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Frequency of *TERT* Promoter Mutations in Prostate Cancer

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TERT · Promoter · Mutation · SNaPshot analysis · Sanger sequencing · Prostate cancer

Abstract
Objective: Recently, recurrent mutations within the core promoter of the human telomerase reverse transcriptase *(TERT)* gene generating consensus binding sites for ETS transcription factor family members were described in melanomas and other malignancies (e.g. bladder cancer, hepatocellular carcinoma). These mutations were discussed as early drivers for malignant transformation. In prostate cancer (PrCa) TERT expression has been associated with a poor prognosis and higher risk for disease recurrence. The underlying mechanisms for high TERT expression in PrCa have still not been clarified. To date, data on *TERT* promoter mutation analysis in PrCa are sparse. Therefore, we performed sequence analysis of the core promoter region of the *TERT* gene in an unselected cohort of prostate tumors. ***Methods:*** Sections from 167 formalin-fixed, paraffin-embedded and cryopreserved prostate tumors were microdissected and used for DNA isolation. The mutation hotspot region within the *TERT* core promoter (–260 to +60) was analyzed by direct Sanger sequencing or SNaPshot analysis. ***Results:*** All cases were analyzed successfully. Mutations within the core promoter of the *TERT* gene were not detected in any of the cases with all tumors exhibiting a wild-type sequence. ***Conclusion:*** *TERT* core promoter mutations reported from several other malignancies were not detected in our unselected cohort of PrCa. These data indicate that alterations within the core promoter of the *TERT* gene do not play an important role in prostate carcinogenesis.

Introduction

The search for recurrent mutations in single genes within tumor genomes is driven by the hope of uncovering important driver alterations that lead to a better understanding of carcinogenesis. The availability of new and sensitive high-throughput sequencing technologies sped up this search and led to the identification of new mutation hotspots in a variety of cancer entities. Although a new definition of a recurrent gene mutation was

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suggested in terms of combining recurrent alterations on the scale of a pathway rather than only one single gene, discovering new recurrent gene mutations is still a worthwhile approach in cancer research [1].

Recently, mutations within the promoter of the telomerase reverse transcriptase *(TERT)* gene that encodes the catalytic subunit of the telomerase were identified in familial and sporadic melanoma with a high frequency [2]. The immortality of cells is still a classical hallmark of tumors and reactivation of telomerase leading to telomere maintenance remains a fundamental process in carcinogenesis. Alterations within the coding region of the *TERT* gene are a rare event in cancers. Therefore, the identification of recurrent mutations within the core promoter of the *TERT* gene leading to new binding motifs for transcription factors of the ETS family attracted great interest among the cancer research field [3]. The consequences of these mutations are still not completely understood but they lead to a 2- to 4-fold increased transcriptional activity in vitro [4]. Subsequently, these mutations were found in several other malignancies, e.g. bladder carcinoma, thyroid cancer or cancers of the nervous system, and were discussed as early drivers for malignant transformation [3].

Expression and reactivation of telomerase has also been described as an important feature of prostate cancer (PrCa). Telomerase activity was found in up to 100% of analyzed PrCa cases [5]. Interestingly, high expression of telomerase components does not always result in mandatory telomerase activity [6]. In addition, a significant association between *TERT* expression and aggressive behavior of prostate tumors has been reported [7]. Recently, promising in vitro data were published showing telomerase as an important target of an antiandrogen therapy for PrCa, and the usefulness of boron derivatives as a telomerase inhibitor in PrCa cells [8, 9]. These data suggest telomerase inhibition as a reasonable therapeutic approach for the treatment of PrCa. The molecular and cellular pathways involved in telomerase reactivation in PrCa are still not clear. Expression of *TERT* and the activity of telomerase were shown to be regulated by androgen receptor (AR) signaling whereas exogenous expression of AR surprisingly led to inhibition of *TERT* transcription in PrCa cells [10, 11]. The genomic region of the *TERT* gene (chromosome 5p15.33) was not described as a region containing copy number alterations in prostate tumors, making gene amplification as a mechanism for *TERT* expression in PrCa unlikely [12]. Less is known about *TERT* promoter mutations in PrCa. To date only three studies with a combined number of 49 prostate tumors have reported sequence analysis of the *TERT* promoter and found no evidence for involvement of *TERT* promoter mutations in PrCa [13–15]. These data already indicate that the cellular mechanisms of telomerase reactivation in PrCa are only poorly understood and further clarification is needed. As *TERT* promoter mutations are a potential mechanism for a possible telomerase reactivation we wanted to further the discussion of this topic for PrCa. We therefore analyzed the core promoter region of the *TERT* gene containing the reported mutation hotspots in the largest series of PrCa examined to date.

Table 1. Characteristics of the study patients

PrCa cases, n	167
Age, years	
Range	46–87
Median	66
Mean	64.9±6.7
Stage, n	
Organ-confined disease	76
Non-organ-confined disease	89
No data available	2
Gleason score	
Range	3–10
Median	7
Gleason sum, n	
<7	52
7	54
>7	54
No data available	7

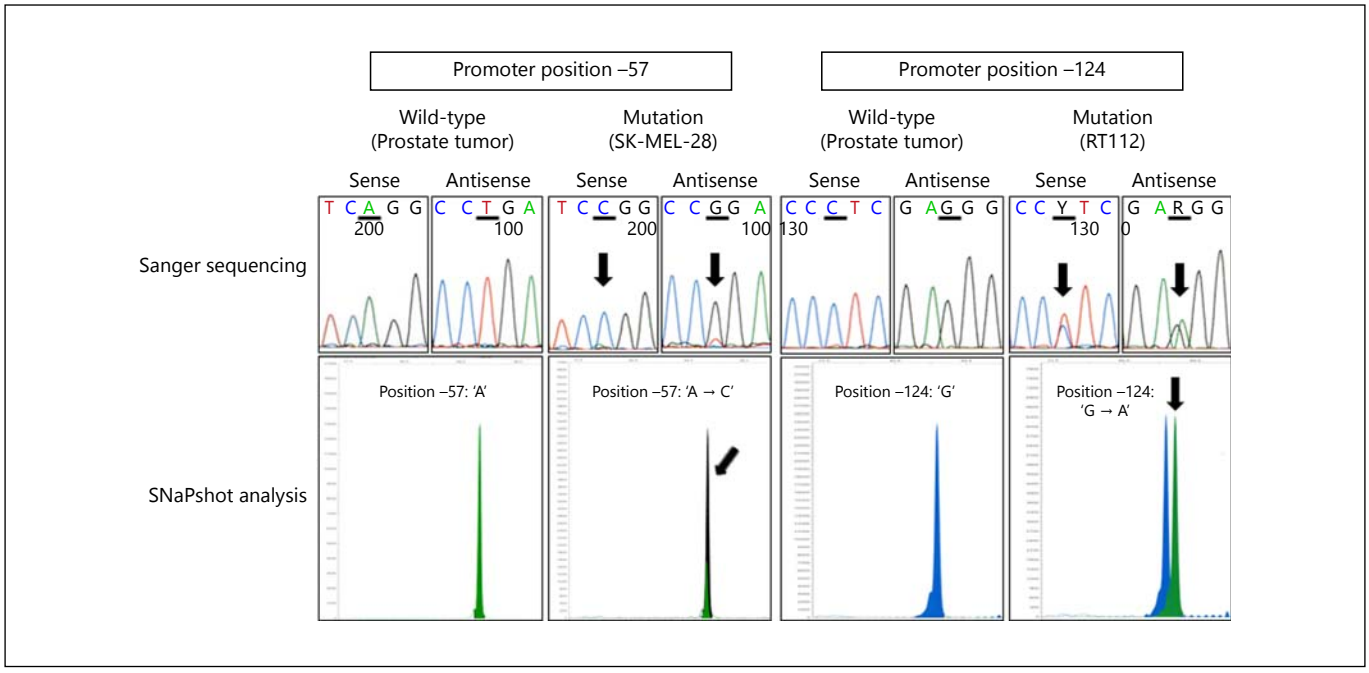
Materials and Methods

Patients and Tissue Samples

Overall, 167 unselected, archival prostate tumors (formalin-fixed and paraffin-embedded tissue samples, n = 119; snap-frozen tissue samples, n = 48) were investigated. All patients were Caucasians. The tumors were diagnosed according to the WHO classification of prostate tumors and staged according to the TNM system [16, 17]. The characteristics of the study participants are shown in table 1. Prior institutional review board (University Hospital Erlangen) approval was obtained for molecular analysis on archival material.

Tissue Microdissection and DNA Isolation

DNA was extracted from prostate tumors after precise manual microdissection (purity of tumor cells >85%) of serial sections (5 µm) using the High Pure PCR Template Preparation Kit (Roche GmbH, Mannheim, Germany) according to the manufacturer's instructions. DNA quality and quantity was determined using the Synergy 2 Multi-Detection Reader (BioTek, Bad Friedrichshall, Germany) according to the manufacturer's instructions.



Color version available online

Fig. 1. Representative examples for Sanger sequencing and SNaPshot analysis of the promoter mutation hotspots at –57 and –124. Upper lane: Sanger sequencing of DNA from a prostate tumor showing a wild-type sequence for *TERT* promoter position –57 (A) and position –124 (G). DNA from the melanoma cell line SK-MEL-28 showing 'A → C' mutation at position –57. DNA from the bladder cancer cell line RT112 showing 'G → A' mutation at position –124. Lower lane: corresponding SNaPshot analyses. Arrows indicate mutations in the promoter sequence.

TERT Promoter Analysis Using Sanger Sequencing

A region of the core promoter (–260 to +60) of the *TERT* gene containing the described mutation hotspots was amplified by PCR using primers (sense: 5′- att cgc ggg cac aga cgc -3′; antisense: 5′- tcg cgg tag tgg ctg cgc -3′) obtained from Metabion (Martinsried, Germany) in a total volume of 25 µl containing approximately 150 ng DNA, 0.2 mM dNTP (Promega, Mannheim, Germany), 0.18 µM primers, 5% DMSO and 0.0025 U/µl GoTaq (Promega). The thermal cycling conditions were as follows: initial denaturation for 3 min at 95°C, 45 cycles of denaturation at 94°C for 1 min, annealing at 69.3°C for 1 min, elongation at 72°C for 1 min and final primer extension at 72°C for 10 min. Gradient PCR was used for the optimization of cycling conditions. After amplification, PCR products (size 335 bp) were purified using the Qiagen Dye Ex 2.0™ Spin Kit according to the manufacturer's conditions. Sequence analysis was performed with PCR primers using a Big Dye Terminator v.1.1 Cycle Sequencing Kit and an ABI 3500 Genetic Analyzer (both Applied Biosystems, Foster City, Calif., USA).

TERT Promoter Analysis Using SNaPshot Analysis

A previously reported SNaPshot assay (Life Technologies Corp., Carlsbad, Calif., USA) was used for the detection of hotspot mutations at positions –57, –124 and –146. Capillary electrophoresis and detection of fluorescence-labeled products were performed using an Applied Biosystems ABI 3500 Genetic Analyzer. A detailed description of the method can be found elsewhere [18].

Cell Lines Used as Positive Controls for TERT Mutation Analysis

The malignant melanoma cell line SK-MEL28 derived form a 51-year-old male patient was kindly donated by Prof. Dr. A. Bosserhoff (Institute of Biochemistry and Molecular Medicine, FAU Erlangen-Nuremberg, Germany). SK-MEL28 cells showed a –57 A → C mutation of the *TERT* promoter. The urothelial carcinoma cell line RT112 derived from a female patient with a transitional cell carcinoma of the urinary bladder was purchased from the German collection of microorganisms and cell cultures (Leibniz Institute DMSZ, Braunschweig, Germany). RT112 cells harbor a –124 C → T mutation of the *TERT* promoter [4].

Results

Analysis of previously reported mutation hotspots within the core promoter of the *TERT* gene was successfully performed in all available cases. Mutation analysis using Sanger sequencing could be performed in 108/167 cases (fig. 1). In 59/167 cases the core promoter region could not be amplified due to insufficient DNA quality (e.g. DNA degradation, low DNA concentration). In these cases SNaPshot analysis of mutation sites at –57,

–124 and –146 was performed (fig. 1). Promoter mutations were not detected in any of the samples with all cases exhibiting a wild-type sequence.

Discussion

In the present study we performed sequence analysis of the core promoter region of the *TERT* gene in a cohort of 167 PrCa samples. The results of our study suggest that *TERT* promoter mutations are not involved in the development of PrCa as no mutations were detected in any of the investigated cases. These data are in line with previously published studies on only very small cohorts and corroborate the minor importance of *TERT* promoter alterations in PrCa [13–15]. Meanwhile, a study investigating the whole genomes of 57 PrCa cases has been published [19]. Apart from already known data from exome analyses, this study displayed the spectrum of whole-genome alterations in prostate tumors. Here only one *TERT* missense mutation was detected (p.R819C) but no promoter mutations were reported. This study also strengthens our findings and should, together with our data, finalize the discussion on *TERT* mutations in PrCa.

There are several lines of evidence that genomic variations but not mutation might influence TERT expression and disease risk in PrCa. In a large case-control study an intronic single nucleotide polymorphism (SNP) in the *TERT* gene (rs2242652, C 8992 T) was found that was strongly associated with increased PrCa risk. Because of this strong correlation it was suggested that the SNP might have a functional relevance. Indeed, further evaluation showed increased TERT expression associated with the presence of SNP variants in benign prostate tissue from patients who underwent radical prostatectomy [20, 21]. This increased TERT expression might provide a possible predisposition for PrCa. Another influence on *TERT* expression might be length polymorphisms in variable number tandem repeats (VNTRs). Recently, it was shown that the *TERT* gene contains five VNTRs that are located within introns 2 and 6. A large case-control study found a significantly higher PrCa risk for individuals carrying rare VNTR2-2nd alleles than for individuals with common alleles. These VNTRs were also discussed as having an enhancer function for gene transcription. In vitro studies on PrCa cell lines analyzing the activity of the *TERT* promoter in combination with different VNTR variants clearly showed an increased luciferase activity for the VNTR2-2nd variants [22, 23]. These effects might also be expected for *TERT* expression and could also increase the individual risk for PrCa.

Besides these genomic influences, TERT expression is also regulated by several cellular processes in PrCa. Matsumura et al. [24] analyzed the impact of the phosphorylation status of Fas-associated death domain-containing protein (FADD) on TERT expression in PrCa. FADD has a crucial role in the formation of the death-inducing signaling complex and is also involved in cell cycle regulation. The phosphorylated form of FADD was highly expressed in PrCa with a lower Gleason score and was inversely associated with a shorter recurrence-free survival after prostatectomy. In parallel, cases with high levels of phosphorylated FADD also showed only low TERT expression, suggesting a direct influence of FADD phosphorylation on TERT expression. Shimada et al. [25] found significant differences between FADD phosphorylation levels and clinicopathological outcomes for Gleason scores 3 + 4 and 4 + 3. These data indicate that Gleason 4 + 3 tumors should be considered as high-risk tumors and stimulating agents that drive transition from nonphosphorylated to phosphorylated FADD (e.g. paclitaxel) might be considered as therapeutic options. High TERT expression correlates with aggressive PrCa levels of TERT and nonphosphorylated FADD, which might represent potent biomarkers for the biological behavior of PrCa. Furthermore, different factors can regulate TERT expression positively or negatively. Several transcription factors (e.g. SP1), hormones (e.g. androgen) and the PI3K/Akt and MAP kinase pathways can upregulate *TERT* transcription [reviewed in 26]. In addition, the downregulation of *TERT* by six microRNAs (let-7g*, miR-133a, miR-138–5p, miR-342–5p, miR-491–5p and miR-541–3p) has recently been reported [27].

In summary, high expression of *TERT* is unlikely to be caused by promoter mutations or other genomic alterations in PrCa. *TERT* expression is more likely influenced by diverse cellular pathways resulting in increased cell cycle activity and proliferation.

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Disclosure Statement

The authors have no conflict of interest.

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