% for TC, and 50.0% for CC (Fig. 2B). No

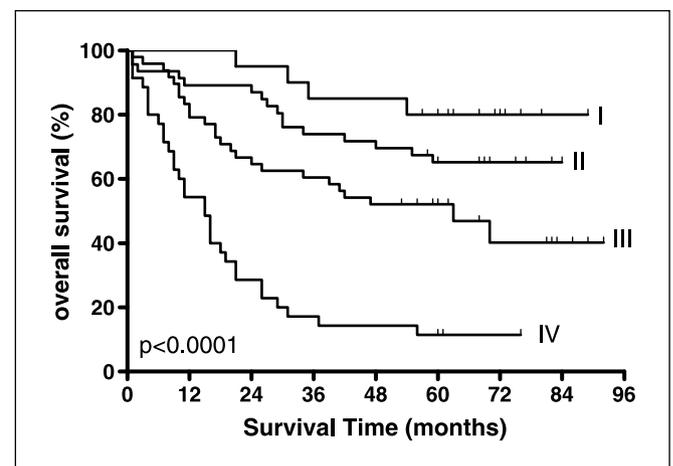


Fig. 1. Overall survival for the period of complete follow-up based on Kaplan-Meier curves for 151 patients with colorectal cancer based on different UICC stages.

association between survival and genotype was detected for UICC stages III to IV (Fig. 2C).

Because it has recently been suggested that associations reported for the T393C polymorphism could be confounded by SNPs from the 5' or 3' haplotype block (17), we chose one SNP in the 5' block (T2291C) and one SNP in the 3' block (T18830C) and calculated the linkage disequilibrium between these SNPs and T393C. In addition, we examined their possible

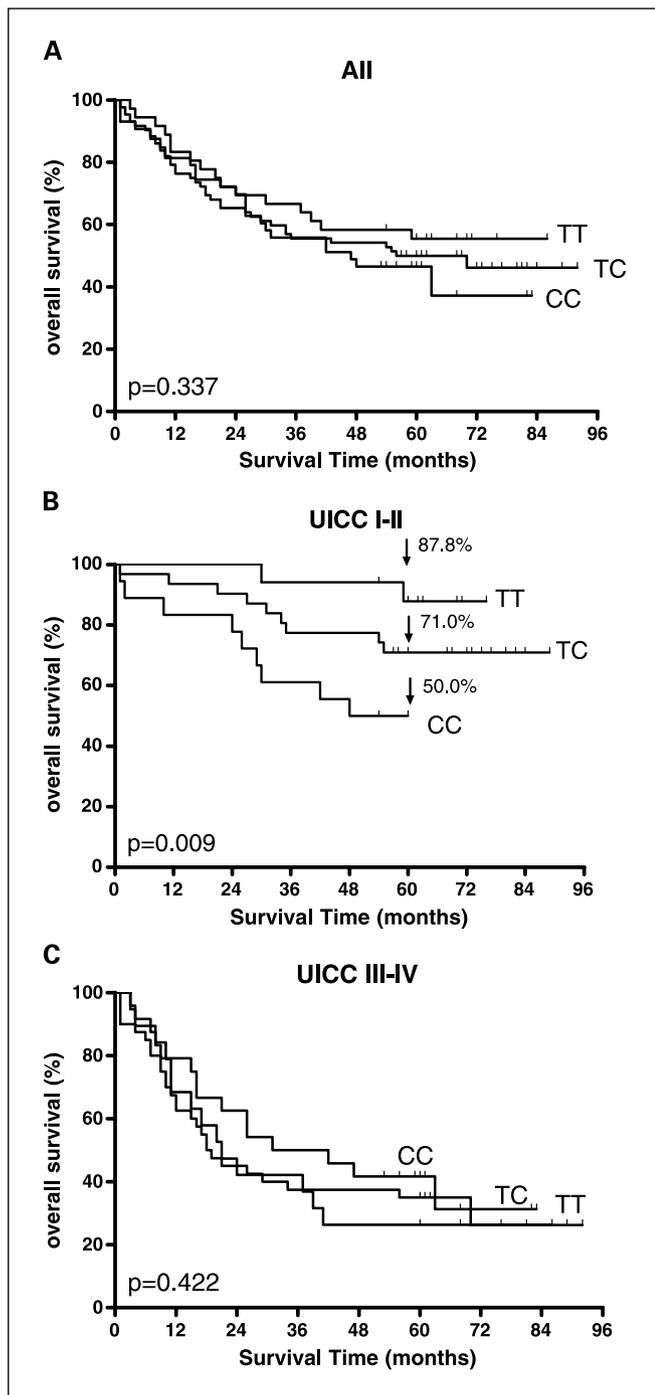
associations with survival both using these SNPs separately as well as by haplotype analysis. As shown in Table 2, the T393C and T2291C are in linkage disequilibrium ( $\chi^2 = 11.6$ ;  $|D'| = 0.45$ ;  $P < 0.01$ ). However, Kaplan-Meier and log-rank statistics using the T2291C SNP alone as well as in haplotype combination with the T393C SNP revealed no significant associations with the clinical outcome at any UICC stage (not shown). The T393C and T18830C SNPs were also in linkage disequilibrium, which was weaker compared with the T2291C ( $\chi^2 = 7.93$ ;  $|D'| = 0.27$ ;  $P < 0.05$ ; Table 2). No significant association of the T18830C SNP to progression of colorectal cancer could be detected. Moreover, no additional effect or interaction was observed when the T393C and T18830C haplotype was investigated (not shown).

Multivariate analysis including age at diagnosis, UICC stage, grade, gender, and T393C genotypes revealed that the T393C polymorphism was an independent risk factor for death in UICC stages I to II (Table 3). Patients with CC genotype had an HR of 12.1 (95% CI, 2.02-72.6;  $P = 0.006$ ) compared with the reference group consisting of T393 homozygous individuals. Heterozygous patients were at intermediate risk (HR, 2.51; 95% CI, 0.45-14.1;  $P = 0.190$ ). The T2291C and T18830C SNPs as well as the corresponding haplotypes with the T393C SNP were not significantly associated with clinical outcome in this multivariate model (not shown).

**Characterization of GNAS1 mRNA folding containing the T393C SNP.** Previous studies suggest a relationship between mRNA secondary structure and gene expression (24, 25). We therefore hypothesized that the T393C substitution may result in changes of mRNA folding. Using MFOLD (26), we modeled the effect of the T393C polymorphism GNAS1 mRNA folding structures of the coding region. Partial mRNA folding structures of 393T and 393C mRNA are shown in Fig. 3. The T>C substitution causes an obvious change in the mRNA folding structures suggesting differences in Gas mRNA stability due to this nucleotide exchange. This is consistent with experimental data showing altered mRNA expression in different T393C genotypes (13).

## Discussion

Although UICC stages generally correlate with outcome in a large percentage of patients, more precise prediction of the individual course would facilitate clinical decision making (e.g., about appropriateness of adjuvant therapy). Unfortunately, biological markers for colorectal cancer describing properties of the tumor itself have yielded inconclusive results (8-10). One of the aims of the present study was, therefore, to investigate whether a genetic host factor, the common T393C polymorphism in the gene GNAS1, may be predictive for survival in patients with colorectal cancer. The results of the study clearly indicate that mortality in UICC stages I to II was significantly increased in CC genotypes compared with TC or TT genotypes whereas no association with the genotype was observed in stages III to IV. Furthermore, the T393C polymorphism was shown to be an independent risk factor for clinical outcome when using UICC stages and grading as covariates with the highest risk for an unfavorable clinical course in CC genotypes (Table 3). Finally, CC genotypes presented at an advanced pathologic stage at first diagnosis ( $P = 0.024$ ; see Table 1).



**Fig. 2.** Overall survival for the period of complete follow-up based on Kaplan-Meier curves for 151 patients with colorectal cancer on different T393C genotypes. *A*, all UICC stages. *B*, UICC stages I to II. *C*, UICC stages III to IV. *P* values for log-rank statistics were calculated for linear comparison of all genotypes.

**Table 2.** Linkage analysis between T2291 and T393C, and T18830C and T393C

		T393C			Total		Haplotype	
		CC	TC	TT			C	T
T2291C	CC	17	35	24	76 (51.7)	C	0.31	0.40
	TC	16	32	10	58 (39.5)	T	0.21	0.07
	TT	9	3	1	13 (8.8)	D'		0.45
	Total	42 (28.6)	70 (47.6)	35 (23.8)	147	P		<0.01
T18830C	CC	19	18	9	46 (31.1)	C	0.34	0.22
	TC	16	41	20	77 (52.0)	T	0.15	0.27
	TT	6	7	12	25 (16.9)	D'		0.27
	Total	41 (27.7)	71 (48.0)	36 (24.3)	148	P		<0.05

The T393C polymorphism is located within a recombination hotspot which was supposed to be in linkage equilibrium with two haplotype blocks, one 5' and one 3' of the T393C polymorphism (17). Therefore, one SNP (T2291C) of the 5' block and one SNP (T18830C) of the 3' block were chosen to analyze linkage disequilibrium; a weak but significant linkage disequilibrium was found between each SNP and the T393C polymorphism (Table 2). However, neither the T2291C nor the T18830C polymorphism was associated with survival of colorectal cancer patients, alone or in combination with the T393C SNP. Together with our previous observation that the T393C polymorphism is a predictive marker for clinical outcome in bladder cancer (13), the present results strongly support its potential role as a possible general genetic marker for tumor progression.

We have recently shown that the *GNAS1* TT genotype is associated with increased *Gas* mRNA expression in different tissues (13). From the above-mentioned observations, we hypothesized that the T393C exchange itself could have an effect on mRNA stability. Interestingly, determinants of mRNA stability have been described in the coding region of some genes (27–29). We found that the T>C substitution at position 393 changes the mRNA folding structures predicted by MFOLD (Fig. 3). Therefore, genotype-dependent differences in mRNA decay due to altered secondary structure could finally cause differences in *Gas* mRNA expression (13). Functional studies will have to verify this hypothesis.

Finally, it still may be speculated that yet unidentified functional SNPs in *GNAS1* are in strong linkage disequilibrium with T393C and thus exert an effect on transcription of *GNAS1*.

**Table 3.** Factors influencing the risk of death by univariate and multivariate Cox-regression analysis

Variable	Multivariate analysis											
	All				UICC I-II				UICC III-IV			
	P*	P	HR	95% CI	P*	P	HR	95% CI	P*	P	HR	95% CI
Age	0.908	0.381	1.01	0.99-1.04	0.514	0.029	1.07	1.01-1.13	0.520	0.816	1.00	0.97-1.03
T393C												
TT			1 <sup>†</sup>				1 <sup>†</sup>				1 <sup>†</sup>	
TC	0.480	0.897	1.04	0.56-0.92	0.190	0.295	2.51	0.45-14.1	0.897	0.951	0.98	0.50-1.93
CC	0.332	0.507	1.25	0.65-2.38	0.023	0.006	12.1	2.02-72.6	0.438	0.661	0.84	0.39-1.81
UICC												
I			1 <sup>†</sup>				1 <sup>†</sup>					
II	0.219	0.183	2.34	0.67-8.20	0.236	0.408	1.73	0.47-6.38				
III	0.021	0.019	4.28	1.26-14.5							1 <sup>†</sup>	
IV	<0.001	<0.001	13.8	3.98-47.9					<0.001	<0.001	2.84	1.57-5.13
Grade												
1			1 <sup>†</sup>				1 <sup>†</sup>				1 <sup>†</sup>	
2	0.199	0.667	1.12	0.67-1.89	0.948	0.169	0.47	0.16-1.38	0.240	0.628	1.17	0.62-2.23
3	0.001	0.328	1.45	0.69-3.04	0.309	0.109	6.93	0.65-74.1	0.067	0.326	1.50	0.67-3.40
Gender												
Female			1 <sup>†</sup>				1 <sup>†</sup>				1 <sup>†</sup>	
Male	0.309	0.714	1.10	0.66-1.83	0.684	0.102	2.65	0.82-8.55	0.093	0.811	0.93	0.51-1.69

\*Univariate analysis.

†Reference group.

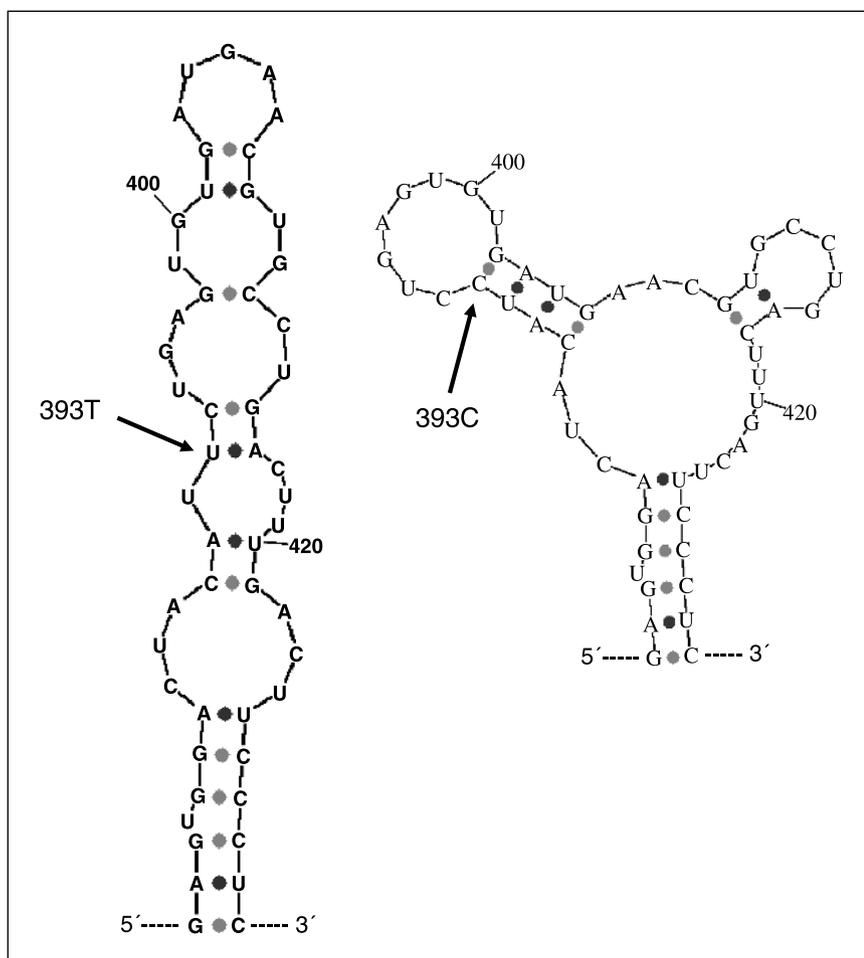


Fig. 3. *GNAS1* mRNA folding structures predicted by MFOLD. The mRNA sequence carrying the T393C polymorphism was used for secondary folding structure model building by the use of the computer program MFOLD (26).

*In vitro* experiments suggest that increased expression of  $G\alpha_s$  is associated with enhanced apoptosis (14–16). The second messenger, cyclic AMP, which is generated by activated  $G\alpha_s$ , seems to play a major role in this proapoptotic process. An increased concentration of the intracellular second messenger cyclic AMP promotes apoptosis in several cell types including leukemic cells (30), ovarian cancer cells (31), and lymphoma cells (32). Increased  $G\alpha_s$  expression in tissues of patients with TT genotypes may therefore confer an enhanced apoptosis in 393T allele carriers. Hypothetically, this mechanism may contribute to a more favorable clinical course in cancer patients. This hypothesis, although attractive, remains to be supported by additional functional studies which, however, were beyond the scope of the present report.

A major finding of the present study is that genotypes of the T393C polymorphism are associated with survival of colorectal cancer patients only in UICC stages I to II, but not in stages III to IV. Because the T393C polymorphism is expected to exert an effect on early events of intracellular signal transduction (i.e., receptor-G protein coupling and downstream signaling events),

this effect is hypothetically expected only in earlier cancer stages, in which the cell cycle is still under control of the “normal” signal transduction machinery. Once cell cycle regulation, migration, and proliferation of cancer cells are “out of control” due to accumulating somatic mutations in cell cycle-controlling proteins, as expected in higher-grade tumors, alterations in the efficacy of early signal transduction may have little or no effect. Despite these considerations, the effects of the T393C polymorphism are relatively strong, as seen from the high HR associated with the CC genotype, which is comparable to that of UICC staging for all patients or tumor grade in patients with tumors UICC I to II (see Table 3).

Both the results of the present study and previous findings (13) strongly suggest a role of the T393C polymorphism in tumor progression. Nevertheless, it has to be emphasized that in both studies a limited number of patients were investigated, which became especially evident when subgroups were analyzed. Therefore, although our findings support the concept of a role of genetic host factors in tumor progression, further independent studies are necessary to confirm their validity.

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