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MNS16A tandem repeat minisatellite of human telomerase gene and prostate cancer susceptibility

Philipp Hofer[1], Julia Zerelles[1], Andreas Baierl[2], Stephan Madersbacher[3], Georg Schatzl[4], Agnes Maj-Hes[5], Hedwig Sutterlüty-Fall[1] and Andrea Gsur[1,*]

[1]Department of Medicine I, Institute of Cancer Research, Comprehensive Cancer Center Vienna, Medical University of Vienna, A-1090 Vienna, Austria, [2]Department of Statistics and Operations Research, University of Vienna, A-1010 Vienna, Austria, [3]Department of Urology and Andrology, Sozialmedizinisches Zentrum Ost, A-1220 Vienna, Austria, [4]Department of Urology, Medical University of Vienna, A-1090 Vienna, Austria and [5]Department of Urology, Sozialmedizinisches Zentrum Süd, A-1100 Vienna, Austria

*To whom correspondence should be addressed. Tel: +43 1 40160 57541; Fax: +43 1 40160 957506; Email: andrea.gsur@meduniwien.ac.at

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Telomere dysfunction is an early event in the development of prostate cancer and telomerase (TERT) activity is detectable in the majority of prostate cancers. Genetic variation in *TERT* and its regulatory elements may influence prostate carcinogenesis. MNS16A, a functional polymorphic tandem repeat minisatellite of *TERT*, has been studied in several malignancies. We determined MNS16A genotypes in an Austrian case–control study for the first time in the context of prostate cancer, comprising 1165 prostate cancer cases and 674 benign prostate hyperplasia controls with PCR. In addition to the five reported variable number of tandem repeats (VNTRs), we identified VNTR-212, a rare variant, for the first time in a European population. Multiple logistic regression analysis revealed no differences in genotype distribution between cases and controls. However, in stratified analysis, MNS16A VNTR-274 (OR = 0.25, 95% CI = 0.06–0.79, *P* = 0.016) and genotype 274/302 (OR = 0.13, 95% CI = 0.01–0.58, *P* = 0.005) were associated with a significantly decreased risk of prostate cancer in the age group >70 years. Our finding of a MNS16A genotype conferring a protective effect against prostate cancer in older men suggests a potential role of this polymorphism in prostate cancer susceptibility but demands to be validated in further studies.

Introduction

Prostate cancer (PC) is the second most common cancer in men worldwide with 900 000 newly diagnosed cases in 2008 (1). Since the early 1990s, in Austria such as in many other developed countries, a strong increase in PC incidence rates due to widely used prostate-specific antigen (PSA) screening has been observed concomitant only with minute decline in PC mortality (2,3). Although a gradual decline in PC incidence was observed in Austria since 2003, this trend might largely be attributed to the earlier identification of PCs, including non-symptomatic indolent PCs. Overdiagnosis and overtreatment of clinically insignificant PCs are important issues in PC management (4). Therefore, the identification of biomarkers for PC beside the established clinical markers such as PSA and Gleason score is required.

So far genome-wide association studies (GWAS) identified >40 PC susceptibility loci, the highest number in common cancers [recently reviewed in ref. (5)]. One of them is the telomerase reverse transcriptase (*TERT*) locus on chromosome 5p15.33 associated with several malignancies beside PC underlining the importance of this locus in tumorigenesis (5–7).

Telomeres, the distal parts of eukaryotic chromosomes, are DNA–protein complexes that protect the chromosomal ends and consist of characteristic TTAGGG repeats in humans (8,9). Telomerase activity and maintenance of telomere length are hallmarks in human tumorigenesis. Telomere attrition, a consequence of cellular aging and the end-replication problem, can be counterbalanced by reactivation of telomerase enzymatic activity allowing cells to attain an unlimited proliferative potential. Dependent on a RNA component (*TERC*) and several other additional proteins and cofactors, the catalytic subunit of human telomerase (*TERT*) forms a multiprotein complex capable to add telomeric repeat sequence $(TTAGGG)_n$ to the ends of shortened chromosomes (10). Telomerase activity is usually absent in somatic human cells but present in germline cells as well as in the majority of cancer cells and *in vitro* immortalised cells (11–13). Telomere dysfunction is an early event in prostate carcinogenesis (14) and telomerase activity has been detected in the majority of PCs (15).

MNS16A, a polymorphic tandem repeat minisatellite of *TERT*, located downstream of exon 16 of *TERT* gene and upstream in the putative promoter region of an antisense *TERT* transcript, was first identified by Wang *et al*. (2003) (16). This minisatellite was shown to have promoter activity dependent on the number of tandem repeats. The structure of MNS16A was found to be characterised by two repeat elements forming a 23 bp, or when separated by a CAT trinucleotide insertion, a 26 bp core sequence. The sequence containing the CAT insert represents a transcription factor binding site for GATA-1. Four different variable number of tandem repeats (VNTRs) VNTR-243, VNTR-274, VNTR-302 and VNTR-333, named on the basis of their PCR fragment size, have been described (16). Recently, we identified in our colorectal cancer study a further rare variant namely VNTR-364 (17). Furthermore, in this study, we found the MNS16A VNTR-274 associated with a 2.7-fold increased colorectal cancer risk. This finding prompted us to investigate the influence of MNS16A VNTRs also on PC susceptibility in a hospital-based case–control study comprising 1165 PC patients and 674 controls with benign prostatic hyperplasia (BPH).

Materials and methods

Study population

In the ongoing Molecular Epidemiology Study of Prostate Cancer, 1839 participants, all of them Caucasians, were recruited from the departments of urology of three hospitals located in Vienna, Austria ('Sozialmedizinisches Zentrum Ost', 'Sozialmedizinisches Zentrum Süd' and the Medical University of Vienna). Cases (n = 1165) were patients

with newly diagnosed, histologically verified PC. The diagnosis of PC was obtained by transrectal ultrasound (TRUS)-guided biopsies in all patients. The indication for PC was either a suspicious finding on digital rectal examination (DRE) and/or elevated serum PSA levels. Further diagnostic work-up in these patients included a nuclear bone scan. The control group (n = 674) consisted of men with lower urinary tract symptoms due to BPH/benign prostatic enlargement, recruited in Vienna. In controls, PC was excluded either clinically by negative DRE and negative serum PSA according to age-specific reference values or histologically by TRUS-guided biopsies or transurethral resection of the prostate. Seventy-six percent of controls received a biopsy of the prostate. Written informed consent was obtained from all participants, and research protocols were approved by the institutional review boards.

Genotyping

Genomic DNA was purified from peripheral blood according to the QIAamp® DNA Blood Midi Spin Protocol (QIAGEN, Valencia, CA).

Twenty microlitre PCR reactions contained 40 ng genomic DNA, GeneAmp 1× PCR Buffer II, 1.5 mM $MgCl_2$, 150 nM deoxyribonucleotide mix, 0.3 units AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA) and 350 nM forward and reverse primers (VBC-BIOTECH Service, Vienna, Austria). MNS16A-specific primers were used as described by Wang *et al.* (16). PCR amplification was performed on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) with the following thermal profile: initial 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 65°C and 30 s at 72°C; with a final amplification step of 10 min at 72°C. MNS16A genotypes were determined by electrophoretical separation of PCR products in ethidium bromide-stained 2.5% agarose gels. Genotyping was performed blinded to case–control status and 15% of randomly chosen samples were re-genotyped for quality control.

Cloning

TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA) containing pCR®II-TOPO® vector was applied as described by the manufacturer. Plasmid DNA purification of positive clones was performed with Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI). For determination of insert sequence of different MNS16A VNTRs, a sequencing service was employed (Microsynth AG, Balgach, Switzerland). Sequence analysis was performed with Clone Manager Professional software version 9.0 (Scientific and Educational Software, Cary, NC, 1994–2007).

Statistical analysis

Genotypic counts of the control group were tested for Hardy–Weinberg equilibrium (HWE) using a chi-square test. Associations of MNS16A genotypes with risk of PC were estimated by computing odds ratios (ORs) and 95% confidence intervals (CIs) by logistic regression analysis with bias correction (R package logistf) (18). The application of Firth's modified score procedure in logistic regression analysis corrects for the well-known problem of small sample bias (19) and provides estimates in case of complete separation. In the case at hand, complete separation occurs if a certain genotype is never observed neither in cases nor in controls.

Age and smoking status were considered as confounding factors. Smoking status (current, former, never) did not prove to be a significant factor for the development of PC, but all regression models contain a linear and quadratic age term. Calculations were performed for MNS16A VNTRs, MNS16A genotypes and the MNS16A SL classification system, frequently applied in literature. VNTR-212, VNTR-243 and VNTR-274 were classified as short (S) variants and VNTR-302, VNTR-333 and VNTR-364 were classified as long (L) variants. On basis of this classification, MNS16A genotypes were assigned to SS, SL or LL genotype groups. MNS16A VNTRs, genotypes and SL groups were included as factors with the most common VNTR or genotype as reference category. Stratified analysis was performed for three equally spaced age groups in order to investigate the hypothesis of age-specific genetic mechanisms in the development of PCs. The observed age at diagnosis of 99% of patients ranged between 40 and 85 years. Hence, age groups were defined as '<55 years', '55–70 years' and '>70 years', respectively. Further subgroup analysis was conducted dividing the case group into patients with aggressive and non-aggressive form of PC. Aggressiveness was defined on basis of Gleason score with Gleason scores ≥7 classified as aggressive and Gleason scores <7 as non-aggressive PC. Additionally, patients with Gleason score 7 and patients with Gleason score 8–10 were also considered as separate groups in stratified analysis. All tests were two-sided and P values <0.05 were considered statistically significant. All statistical analyses were performed with R version 2.15.0 (20).

Results

Study population

Selected characteristics of the study population are presented in Table I. A total of 1839 participants of our Molecular Epidemiology of Prostate Cancer study was genotyped, including 1165 PC cases and 674 BPH controls. A total of 1137 cases and 650 controls were eligible for statistical analysis. Reduction in sample number was due to missing histology or covariate data.

The mean age of the case group (63.8 years) was significantly lower than the mean age of the control group (67.4 years, chi-square test, $P < 2.1 \times 10^{-15}$). Smoking status did not significantly differ between case and control group (chi-square test, $P = 0.469$). Therefore, statistical models were not adjusted for smoking status.

Genotyping

In total, 1839 individuals were genotyped by standard PCR followed by electrophoretical separation. Genotyping was successful in 98.1% of all attempts. For quality control, 15% of the samples were randomly selected and re-genotyped.

Table I. Selected characteristics of the study population

	PC case	PC Gleason score <7	PC Gleason score ≥7	BPH control
	n (%)	*n* (%)	*n* (%)	*n* (%)
Age (years)				
≤50	55 (4.8)	32 (6.3)	21 (4.3)	18 (2.8)
51–60	308 (27.1)	136 (26.9)	127 (25.9)	133 (20.5)
61–70	563 (49.5)	259 (51.3)	244 (49.8)	270 (41.5)
71–80	190 (16.7)	73 (14.5)	90 (18.4)	181 (27.8)
>80	21 (1.8)	5 (1.0)	8 (1.6)	48 (7.4)
Total	1137 (100.0)	505 (100.0)	490 (100.0)	650 (100.0)
Mean (SD[a])	63.8 (7.7)	63.4 (7.6)	63.9 (7.5)	67.4 (8.9)
Smoking				
Never	435 (41.4)	188 (40.7)	189 (41.7)	238 (38.4)
Former	465 (44.3)	203 (43.9)	199 (43.9)	291 (47.0)
Current	150 (14.3)	71 (15.4)	65 (14.3)	90 (14.5)
Smoking rate[b]	615 (58.6)	274 (59.3)	264 (58.2)	381 (61.5)
Total	1050 (100.0)	462 (100.0)	453 (100.0)	619 (100.0)

[a]SD, standard deviation.
[b]Current and former smokers.

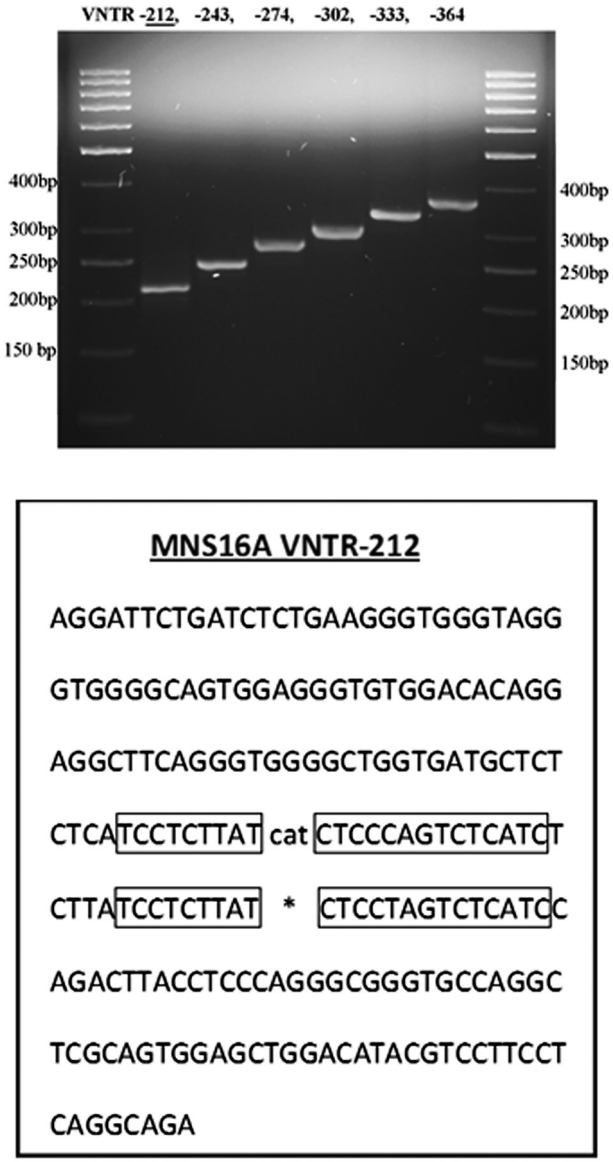

Fig. 1. VNTRs of TERT MNS16A. Six different MNS16A VNTRs were named on basis of PCR fragment size. Nucleotide sequence of VNTR-212 is given with characteristic repeat elements indicated by boxes.

The results of the repeat measurements were in complete congruence with the preceding genotype results.

Six different VNTRs of MNS16A were detected (MNS16A VNTR-212, VNTR-243, VNTR-274, VNTR-302, VNTR-333 and VNTR-364; Figure 1), occurring in 11 different genotype patterns. The genotype frequencies were 42.47% (302/302), 10.16% (243/243), 40.74% (243/302), 2.68% (302/333), 1.12% (243/274), 2.01% (274/302), 0.56 (243/333), 0.06% (302/364), 0.11% (274/333), 0.06% (212/302) and 0.06% (274/274), respectively. Genotypes 302/364 (n = 1), 274/333 (n = 2), 212/302 (n = 1) and 274/274 (n = 1) are very rare variants and were therefore excluded from statistical models. Genotype distributions of PC cases and BPH controls are given in Table II. Controls were tested for Hardy–Weinberg equilibrium and showed no significant deviation (P = 0.49).

Logistic regression analysis revealed no significantly different distribution of MNS16A genotypes between PC cases and BPH controls (Table III). Also the stratified analysis for Gleason score showed no significant differences in genotype distribution between PC cases with Gleason score <7 and BPH controls or between PC cases with Gleason score ≥7 and BPH controls. Furthermore, no differences in genotype distribution between PC cases with Gleason score 7 or >7 and BPH controls were observed (data not shown). However, for the age group >70 years, MNS16A VNTR-274 (OR = 0.25, 95% CI = 0.06–0.79, P = 0.016) and genotype 274/302 (OR = 0.13, 95% CI = 0.01–0.58, P = 0.005) were associated with a significantly decreased risk of PC (Table IV).

Cloning

Sequence of VNTR-212 was confirmed by cloning and sequencing (Figure 1). VNTR-212 is the shortest known MNS16A VNTR and is by 31 bp shorter than VNTR-243. It can be described as a VNTR-243 that lost one of its tandem repeats separated by CAT trinucleotide and hence, of all six reported MNS16A VNTRs, carries the smallest number of GATA-1 transcription factor binding sites.

Discussion

MNS16A, a functional minisatellite of *TERT*, discovered in 2003 by Wang *et al*. (16), has been investigated in several malignancies such as lung (16,21–23), breast (24), brain (25–27) and colorectal cancer (17) so far. This study is the first investigating genetic associations of MNS16A concerning PC. In our Austrian hospital-based case–control study comprising 1165 PC cases and 674 BPH controls, we observed no statistically significant association of MNS16A genotypes with risk of PC in the overall patient group. Stratified analysis for Gleason score was performed for aggressive PCs with Gleason score ≥7 and non-aggressive PCs with Gleason score <7. Because Gleason score 7 is a relatively heterogeneous histological group (28), we considered Gleason score 7 also as distinct group in statistical analyses. However, we observed no significant differences in genotype distribution of aggressive PCs and PCs with less aggressive form. However, in stratification for age, an association of MNS16A VNTR-274 (OR = 0.25, P = 0.016) and the MNS16A genotype 274/302 (OR = 0.13, P = 0.005) with a significantly decreased risk of PC was found in the age group >70 years suggesting a protective effect of VNTR-274 in older patients.

In accordance with our findings, Zhang *et al*. (29) recently reported a reduced risk of nasopharyngeal carcinoma (NPC) for carriers of VNTR-274 and 274/302 genotype. In this case–control study of 855 NPC patients and 1036 cancer-free controls, a reduced risk for SL (OR = 0.73, P = 0.037) and SS + SL (OR = 0.71, P = 0.025) genotype groups, containing the VNTR-274 variant allele, was observed when compared with the LL reference. Likewise, VNTR-274 (OR = 0.65, P = 0.058) and 274/302 genotype (OR = 0.64, P = 0.059) were associated with a lower risk of NPC when compared with the wild-type VNTR-302 and the wild-type genotype 302/302, respectively. The protective effect of reduced NPC risk conferred by SS + SL genotype was also more pronounced in older patients (≥45 years) and correlated with lower levels of TERT protein expression measured by immunohistochemistry assay in patients with SL genotypes. However, MNS16A genotypes did not correlate with progression of NPC.

In contrast, we previously reported a significant association of VNTR-274 with an increased risk of colorectal cancer

Table II. Genotype distributions of TERT MNS16A

Genotype	SL classification	PC case	PC Gleason score <7	PC Gleason score ≥7	BPH control
		n (%)	*n* (%)	*n* (%)	*n* (%)
302/302	LL	474 (41.7)	215 (42.6)	200 (40.8)	287 (44.2)
243/243	SS	124 (10.9)	56 (11.1)	54 (11.0)	58 (8.9)
243/302	SL	472 (41.5)	202 (40.0)	209 (42.7)	258 (39.7)
302/333	LL	27 (2.4)	14 (2.8)	10 (2.0)	21 (3.2)
243/274	SS	13 (1.1)	4 (0.8)	7 (1.4)	7 (1.1)
274/302	SL	18 (1.6)	11 (2.2)	5 (1.0)	18 (2.8)
243/333	SL	9 (0.8)	3 (0.6)	5 (1.0)	1 (0.2)
Total		1137 (100.0)	505 (100.0)	490 (100.0)	650 (100.0)

Rare genotypes 302/364, 274/333, 212/302 and 274/274 were excluded from statistical models.

Table III. Association of TERT MNS16A genotype and PC risk

MNS16A	PC case	BPH control	OR (95% CI)[a]	*P* value[b]
	n (%)	*n* (%)		
Genotypes				
302/302	474 (41.7)	287 (44.2)	1	
243/243	124 (10.9)	58 (8.9)	1.27 (0.90–1.81)	0.182
243/302	472 (41.5)	258 (39.7)	1.13 (0.91–1.41)	0.261
302/333	27 (2.4)	21 (3.2)	0.74 (0.41–1.36)	0.332
243/274	13 (1.1)	7 (1.1)	1.00 (0.40–2.65)	0.992
274/302	18 (1.6)	18 (2.8)	0.67 (0.33–1.33)	0.248
243/333	9 (0.8)	1 (0.2)	3.12 (0.71–29.31)	0.144
Total	1137 (100.0)	650 (100.0)		
VNTRs				
302	1465 (64.4)	871 (67.0)	1	
243	742 (32.6)	382 (29.4)	1.15 (0.99–1.34)	0.068
274	31 (1.4)	25 (1.9)	0.75 (0.44–1.31)	0.313
333	36 (1.6)	22 (1.7)	0.90 (0.53–1.56)	0.696
Total	2274 (100.0)	1300 (100.0)		
SL system				
LL	501 (44.1)	308 (47.4)	1	
SL	499 (43.9)	277 (42.6)	1.14 (0.92–1.40)	0.229
SS	137 (12.0)	65 (10.0)	1.26 (0.91–1.77)	0.167
SL+SS	636 (55.9)	342 (52.6)	1.16 (0.95–1.42)	0.136

[a]Odds ratio with 95% confidence interval were adjusted for age.
[b]$P < 0.05$ was considered significant.

Table IV. Association of MNS16A and PC risk in the age group >70 years

MNS16A	PC case	BPH control	OR (95% CI)[a]	*P* value[b]
	n (%)	*n* (%)		
Genotypes				
302/302	91 (43.1)	94 (41.0)	1	
243/243	21 (10.0)	22 (9.6)	0.85 (0.43–1.71)	0.655
243/302	92 (43.6)	92 (40.2)	1.10 (0.72–1.69)	0.663
302/333	4 (1.9)	8 (3.5)	0.51 (0.14–1.65)	0.265
243/274	2 (0.9)	2 (0.9)	0.99 (0.13–7.70)	0.996
274/302	**1 (0.5)**	**11 (4.8)**	**0.13 (0.01–0.58)**	**0.005**
243/333	0 (0.0)	0 (0.0)		
Total	211 (100.0)	229 (100.0)		
VNTRs				
302	279 (66.1)	299 (65.3)	1	
243	136 (32.2)	138 (30.1)	1.04 (0.76–1.40)	0.821
274	**3 (0.7)**	**13 (2.8)**	**0.25 (0.06–0.79)**	**0.016**
333	4 (0.9)	8 (1.7)	0.52 (0.14–1.64)	0.269
Total	422 (100.0)	458 (100.0)		
SL system				
LL	95 (45.0)	102 (44.5)	1	
SL	93 (44.1)	103 (45.0)	1.03 (0.68–1.56)	0.898
SS	23 (10.9)	24 (10.5)	0.91 (0.47–1.76)	0.769
SL + SS	116 (55.0)	127 (55.5)	1.00 (0.68–1.49)	0.992

[a]Odds ratio with 95% confidence interval were adjusted for age.
[b]$P < 0.05$ was considered significant (bold).

(17). Also, in a Chinese breast cancer study (22), 274/302 genotype was associated with a significantly increased risk of axillary lymph node metastasis. These findings suggest a potential relevance of VNTR-274 in susceptibility of these cancers but require further investigation. Although MNS16A studies concerning several malignancies have been conducted so far, the findings on clinical relevance regarding both risk and survival time are conflicting (recently discussed) (17). Different study designs and selection bias as well as different genotyping methods and the differences of genotype frequencies between Asian and Caucasian populations may contribute to these discrepancies. Although the effect of MNS16A genotype on occurrence of disease is thought to act via modulating the repression of *TERT* expression, telomerase reactivation is gradually important or required in tumorigenesis of different cancers. Differences in molecular mechanisms in pathogenesis of different malignancies make it difficult to compare these findings. In prostate carcinogenesis, telomere dysfunction, a driver of genetic instability, is an early event in tumour development and telomerase activity is present in the majority of PCs [reviewed in ref. (30)]. It can be hypothesised that VNTR-274 by promoting *TERT* repression over time delays onset of PC and thereby confers a protective effect for patients carrying VNTR-274, or the 274/302 genotype, respectively.

A strength of our study is the high quality control group, especially important in the context of PC, a malignancy highly frequent in older men. Controls were patients with histologically confirmed BPH, known to be free of PC or BPH patients where PC was clinically excluded by negative DRE and negative serum PSA. Selection bias was omitted by hospital-based recruitment of both cases and controls. However, a limitation was limited sample size in subgroup analysis and significant associations depended on a relatively small number of subjects with this genotype.

More than 40 PC susceptibility loci on various chromosomes have been identified by GWASs so far underlining the strong genetic influence on PC development. One of these loci is 5p15 harbouring variants rs2242652 of *TERT* (7), rs401681 and rs2736098 (6). Besides single nucleotide polymorphisms, associations of a further polymorphic minisatellite of *TERT*, VNTR 2-2nd with PC susceptibility were reported (31). Genetic variants of *TERT* could predispose to cancer by causing telomere dysfunction leading to chromosomal instability and thereby driving tumour formation. It could be worth investigating whether these risk variants of *TERT* locus interact with MNS16A and other tandem repeat polymorphisms of the human telomerase gene (32).

Because telomerase and telomeres play an important role in PC development, *TERT* expression and telomere length were discussed as markers for PC. Tumour telomere DNA content was associated with recurrence of PC, independent of age, Gleason score or lymph nodal status by Fordyce *et al.* (33). Recently, the combined analysis of *TERT* expression with *TMPRSS2-ERG* [transmembrane protease, serine 2–v-ets erythroblastosis virus E26 oncogene homologue (avian)] fusion, one of the most common genetic rearrangements observed in PC, was suggested as predictor of biochemical recurrence of PC after radical prostatectomy (34). Nevertheless, no single independent marker is likely to significantly augment the level of diagnostic and prognostic accuracy in the management of PC (35). However, the combination of clinically established markers with markers recently identified by proteomic screens and DNA methylation profiling, presently being under validation, may substantially improve the present situation. Our report of *TERT* MNS16A genotype 274/302 and VNTR-274 conferring a protective effect against PC in older men indicates a potential role of this polymorphism in prostate carcinogenesis and demands to be validated in further studies.

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