The *GNAS1* T393C Polymorphism Predicts Survival in Patients with Clear Cell Renal Cell Carcinoma

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Abstract

Purpose: G proteins mediate signaling from cell surface receptors to specific intracellular proteins. *In vitro* cancer cell line studies revealed a link between the Gαs protein and proapoptotic processes. We have recently shown that TT genotypes of the *GNAS1* T393C polymorphism display increased transcription of Gαs and a more favorable clinical course in bladder and colorectal cancer patients compared both with TC or CC genotypes.

Experimental Design: In the present study, 150 patients with clear cell renal cell carcinoma surgically treated by nephrectomy with curative intent were retrospectively genotyped to elucidate a potential association between T393C genotypes and clinical outcome.

Results: The C-allele frequency in the renal cell carcinoma patient group was 0.51, which is not significantly different from that of a healthy blood donor group. Kaplan-Meier curves for tumor progression, development of metastasis, and tumor-related death showed a significant association of the T393C polymorphism with outcome (5-year cancer-specific survival rates: TT, 91%; TC, 81%; CC, 69%; $P = 0.015$). Multivariate Cox proportional analysis of a 10-year follow-up confirmed the T393C polymorphism as an independent prognostic factor in clear cell renal cell carcinoma. Homozygous CC patients were at highest risk for progression (hazard ratio, 2.48; $P = 0.009$) or tumor-related death (hazard ratio, 3.15; $P = 0.018$) compared with T-allele carriers.

Conclusion: Our results show that besides tumor stage, lymph node status, and tumor grade, the *GNAS1* T393C status is a novel independent host factor for disease progression in patients with clear cell renal cell carcinoma and provides further evidence for the T393C polymorphism as a general prognostic tumor marker.

Renal cell carcinoma is the most common malignant kidney tumor in adults. It accounts for ~3% of all human malignancies. Among cancers of the urinary system, renal cell carcinoma is associated with the worst clinical outcome (1). The incidence of renal cell carcinoma is increasing and it is estimated that renal cell carcinoma accounts worldwide for 95,000 cancer-related deaths per year (2). Complete surgical resection is considered the only effective treatment for patients with clinically localized renal cell carcinoma. However, 20% to 40% of patients will show, even after curative nephrectomy, tumor recurrence, and/or development of metastasis. Therefore, more precise prediction of long-term cancer-free survival immediately after resection of clinically localized disease would be valuable for the establishment of follow-up protocols and for identifying patients with a high risk of tumor progression. Although conventional prognostic factors, such as tumor stage and grade, are useful, other novel prognostic variables will be needed that provide additional predictive value (3).

Renal cell carcinoma is a morphologically and genetically heterogeneous tumor type that includes, among several rare entities, four major subtypes, namely clear cell, papillary, chromophobe, and collecting duct (Bellini duct) carcinomas (4–6). The clear cell subtype accounts for ~70% to 80% of all renal cell carcinomas (6). Clear cell renal cell carcinoma is thought to arise from the proximal tubule and presents as both a hereditary and a sporadic form. Hereditary clear cell renal cell carcinoma occurs in patients with Von Hippel-Lindau (VHL) disease as a result of germ line mutations in the *VHL* tumor suppressor gene located on the short arm of chromosome 3 (3p25; ref. 7). Somatic mutations in the *VHL* gene are also thought to play a role in the development of sporadic clear cell renal cell carcinoma, because a high number of patients displays loss of heterozygosity due to either mutation or inactivation by hypermethylation of the other allele (8, 9). Moreover, the presence of *VHL* alterations (mutation or hypermethylation) may be associated with better outcomes in patients with clear cell renal cell carcinoma treated by potentially curative nephrectomy (10). However, determination of somatic VHL alterations is laborious and requires sequencing of the whole gene (11).

We have recently shown that genotypes of the single nucleotide polymorphism T393C in the gene *GNAS1*, encoding

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Table 1. Genotype distribution, demographic characteristics, grade, stage, and nodal status of the tumor at primary diagnosis in 150 patients with renal cell carcinoma

	All	TT	TC	CC	*P*
n (%)	150	34 (22.7)	79 (52.7)	37 (24.6)	
Mean age (y ± SD)	60.9 ± 11.0	64.0 ± 10.1	60.5 ± 10.8	58.8 ± 12.0	0.048
Median follow-up, mo (range)	38 (1-180)	37 (1-118)	51 (1-180)	29 (1-117)	0.725*
Gender, male/female	101/49	17/17	58/21	26/11	0.047
Grading	121	28 (23.1)	64 (52.9)	29 (24.0)	
G1	21	7 (33.3)	10 (47.6)	4 (19.0)	
G2	82	18 (22.0)	45 (54.9)	19 (23.2)	
G3	18	3 (16.7)	9 (50.0)	6 (33.3)	0.161
Staging					
I	19	7 (36.8)	8 (42.1)	4 (21.1)	
II	68	16 (22.9)	33 (47.1)	21 (30.0)	
III-IV	63	11 (18.0)	38 (62.3)	12 (24.7)	0.579
Nodal status					
0	121	30 (24.8)	62 (51.2)	29 (24.0)	
≥1	29	4 (13.8)	17 (58.6)	8 (27.6)	0.305

NOTE: Value expressed as absolute numbers with percentages in parentheses. *P* values were calculated using χ^2 test for categorical variables and linear ANOVA for continuous variables.

*Kruskal-Wallis test for nonparametric variables.

the ubiquitously expressed Gαs subunit of heterotrimeric G proteins, predict the outcome of patients with urothelial carcinoma and sporadic colorectal cancer (12, 13). Patients with the TT genotype showed a prolonged survival compared with patients with either TC or CC genotypes. In TT genotypes, Gαs mRNA expression was found to be increased not only in bladder carcinoma tissue but also in human heart and fat cell specimens (12), which may result from altered mRNA stability associated with different genotypes (13). Data from *in vitro* experiments suggest that increased expression of Gαs enhances apoptosis (14–16). Hence, it is tempting to hypothesize that increased Gαs expression with concomitantly enhanced apoptosis associated with the TT genotype may be related to the prolonged survival observed in both bladder and colorectal cancer.

The aim of the present study was to provide further evidence that the T393C-dependent altered Gαs expression is not only associated with the prediction of outcome in patients with bladder and colorectal cancer, but may represent a more general progression marker 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 clinical outcome in a series of 150 patients with clear cell renal cell carcinoma.

Materials and Methods

Patients. The present series comprised 150 consecutive patients who were treated for clear cell renal cell carcinoma at the Department of Urology of the University Hospital Essen, Germany. Entry criteria were histopathologic diagnosis of clear cell kidney cancer, availability of tumor material or blood for DNA extraction, and the potential for follow-up. All tumors were classified as clear cell renal cell carcinoma according to the definition of the recently published WHO classification (4). The tumors were staged and graded by standard histologic analysis according to the tumor-node-metastasis classification (17). Because some patients were assigned to resection of solitary metastasis or systemic therapy due to progression after primary nephrectomy in different hospitals, grading of the primary tumor was not available for all cases. The medical records of all patients were reviewed and follow-up data were collected in cooperation with the assigning urologist and/or family physician. The data were evaluated for the end-points "tumor progression," "development of metastasis," "tumor-related death," and "overall survival." The cause of death was determined using hospital files and information from the responsible urologist/family physician. The most recent clinical assessment was used to define the follow-up period. Progression was defined as an increase in stage, occurrence of metastasis, or local recurrence after radical nephrectomy, or death due to renal cell carcinoma. For patients initially lost for follow-up, the local municipal registry was consulted to assess survival.

Healthy blood donors. The control group consisted of 255 age- and sex-matched healthy Caucasian individuals who were randomly recruited at the local Institute of Transfusion Medicine, University Hospital of Essen. The control sample consisted of 161 males and 94 females and the mean age was 56.7 ± 4.4 years. Details of this control group have been published previously (18).

Genotyping. DNA was extracted from frozen tumor tissue specimens or whole blood using a commercially available kit (QIAAmp, Qiagen, Hilden, Germany). Genotypes of the T393C polymorphism were determined by PCR using the following primers: forward primer, 5′-CTCCTAACTGACATGGTGCAA-3′ and reverse primer, 5′-TAAGGCCA-CACAAGTCGGGGT-3′. After denaturation at 94°C, 35 cycles of DNA amplification were done using *Taq* PCR Mastermix (Eppendorf, Hamburg, Germany) at 94°C for 45 seconds, 58°C for 40 seconds, and 72°C for 45 seconds. The 345 bp PCR products were digested using the restriction enzyme *Fok*I and analyzed on a 2% agarose gel. The unrestricted products (345 bp) represent the TT genotype; the completely restricted products (259 and 86 bp) represent the CC genotype.

Statistical analysis and presentation of data. The clinical outcomes analyzed in the study were overall survival, cancer-related death, time to first progression, and time to first metastasis. Kaplan-Meier plots and

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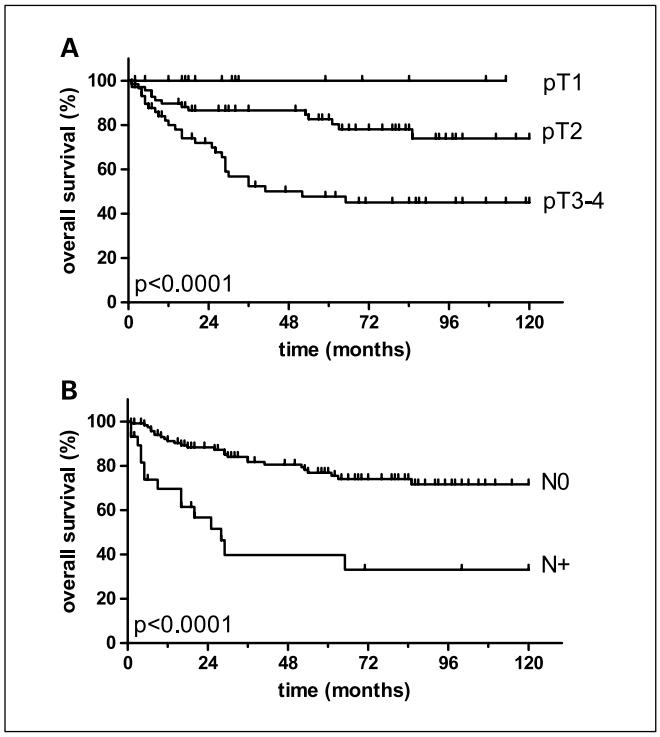

Fig. 1. Overall survival based on Kaplan-Meier curves for 150 patients with clear cell renal cell carcinoma based on different tumor stages (*A*) or lymph node status (*B*).

the log-rank test for trend were used to retrospectively evaluate the relationship between tumor stage, lymph node status, T393C genotypes, and outcome from the date of primary diagnosis to a 10-year follow-up. Log-rank tests for T393C genotypes were adjusted for pathologic stage. Multivariate models of clinical follow-up were established using clinical and pathologic variables known as predictors of prognosis in clear cell renal cell carcinoma (19), including the genotypes of the T393C polymorphism. A backward stepwise Cox proportional hazard model was used to calculate hazard ratios (HR), 95% confidence interval (95% CI), and *P* values (20). Age and gender were also included because they were significantly associated with T393C genotypes.

Contingency tables and the Pearson's χ^2 test were used to compare categorical variables using T393C genotypes as indicated. Because the T393C polymorphism displays a gene-dose effect (12, 13), linear ANOVA was used for comparison of continuous variables where appropriate. Differences were regarded significant at $P < 0.05$. All statistical analysis was done using SPSS 11.0 (SPSS, Chicago, IL) or Graphpad Prism 4.0 (Graphpad Software, San Diego, CA). Continuous variables are given as means ± SD.

Results

Genotype distribution and subject characteristics. Demographic characteristics and tumor grade and stage in the whole case group and by T393C genotypes are displayed in Table 1. The mean age was 60.1 years (range 31-87 years) and median follow-up time was 38 months (range 1-180 months). Distributions of tumor stage and grade of the whole sample were compatible with those reported in the literature (21, 22). The frequency of the C allele (f_C) in the patients group was 0.51 and genotype distribution was compatible with the Hardy-Weinberg equilibrium. Genotypes and allele frequencies were compared with those from 255 age and sex-matched healthy white blood donors. Genotype distribution (TT, $n = 62$; TC, $n = 125$; CC, $n = 68$) as well as C-allele frequency ($f_C = 0.51$) was not significantly different from that of the patients group, which argues against an association of T393C genotypes with an increased risk for renal cell carcinoma. Stage, grade, and lymph node status were not associated with genotypes (Table 1). However, the comparison of age at diagnosis and gender showed a borderline significant association with T393C genotypes. The CC genotype seems to be associated with younger age at primary diagnosis and a higher proportion of males compared with TC and TT genotypes (Table 1).

To confirm that our sample was representative for patients with renal cell carcinoma, we calculated Kaplan-Meier curves for overall survival depending on the pathologic stage and

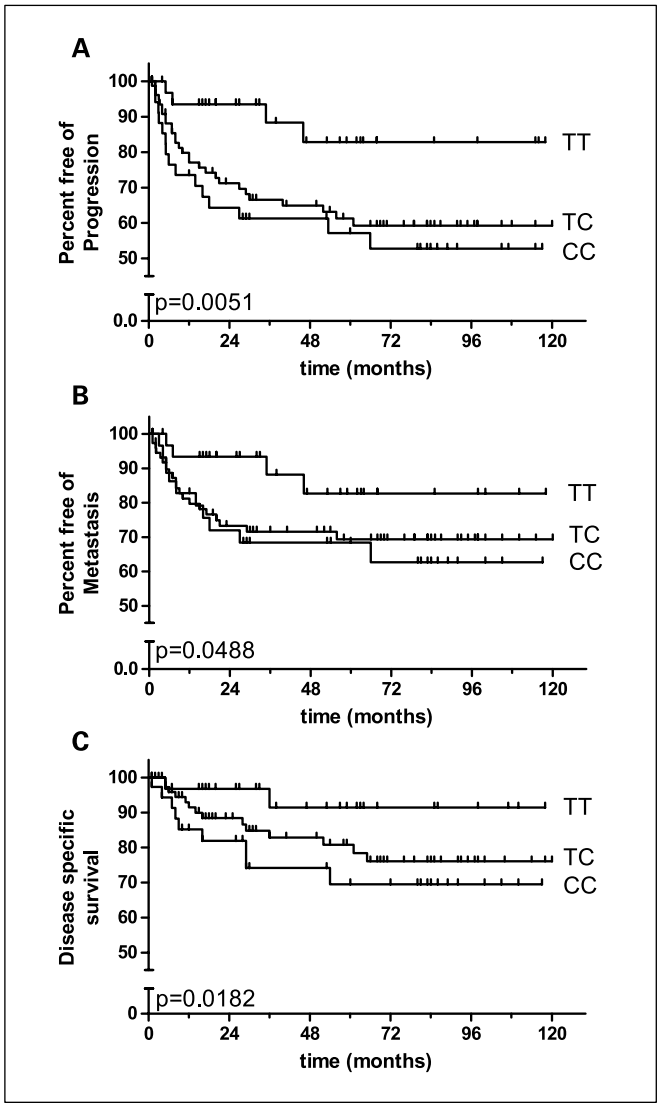

Fig. 2. Progression (*A*), metastasis (*B*), and tumor-specific survival (*C*) based on Kaplan-Meier curves for 150 patients with clear cell renal cell carcinoma on different T393C genotypes. *P* values for log-rank statistics were adjusted for pathologic stage.

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lymph node status. As shown in Fig. 1, overall survival was significantly dependent on pathologic stage (5-year survival, pT_1, 100%; pT_2, 82%; pT_{3-4}, 48%; $P < 0.0001$) and lymph node status (N_0, 77%; N_+, 40%; $P < 0.0001$), and computed values were compatible with published data (21, 23, 24).

Clinical outcome by T393C genotypes. Progression, metastasis, and cancer-related survival dependent on T393C genotypes were analyzed using Kaplan-Meier survival curves (Fig. 2). As pathologic stage is regarded as one of the best prognostic factors in renal cell carcinoma (25), log-rank tests for clinical outcome were adjusted for this variable.

Progression was significantly dependent on the T393C genotype with 393C homozygous patients having a significantly higher risk for tumor progression than T393 homozygous and heterozygous patients ($P = 0.005$; Fig. 2A). The HR for CC versus TT was 3.8 (95% CI, 1.3-8.6, $P = 0.010$); for CT versus TT, the HR was 3.1 (95% CI, 1.1-5.1, $P = 0.023$). Time to metastasis ($P = 0.049$) and cancer-related survival ($P = 0.018$) were also significantly associated with the T393C polymorphism. Although 393C homozygous patients were at higher risk for the development of metastases (CC versus TT: HR, 2.7; 95% CI, 0.9-7.4; $P = 0.078$) or death from renal cell carcinoma (CC versus TT: HR, 4.3; 95% CI, 1.1-11.4; $P = 0.039$), T393 homozygosity was associated with a protective effect. Heterozygous subjects were at intermediate risk (HR for tumor-related death, 2.9; $P = 0.14$; HR for metastases, 2.4; $P = 0.10$; Fig. 2B and C). Five-year survival rates were calculated as follows: TT, 91%; TC, 81%; CC, 69% ($P = 0.015$).

To investigate whether genotypes of the T393C polymorphism are independent risk factors for outcome in patients with clear cell renal cell carcinoma, multivariate analysis was done based on two different Cox proportional hazard models (Table 2). Model A included 121 patients for which grading was available. Model B comprised all 150 patients without grading being included in the analysis. Furthermore, age and gender, which showed significant associations with T393C genotypes (Table 1), were included into the models and TT and TC genotypes were combined (T+ allele) to achieve a substantial number of patients as a reference group. Multivariate analysis revealed grading to be the best prognostic factor for clinical outcome followed by pathologic staging (Table 2). Most interestingly, in both models, the T393C polymorphism was an independent risk factor for progression, metastasis, and tumor-related death. HRs for patients with CC genotype ranged between 2.05 and 3.15 for progression, metastasis, and tumor-related death compared with the reference group consisting of 393T allele carriers.

Discussion

Current efforts in cancer research are focused on the detection and validation of biomarkers and genetic markers that are hoped to permit both to individually predict tumor behavior and to better stratify patients into more refined risk categories, ultimately permitting to design and target therapies to carefully selected patient populations (26). The majority of markers are related to properties of the tumor tissue itself, e.g., somatic mutations or differential expression of genes or proteins. However, difficulties in standardization of, e.g., immunohistochemical biomarkers often prevent their routine application in clinical practice (10, 27, 28). The aim 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 clear cell renal cell carcinoma. In the present study, the most common type of renal cell carcinoma, namely the clear cell renal cell carcinoma, has been investigated. Our data indicate that progression, metastasis, and cancer-related mortality was significantly increased in CC genotypes compared with 393T allele carriers, and multivariate analysis confirmed the T393C polymorphism to be an independent prognostic factor for clinical outcome.

Table 2. Multivariate Cox proportional hazard model for progression, metastasis, and tumor-related death in 121 patients (model A) and 150 patients (model B, without grading) with clear cell renal cell carcinoma

	Progression		Metastasis		Tumor-related death	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Model A						
Grade, 3/1-2	4.21 (2.00-8.85)	<0.001	5.99 (2.58-13.9)	<0.001	6.38 (2.14-19.0)	0.001
Stage, III-IV/I-II	2.53 (1.19-5.39)	0.016	1.92 (0.79-4.65)	0.151	4.01 (1.30-12.8)	0.016
Nodal status, $N_+/0$	2.29 (1.15-4.53)	0.018	2.21 (0.93-5.25)	0.073	2.41 (0.93-6.21)	0.069
Age, >56/≤56 y	1.47 (0.75-2.89)	0.262	1.83 (0.87-3.84)	0.113	1.46 (0.53-4.00)	0.459
Sex, male/female	0.99 (0.49-1.97)	0.973	1.26 (0.51-3.01)	0.620	1.43 (0.54-3.79)	0.470
T393C, CC/T+	2.48 (1.26-4.90)	0.009	2.27 (0.97-5.33)	0.060	3.15 (1.22-8.14)	0.018
Model B						
Stage, III-IV/I-II	3.60 (1.91-6.78)	<0.001	3.41 (1.64-7.07)	0.001	6.22 (2.38-16.3)	<0.001
Nodal status, $N_+/0$	2.85 (1.48-5.47)	0.002	3.02 (1.34-6.81)	0.008	3.04 (1.26-7.37)	0.013
Age, >56/≤56 y	1.11 (0.58-2.12)	0.763	1.42 (0.69-2.94)	0.333	0.75 (0.27-1.94)	0.547
Sex, male/female	1.12 (0.62-2.22)	0.629	0.87 (0.38-2.00)	0.747	0.96 (0.39-2.34)	0.924
T393C, CC/T+	2.21 (1.16-4.19)	0.015	2.05 (0.93-4.53)	0.077	2.98 (1.26-7.05)	0.013

NOTE: Tstage (T_{3-4} versus T_{1-2}), nodal status (≥1 versus no positive lymph nodes), tumor grade (3 versus 1-2), age (>56 versus ≤56 years), and T393C genotypes (393C homozygous versus T393 allele carriers).

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The T>C substitution at position 393 changes the mRNA folding structures (13). Therefore, genotype-dependent differences in mRNA decay due to altered secondary structure could finally cause differences in Gαs mRNA expression, which was previously reported for different tissues (12). Together with our previous observation showing that the T393C polymorphism is a predictive marker for clinical outcome in bladder and colorectal cancer (12, 13), the present results strongly support its potential role as a possible genetic marker for tumor progression in other cancer types. *In vitro* experiments suggest that increased expression of Gαs is associated with enhanced apoptosis (14–16). The second messenger cyclic AMP, which is generated subsequently to activation of Gαs, seems to play a major role in this proapoptotic process. An increased concentration of cyclic AMP promotes apoptosis in several cell types, e.g., leukemic cells (29) and ovarian cancer cells (30). Interestingly, activated Gαs or cyclic AMP have also been shown to suppress Ras-dependent activation of Raf (31, 32). The Ras/Raf signaling pathway is an important mediator of tumor cell proliferation and angiogenesis, and a novel potent Raf inhibitor shows promising results in patients with renal cell carcinoma (33). It would, therefore, be of interest to investigate whether patients with specific T393C genotypes would especially benefit from such novel anticancer drugs. This could ultimately help to individually tailor drug treatment with regard to both efficacy and safety, which is the ultimate goal of pharmacogenetics in terms of more individualized therapeutic strategies.

Both the results of the present study and two previous independent reports (12, 13) strongly suggest a role of the T393C polymorphism in tumor progression. Nevertheless, it has to be emphasized that, in the present study, a limited number of patients was investigated. Therefore, although our findings support the concept of the T393C polymorphism to be a genetic host factor predictive of tumor progression, further independent studies will have to confirm this hypothesis.

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