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Analysis of MNS16A VNTR polymorphic sequence variations of the *TERT* gene and associated risk for development of bladder cancer

Iqra Anwar[a,b], Arshad A. Pandith[a,*], Mohammad S. Wani[c], Hyder Mir[d], Meena Godha[b], Aabid Koul[a], Zafar A. Shah[e], Usma Manzoor[a], Ina Amin[a], Iqbal Qasim[a]

[a]Advanced Centre for Human Genetics, Sher-I-Kashmir Institute of Medical Sciences (SKIMS), Srinagar, Jammu & Kashmir, India; [b]School of Life Sciences, Jaipur National University, Jaipur, Rajasthan, India; [c]Department of Urology and Kidney Transplant, SKIMS, Jammu & Kashmir, India; [d]Department of Internal Medicine, SKIMS, Jammu & Kashmir, India; [e]Immunology and Molecular Medicine, SKIMS, Srinagar, Jammu & Kashmir, India

Abstract

Background: The MNS16A variable number tandem repeat (VNTR) polymorphism of the human telomerase reverse transcriptase (*hTERT*) gene acts as a regulator of *hTERT* promoter activity and has been shown to have a role in the predisposition toward various cancers. The current study aimed to investigate the association between MNS16A VNTR alleles and genetic predisposition to bladder cancer in the Kashmir region of northern India.

Materials and methods: A total of 130 patients with bladder cancer and 170 age- and gender-matched healthy controls were included in this study. Primer-specific polymerase chain reaction was used to genotype the different variants of VNTR alleles of the MNS16A VNTR polymorphism.

Results: Short allele VNTR-243 (SS) genotype frequency significantly differed between cases (9.23%) and controls (3.52%) (OR = 3.08 [95% CI = 1.10–8.61], $p = 0.042$). The VNTR-243 short allele (S) was found significantly more frequent in bladder cancer cases (28.46%) than controls (20.88%) (OR = 1.50 [95% CI = 1.03–2.19], $p = 0.034$). Likewise, the long allele (LL) *hTERT* MNS16A genotype was distributed more frequently in low stage disease versus high stage disease (60.29% vs. 39.70%) (OR = 0.79 [95% CI = 0.39–1.60], $p = 0.595$).

Conclusion: The MNS16A VNTR short allele (S) was associated with a higher risk for bladder cancer in our population as compared to long alleles.

Keywords: Alleles; Bladder cancer; Kashmir; MNS16A VNTR; Polymerase chain reaction

1. Introduction

Bladder cancer, the most common malignancy of the urinary tract, is considered to be a diverse disease with different morphologic and clinical manifestations.[1] It is the 4th most prevalent malignancy and the 2nd most commonly diagnosed urologic cancer in men.[2] It is characterized by high rates of recurrence and poor prognosis, displaying an increase in incidence with increasing age.[3] The etiology of bladder cancer is multifactorial, with smoking known to be the most significant risk factor for development of bladder malignancy, while other risk factors include exposure to certain dyes, drugs, or arsenic, and certain chronic infections.[4] Genetic studies have identified a variety of genetic changes that occur during urothelial transformation that are associated with different clinical outcomes, including *FGFR3*, *HRAS*, *NRAS* activation in non-invasive bladder cancers and *TP53*, *Rb1*, *E2F3* in muscle invasive bladder cancer.

The telomeres present at the ends of chromosomes help to maintain the integrity of the genomic structure.[5] The presence of a single stranded extension and protein complex with tandem nucleotide repeats of the hexamer TTAGGG in telomeres prevents chromosome degradation and stops fusions and rearrangements in eukaryotic chromosomes.[6–9] Human telomerase reverse transcriptase gene (*hTERT*), present in region 5p15.33, encodes a ribonucleoprotein enzyme that extends the chromosome ends which are shortened with each cell division. [8] Mutations that occur in coding regions of the *hTERT* gene have important effects on telomerase activity and telomere length.[10,11] It has been reported that control of telomerase action and telomere length is closely related to tumor formation in humans. Recent evidence suggests that genetic variation in the 5p15.33 region of the *hTERT* gene might play a role in regulating the risk of developing cancer.[10,12] Studies have proposed the effects of *hTERT* gene variants in conferring increased risk to hematological malignancies.[10] Other evidence from genome-wide association studies (GWAS) has indicated the presence of a

* Corresponding Author: Arshad A. Pandith, Advanced Centre for Human Genetics, Sher-I-Kashmir Institute of Medical Sciences, Srinagar 190011, Jammu & Kashmir, India. E-mail address: arshaajiz@gmail.com, saleemwani71@gmail.com (A.A. Pandith).

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strong association between the locus at 5p15.33 and some cancers, including adenocarcinoma,[13] basal cell carcinoma, pancreatic cancer,[14] and lung cancer.[15,16] In addition, "risky" alleles present in this region have been reported to be significantly related to the occurrence of glioma,[17] bladder cancer,[18,19] and prostate cancer.[20] In terms of bladder cancer, activating mutations in the core promoter of the *hTERT* gene are the most common mutations identified, with a frequency of 55.6%–82.8%. Additionally, these mutations have been found to be independent of the stage and grade of disease.[19] Polymorphic tandem repeat minisatellites of TERT, called MNS16A, were first identified in a study of lung cancer patients.[21] MNS16A, an antisense copy of the *hTERT* gene, is located down-stream of exon 16 of the *hTERT* gene and upstream in the putative promoter region of an antisense *hTERT* transcript. MNS16A has 2 repeat elements forming a 23bp core sequence or a 26bp core sequence with a CAT insertion representing a transcription factor binding site for GATA-1. On the basis of polymerase chain reaction (PCR) fragment size, two different variable number of tandem repeats (VNTRs) alleles were named VNTR-302 and VNTR-243.[8] In addition, two other rare alleles, VNTR-272 and VNTR-333, were also found in cancer cell lines. Classification of VNTR-243 and VNTR-272 as short (S) alleles, and VNTR-302 and VNTR-333 as long (L) alleles, was introduced for the purposes of statistical analysis by Wang et al.[21] The MNS16A variable number tandem repeat (VNTR) functional polymorphism has been investigated in various studies including nasopharyngeal carcinoma,[22] colorectal cancer,[8,23] lung cancer,[21,24] prostate cancer,[23,25] bladder cancer,[26] and in the normal human life span.[27] In this study, we aimed to determine whether the MNS16A VNTR polymorphism of the *hTERT* gene was associated with bladder cancer risk in a Kashmiri population.

2. Material and methods

2.1. Study population

The present study enrolled 130 patients with bladder cancer and 170 healthy controls (129 males and 41 females) who were free of any type of malignancy. Bladder cancer cases included 103 (79%) males and 27 (21%) females, a ratio of 4:1; 67% were smokers and 33% nonsmokers. Controls were matched to cases, and no gender, age, or smoking-related differences were observed between the 2 groups ($p > 0.05$). Subjects (cases and controls) were randomly recruited from the Department of Urology, SK Institute of Medical Sciences (SKIMS), Jammu &Kashmir, India, and were studied prospectively. All cases were selected after confirmation of transitional cell carcinoma by histopathological examination. This study was approved by the Ethics Committee of SK Institute of Medical Sciences, and written informed consent was obtained from all patients prior to participation in this study. Peripheral blood samples (5 mL) and corresponding tumor tissue samples were collected from Department of Urology and Kidney Transplant (SKIMS), and were preserved at −20°C for analysis.

2.2. Genotyping and allele confirmation

DNA was extracted from both peripheral blood and tumor tissues using the phenol-chloroform methodanda DNA extraction kit (Zymo Research Corporation, USA). PCR was employed to amplify the genomic variants of the MNS16A VNTR polymorphism. For amplification of *hTERT* MNS16A, a 25 μL reagent solution was used containing genomic DNA: 250 ng/mL, 1× PCR buffer: 100 mM Tris–HCl, pH 8.3; 500 mM KCl; 15 mM $MgCl_2$; deoxyribonucleotide triphosphate (Biotools, B&M Labs, Madrid, Spain): 10 mM dATP; 10 mM dCTP; 10 mM dGTP; 10 mM dTTP, 10 pM primers (Sigma–Aldrich, USA): and *Taq DNA polymerase* 5 U/μL (Biotools, Madrid, Spain). The set of primers used for the MNS16A amplification encompassing the region with forward primer sequence was 5'-AGGATTCT-GATCTCTGAAGGGTG-3' (sense) and reverse primer was 5'-TCTGCCTGAGGAAGGACGTATG-3' (antisense).[28] The PCR protocol was as follows; an initial denaturation at 95°C for 5 min, followed by denaturation for 35 cycles at 95°C for 30 s, and annealing at 60°C for 30 s, at 72°C for 1 min, and extension at 72°C for 10 min. The PCR products were further visualized on 3% high resolution agarose gel under a UV transilluminator using Safe-T staining (ethidium bromide alternative).[16] The representative picture of *hTERT* MNS16A is shown in Supplementary Figure 1, http://links.lww.com/CURRUROL/A4. To ensure quality control, distilled water was used instead of DNA as a negative control.

2.3. Statistical analysis

Statistical analysis was done by using IBM Statistics SPSS software 23.0. Cases and controls were compared using the chi square test for categorical variables like gender and age demographic variables. A chi-square goodness-of-fit test was employed to evaluate whether the polymorphisms were in Hardy-Weinberg equilibrium between cases and controls. Odds ratios (OR) were used as estimates of relative risk, and 95% confidence intervals (CI) were calculated to estimate the association between certain genotypes or other related risk factors and bladder cancer. Statistical significance level was set at a p value < 0.05.

3. Results

In the present study, a total of 300 subjects (130 bladder cancer cases and 170 controls) were studied. Selected demographic characteristics of the case and control groups are demonstrated in Table 1. No significant difference was observed between groups in terms of gender, age or smoking ($p > 0.05$). The bladder cancer group consisted of 103 (79.23%) men and 27 (20.76%) women,

Table 1

Demographic characteristics of bladder cancer cases and healthy controls.

Variable	Cases (n = 130)	Controls (n = 170)	p
Age, yr			
<50	46 (35.38%)	60 (35.29%)	0.920
≥50	84 (64.61%)	110 (64.70%)	
Gender			
Female	27 (20.76%)	41 (24.11%)	0.578
Male	103 (79.23%)	129 (75.88%)	
Geographic area			
Rural	99 (76.15%)	112 (65.88%)	0.056
Urban	31 (23.84%)	58 (34.11%)	
Smoking status			
Never	42 (32.30%)	73 (42.94%)	0.072
Ever	88 (67.69%)	97 (57.05%)	
Histologic type			
GI/GII	72 (55.38%)		
GIII/GIV	58 (44.61%)		
Tumor stage			
pTa/pT1	75 (57.69%)		
pT2/higher	55 (42.30%)		

Table 2

Genotypic/allelic distribution of *hTERT* MNS16A polymorphisms in bladder cancer cases and healthy controls.

MNS16A		Cases (n = 130)	Controls (n = 170)	OR (95% CI)	*p*
Long allele (LL)	302	68 (52.30%)	105 (61.76%)	Reference	Reference
Long short allele (LS)	302/243	50 (38.46%)	59 (34.70%)	1.30 (0.80–2.12)	0.321
Short allele (SS)	243	12 (9.23%)	6 (3.52%)	3.08 (1.10–8.61)	**0.042**
L allele	302	186 (71.53%)	269 (79.11%)	Reference	Reference
S allele	243	74 (28.46%)	71 (20.88%)	1.50 (1.03–2.19)	**0.034**

CI = confidential interval; OR = odds ratio.

while the control group had 129 (75.88%) men and 41 (24.11%) women. There were 42 (32.30%) nonsmokers and 88 (67.70%) smokers in the bladder cancer group. Among bladder cancer cases, 75 (57.69%) cases had lower stage disease and 55 (42.30%) had higher stage disease; 72 (55.38%) had low grade disease and 58 (44.61%) had high grade disease. On stratification of bladder cancer cases, *hTERT* MNS16A LL genotype was present more often in low grade cases (G-I/G-II) as compared to high grade cases (G-III/G-IV), 55.88% versus 44.11%, respectively (OR = 0.95 [95% CI = 0.47–1.91], $p = 1.00$).

The observed genotype frequencies for the MNS16A variant were in Hardy-Weinberg equilibrium (HWE) with controls ($p = 0.511$).

The results of genotyping are shown in Table 2, where different sizes of MNS16A (VNTR) alleles were analyzed and grouped as LL, LS, and SS on the basis of fragment size. Among these different set of alleles, we found VNTR-302 (LL) allele to be the most common allele in both cases (71.53%) and controls (79.11%). VNTR-243 (SS) allele was the 2nd most common, at a frequency of 28.46% in bladder cancer cases and 20.88% in controls. On the other hand, the VNTR-333 allele was found at the lowest frequency. In our study, the genotype frequencies for 302/302, 302/243, and 243/243 were 52.30%, 38.46%, and 9.23% in bladder cancer patients and 61.76%, 34.70%, and 3.52% in controls, respectively ($p < 0.05$). Additionally, of very rare genotypes 333/243, 333/302, 243/274, and 302/274, in our study only 333/302 was found in both cases and controls, 0.76% versus 1.17%, respectively. The short allele VNTR-243 (SS) genotype frequency was significantly different between cases and controls, at 9.23% versus 3.52%, respectively (OR = 3.08 [95% CI = 1.10–8.61], $p = 0.042$). Furthermore, distribution of the VNTR-243 allele was found to be significantly different between the 2 groups (28.46% in cases vs. 20.88% in controls) (OR = 1.50 [95% CI = 1.03–2.19], $p = 0.034$). No association was found between *hTERT* MNS16A genotype variation and gender or any other characteristic (Table 3). Likewise, *hTERT* MNS16A LL genotype was distributed more frequently in cases with low stage disease as compared to cases with high stage disease, 60.29% versus 39.70%, respectively (OR = 0.79 [95% CI = 0.39–1.60], $p = 0.595$). In addition, the frequency of combined genotype (LS+SS) was present more often in cases with both low grade and low stage disease (54.83%) compared to cases with both high grade and high stage disease (45.16%). We then performed Cox-Regression hazard analysis to determine whether the SS variant of *hTERT* MNS16A was independently associated with an increased risk of bladder carcinogenesis and not influenced by other variables. Multivariate analysis adjusted for physiological (age, gender, geographic area, smoking status, and pesticide exposure) and pathologic characteristics (disease grade and stage) confirmed that the *hTERT*-SS variant was an independent risk factor for bladder cancer; carriers

Table 3

Genotypic distribution of hTERT MNS16A gene polymorphisms in bladder cancer cases and healthy controls with respect to different clinicopathological characteristics.

Parameter	Cases (n = 130)	LL	LS+SS	Controls (n = 170)	LL	LS+SS	Adjusted OR (95% CI)	*p*
Overall genotype		68 (52.3%)	62 (47.6%)		105 (61.7%)	65 (38.23%)	1.47 (0.92–2.33)	0.125
Age, yr								
<50	46 (35.38%)	23 (33.82%)	23 (37.09%)	60 (35.29%)	30 (28.57%)	30 (46.15%)	1 (0.46–2.15)	1.00
≥50	84 (64.61%)	45 (66.17%)	39 (62.90%)	110 (64.7%)	75 (71.42%)	35 (53.84%)	1.85 (1.03–3.34)	0.052
Sex								
Male	103 (79.23%)	55 (80.88%)	48 (77.41%)	129 (75.8%)	76 (72.38%)	53 (81.53%)	1.25 (0.74–2.11)	0.425
Female	27 (20.76%)	13 (19.11%)	14 (22.58%)	41 (24.11%)	29 (27.61%)	12 (18.46%)	2.60 (0.94–7.15)	0.077
Smoking status				73 (42.94%)				
Never	42 (32.30%)	23 (33.82%)	19 (30.64%)	97 (57.05%)	44 (41.90%)	29 (44.61%)	1.25 (0.58–2.70)	0.694
Ever	88 (67.69%)	45 (66.17%)	43 (69.35%)		61 (58.09%)	36 (55.38%)	1.61 (0.90–2.91)	0.136
Geographic area								
Rural	99 (76.15%)	49 (72.05%)	50 (80.64%)	112 (65.8%)	71 (67.61%)	41 (63.07%)	1.76 (1.01–3.06)	0.051
Urban	31 (23.84%)	19 (27.94%)	12 (19.35%)	58 (34.11%)	34 (32.38%)	24 (36.92%)	0.89 (0.36–2.18)	0.825
Histologic type								
GI/GII	72 (55.38%)	38 (55.88%)	34 (54.83%)					
GIII/GIV	58 (44.61%)	30 (44.11%)	28 (45.16%)				0.95 (0.47–1.91)	1.00
Tumor stage								
pTa/pT1	75 (57.69%)	41 (60.29%)	34 (54.83%)					
pT2/higher	55 (42.30%)	27 (39.70%)	28 (45.16%)				0.79 (0.39–1.60)	0.595

LL = long allele; LS = long short allele; SS = short allele; CI = confidential interval; OR = odds ratio.

of this variant had increased risk of developing bladder cancer more than 3 times higher than that of non-carriers (HR = 3.34, $p = 0.04$) (Supplementary Table 1, http://links.lww.com/CURRUROL/A3).

4. Discussion

Currently, increased telomerase activity is regarded as a prominent risk factor for different types of cancer that plays avital role in their growth and progression.[16,18] Various studies have investigated functional polymorphic variations impacting the expression or function of the *hTERT* gene which increase susceptibility for bladder cancer.[18,19] The association between MNS16A VNTR and different cancers, including breast, glioblastoma multiforme, and non-small cell lung cancer, has been investigated with variable findings with respect to unique VNTRs depending on their lengths.[24,28,29]

In the present study, the impact of *hTERT* variants on bladder cancer risk was analyzed in one ethnic population of the Kashmir region of northern India. The current study found a significantly increased frequency, around 3-fold higher, of the short allele VNTR-243 (SS) genotype in cases as compared to controls (9.23% vs. 3.52% respectively) ($p = 0.042$). This finding supports the hypothesis that VNTR-243 (SS) confers an increased risk for bladder cancer.

A previous study investigating lung cancer confirmed that *hTERT* mRNA expression is regulated by MNS16A.[30] Additionally, VNTR-SS allele was significantly associated as a risk factor for colorectal cancer compared with the VNTR-302 wild-type by Hofer et al.[8] Yan Wang et al also found that the SS genotype (243/243) of MNS16A in Chinese women with breast cancer was significantly related with an increased risk of breast cancer.[28]

Therefore, based on our study and other studies noted above, a plausible conclusion can be reached that the MNS16A short allele has a role in predisposition for cancer. As per Wang et al.[21] the *hTERT* gene locus demonstrated that MNS16A short alleles are correlated with increased *hTERT* expression and telomerase activity compared with long alleles.[21,29] Another study in a non-Hispanic white population found the VNTR-243 S allele to be significantly more frequent in glioma cases (28.46%) than controls (20.88%) ($p = 0.034$).[28] Furthermore, a study on lung cancer with respect to MNS16A polymorphic analysis included a large number of samples, with 937 patients and 943 healthy controls.[16] This study showed the SS form of VNTR-243 was associated with an elevated risk of lung cancer. On the contrary, despite the fact that MNS16A polymorphic variation has been studied in numerous contexts related to a number of different cancers,[8,21–25] results are conflicting, either due to ethnic variations or some other technical reasons. For example, a study conducted in China found no significant differences between MNS16A polymorphisms among pericentenarian and normal controls.[9] Another study on MNS16A polymorphisms in prostate cancer cases and controls with benign prostatic hyperplasia showed no significant association with any variant form.[25] In a study on bladder cancer, SongulDiler et al[26] found comparable distributions of MNS16A VNTR S/L alleles and SS/LL genotypes among bladder cancer cases and controls with no significant differences. Nonetheless, one common finding in our study and their study was a similar frequency of MNS16A SS genotype, 9.2% versus 10%, respectively.

In sum, given the plausible role of the S allele of MNS16A to confer risk for different cancers, as demonstrated in the current study and relevant studies noted above, this allele shows promise as a potential relevant tumor risk marker. This is further supported by the finding that the MNS16A S allele is linked with enhanced *hTERT* expression, which signifies that MNS16A genetic variation is a potential factor that affects *hTERT* mRNA expression[29] and therefore influences the risk for various cancers, in particular bladder cancer. No single clinical or pathological parameter such as smoking status, histological type, tumor stage, or geographic area were found to be associated with different genotypes of MNS16A ($p > 0.5$), although age < 50 years approached statistical significance ($p = 0.052$). These results are consistent with other studies on breast cancer conducted by Hashemi et al.[31] and bladder cancer conducted by Diler et al.[26] where all clinical and pathological parameters showed no association.

It is known that genetic polymorphic variations demonstrate variable prevalences in different ethnic groups. The frequency of MNS16A in the current study showed similarities and discrepancies with other ethnic populations, as depicted in Table 4. The

Table 4

Comparison of various studies regarding *hTERT* MNS16A polymorphisms.

			Cases				Controls				
Author	Year	Cancer type	LL	LS	SS	Total	LL	LS	SS	Total	Significance
Songül Diler[26]	2020	Bladder	29	34	7	70	47	65	8	120	No
Luo Wang[21]	2003	Lung	30	17	6	53	33	29	10	72	No
Philipp Hofer[8]	2011	CRC	36	8	44	88	770	195	747	1712	Yes
Hashemi[31]	2014	Breast	115	136	15	266	66	153	5	224	Yes
Luo Wang[29]	2006	GBM	133	132	34	299	106	98	20	224	Yes
Yan Wang[28]	2008	Breast	860	141	5	1,006	984	107	4	1095	Yes
Yang Zhang[22]	2011	NPC	725	71	2	798	891	121	7	1019	Yes
Martino[33]	2016	Renal cell	116	106	20	242	148	201	71	420	Yes
Wysoczanska[34]	2015	Lymphoma	28	37	10	75	53	54	19	126	No
Zagouri[35]	2012	Breast	50	36	27	113	63	29	32	124	No
Jin[16]	2011	Lung	820	110	7	937	840	101	2	943	No
Andersson[32]	2009	Glioma	282	277	89	648	650	560	149	1359	Yes
Carpentier[36]	2007	Glioma	126	174	52	352	133	144	28	305	Yes
Current study	2020	Bladder	68	50	12	130	105	59	6	170	Yes

CRC = colorectal cancer; GBM = Glioblastoma; NPC = nasopharangeal cancer; LL = long allele; LS = long short allele; SS = short allele.

frequencies of different alleles of MNS16A observed for cases in our population correlated most closely with studies conducted on different cancers in Caucasian populations[25,32,33] and ethnic groups of Turkish descent.[26] Although the frequency of different alleles in our control group correlated closely with those reported by Martino et al,[33] the overall distribution varied considerably across all included studies (Table 4).[8,16,21,22,26,28,29,31–36] This shows considerable variation in MNS16A alleles among different ethnic populations and makes it an interesting genetic polymorphism to be investigated as a risk factor for different cancers.

5. Conclusion

This study found that both MNS16A VNTR short allele (S) and genotype (SS) are associated with an increased risk for bladder cancer in our study population as compared to long alleles. These findings needs to be replicated in more studies on urinary tract cancers.

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Statement of ethics

This study was approved by the Ethics Committee of SK Institute of Medical Sciences, and written informed consent was obtained from all patients prior to participation in this study. All procedures involving human subjects were conducted in compliance with the ethical standards of the institutional and/ or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of interest statement

The authors declare no conflicts of interest.

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Author contributions

Arshad A. Pandith: Design of the study, conception of and writing the draft manuscript and figures;
Iqra Anwar: Data interpretation, conducting experiments, statistical analysis;
Mohammad S. Wani: Provided samples, revisions to the draft manuscript;
Usma Manzoor, Ina Amin, Hyder Mir, Aabid Koul, Meena Godha, Iqbal Qasim, Zafar A. Shah: Assisted in conducting experiments.
Iqra Anwar and Arshad A. Pandith contributed equally.

Data avaliability

All data that support the results and conclusions of this manuscript will be made accessible to any eligible researcher.

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