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ASSESSMENT OF POSSIBLE ASSOCIATION BETWEEN RS378854 AND PROSTATE CANCER RISK IN THE SERBIAN POPULATION

G. BRAJUŠKOVIĆ[1], ZORANA NIKOLIĆ[1], A. KOJIĆ[1], DUŠANKA SAVIĆ PAVIĆEVIĆ[1], SNEŽANA CEROVIĆ[2], S. TOMOVIĆ[3], NATAŠA FILIPOVIĆ[4], VINKA VUKOTIĆ[4] and STANKA ROMAC[1]

[1] *University of Belgrade, Faculty of Biology,* 11000 Belgrade, Serbia
[2] *Institute of Pathology and Forensic Medicine, Military Medical Academy,* 11000 Belgrade, Serbia
[3] *Clinic of Surgery, Clinical Center "Zvezdara",* 11000 Belgrade, Serbia
[4] *Clinic of Urology, Clinical Center "Dr Dragiša Mišović",* 11000 Belgrade, Serbia

Abstract - Prostate cancer (PCa) is the second most commonly diagnosed cancer among men worldwide. Despite its high incidence rate, the molecular basis of PCa onset and its progression remains little understood. Genome-wide association studies (GWAS) have greatly contributed to the identification of single nucleotide polymorphisms (SNP) associated with PCa risk. Several GWAS identified 8q24 as one of the most significant PCa-associated regions. The aim of this study was to evaluate the association of SNP rs378854 at 8q24 with PCa risk in the Serbian population. The study population included 261 individuals diagnosed with PCa, 257 individuals diagnosed with benign prostatic hyperplasia (BPH) and 106 healthy controls. Data quality analysis yielded results showing deviations from Hardy-Weinberg equilibrium in groups of PCa patients and BPH patients as well as in the control group. There was no significant association between alleles and genotypes of the genetic variant rs378854 and PCa risk in the Serbian population.

Key words: Prostate cancer, association study, polymorphism, single nucleotide

INTRODUCTION

Prostate cancer (PCa) is the second most commonly diagnosed malignant disease among men, and the sixth leading cause of cancer-related death in the male population (Ferlay et al., 2010). The number of new cases of PCa diagnosed in the Serbian population showed a 65% increase in the period between 1999 and 2009, according to data obtained from central Serbia (Vukičević et al., 2002; Miljuš et al., 2011). During this period, PCa remained the third most common cancer among males in the Serbian population (Vukičević et al., 2002; Miljuš et al., 2011). Predictions made in the American population indicate that projected PCa incidence will increase by approximately 45% from 2010 to 2030 (Smith et al., 2009). In the United Kingdom, the increase in number of diagnosed PCa from 2007 to 2030 was predicted to reach 69%, which would make this cancer the most common malignant disease in men (Mistry et al., 2011). The only known risk factors for developing PCa, besides increasing age, are ethnic background and positive family history, which are considered to reflect the underlying genetic factors involved in PCa etiology (Hsing and Chokkalingam, 2006).

As in other malignant diseases, the initiation and progression of PCa involve a progressive accumulation of genetic and epigenetic alterations. Numerous chromosomal rearrangements and copy number alterations, along with somatic point mutations, have been associated with prostate carcinogenesis (Shen

and Abate-Shen, 2010). Furthermore, global and gene-specific changes in histone modifications and DNA methylation patterns are believed to be involved in PCa etiology (Li et al., 2005).

Apart from these somatic alterations, numerous genetic polymorphisms have been identified as factors affecting the process of malignant transformation in prostatic tissue. Genome-wide association studies (GWAS) have greatly contributed to the identification of single nucleotide polymorphisms associated with PCa risk (Amundadottir et al., 2006; Freedman et al., 2006; Gudmundsson et al., 2007a; Gudmundsson et al., 2007b; Haiman et al., 2007; Yeager et al., 2007; Eeles et al., 2008; Gudmundsson et al., 2008; Thomas et al., 2008; Al Olama et al., 2009; Eeles et al., 2009; Gudmundsson et al., 2009; Yeager et al., 2009; Takata et al., 2010). The first study of this type was performed in 2006 and led to the identification of association between several SNPs at 8q24 and PCa risk (Amundadottir et al., 2006). Afterwards, other loci in this region were identified as PCa susceptibility loci. Polymorphisms at 8q24 were also evaluated for association with the clinicopathological features of PCa. Several SNP showed significant association with a higher prostate-specific antigen (PSA) level at diagnosis, higher Gleason score, higher tumor stage, younger age at onset or greater disease aggressiveness in various populations (Armundadottir et al., 2006; Zheng et al., 2007; Penney et al., 2009; Bao et al., 2010; Lange et al., 2012).

The mechanism by which polymorphisms at 8q24 affect PCa onset and progression remains largely unknown. Since these polymorphisms reside within a region devoid of characterized protein-coding genes, a great effort has been made to evaluate the presence of transcribed sequences in this region, as well as the regulatory sequences of adjacent genes (Huppi et al., 2008; Pomerantz et al., 2009; Sotelo et al., 2010; Wasserman et al., 2010; Prensner et al., 2011; Ting et al., 2012). The closest annotated protein-coding genes are the family with sequence similarity 84, member B gene (*FAM84B*), and the proto-oncogene v-*myc* myelocytomatosis viral oncogene homolog (avian) (c-*MYC*). Somatic amplification of the chromosomal region containing *MYC*, causing its overexpression, was detected in advanced and recurrent PCa (Jenkins et al., 1997). MYC protein was also found to be upregulated in prostatic intraepithelial neoplasia (PIN), suggesting its involvement in PCa initiation (Gurel et al., 2008). Evidence for this hypothesis was also obtained in a study that showed that *MYC* overexpression is sufficient to drive normal human prostatic epithelial cells to malignant phenotype within a tissue recombination model (Williams et al., 2005). Functional variants located within *cis*-regulatory elements of *MYC* could possibly underlay the observed association of numerous loci at 8q24 with PCa risk and aggressiveness by modifying its expression patterns. Findings regarding several MYC enhancers identified at 8q24 in the intervals associated with PCa risk are supportive of this supposition (Sotelo et al., 2010; Wasserman, 2010; Ting et al., 2012). Another gene frequently amplified in PCa, along with *MYC*, is the plasmacytoma variant translocation 1 (*PVT1*) oncogene, which encodes a long non-coding RNA (lincRNA) and is a host gene for several miRNAs whose functions are largely unknown (Meyer et al., 2011). A recent study conducted by Meyer et al. (2011) identified rs378854 as a novel functional variant associated with PCa risk. The G allele of rs378854 was shown to reduce the binding of the Ying Yang 1 (YY1) transcription regulator to the regulatory element of *PVT1*, causing the increase in its expression but not influencing the expression of *MYC* (Meyer, 2011).

This study is the first to evaluate the possible association of rs378854 with PCa risk in the Serbian population. It is also the first study to include the assessment of the association between rs378854 and PCa progression in this population.

MATERIALS AND METHODS

The study used peripheral blood samples obtained from patients treated in the period 2009-2012 at the Clinical Center "Dr Dragiša Mišović Dedinje" and Clinical Center "Zvezdara". Research was conducted with the approval of the ethics committees of these medical institutions.

The study included 261 individuals diagnosed with PCa and 257 individuals diagnosed with benign prostatic hyperplasia (BPH), who provided peripheral blood samples, as well as 106 healthy individuals comprising the control group from whom buccal swabs were obtained. The diagnoses of PCa and BPH were made using standard clinical procedure, which included digital rectal examination, transrectal ultrasonography, abdominal and pelvic ultrasound, bone scintigraphy and radiography, serum PSA level and biopsy of the prostate. Serum PSA levels were determined by the Hybritech method of monoclonal immunoassay. Information about the prostate-specific antigen serum level, Gleason score and clinical stage were available for individuals diagnosed with PCa. The clinical stage of cancer was determined according to the TNM classification system.

Patients diagnosed with PCa were classified into groups based on the values of standard prognostic parameters – PSA at diagnosis (PSA <10 ng/ml; 10 ng/ml≤ PSA <20 ng/ml; PSA >20 ng/ml), Gleason score (GS <7; GS =7; GS >7) and clinical stage (T1; T2; T3/T4). The presence of metastases was an additional parameter used for the classification of patients with PCa into two groups. Based on the risk of localized cancer progression, three groups of patients were formed according to D'Amico criteria (D'Amico et al., 1998). These groups were defined as low-risk (PSA <10 ng/ml, GS <6, and clinical stage T1-T2a), intermediate-risk (PSA 10–20 ng/ml or GS =7 or clinical stage T2b-T2c), and high-risk (PSA >20 ng/ml or GS >7 or stage T3/T4) (D'Amico et al., 1998). Since patients with metastases were included in the study, the criteria were modified to include this subset into the high-risk group. Patients were also selected into the low-risk (Gleason score <7 and stage T1-T2) and high-risk (Gleason score ≥7 or stage T3/T4 or metastases) groups according to Medeiros et al. (2002).

Genomic DNA was isolated from peripheral blood and buccal swabs using QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturers' protocol. The 303 bp of sequence surrounding rs378854 was amplified by PCR from isolated genomic DNA. Sequences of the primers used for PCR amplification are the following ones: forward primer - 5'-TCTGCAGATTCACAGCTCTTT-3'; reverse primer - 5'-AATGCCTCACCTTGCTTTG-3'. For the PCR step, forward primer was labeled at 5' end with FAM™. The PCR reaction mixture (final volume 15 µl per single reaction) contained 1.5 µl of 10X PCR buffer A (with 15 mM $MgCl_2$, *Kapa Biosystems*, Woburn, MA, USA), dNTPs at final concentration of 200 µM each (*Fermentas*, Hunover, MD, USA), primers at final concentration of 0.2 µM each (*Applied Biosystems*, Foster City, CA, USA), *Taq* DNA polymerase (*Kapa Biosystems*, Woburn, MA, USA) at final concentration of 0.04 U/µl, 2 µl of genomic DNA (1-20 ng) and nuclease-free water (*Serva*, Westbury, NY, USA). The PCR thermocycling conditions were as follows: 97°C for 3 min, followed by 35 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min. Final extension was performed at 72°C for 10 min, after which the reaction mixture was incubated at 20°C for 20 min.

PCR products (3.33 µl) were digested with 1 U of restriction enzyme *MspI* (*HpaII*) (Fermentas, Hunover, MD, USA) for 24 h in a reaction volume of 5 µl, which also included 0.5 µl of 10X buffer R (Fermentas, Hunover, MD, USA) and nuclease-free water (Serva, Westbury, NY, USA). Digestion of the PCR products with *MspI* resulted in the formation of a different set of fragments depending on the rs378854 genotype (GG: 113 bp, 88 bp, 66 bp and 36 bp; GA: 149 bp, 113 bp, 88 bp, 66 bp and 36 bp; AA: 149 bp, 88 bp and 66 bp).

The loading mixture for gel electrophoresis which included 8.7 µl of Hi-Di formamide (Applied Biosystems, Foster City, CA, USA) and 0.3 µl of Gene Scan 500 Liz Size Standard (Applied Biosystems, Foster City, CA, USA) was mixed with 1.1 µl of restriction digestion product. Samples were denaturized at 95°C for 5 min. Fragments were separated by capillary gel electrophoresis using a 3130 Genetic analyzer (Applied Biosystems, Foster City, CA, USA). The results of electrophoresis were analyzed by GeneMapper (Applied Biosystems, Foster City, CA, USA). The detected fragment length for

Table 1. Classification of patients with prostate cancer based on values of standard prognostic parameters regarding disease progression.

Standard prognostic parameter	Prostate cancer patients n (%)
PSA at diagnosis	
<10 ng/ml	81 (31.3)
10-20 ng/ml	68 (26.2)
>20 ng/ml	110 (42.5)
Unknown	2
Gleason score	
<7	145 (57.5)
=7	72 (28.6)
>7	35 (13.9)
Unknown	9
Clinical stage	
T1	40 (18.2)
T2	113 (51.4)
T3/T4	67 (30.4)
Unknown	41
Metastases	
Detected	72 (32.7)
Not detected	148 (67.3)
Unknown	41

the rs378854 GG genotype was 113 bp. For the GA genotype fragment, lengths were 149 bp and 113 bp, while for the AA genotype the detected fragment was 149 bp long.

Statistical analysis of SNP association was performed using PLINK statistical software (Purcell et al., 2007). Hardy-Weinberg equilibrium (HWE) was assessed using the exact test (Wigginton et al., 2005) implemented in PLINK. The Cochran-Armitage trend test was used to assess the association of rs378854 with PCa risk and progression under an additive genetic model. The odds ratio (OR) was calculated as a measure of the effect size of G allele. A 5% level of significance was used in the analysis.

RESULTS

The genotyping of rs378854 was successful in the 106 control subjects, 257 patients with BPH and 261 patients with PCa. Clinical and pathological features of the group of patients diagnosed with PCa are shown in Table 1. The majority of subjects had an elevated serum PSA over 20 ng/μl, while less than 15% of subjects had a high Gleason score. About 30% of patients had advanced stage PCa. In addition, most of the patients were diagnosed with high-risk PCa according to both D'Amico criteria (D'Amico et al., 1998) and Medeiros et al. (2002) (Table 2).

The allele and genotype frequencies of rs378854 in the groups of patients diagnosed with PCa and BPH, as well as in the control group, are shown in Table 3. The frequencies of rs378854 genotypes were inconsistent with HWE between both groups of patients with PCa and BPH as well as among the controls (results not shown). Due to this observation, a statistical method that does not assume HWE was

Table 2. Classification of patients with prostate cancer based on risk of disease progression.

Risk group	Prostate cancer patients n (%)
D'Amico risk criteria	
Low-risk	16 (6.4)
Intermediate-risk	87 (34.9)
High-risk	146 (58.6)
Unknown	12
Medeiros et al. risk criteria	
Low-risk	96 (40.3)
High-risk	142 (59.7)
Unknown	23

Table 3. rs378854 A allele frequencies and genotype distributions in groups of patients with prostate cancer, benign prostatic hyperplasia and in the control group.

Group	Allele A frequency	Genotype frequencies		
		GG	GA	AA
Prostate cancer	0.278	0.444	0.341	0.215
Benign prostatic hyperplasia	0.264	0.471	0.327	0.202
Controls	0.302	0.396	0.349	0.255

Table 4. Association of G allele and genotypes of rs378854 with prostate cancer risk.

Comparison	P value	Per allele OR (95% CI)
PCa *vs.* BPH	0.527	0.92 (0.72-1.18)
PCa *vs.* controls	0.328	1.20 (0.87-1.66)
BPH *vs.* controls	0.160	1.30 (0.94-1.81)

Abbreviations: OR-odds ratio; CI-confidence interval.

used to test the possible association of rs378854 with PCa risk (Table 4). No significant association of rs378854 with PCa risk was determined in comparing genotype distributions in patients with PCa and controls. Comparison of genotype distributions among the patients with PCa and BPH also yielded no evidence of association between rs378854 and PCa risk. Furthermore, no significant association was observed between rs378854 and the risk of BPH (Table 4).

The frequencies of rs378854 A allele and genotype distributions in the groups of patients formed according to the values of standard prognostic parameters regarding the progression of PCa and the risk of progression assessed according to D'Amico et al. (1998) and Medeiros et al. (2002), are summarized in Table 5 and Table 6, respectively. Due to deviations from HWE, the Pearson chi-square test could not be used to analyze 3x3 contingency tables. For this reason, differences between the groups of patients formed according to the values of standard prognostic parameters of PCa progression were analyzed using the Cochran-Armitage trend test on 2x3 contingency tables. These tests showed no evidence of the supposed association (Table 7). Also, no statistically significant association between rs378854 and the presence of metastases was determined (Table

Table 5. rs378854 A allele frequencies and genotype distributions in groups of patients with prostate cancer formed according to values of standard prognostic parameters.

Comparison	Allele A frequency	Genotype frequencies		
		GG	GA	AA
PSA at diagnosis				
<10 ng/ml	0.426	0.407	0.333	0.259
10-20 ng/ml	0.397	0.412	0.382	0.206
>20 ng/ml	0.350	0.491	0.318	0.191
Gleason score				
<7	0.369	0.448	0.366	0.186
=7	0.423	0.458	0.236	0.306
>7	0.386	0.371	0.486	0.143
Clinical stage				
T1	0.388	0.425	0.375	0.200
T2	0.403	0.416	0.363	0.221
T3/T4	0.328	0.507	0.328	0.164
Metastases				
Detected	0.354	0.486	0.319	0.194
Not detected	0.388	0.425	0.372	0.203

Table 6. rs378854 A allele frequencies and genotype distributions in groups of patients formed according to criteria for assessment of the risk of prostate cancer progression described by D'Amico et al. (1998) and Medeiros et al. (2002).

Group	Allele A frequency	Genotype frequencies		
		GG	GA	AA
D'Amico et al. (1998) risk criteria				
Low-risk	0.344	0.438	0.438	0.125
Intermediate-risk	0.431	0.391	0.356	0.253
High-risk	0.356	0.486	0.315	0.199
Medeiros et al. (2002) risk criteria				
Low-risk	0.385	0.427	0.375	0.198
High-risk	0.387	0.451	0.324	0.225

7). Similar results were obtained in analysis of the association between rs378854 and the risk of PCa progression. Differences in genotype frequencies between the groups of patients formed according to D'Amico criteria (D'Amico et al., 1998) did not reach statistical significance (Table 8). The results of the applied statistical tests also provided no evidence for an association of rs378854 with the risk of PCa progression assessed according to Medeiros et al. (2002) (Table 8).

DISCUSSION

Over the past decade, GWAS have had substantial success in identifying genetic variants associated with complex diseases. The first GWAS of PCa yielded evidence of an association between the genetic variant at 8q24 and the risk of developing prostate malignancy (Amundadottir et al., 2006). Since then, five different regions within the 8q24 gene desert have been reported by GWAS to be associated with PCa risk, of which some also confer the risk of breast, ovarian, colorectal or bladder cancer (Ghoussaini et al., 2008; Wasserman et al., 2010). Molecular mechanisms underlying the association of variants within 8q24 with different malignant diseases are still largely unknown. Several lines of evidence suggest a possible effect of SNP at 8q24 on *MYC* gene expression (Ahmadiyeh et al., 2010; Wasserman et al., 2010). *MYC* was found to be upregulated in both prostate and breast tumor

Table 7. Association of rs378854 with standard prognostic parameters regarding prostate cancer progression and the presence of metastases.

Comparison	P value	Per allele OR (95% CI)
PSA at diagnosis		
10-20 ng/ml *vs.* <10 ng/ml	0.654	1.13 (0.71-1.79)
>20 ng/ml *vs.* <10 ng/ml	0.188	1.38 (0.91-2.09)
>20 ng/ml *vs.* 10-20 ng/ml	0.426	1.22 (0.79-1.90)
Gleason score		
=7 *vs.* <7	0.338	0.80 (0.53-1.20)
>7 *vs.* <7	0.810	0.93 (0.54-1.59)
>7 *vs.* =7	0.648	1.17 (0.65-2.10)
Clinical stage		
T2 *vs.* T1	0.831	0.94 (0.56-1.58)
T3/T4 *vs.* T1	0.431	1.29 (0.73-2.30)
T3/T4 *vs.* T2	0.209	1.38 (0.88-2.16)
Metastases		
Detected *vs.* Not detected	0.532	1.16 (0.77-1.75)

Abbreviations: OR-odds ratio; CI-confidence interval.

Table 8. Association of rs378854 with the risk of prostate cancer progression assessed according to D'Amico et al. (1998) and Medeiros et al. (2002).

Comparison	P value	Per allele OR (95% CI)
D'Amico et al. (1998) risk criteria		
Intermediate-risk *vs.* Low-risk	0.409	0.69 (0.31-1.52)
High-risk *vs.* Low-risk	0.902	0.95 (0.44-2.04)
High-risk *vs.* Intermediate-risk	0.159	1.37 (0.93-2.01)
Medeiros et al. (2002) risk criteria		
High-risk *vs.* Low-risk	0.970	0.99 (0.68-1.45)

Abbreviations: OR-odds ratio; CI-confidence interval.

samples and is thought to be implicated in PCa initiation, as well as in the progression of disease (Jenkins et al., 1997; Bièche et al., 1999; Gurel et al., 2008). *FAM84B*, a nearby gene, has also been proposed to be a PCa susceptibility gene. *FAM84B* was shown to be overexpressed in PCa, mainly in the early stages, and is suggested to be a target of somatic amplification (Solé et al., 2008). A processed pseudogene POU class 5 homeobox 1B (*POU5F1B*) located at 8q24 was also found to be significantly overexpressed in PCa compared to surrounding normal prostatic tissue (Kastler et al., 2010). Reported overexpression in PCa and its homology with the octamer-binding transcription factor 4 (*OCT4*) gene involved in the maintenance of the pluripotency of stem cells qualify *POU5F1B* as a plausible PCa susceptibility candidate gene (Kastler et al., 2010). Recently, several non-coding RNA genes have been identified at 8q24 and considered for possible involvement in prostate carcinogenesis. Altered expression patterns in PCa and/or PIN have been shown for lincRNA genes, such as *PVT1*, prostate cancer non-coding RNA 1 (*PRNCR1*) and prostate cancer associated transcript 1 (*PCAT1*), while for microRNA (miRNA)-coding genes such results have not yet been obtained (Kim et al., 2007; Pomerantz et al., 2009; Chung et al., 2011; Meyer et al., 2011; Prensner et al., 2011).

A study by Meyer et al. (2011) showed a significant association between PCa risk and the functional

variant rs378854, which confers increased expression of *PVT1*. The G allele of rs378854 reduces the binding of the YY1 transcription repressor to the regulatory element within 8q24 that was shown to affect the expression of *PVT1*, but not the adjacent *MYC* gene. These findings were reported for both European-Americans and African-Americans, even though risk allele frequencies differ significantly between these ethnic groups. There was also no evidence of rs378854 affecting the expression patterns of miRNAs hosted within the *PVT1* gene (Meyer et al., 2011). Furthermore, the expression on hsa-mir-1208, a miRNA located downstream of *PVT1*, was also found to be affected by the rs378854 genotype (Meyer et al., 2011).

Our study evaluated the possible association between rs378854 and PCa risk and progression in the Serbian population. Due to the observed deviation from HWE in both groups of patients with PCa and BPH, as well as in the control group, the results were examined for miscalling genotypes that showed no obvious errors in the genotyping procedure. Further reasonable explanations for the discordance with HWE include possible duplications creating pseudo-SNPs (Leal, 2005). Apart from duplication, deletion polymorphisms in the amplified region could influence the genotyping results causing a misinterpretation of heterozygotes as homozygotes (Balding, 2006). Other factors with possible influence on the deviations from HWE are natural selection and genetic drift, as well as population admixture and inbreeding (Schaid and Jacobsen, 1999; Balding, 2006).

In contrast to the results of Meyer et al. (2011), our study showed no evidence of association between rs378854 and PCa risk. This SNP was identified as a functional genetic variant in perfect LD with the previously reported a PCa susceptibility variant rs620861 (heterozygote OR = 1.17, 95% CI 1.10-1.24; homozygote OR = 1.33, 95% CI 1.21-1.45) (Meyer, 2011; Yeger, 2009). Association between rs620861 and PCa risk was found in a GWAS. It involved 10,286 PCa cases and 9,135 controls of European ancestry (Yeger, 2009). Therefore, the small sample size compared to the number of subjects involved in the previous study could influence the results obtained in the Serbian population and underlie the lack of replication of GWAS results. Considering the observed HWE deviations, the discordance of our results with those previously reported could also be due to population characteristics, aside from methodological errors and genetic alterations (Balding, 2006; Schaid and Jacobsen, 1999).

Analysis of the effect of the rs378854 G allele on PCa progression yielded no evidence to support the supposed association. These data concur with the results obtained in the studies by Al Olama et al. (2009) and Lindstrom et al. (2011) that showed no evidence of association of rs620861 (in perfect LD with rs378854) with tumor grade and stage. Another result of the study conducted by Al Olama et al. (2009) is a weak association of rs620861 with PSA level, which was not confirmed in our analysis involving rs378854. Furthermore, Klein et al. (2012) showed no evidence for the association between rs620861 and PCa aggressiveness as evaluated under three different definitions.

Further conclusions about an association between rs378854 and PCa in the Serbian population cannot be made without a final exclusion of genotyping error by retyping the SNP using different methodology, preferably bidirectional sequencing. A larger number of subjects will be required to determine if sample size affects the observed results.

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