Experimental and Molecular Pathology 99 (2015) 145–150

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Assessment of association between genetic variants in microRNA genes *hsa-miR-499*, *hsa-miR-196a2* and *hsa-miR-27a* and prostate cancer risk in Serbian population

Z. Nikolić [a], D. Savić Pavićević [a], N. Vučić [a], S. Cidilko [a], N. Filipović [b], S. Cerović [c], V. Vukotić [b], S. Romac [a], G. Brajušković [a,*]

[a] University of Belgrade, Faculty of Biology, Belgrade, Serbia
[b] Department of Urology, Clinical Centre "dr Dragiša Mišović", Belgrade, Serbia
[c] Institute of Pathology, Military Medical Academy, Belgrade, Serbia

ARTICLE INFO

Article history:
Received 2 June 2015
Accepted 18 June 2015
Available online 22 June 2015

Keywords:
Prostate cancer
MicroRNA
Association study
miR-499
miR-196a2
miR-27a

ABSTRACT

Due to their potentially functional significance, genetic variants within microRNA genes have been recognized as candidates for cancer-related genetic biomarkers. Among the most extensively studied so far are rs3746444, rs11614913 and rs895819. Nevertheless, only few previous studies in Asian population analyzed the association of rs3746444 and rs11614913 with prostate cancer (PCa) risk, while rs895819 was not evaluated in relation to this issue. The aim of this study was to assess the possible association between these genetic variants and PCa risk and progression in Serbian population. 355 samples of peripheral blood were obtained from the patients with PCa and 353 samples from patients with benign prostatic hyperplasia (BPH). 312 volunteers derived from general population who gave samples of buccal swabs were included in the control group. Genotyping of rs3746444, rs11614913 and rs895819 was performed by using PCR-RFLP method, HRM analysis and allele-specific PCR, respectively. Allelic and genotypic associations were evaluated by unconditional linear (for serum PSA level in PCa patients) and logistic regression method with adjustment for age.

Minor allele C of rs895819 was found to be associated with the increased risk of developing PCa under dominant ($P = 0.035$; OR = 1.38, 95%CI 1.02–1.86) and overdominant ($P = 0.04$; OR = 1.37, 95%CI 1.01–1.85) genetic model. Same genetic variant was found to be associated with the clinical stage of localized PCa, as well as with the presence of distant metastases. Allele G of rs3746444 was also shown to be associated with the decreased risk of PCa progression. According to our data, rs3746444 qualifies for a genetic variant potentially associated with PCa aggressiveness in Serbian population. Furthermore, our study provided the first evidence of association between rs895819 and PCa risk, as well as for its genetic association with the presence of distant metastases among PCa patients.

1. Introduction

According to recent projections based on GLOBOCAN 2012 report, global cancer burden is expected to substantially increase within the next decade (Ferlay et al., 2015). Being the second leading cancer in terms of incidence and ranking sixth among causes of cancer-related death worldwide, prostate cancer (PCa) is due to significantly contribute to this growing public health problem (Bray et al., 2012; Ferlay et al., 2015). Among diagnosed tumors, clinically inapparent latent prostate adenocarcinomas which do not require aggressive therapy, but active surveillance, represent a significant proportion (Van der Kwast and Roobol, 2013). Therefore, identification of genetic variants contributing not only to cancer susceptibility, but also to PCa aggressiveness has become one of the imperatives in research activity regarding prostate carcinogenesis. These genetic variants are considered to be potential nonstandard prognostic parameters of PCa that could be implemented in algorithms used for assessing the risk of PCa development, as well as for evaluating the risk of PCa progression and outcome prediction (Goh and Eeles, 2014; Helfand and Catalona, 2014).

Although large genome-wide association studies (GWAS) have made the major contribution to identifying genetic factors contributing to prostate cancer susceptibility, candidate-gene based approaches have also revealed multiple PCa associated regions (Goh and Eeles, 2014). Due to accumulating evidence of the involvement of regulatory mechanisms based on the activity of non-coding RNAs in prostate carcinogenesis,

Abbreviations: PCa, prostate cancer; BPH, benign prostatic hyperplasia; GWAS, genome-wide association study; PSA, prostate-specific antigen; GS, Gleason score; PCR–RFLP, restriction fragment length polymorphism analysis of PCR-amplified fragments; HRMA, high resolution melting analysis; HWE, Hardy–Weinberg equilibrium; OR, odds ratio; CI, confidence interval; AIC, Akaike information criterion.

* Corresponding author.
E-mail address: brajuskovic@bio.bg.ac.rs (G. Brajušković).

http://dx.doi.org/10.1016/j.yexmp.2015.06.009
0014-4800/

genetic variants in microRNA genes have been recognized as potential PCa susceptibility loci (Bolton et al., 2014; George et al., 2011; Nikolić et al., 2014; Xu et al., 2010). These genetic variants could influence microRNA biogenesis, stability of mature microRNA molecules, efficiency of target gene regulation, as well as target specificity, therefore, leading to aberrant expression of numerous oncogenes and tumor-suppressors (George and Mittal, 2010; Ryan et al., 2010).

Among the most extensively analyzed genetic variant in microRNA genes are rs3746444 in *hsa-miR-499* and rs11614913 in *hsa-miR-196a2* (Ma et al., 2013). The minor allele of genetic variant rs3746444 was found to cause a mismatch in the stem region of miR-499 precursor, thus potentially leading to a secondary structure alteration affecting biogenesis of mature microRNA molecules. Being located in the segment of hsa-miR-499 gene encoding the seed region of the mature passenger microRNA strand (hsa-miR-499-3p), rs3746444 was hypothesized to affect its function by altering interactions with target mRNAs and target specificity (Chen et al., 2014; Hu et al., 2009). Therefore, mature passenger microRNAs originating from hsa-miR-499 gene which bear different allelic variants could have a significantly discordant set of target mRNAs. Genetic variant rs11614913 is located in the region of *hsa-miR-196a2* encoding the passenger strand of the mature miR-196a2 (hsa-miR-196a2-3p). Thus, it was hypothesized that this genetic variant could affect the interaction between hsa-miR-196a2-3p and target mRNAs by altering the sequence of this potentially functional microRNA molecule. Furthermore, by inducing secondary structure changes, rs11614913 could affect the processing of miR-196a2 precursors (Hu et al., 2009; Ryan et al., 2010). Evidence to support this hypothesis were obtained in expression analyses showing significant differences in mature miR-196a2 levels associated with different rs11614913 genotypes (Hu et al., 2008). Due to these potential functional consequences, rs3746444 and rs11614913 were selected for numerous case–control studies on different types of malignant diseases which provided contrasting and inconclusive results (Ma et al., 2013). Not only that these genetic variants were found to be associated with the risk of developing multiple types of cancer, but also to be related to cancer prognosis (Ahn et al., 2013; Hu et al., 2008; Wang et al., 2013).

In spite of the wide range of their potential functional significance, the effect of genetic variants in genes encoding microRNA on PCa susceptibility and progression was assessed in only few studies conducted in Asian populations and our previous study involving PCa patients from Serbia (European origin). These earlier studies have provided discordant results regarding the effects of genetic variants in *hsa-miR-146a* (Nikolić et al., 2014; Parlayan et al., 2014; Xu et al., 2010) and *hsa-miR-196a2* (George et al., 2011; Parlayan et al., 2014), while for rs3746444 in *hsa-miR-499* gene analysis of association with PCa risk was conducted in a single study (George et al., 2011). The obtained findings emphasize the need for additional analyses in multiple European and non-European populations in order to make definite conclusions. To date, no other microRNA genetic variants have been analyzed in respect to their association with PCa. Nevertheless, rs895819 stands out due to its association with multiple other malignant diseases, as well as for its potential significance in regulating the biogenesis and/or function of PCa-related miR-27a (Fletcher et al., 2012; Goh and Eeles, 2014; Xu et al., 2013). This genetic variant has been identified as a SNP affecting the sequence of the miR-27a pre-microRNA terminal loop and altering its secondary structure. Therefore, rs895819 was hypothesized to influence the processing of miR-27a precursors by Drosha enzyme (Sun et al., 2010; Zeng et al., 2005). Mature miR-27a was previously found to be aberrantly expressed in PCa, leading to enhanced malignant cell growth (Fletcher et al., 2012; Porkka et al., 2007; Szczyrba et al., 2010; Volinia et al., 2006). Also, it was shown that this microRNA is androgen-regulated and stimulates the androgen signalization in a positive feedback loop (Fletcher et al., 2012).

The aim of this study was to evaluate the association of genetic variants rs11614913 in *hsa-miR-196a2* and rs3746444 in *hsa-miR-499* with PCa risk and progression in Serbian population. To our knowledge, this is the first European population in which a case–control study on this subject was performed. Since there were no reports on the relation of rs895819 with PCa risk, we have also conducted the first study designed to provide data referring to this issue. Furthermore, we assessed the association of rs895819 with standard prognostic parameters of PCa, as well as with the risk of PCa progression.

2. Material and methods

The study used peripheral blood samples obtained from the patients treated in the period between 2009 and 2013 at Clinical Centre "Dr Dragiša Mišović Dedinje", Belgrade, Serbia. Research was conducted with the approval of ethics committees of this medical institution. Experiments are in accordance with the Helsinki Declaration of 1975.

355 samples of peripheral blood were obtained from patients with PCa and 353 samples from patients with benign prostatic hyperplasia (BPH). The control group comprised 312 healthy volunteers who gave samples of buccal swabs. Written informed consent was obtained from all participants. The exclusion criteria for potential controls were the presence of any self-reported diseases and family history of PCa. Mean ages for PCa and BPH patients and controls were 69.96, 68.13 and 68.27 years, respectively. Diagnoses of PCa and BPH were made by using standard clinical procedure which included digital rectal examination, transrectal ultrasonography, abdominal and pelvic ultrasound, bone scintigraphy and radiography, serum prostate-specific antigen (PSA) level and biopsy of the prostate.

Patients with PCa were selected 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). Two groups of patients were formed based on the presence of distant metastases. Based on the risk for localized cancer progression, three groups of patients were formed, according to D'Amico criteria (D'Amico et al., 1998) and as recommended by European Association of Urology (EAU). Groups were defined as low-risk (PSA < 10 ng/ml, GS < 7, 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 high risk group. During the genotyping procedure, investigators were blinded for the clinical status of subjects.

Genomic DNA was isolated from peripheral blood and buccal swab samples using the QIAamp® DNA Mini Kit (*QIAGEN*, Hilden, Germany) following the manufacturers' protocol.

Genotyping of rs3746444 was performed using PCR–RFLP method. Primers used to amplify a segment of DNA surrounding rs3746444 were previously published by Hu et al. (2008). PCR protocol is presented as Supplementary information 1. The expected length of PCR products was 146 bp.

10 μl of PCR products were digested at 55 °C overnight with 1 U of BclI enzyme (*Fermentas*, Hunover, MD, USA) per single reaction (15 μl reaction mixture). Digested products were separated by 3% agarose gel electrophoresis. The expected lengths of fragments resulting from restriction digest were 120 bp and 26 bp for AA genotype, 146 bp, 120 bp and 26 bp for AG genotype, and 146 bp for GG genotype.

Genotyping of rs11614913 was performed by High Resolution Melting Analysis (HRMA). Primers used to amplify 81 bp long segment of DNA surrounding this genetic variants were previously described by Qi et al. (2014). The protocol for HRMA is presented as Supplementary information 2.

For genotyping rs895819, allele-specific PCR was optimized. Primers were designed by using BatchPrimer3 v1.0 software (BatchPrimer3; You et al., 2008). For each sample, two PCR reaction mixtures were prepared containing different allele-specific primer, a common forward primer and a common reverse non-allele-specific primer (Table 1) used to generate amplicon which acts as internal positive control. PCR