

## GNAS1 T393C POLYMORPHISM AND DISEASE PROGRESSION IN PATIENTS WITH MALIGNANT MELANOMA

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### Abstract

**Background:** Once metastasized, despite a variety of therapeutic options, the prognosis of patients with malignant melanoma (MM) is still poor. Therefore, the search for reliable markers to identify patients with high risk of disease progression is of high clinical importance. We have recently shown that TT genotypes of the single-nucleotide polymorphism (SNP) T393C in the gene *GNAS1* are significantly associated with better outcome in a variety of carcinomas.

**Patients:** In the present study we assessed whether the T393C SNP is also related to the clinical course in MM. 328 patients with MM were retrospectively genotyped and genotypes were correlated with clinical outcome.

**Results:** While the allele frequency in the MM group (fC 0.52) did not significantly differ from that of healthy blood donors, the T393C SNP was associated with tumor progression of MM. Carriers of the C-allele showed a significantly more severe tumor progression as estimated from the time period to develop metastasis (HR 2.2, 95% CI 1.1-3.2, p = 0.017). Proportions of 5-year metastasis-free intervals were 87.1% for TT genotypes and 66.0% for C-allele carriers. Moreover, multivariable Cox regression analysis including tumor stage and melanoma subtype proved the T393C polymorphism to be an independent factor for metastasis (p = 0.012).

**Conclusions:** In summary, the *GNAS1* T393C SNP represents a genetic host factor for predicting tumor progression also in patients with MM; genotyping of this SNP may contribute to better define patients who could benefit from an early individualized therapy.

**Key words:** polymorphism, G Protein, melanoma, prognostic factor

### INTRODUCTION

Early detection of metastasis is one of the aims of follow-up examination in patients with malignant melanoma since once metastasized, despite a variety of

therapeutic options e. g. chemo- and/ or immunotherapy, patients suffering from malignant melanoma show an unfavorable prognosis. Therefore, it would be most desirable to identify patients with high risk of disease progression as early as possible [1]. Since laboratory tests are easy to handle and modestly expensive, a variety of different serum factors has been investigated for their potential relevance as prognostic markers in malignant melanoma [2-4]. To date, none of these markers is routinely used in the clinical setting. The most promising one at present, S100-protein, has been demonstrated in several studies to be a valid and reliable predictive marker for clinical progression and response to therapy in melanoma patients with advanced stages of disease [5, 6]. However, a major disadvantage of S100-protein is its poor prognostic impact in early-stage melanoma patients [6-8].

The identification of new prognostic markers may help to better distinguish low from high risk disease apart from the classical histopathologic and clinical criteria and may unravel new targets for therapy. The mechanism of malignant transformation of melanocytes is poorly understood, but growth factors and their receptors play an important role in the progression of melanoma [9]. Moreover, alterations in the mitogen-activated protein kinase pathway are almost uniformly present in melanoma. While RAS itself is only infrequently mutated in melanoma [10] the downstream BRAF gene of the mitogen activated protein kinase pathway is mutated in 60% to 70% of superficial spreading melanomas [11]. On the other hand, a genetic host factor may offer a better stratification of patients into more refined risk categories without the need for tumor tissue. For example, the A61G single nucleotide polymorphism (SNP) in the EGF gene which operates via RAS and BRAF in melanocytic tumors has recently been associated with risk for metastasis in melanoma patients [12].

We have recently shown that the T393C polymorphism in the gene *GNAS1*, which encodes the G<sub>s</sub> subunit of heterotrimeric G protein, is significantly associated with the clinical outcome of patients suffering from bladder cancer [13], colorectal cancer [14], chronic lymphocytic leukaemia [15], and renal cell carcinoma [16]. We also demonstrated that the T allele of

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*GNAS1* T393C polymorphism is associated with increased *Gαs* mRNA expression in a variety of tissues, including tumor tissue [14]. In vitro experiments suggest that an increased expression of *Gαs* is associated with enhanced apoptosis and that the second messenger cAMP, which functions downstream of G proteins, plays a major role in this pro-apoptotic process [17-20]. Hence, an increased *Gαs* expression with concomitantly enhanced apoptosis may be also associated with better survival in melanoma patients with TT genotypes.

MATERIALS AND METHODS

PATIENTS

This study comprises a total of 328 consecutive malignant melanoma patients (with a mean age of 54.2 ± 16.4 years) treated at the Department of Dermatology and Allergology, Ruhr-University Bochum (Bochum, Germany) between 1995 and 2000 with disease progression being continuously documented. The clinical staging was performed according to the American Joint Committee on Cancer (AJCC 1997). Briefly, stages I and II included patients with primary melanoma, stage III included patients with regional

lymph node and/or in-transit metastases and stage IV included patients with distant metastases. Patients were treated according to therapy protocols of the Dermatologic Cooperative Oncology Group (DeCOG) including cytostatic drugs (dacarbazine, cisplatin, temozolomide, vincristine) and immunomodulatory agents [interferon(IFN)] in different combinations and schedules. Follow-up was performed in at least 3-months intervals including physical examination, x-ray of the chest, ultra- sound of the abdomen and lymph nodes as well as blood chemistry. Patients in advanced disease stages additionally underwent computer tomography of the brain and szintigraphy of the skeleton. Detailed clinical characteristics of the study patients are summarized in Table 1. The retrospective analysis and genotyping of DNA from paraffin-embedded tissues in an anonymous form was approved by the ethical committee of the University Hospital Essen (Essen, Germany).

GENOTYPING

DNA was extracted from paraffin-embedded tissue samples using a commercially available kit (QIAAmp, Qiagen, Hilden, Germany). Genotypes of the T393C

Table 1.

	All	TT	TC	CC	P
N (%)	328	69 (21.0)	149 (45.4)	110 (33.5)	
Gender (m/f)	167/161	30/39	86/63	51/59	0.074
Mean age at diagnosis	54.2	55.0	54.4	53.4	0.517
UICC Category.					
I	202 (61.6)	45 (22.3)	85 (42.1)	72 (35.6)	
II	72 (22.0)	13 (18.1)	34 (47.2)	25 (34.7)	
III	47 (14.3)	9 (19.1)	30 (63.8)	8 (17.0)	
IV	7 (2.1)	2 (28.6)	0 (0)	5 (71.4)	0.683
Type					
SSM	153	31	71	51	
NM	38	8	16	14	
LMM	34	9	14	11	
ALM	30	7	16	7	
UCM	73	14	32	27	
Tumor thickness (mm)					
<0,75	191 (58.2)	40 (20.9)	80 (41.9)	71 (37.2)	
0,75-1,5	55 (16.8)	14 (25.5)	27 (49.1)	14 (25.5)	
1,5- 4	58 (17.7)	8 (13.8)	31 (53.4)	19 (32.8)	
>4	24 (7.3)	7 (29.2)	11 (45.8)	6 (25.0)	0.385
Clark level					
I	51 (15.5)	10 (19.6)	22 (43.1)	19 (37.3)	
II	40 (12.2)	10 (25.0)	15 (37.5)	15 (37.5)	
III	114 (34.8)	24 (21.1)	50 (43.9)	40 (35.1)	
IV	92 (28.0)	20 (21.7)	45 (48.9)	27 (29.1)	
V	10 (3.0)	2 (20.0)	6 (60.0)	2 (20.0)	0.333
Node					
0	305 (93.0)	66 (21.6)	135 (44.3)	104 (34.1)	
1	13 (4.0)	3 (23.1)	6 (46.2)	4 (30.8)	
2	10 (3.0)	0 (0)	8 (80.0)	2 (20.0)	0.865
Distant metastasis					
No	321 (97.9)	67 (20.9)	149 (46.4)	105 (32.7)	
Yes	7 (2.1)	2 (28.6)	0 (0,0)	5 (71.4)	0.266

M; male; f, female; SSM, superficial spreading melanoma; NM, nodular melanoma; LMM, lentigo maligna melanoma; ALM, acral lentiginous melanoma; UCM, unclassified melanoma

polymorphism were determined by PCR using the following primers: forward primer 5'-CTCCTAACTGACATGGTGCAA-3' and reverse primer 5'-TAAGGCCACACAAGTCGGGGT-3'. After denaturation at 94°C, 35 cycles of DNA amplification were performed using Taq PCR Mastermix (Eppendorf, Hamburg, Germany) at 94° for 45 sec, 58°C for 40 seconds, and 72° for 45 seconds. The 345 bp PCR products were digested using the restriction enzyme FokI and analyzed on a 2% agarose gel. The unrestricted products (345 bp) represented the TT genotype; the completely restricted products (259 and 86 bp) represented the CC genotype.

#### STATISTICAL ANALYSIS

The clinical outcome analyzed in this study was survival, and metastasis dependent on UICC stages, melanoma subtypes, and T393C genotypes. Kaplan-Meier plots and the log-rank test were used to evaluate the relationship between UICC stages, melanoma subtypes or T393C genotypes, and clinical outcome from the date of the primary diagnosis to the end of follow-up. The effects of gender, age at diagnosis, UICC stages, 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-square test were used for categorical variables using T393C genotypes. Linear ANOVA was used for comparison of continuous variables where appropriate. Differences were regarded significant at  $P < 0.05$ . All statistical analyses were done using SPSS 13.0 (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

##### GENOTYPE DISTRIBUTION

Demographic characteristics and tumor stage in the whole patient group and by T393C genotypes are displayed in Table 1. The frequency of the C allele in the patient group was 0.52 and genotype distribution was compatible with the Hardy-Weinberg equilibrium. Genotypes and allele frequency of patients were comparable with those of healthy white blood donors; details of this control group have been published previously [21]. This argues against an association of T393C genotypes with an increased susceptibility for malignant melanoma. We found no genotype association with age at time of first diagnosis. Moreover, genotypes were not significantly associated with tumor localization, UICC classification, or tumor thickness (Table 1).

##### T393C GENOTYPE AND CLINICAL FOLLOW UP

During follow up, 85 patients (25.9%) experienced metastases and 88 (28.1%) died. In order to confirm that our sample was representative for patients with

malignant melanoma we calculated Kaplan-Meier curves for overall survival depending on the UICC stages and tumor subtype. As shown in Fig. 1 A-B, overall survival was significantly dependent upon AJCC stage and tumor type. Five-year survival rates were 90.4% for UICC I, 77.1% for UICC II, 52.4% for UICC III, and 28.6% for UICC IV. Regarding tumor types, 5-year survival rates were 83.4% for SSM, 73.3% for NM, 87.3% for LMM, 75.0% for ALM and 79.4% for UCM. These findings are comparable with those from the literature [22].

Concerning genotypes of the T393C polymorphism we observed no association with overall survival. However, we detected a significant allele-dependent influence upon tumor progression. *GNAS1* 393C allele carriers displayed a higher risk for metastasis (*C versus TT*: 2.2, 95% CI 1.1-3.2,  $p = 0.017$ ) compared to T393 homozygous patients. Proportions of 5-year metastasis free intervals were 87.1% for TT genotypes and 66.0% for C-allele carriers, respectively (Fig. 2).

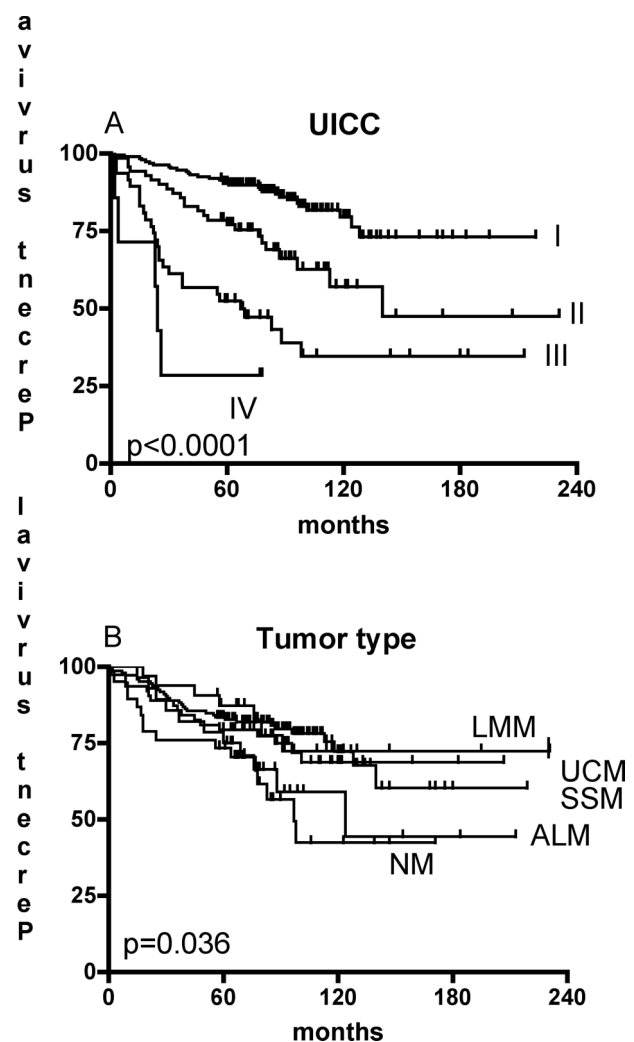


Fig. 1. Kaplan-Meier curves for overall survival in 328 patients with malignant melanoma based on the UICC stage (A) and tumor subtype (B). SSM, superficial spreading melanoma; NM, nodular melanoma; LMM, lentigo maligna melanoma; ALM, acral lentiginous melanoma; UCM, unclassified melanoma.

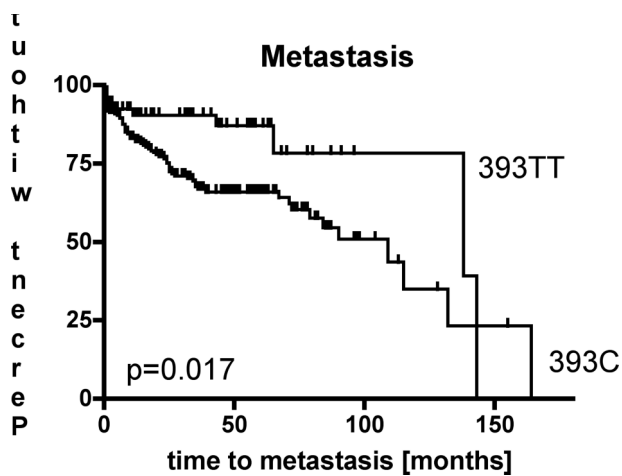


Fig. 2. Kaplan-Meier curves for metastasis-free survival in 328 patients with malignant melanoma based on T393C alleles.

Table 2. Multivariable Cox proportional hazard model for metastasis in 328 patients with malignant melanoma.

	Metastasis		
	Hazard ratio	95% CI	p
Age at diagnosis	1.0	0.98-1.02	0.802
Gender			
Male	1*		
Female	0.96	0.61 - 1.53	0.866
UICC Stage			
I	1*		
II	1.91	0.96 - 3.81	0.067
III	12.19	6.30 - 23.56	<0.001
IV	21.47	6.03 - 76.49	<0.001
MM Type			
SSM	1*		
NM	1.89	0.92 - 3.88	0.081
LMM	2.75	1.14 - 6.2	0.024
ALM	1.56	0.74 - 3.33	0.239
UCM	2.38	1.23 - 4.58	0.010
T393C			
TT	1*		
C	2.55	1.22 - 5.32	0.012

\*reference group. CI, confidence interval. SSM were chosen as reference because they represent the largest subgroup of melanomas. SSM, superficial spreading melanoma; NM, nodular melanoma; LMM, lentigo maligna melanoma; ALM, acral lentiginous melanoma; UCM, unclassified melanoma

In the multivariable analysis with gender, age at diagnosis, tumor stage, and melanoma subtype, the T393C polymorphism was an independent marker for tumor progression. The hazard ratio for patients with C-alleles was 2.55 for metastasis compared to homozygous 393T allele carriers (Table 2).

### DISCUSSION

Although UICC stages generally correlate with outcome in a large percentage of cancer patients, refined prediction of the individual disease course would facilitate clinical decision making, e.g. early requirement of

adjuvant therapy. The familial melanoma syndromes are associated with germ line mutations in three highly penetrant gene products: p16, alternate reading frame, and cyclin-dependent kinase 4. Certain variants in a low-penetrance gene, MC1R, the melanocortin 1 receptor gene, increase melanoma risk to a lesser extent and act as genetic modifiers when cosegregating with a deleterious p16 gene [23]. Since the immune response against malignant melanoma cells can be influenced by cytokines with potentially inhibitory effects on tumor cell growth, SNPs in cytokine genes associated with reduced cytokine production are expected to influence susceptibility to cancer, including malignant melanoma. Genotypes associated with reduced production of pro-inflammatory and immunomodulatory TNF-alpha, IFN-gamma, and IL-6, and anti-inflammatory IL-10 and TGF-beta1 could be involved in mechanisms of cancer progression and escape from immunosurveillance [24]. Genotypic variations in the IL10 promoter are significantly associated with an increased risk for malignant melanoma and may be associated with a poor outcome [25]. One of the aims of the present study was, therefore, to investigate whether the common T393C SNP in the gene *GNAS1*, may be predictive for survival or disease-free survival in patients with malignant melanoma.

The mean age of the patients of the present malignant melanoma series was 54.2 years and the median follow-up time was 80 months. Distribution of tumor stage and subtype of the whole series were compatible with data reported in the literature. Results of the German Central Register of Malignant Melanoma showed a mean age of 58.1 year and a similar distribution of subtypes as reported here [26]. Beside the role of tumor stage and tumor thickness as the most important prognostic factor in patients with malignant melanoma, our data suggest a potential predictive use of the *GNAS1* T393C SNP as an independent prognostic factor in malignant melanoma. C-allele carriers have an increased risk for tumor progression when compared to patients with the homozygous 393TT genotype, the latter group showing both a longer relapse-free survival and a later requirement for secondary therapy. Together with our previous observations showing that the T393C SNP is a predictive marker for outcome in bladder cancer [13], renal cell carcinoma [16] and colorectal cancer [14], the present results confirm the potential role of this SNP as a universal genetic marker for predicting tumor progression. The *GNAS1* TT genotype is associated with increased *Gαs* mRNA expression in different tissues [14] which led us to hypothesize that the T393C exchange itself could have an effect upon mRNA stability. Interestingly, determinants of mRNA stability have been described in the coding region of some other genes [27-29]. Different in vitro experiments suggest that increased expression of *Gαs* is associated with enhanced apoptosis [17-19]. The second messenger cyclic AMP, which is generated subsequently to activation of *Gαs*, seems to play a major role in this pro-apoptotic process. Cyclic AMP can augment or suppress ERK activity, depending on the cell type. As an example, CRAF is inhibited by protein kinase A, which is one of the downstream effectors of cAMP

[30]. Cyclic AMP is produced in response to melanocytic agonists such as  $\alpha$ -melanocyte-stimulating hormone acting through the melanocortin 1 receptor [31] resulting in activation of G $\alpha$ s. Although this is only able to weakly stimulate proliferation, cAMP is closely associated with melanocyte differentiation (e. g. stimulation of melanin synthesis) [32].

A recent report has shown that cAMP blocks CRAF activity in melanocytes resulting in suppression of the oncogenic activity of CRAF in these cells [33]. This process could be of particular importance in TT genotypes in which G $\alpha$ s expression is increased. However, CRAF is not required for MEK/ERK signalling in melanoma cells when BRAF is mutated [34, 35]. Late mutations in the BRAF gene could, therefore, mitigate the T393C genotype effect which may explain the lack of genotype-dependent differences in overall survival. This hypothesis is supported by observations from studies in different types of cancer in which BRAF mutations are rarely events and in which then the T393C SNP affects overall survival [36]. The goal of ongoing studies is to unravel both the detailed molecular mechanisms by which the silent C393T SNP contributes to differential G $\alpha$ s expression in human tissues and how this may change the phenotype of different tumor cells.

The authors state no conflict of interest.

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