

## MNS16A tandem repeats minisatellite of human telomerase gene: a risk factor for colorectal cancer

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**Telomerase reactivation and expression of human telomerase gene [human telomerase reverse transcriptase (hTERT)] are hallmarks of unlimited proliferation potential of cancer cells. A polymorphic tandem repeats minisatellite of hTERT gene, termed MNS16A was reported to influence hTERT expression. To assess the role of MNS16A as potential biomarker for colorectal cancer (CRC), we investigated for the first time the association of MNS16A genotypes with risk of colorectal polyps and CRC. In the ongoing colorectal cancer study of Austria (CORSA), 3842 Caucasian participants were recruited within a large screening project in the province Burgenland including 90 CRC cases, 308 high-risk polyps, 1022 low-risk polyps and 1822 polyp free controls verified by colonoscopy. MNS16A genotypes were determined by polymerase chain reaction from genomic DNA. Associations of MNS16A genotypes with CRC risk were estimated by logistic regression analysis computing odds ratios (ORs) and 95% confidence intervals (CIs). We identified five different variable number of tandem repeats (VNTRs) of MNS16A including VNTR-364, a newly discovered rare variant. VNTR-274 allele was associated with a 2.7-fold significantly increased risk of CRC compared with the VNTR-302 wild-type (OR = 2.69; 95% CI = 1.11–6.50; *P* = 0.028). In our CORSA study, the medium length VNTR-274 was identified as risk factor for CRC. Although, this population-based study herewith reports the largest cohort size concerning MNS16A thus far, further large-scale studies in diverse populations are warranted to confirm hTERT MNS16A genotype as potential biomarker for assessment of CRC risk.**

### Introduction

Telomeres are highly conserved functional structures at the distal parts of eukaryotic chromosomes protecting them from degradation and end-to-end fusion (1). Telomerase gene [human telomerase reverse transcriptase (hTERT)], located on chromosome band 5p15.33 (2) encodes a ribonucleoprotein enzyme that extends chromosome ends, which have been shortened during successive cycles of cell division (3). Telomerase holoenzyme consists of a catalytic subunit (hTERT), a functional RNA subunit serving as template for telomeric DNA synthesis (human telomerase RNA), other telomerase components and several telomerase-associated proteins (e.g. reviewed in ref. 4). Telomerase activity is usually absent in somatic human cells but present in >90% of cancer cells and *in vitro* immortalized cells (5,6) underlining the importance of telomerase in tumorigenesis and cellular ageing.

**Abbreviations:** CI, confidence interval; CORSA, colorectal cancer study of Austria; CRC, colorectal cancer; FOBT, faecal occult blood test; GBM, glioblastoma multiforme; HR, hazard ratio; hTERT, human telomerase reverse transcriptase; HWE, Hardy–Weinberg equilibrium; NSCL, non-small cell lung cancer; OR, odds ratio; PCR, polymerase chain reaction; VNTR, variable number of tandem repeat.

Several studies correlated length of telomeres with hTERT expression in colorectal cancer (CRC) (7–14) and genome-wide association studies recently associated TERT-CLPTM1L locus with several cancer types including lung, urinary bladder, cervix and prostate cancer (15).

In 2003, Wang *et al.* (16) first reported in a lung cancer study the identification of a polymorphic tandem repeats minisatellite of hTERT termed MNS16A. This minisatellite is located down-stream of exon 16 of hTERT gene and up-stream in the putative promoter region of an antisense hTERT transcript. Detection of antisense messenger RNA of hTERT suggested a possible role in regulation of human telomerase expression. MNS16A was found to have two repeat elements forming a 23 bp core sequence or a 26 bp core sequence with a CAT insertion representing a transcription factor binding site for GATA-1. Two different variable number of tandem repeats (VNTRs) alleles, VNTR-302 and VNTR-243 were named on basis of their polymerase chain reaction (PCR) fragment size. In addition, two other rare alleles (VNTR-272 and VNTR-333) were discovered in cancer cell lines. For statistical analysis, a classification in short alleles (S alleles: VNTR-243 and VNTR-272) and long alleles (L alleles: VNTR-302 and VNTR-333) was introduced by Wang *et al.* (16). Promoter activity was demonstrated to depend on the number of tandem repeats implicating functionality of MNS16A genotype. It was proposed that this minisatellite might act as a repressor for the promoter of the hTERT antisense transcript and an influence of MNS16A on lung cancer susceptibility was suggested. Following studies investigated MNS16A as potential biomarker for glioblastoma (17–19), breast (20) and lung cancer (21,22) but with contradictory findings on clinical relevance regarding both risk and survival time.

In Austria, the incidence of CRC is in the top third within the European Union and CRC is the second most common cause of cancer-related death in women and men nationwide (GLOBOCAN 2008, <http://globocan.iarc.fr/>, accessed 30 September 2010). There is a remarkable decline in CRC incidence rates from east to west in Austria with the highest rates in the province Burgenland (23).

Our ongoing colorectal cancer study of Austria (CORSA) is nested within a large screening project covering the whole province Burgenland. This study comprised 3842 participants and is herewith the largest population concerning MNS16A thus far. Furthermore, in our CORSA study, we investigated MNS16A in the context of CRC for the first time. The purpose of this study was to examine the association of this polymorphic tandem repeats minisatellite with risk of colorectal polyps and CRC as potential biomarker for the identification of high-risk individuals to improve CRC screening.

### Materials and methods

#### Study population

In the ongoing molecular epidemiology, CORSA 3842 Caucasian participants were recruited since May 2002 within a large province-wide screening project in the province Burgenland, Austria. This screening project 'Burgenland against CRC' uses faecal occult blood tests (FOBT) and all inhabitants of Burgenland aged between 40 and 80 years are invited to take part in the screening programme. Participants with a positive faecal occult blood testing receive further diagnostic workup such as colonoscopies. These persons are asked to participate in our 'Molecular epidemiology study of CRC'. Results of colonoscopies are collected in a central database and standardized documentation guidelines are followed. Demographic and anthropometric factors as well as dietary and smoking habits are assessed by a short questionnaire. All subjects gave written informed consent. The study was approved by the institutional review board 'Ethikkommission Burgenland'.

Between 2003 and 2008, the target group of the screening programme consisted of 698 951 female and male inhabitants aged between 40 and 80 years and a total of 33.6% (234 808) participated in the programme. 20 968 individuals

(8.9%) were tested FOBT positive and 13 630 colonoscopies were performed. Within this time interval, 347 cases of CRC 1648 high-risk polyps, 2014 low-risk polyps and 816 hyperplastic polyps were diagnosed.

All participants (100%) in this study had positive FOBT and underwent complete colonoscopy. CRC cases were patients with histologically confirmed, previously untreated CRC, firstly diagnosed within this screening programme ( $n = 90$ ). For statistical analysis, the polyp group consisting of 1330 patients was classified by their histopathology as high-risk ( $n = 308$ ) and low-risk ( $n = 1022$ ) polyps according to the amount of villous elements. Adenomatous villous, adenomatous tubulovillous and co-occurrence of adenomatous tubular with tubulovillous polyps were classified as high-risk polyps, whereas hyperplastic polyps which have no malignant potential and adenomatous tubular polyps were assigned to the low-risk group. The control group comprised 1822 individuals with positive FOBT who underwent colonoscopy but exhibited no pathological findings. Individuals with serious medical conditions at baseline including all malignant diseases were excluded from the study.

#### DNA purification and genotyping of MNS16A

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 10× PCR Buffer II, MgCl<sub>2</sub> Solution (1.5 mM MgCl<sub>2</sub>), deoxyribonucleotide Mix (150 nM dNTPs), AmpliTaq DNA Polymerase (0.3 U) (Applied Biosystems, Foster City, CA) and 350 nM forward and reverse primer (VBC-BIOTECH Service, Vienna, Austria). MNS16A primers were used as described by Wang *et al.* (16) PCR products were amplified on a 2720 Thermal Cycler (Applied Biosystems) by the following thermal profile: 95°C (5 min); 35 cycles of 95°C (30 s), 65°C (30 s), 72°C (30 s) and 72°C (10 min). MNS16A genotypes were determined by electrophoretic 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.

Several PCR products were also loaded on 5% non-denaturing polyacrylamide gels and electrophoretic separation was performed at constant 80 V (50 min). For detection, polyacrylamide gels were stained in a 1:10 000 dilution of VisTra Green™ Nucleic Acid Stain (Amersham Biosciences, Piscataway, NJ).

#### Cloning of MNS16A PCR products and allele confirmation by sequencing

For cloning of size-selected MNS16A PCR products, the TOPO TA Cloning® Kit containing pCR®II-TOPO® vector was applied as described by the man-

ufacturer (Invitrogen, Carlsbad, CA). Plasmid DNA purification of positive clones was performed with Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI). For verification of insert sequence of different VNTRs a sequencing service was engaged (QIAGEN GmbH Genomic Services, Hilden, Germany). Sequence analysis was carried out with Clone Manager Professional version 9.0 (Scientific and Educational Software, Cary, NC, 1994–2007).

#### Statistical analysis

Genotypic counts for each patient group (CRC, high-risk polyp, low-risk polyp and control) were tested for Hardy–Weinberg equilibrium (HWE) using  $\chi^2$ -tests. Due to low cell counts caused by low allele frequencies,  $P$  values for the  $\chi^2$  tests were simulated using a simulation/permutation method implemented in the statistical programming language R (function HWE.test, package genetics). Exact significance tests are not available for situations where more than two alleles occur.

Associations between MNS16A genotypes and CRC risk were estimated by computing odds ratios (ORs) and 95% confidence intervals (CIs) by logistic regression analysis with adjustment for age, sex, smoking (current, former and never) and education. MNS16A genotypes were included as factors with the most common genotype as reference category. Calculations were performed for single alleles, genotypes and SL converted system. For the SL classification system, VNTR-243 and VNTR-274 were regarded as short (S) and VNTR-302, VNTR-333 and VNTR-364 as long (L) variant alleles. All tests were two-sided and  $P$  values <0.05 were considered statistically significant. All statistical analyses were performed with R Version 2.11.1 (R Development Core Team, 2010).

## Results

#### Study population

Characteristics of the study population are given in Table I. A total of 3803 participants of the CORSA study was analysed, consisting of 90 patients with CRC, 308 patients with high-risk polyps, 1022 patients with low-risk polyps and 1822 colonoscopy-negative controls. Reduction of sample number eligible to statistical analysis was due to incomplete histology or confounding data. The mean age of the CRC case group (66.8 years) was significantly higher than the mean age of the control group (61.3 years;  $F$ -test,  $P < 0.0001$ ). The study

**Table I.** Selected characteristics of the study population

	CRC <sup>a</sup> N (%)	High-risk polyp <sup>b</sup> N (%)	Low-risk polyp <sup>c</sup> N (%)	Control <sup>d</sup> N (%)
Age (years)				
≤50	8 (1.5)	45 (8.3)	135 (24.9)	354 (65.3)
51–60	18 (2.4)	63 (8.4)	238 (31.7)	433 (57.6)
61–70	24 (2.2)	115 (10.6)	372 (34.2)	576 (53.0)
71–80	34 (4.3)	80 (10.2)	258 (32.8)	414 (52.7)
≥80	6 (14.3)	2 (4.8)	14 (33.3)	20 (47.6)
Total	90 (100)	305 (100)	1017 (100)	1797 (100)
Mean (95% CI)	66.8 (45.8–87.7)	62.9 (43.2–82.7)	63.2 (43.2–83.2)	61.3 (39.4–83.3)
Sex				
Male	58 (3.3)	205 (11.6)	663 (37.5)	841 (47.6)
Female	32 (2.2)	101 (7.0)	354 (24.5)	957 (66.3)
Total	90 (100)	306 (100)	1017 (100)	1798 (100)
Education				
Elementary	52 (3.2)	155 (9.5)	552 (33.7)	879 (53.7)
Secondary modern	28 (2.3)	120 (9.9)	359 (29.6)	707 (58.2)
Matura	5 (2.5)	13 (6.6)	56 (28.4)	123 (62.4)
University	3 (6.5)	5 (10.9)	15 (32.6)	23 (50.0)
Total	88 (100)	293 (100)	982 (100)	1732 (100)
Smoking				
Current	13 (2.6)	56 (11.0)	198 (39.0)	241 (47.4)
Former	37 (4.0)	93 (10.1)	310 (33.6)	483 (52.3)
Never	40 (2.3)	148 (8.7)	489 (28.7)	1029 (60.3)
Total	90 (100)	297 (100)	997 (100)	1753 (100)

<sup>a</sup>CRC cases.

<sup>b</sup>Adenomatous villous, tubulovillous and villous + tubulovillous polyps.

<sup>c</sup>Adenomatous tubular and hyperplastic polyps.

<sup>d</sup>Polyp-free controls.

population consisted of 45.0% females and 55.0% males. Male subjects were overrepresented in all three case groups (64.4% of CRC, 67.0% of high-risk and 65.2% of low-risk polyps;  $\chi^2$ -test,  $P < 0.0001$ ). In total, 3.3% of men had developed CRC (2.2% of women), 11.6% high-risk and 37.5% low-risk polyps compared with 7.0 and 24.5% of females, respectively.

*Sequencing of MNS16A VNTRs*

Sequencing of cloned MNS16A PCR products yielded in the identification of five individual MNS16A variant sequences displayed in Figure 1. With increasing fragment length, alleles gain one additional tandem repeat. The MNS16A VNTRs-243, -274, -302, -333 and -364 contain 3, 4, 5, 6 and 7 tandem repeats, respectively, with 2, 3, 3, 4 and 5 CAT trinucleotide insertions, respectively. The newly identified MNS16A VNTR-364 represents a '333 allele' with an additional 31 bp insert of one tandem repeat carrying an extra (fifth) CAT trinucleotide. VNTR-364 thereby carries the highest number of GATA-1 transcription factor binding sites. Regarding VNTR-243 and VNTR-302, we found minute differences from the core sequence published by Wang *et al.* (16) apparent in two bases (indicated by grey highlighting, Figure 1). Through checking the sequencing chromatograms, we can exclude sequencing errors. This difference was found in all five fragments we sequenced coming from five different individuals. Thus, the insertion site of additional tandem repeats can be tracked easily.

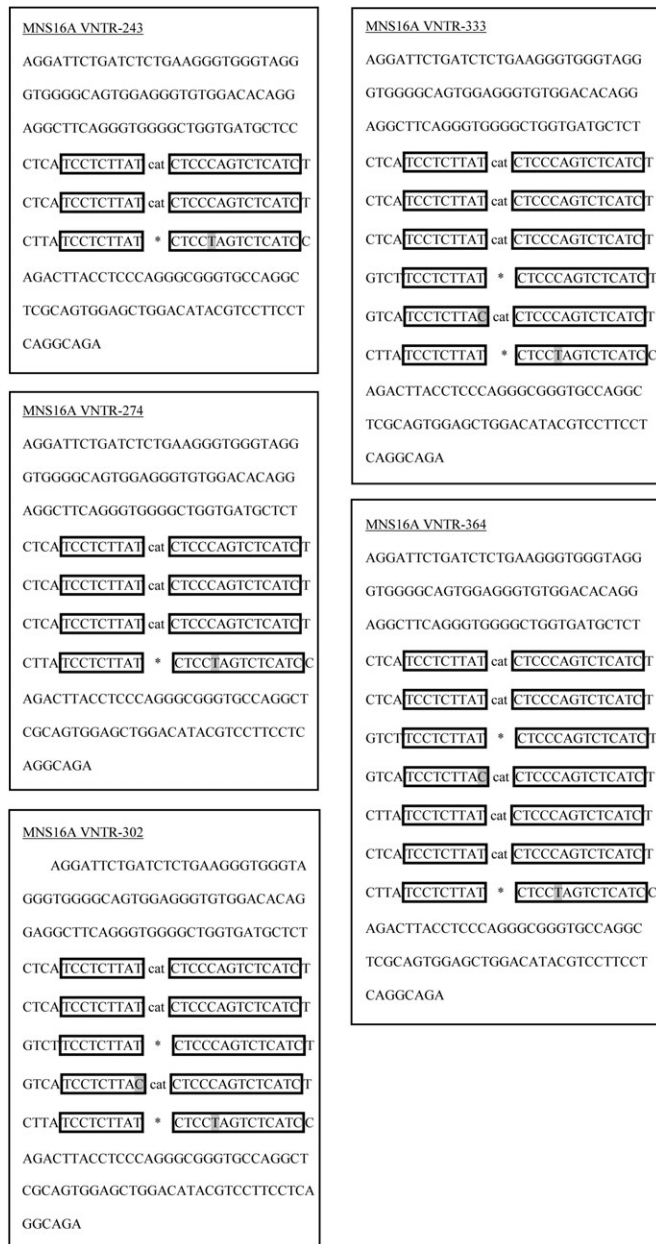
A BLAST search for the MNS16A VNTR-302 using Clone Manager led to a 100% match with hTERT sequence (NT\_006576.16; accessed 02 March 2010). hTERT gene sequence including adjacent 3' sequence was obtained from 'nucleotide' search and alignment in Clone Manager resulted in 100% match of VNTR-302 molecule with hTERT sequence (NG\_009265.1; www.ncbi.nlm.nih.gov). The congruent region of NG\_009265.1 with VNTR-302 was found between bases 47512-47813.

*MNS16A genotyping*

Three thousand eight hundred and forty-two individuals were genotyped for MNS16A alleleotype by electrophoretical separation of PCR products. Genotyping was successful in 99.1% of all attempts. Fifteen per cent of randomly chosen samples were re-genotyped for quality control and results were in 100% accordance with previous findings. In total, five different MNS16A variants were detected emerging in ten different genotype patterns (Figure 2).

Heterozygotes of MNS16A frequently exhibited a weak third band above the two main band signals in agarose gels. When loaded on polyacrylamide gels, only heterozygous samples showed frequently a third band at ~700 bp (data not shown). Also, a mixture of two homozygous samples previously found to exhibit only one distinct band was used as template for PCR, leading to the same additional band in agarose gels supporting the findings of conformational changes of PCR products only being responsible for the additional band signal (20). Furthermore, we demonstrated the existence of VNTR-333 by successful cloning and sequencing.

All genotyped samples with valid histology data were tested for HWE.  $P$  values of  $\chi^2$ -tests for each patient group were 0.980 (control), 0.943 (CRC), 0.884 (high-risk polyp) and 0.981 (low-risk polyp). The frequency distribution of genotypes of this study population was 42.36% for MNS16A genotype pattern 1, 10.07% for 2, 40.55% for 3, 2.50% for 4, 0.95% for 5, 2.47% for 6, 1.03% for 7, 0.03% for 8, 0.03% for 9 and 0.03% for 10 referring to the MNS16A genotype patterns stated in Figure 2. Genotypes 333/333, 302/364 and 274/333 are very rare variants and were found only once each. The variants 302/302 (42.36%) and 243/302 (40.55%) were similarly represented within the study population followed by the homozygous variant 243/243 (10.07%). The three rare variants, represented by only one individual each were excluded from further models. Genotype distribution by histology is given in Table II identifying the highest percentages of CRC for the MNS16A genotypes 243/274 (6.45%), 274/302 (5.56%) and 243/333 (5.71%). Remarkably, all 274 alleles



**Fig. 1.** Sequences of hTERT MNS16A VNTRs. Alleles were named on basis of the amplicon length. CAT indicates insert of CAT trinucleotide between repeat elements, whereas \* represents no CAT insert.

eligible for statistical models were contained within this group of MNS16A genotypes with the highest amount of CRC.

Logistic regression models for the seven considered MNS16A genotypes (allele combinations) could not identify a significantly increased risk for distinct genotypes (Table III). Moreover, calculation of pooled genotype groups (SS, SL and LL groups) as applied in literature could not detect significant differences in genotype distribution of case and control groups.

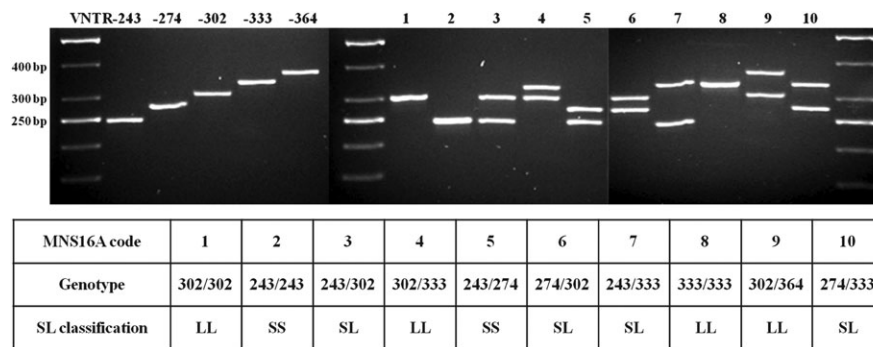
But, logistic regression analysis for single alleles (effects of single alleles not biased by the partner allele) identified MNS16A VNTR-274 as risk factor for CRC. The four alleles (243, 274, 302 and 333) occurring in the seven considered allele combinations were distinctly added up, consequently, yielding in a doubled sample number (3058 allele combinations, 6116 alleles). The 274 allele was associated with a 2.7-fold increased risk of CRC compared with 302 wild-type allele (OR = 2.69; 95% CI = 1.11–6.50;  $P = 0.028$ ).



**Table II.** Genotype distributions of hTERT MNS16A in CRC, high-risk polyp, low-risk polyp and control group

Genotype <sup>a</sup>	SL classification	CRC N (%)	High-risk polyp N (%)	Low-risk polyp N (%)	Control N (%)
302/302	LL	35 (2.68)	127 (9.72)	411 (31.45)	734 (56.16)
243/243	SS	6 (1.97)	22 (7.21)	97 (31.80)	180 (59.02)
243/302	SL	38 (3.06)	116 (9.35)	395 (31.85)	691 (55.73)
302/333	LL	1 (1.47)	11 (16.18)	20 (29.41)	36 (52.94)
243/274	SS	2 (6.45)	1 (3.23)	13 (41.94)	15 (48.39)
274/302	SL	4 (5.56)	8 (11.11)	20 (27.78)	40 (55.56)
243/333	SL	2 (5.71)	3 (8.57)	14 (40.00)	16 (45.71)

<sup>a</sup>Three very rare genotypes (333/333, 302/364 and 274/333) were excluded from statistical models.



**Fig. 2.** Genotype patterns of hTERT MNS16A. Five identified VNTRs were detected in 10 allele combinations named MNS16A genotype patterns 1–10. For SL classification, VNTR-243 and VNTR-274 were considered as short (S) and VNTR-302, VNTR-333 and VNTR-364 as long (L) variant alleles.

## Discussion

In our CORSA study, the hTERT MNS16A VNTR-274 was found to be associated with a significantly increased risk of CRC, suggesting an influence on CRC susceptibility. Also, a novel variant with an additional tandem repeat was discovered carrying a further binding site for the zinc finger transcription factor GATA-1 (16). MNS16A genotypes were determined in this study for the first time in an Austrian population and also for the first time ever in the context of CRC. Furthermore, the strength of this study is that it comprises the largest study population until now consisting of 3842 participants, investigating MNS16A genotypes. A further strength of CORSA is that controls were known to be free of polyps and CRC because all participants underwent complete colonoscopy. Such a high-quality control group, also referred to as ‘supercontrols’ (24) can enhance the statistical power of a study. However, a limitation of this study is, as expected in a population-based screening programme, that the number of CRC patients is rather small.

MNS16A was first identified in the course of a pilot scale hospital-based lung cancer study (16) comprising 53 cases and 72 cancer-free controls. The MNS16A LL genotype was associated with a >2-fold higher risk of lung cancer (OR = 2.18, 95% CI = 0.92–5.20), suggesting an influence of MNS16A genotype on lung cancer susceptibility.

Based on these findings, MNS16A was studied by the same author as potential biomarker for survival in 299 glioblastoma multiforme (GBM) patients (17). The SS genotype was associated with the longest median survival time. SL and LL genotypes were associated with increased risk of death (adjusted hazard ratio, HR = 1.78, 95% CI = 1.11–2.85).

A subsequent study in GBM by Carpentier *et al.* (18) examined MNS16A in 352 patients and 305 controls. The SS genotype was found to be more frequent in patients than in controls, however, in

contrast to previous findings, no significant differences in survival times were found between SS, SL and LL genotypes.

In a large multinational GBM study (19), being part of the international Interphone study participants had been recruited in Denmark, Finland, Sweden and the United Kingdom. MNS16A genotypes of 648 glioma cases, 473 meningioma cases and 1359 controls were determined, but no significant differences in genotype distributions were observed. The HR for the SL genotype was significantly increased (HR = 2.44, 95% CI = 1.56–3.82) and for SS + SL the HR was 2.10 (95% CI = 1.41–3.1), not supporting a (linear) trend towards increasing HR with increasing number of S alleles. The LL genotype had a significantly better survival in GBM patients than the SS + SL group and also the HR for death was significantly increased for SL or SS + SL groups.

In a breast cancer study (20) of ethnic Han Chinese including 1029 cases and 1107 cancer-free controls, all three detected variant alleles were associated with a significantly increased risk of breast cancer. Furthermore, the 271/302 genotype was associated with a significantly elevated risk of axillary lymph node metastasis.

Recently, 808 non-Hispanic White patients with non-small cell lung cancer were genotyped and statistical analysis focused on 221 early-stage I or II non-small cell lung cancer patients displayed significantly shorter median survival times for carriers of SS and SL compared with LL genotypes (21).

The most recent MNS16A related lung cancer study (22) comprised 937 patients and 943 healthy controls. In this Korean study population, VNTR-243 was associated with an increased risk of lung cancer (OR = 1.55, 95% CI = 1.07–2.25), but a dual conflicting role of this MNS16A VNTR was observed because VNTR-243 also seemed to improve survival in patients.

In the Chinese breast cancer cohort (20), no VNTR-333 was detected, although frequently a third band in agarose gels (>302 bp) emerged that was shown to result from conformational changes

**Table III.** Association of hTERT MNS16A genotypes and CRC risk

MNS16A	CRC				CRC + high-risk polyp			CRC + high-risk + low-risk polyp		
	Controls	Cases	OR (95% CI) <sup>a</sup>	P value	Cases	OR (95% CI) <sup>a</sup>	P value	Cases	OR (95% CI) <sup>a</sup>	P value
<b>Alleles</b>	<b>N = 3424,</b> N (%)	<b>N = 176,</b> N (%)			<b>N = 752,</b> N (%)			<b>N = 2692,</b> N (%)		
302	2235 (65.27)	113 (64.20)	1.00		502 (66.76)	1.00		1759 (65.34)		
243	1082 (31.60)	54 (30.68)	0.96 (0.69–1.35)	0.825	218 (28.99)	0.88 (0.74–1.05)	0.160	834 (30.98)	0.96 (0.86–1.08)	0.510
<b>274</b>	<b>55 (1.61)</b>	<b>6 (3.41)</b>	<b>2.69 (1.11–6.50)</b>	<b>0.028</b>	15 (1.99)	1.29 (0.71–2.33)	0.400	48 (1.78)	1.13 (0.76–1.69)	0.546
333	52 (1.52)	3 (1.70)	1.33 (0.40–4.45)	0.640	17 (2.26)	1.62 (0.92–2.87)	0.098	51 (1.89)	1.31 (0.88–1.96)	0.185
<b>Genotypes</b>	<b>N = 1712,</b> N (%)	<b>N = 88,</b> N (%)			<b>N = 376,</b> N (%)			<b>N = 1346,</b> N (%)		
302/302	734 (42.87)	35 (39.77)	1.00		162 (43.09)	1.00		573 (42.57)	1.00	
243/243	180 (10.51)	6 (6.82)	0.68 (0.28–1.66)	0.394	28 (7.45)	0.68 (0.44–1.06)	0.090	125 (9.29)	0.85 (0.66–1.11)	0.236
243/302	691 (40.36)	38 (43.18)	1.09 (0.68–1.77)	0.713	154 (40.96)	0.98 (0.76–1.25)	0.846	549 (40.79)	1.00 (0.85–1.17)	0.968
302/333	36 (2.10)	1 (1.14)	0.66 (0.09–5.09)	0.690	12 (3.19)	1.62 (0.81–3.24)	0.175	32 (2.38)	1.19 (0.72–1.96)	0.509
243/274	15 (0.88)	2 (2.27)	3.97 (0.84–18.77)	0.082	3 (0.80)	1.00 (0.28–3.61)	0.995	16 (1.19)	1.52 (0.73–3.16)	0.265
274/302	40 (2.34)	4 (4.55)	2.48 (0.82–7.52)	0.108	12 (3.19)	1.41 (0.71–2.78)	0.327	32 (2.38)	1.00 (0.61–1.63)	0.997
243/333	16 (0.93)	2 (2.27)	3.12 (0.65–14.94)	0.154	5 (1.33)	1.68 (0.60–4.76)	0.326	19 (1.41)	1.61 (0.81–3.20)	0.177
<b>SL system</b>	<b>N = 1712,</b> N (%)	<b>N = 88,</b> N (%)			<b>N = 376,</b> N (%)			<b>N = 1346,</b> N (%)		
LL	770 (44.98)	36 (40.91)	1.00		174 (46.28)	1.00		605 (44.95)	1.00	
SL	747 (43.63)	44 (50.00)	1.21 (0.76–1.92)	0.417	171 (45.48)	0.98 (0.78–1.25)	0.901	600 (44.58)	1.00 (0.86–1.17)	0.985
SS	195 (11.39)	8 (9.09)	0.87 (0.39–1.93)	0.735	31 (8.24)	0.69 (0.45–1.05)	0.080	141 (10.48)	0.89 (0.70–1.15)	0.378

P values <0.05 were considered statistically significant (bold).

<sup>a</sup>OR were adjusted for age, sex, smoking status and education.

of the heterozygote. We also found conformational changes of the PCR amplicons being responsible for the additional band signal and verified VNTR-333 by successful cloning and sequencing. Furthermore, we identified the very rare new MNS16A variant VNTR-364 containing an additional tandem repeat.

The highest percentages of CRC were observed for the three genotypes 243/274, 274/302 and 243/333. Interestingly, all 274 alleles eligible for statistical models were contained in this group, hinting a particular significance of this VNTR. Logistic regression models for the seven considered MNS16A genotypes did not identify genotypes with significantly increased risk of CRC. Moreover, no differences in genotype distribution in case and control groups were observed by following the conversion of alleles as applied in literature leading to combinations of short (S) and long (L) alleles (SS, SL and LL). But, logistic regression analysis for the single VNTRs identified MNS16A VNTR-274 as risk factor for CRC. The 274 allele was associated with a 2.7-fold increase in CRC risk compared with the 302 wild-type allele.

This finding of an S allele having malignant potential was strongly supported by results of the large Chinese breast cancer study (20) that found the 271/302 variant associated with the highest risk (OR = 1.77, 95% CI = 1.06–2.96) and by single allele consideration also associated the 271 (274 bp) variant with highest risk for breast cancer (OR = 1.67, 95% CI = 1.01–2.76).

Likewise, the results of the study of Andersson *et al.* (19) with the second largest population size supported our finding by associating the SS and SL groups with shortened survival times and increased risk of death, although, it remained unclear whether these effects were due to 274 alleles because the number of 274 alleles in SS and SL groups was not designated (19).

The findings of our study did not conflict with the overall suspicion against the S alleles but, in addition, specified a certain allele by identifying MNS16A VNTR-274 as risk allele for CRC. Regarding distinct alleles separately in calculation rather than combinations of alleles (genotypes) seemed to avoid a ‘diluting effect’ of the partner allele, particularly, in cases where a rare or risk-associated allele occurred with a common and unsuspecting variant such as 243 or 302. By avoidance of the established conversion of a four allele system into a two allele system, we identified a distinct risk allele and thereby offered a possible ex-

planation for conflicting results of previous studies. This seemed to be particularly important since this study did not propose an extremely short (243) or long (333) allele as risk allele but one lying interjacently in terms of length and number of tandem repeats. These findings no longer support a linear correlation between length or number of tandem repeats and cancer risk (as ‘the shorter, the worse’ would describe), but a more complex correlation has to be considered.

In conclusion, the relatively strong significance of the finding of a medium length VNTR being a risk factor and not an extreme length of tandem repeats queries the established mode of statistical analysis using a classification system that combines alleles associated with diverse risks within the same genotype group. Reanalyses of existing genotyping data may be of interest. As the entities of hTERT antisense transcripts, MNS16A mode of regulating hTERT expression needs to be elucidated in detail. If the role of MNS16A polymorphism can be confirmed by large replication studies and also in other malignant diseases, this tandem repeats minisatellite can be expected to become a useful biomarker of risk, outcome or prediction in several malignancies.

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