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THE EFFECT OF EPISTATIC INTERACTIONS BETWEEN GENETIC VARIANTS LOCATED IN MICRORNA AND SILENCING COMPLEX GENES ON PROSTATE CANCER PROGRESSION RISK

Zorana DOBRIJEVIĆ[1,2], Jelena KARANOVIĆ[1], Dušanka SAVIĆ-PAVIĆEVIĆ[1], Goran BRAJUŠKOVIĆ[1*]

[1]University of Belgrade - Faculty of Biology, Belgrade, Serbia
[2]Present address: University of Belgrade – Institute for Application of Nuclear Energy, Belgrade, Serbia

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Previous studies conducted in Asian and European populations have provided evidence of the association between microRNA-related genetic variants and prostate cancer (PCa) risk and/or progression. Nevertheless, the results obtained in these studies are inconsistent, which could be explained by the limitations of single-locus main effect evaluations to detect joint effects of multiple genetic variants, reflected in statistical epistases. Therefore, we conducted the analysis of potential epistatic interactions between variants located in microRNA genes and in genes encoding the components of RNA-induced silencing complex (RISC) in relation with PCa risk/aggressiveness. Raw data on genotyping results from our previous studies involving four microRNA polymorphisms and five variants in RISC genes were subjected to the exclusion of samples based on missing data criterion, followed by the re-evaluation of Hardy-Weinberg equilibrium. Afterwards, these genotyping results were included in the Multifactor dimensionality

Corresponding author: Goran Brajušković, University of Belgrade - Faculty of Biology, Belgrade, Serbia, Phone: + 38162279777; Fax: + 381112639100, *E-mail*: brajuskovic@bio.bg.ac.rs

reduction (MDR) analysis. Permutation testing was conducted in order to assess statistical significance of the best models from MDR tests. MDR tests on the risk of developing PCa yielded statistically insignificant results. Nevertheless, the MDR results for comparison of PCa patients with high and low cancer progression risk were statistically significant for the analysis that included rs11614913, with the 3-locus best model comprising this genetic variant, rs7813 and rs784567. We conclude that statistical epistasis between rs11614913 in *hsa-miR-196a2*, rs7813 in *GEMIN4* and rs784567 in *TARBP2* shows association with the invasiveness of PCa.

Keywords: epistasis; MDR, microRNA, prostate cancer, RISC

INTRODUCTION

Prostate cancer (PCa) is one of the most significant health issues worldwide, accounting for about 13.5% of newly diagnosed cancers in males, according to recent statistics (BRAY *et al*., 2020). In Serbia, PCa ranks fifth on both incidence and the mortality cancer scales, when combining statistics for male and female gender (FITZMAURICE *et al*., 2019). Furthermore, the increasing trend for crude PCa mortality rates was reported in Serbia for the period between 1990 and 2020 (INSTITUTE OF PUBLIC HEALTH OF SERBIA "DR MILAN JOVANOVIĆ BATUT", 2020). Since PCa is a complex multifactorial disease, its molecular basis is still not clearly understood (SCHRECENGOST and KNUDSEN, 2013). To date, numerous lines of evidence suggested the involvement of dysregulation in RNA interference processes in prostate carcinogenesis (KHANMI *et al*., 2015). Therefore, case-control studies on PCa which involved microRNA-related potentially functional genetic variants have been conducted in Asians and in European populations (XU *et al*., 2010; GEORGE *et al*., 2011; LIU et al., 2012; NIKOLIĆ *et al*., 2014; NIKOLIĆ *et al*., 2015; STEGEMAN et al., 2015; CHU *et al*., 2016; HASHEMI *et al*., 2016; NIKOLIĆ *et al*., 2017; KOTARAC *et al*., 2019; KOTARAC *et al*., 2020; DAMODARAN *et al*., 2020). These studies provided evidence of association between the analyzed genetic variants and the PCa risk or disease aggressiveness. Among the analyzed variants are the ones located within genes encoding microRNA molecules, as well as those affecting the function of the components of RNA-induced silencing complex (RISC) (XU *et al*., 2010; GEORGE *et al*., 2011; LIU et al., 2012; NIKOLIĆ *et al*., 2014; NIKOLIĆ *et al*., 2015; STEGEMAN et al., 2015; CHU *et al*., 2016; HASHEMI *et al*., 2016; NIKOLIĆ *et al*., 2017; KOTARAC *et al*., 2019; KOTARAC *et al*., 2020; DAMODARAN *et al*., 2020).

Nevertheless, the results obtained in these studies are inconsistent, which could be partially explained by the epistatic interactions between multiple genes, as well as by the involvement of environmental risk factors (BARNHOLTZ-SLOAN *et al*., 2011). Namely, the statistical approaches used in these previous studies are adequate for the assessment of the main effects of single variants, but do not point out the potential effects of interactions between polymorphisms on PCa risk or invasiveness. In an attempt to overcome the limitations of a single-gene approach, we decided to evaluate the joint effect of selected microRNA-related variants on PCa risk and disease aggressiveness by using Multifactor dimensionality reduction (MDR) as a data-mining method (MOORE *et al*., 2016). The present case-control study included groups of PCa patients and healthy controls from Serbian population which were recruited for our previous studies which evaluated the main effects of same genetic variants on the risk of

developing PCa, as well as on PCa progression (NIKOLIĆ *et al*., 2014; NIKOLIĆ *et al*., 2015; NIKOLIĆ *et al*., 2017).

To our knowledge, the present study is the first one evaluating the ability of statistical epistatic interactions between microRNA-related genetic variants to predict the PCa risk. Since the genetic variants included in the tests of statistical epistases are hypothesized to affect the functions of biologically related genes, the statistically significant results could reflect the real biological epistases.

MATERIAL AND METHODS

The study included 355 patients with PCa and 312 healthy controls previously described in detail in NIKOLIĆ *et al*. (2015). Briefly, for this case-control study in Serbian population, PCa patients were consecutively recruited from the medical institution after giving informed consent. Their clinical data was acquired, including serum prostate-specific antigen (PSA) level at diagnosis, Gleason Score, clinical stage of primary PCa, as well as the presence of metastases. PCa patients were selected into groups with high (Gleason score ≥ 7 or stage T3/T4 or bone metastases) and low risk (Gleason score < 7 and stage T1-T2) for cancer progression according to criteria defined by MEDEIROS *et al*. (2002). Healthy controls enrolled in this study presented no history of prostatic diseases and were age-matched to PCa patients.

Samples were previously genotyped for microRNA genetic variants rs2910164 in *hsa-miR-146a*, rs3746444 in *hsa-miR-499*, rs11614913 in *hsa-miR-196a2* and rs895819 in *hsa-miR-27a* in order to assess their main effects and the results were published by NIKOLIĆ *et al*. (2014; 2015). Also, RISC variants rs3742330 in *DICER1*, rs4961280 in *AGO2*, rs784567 in *TARBP2*, rs7813 in *GEMIN4* and rs197414 in *GEMIN3* were previously genotyped by our research group (NIKOLIĆ *et al*., 2017). The mentioned publications include detailed descriptions of genotyping procedures. Namely, Taqman® SNP Genotyping Assays (Applied Biosystems, Foster City, California, USA) were used for genotyping rs2910164 and rs7813 (NIKOLIĆ *et al*., 2014; NIKOLIĆ *et al*., 2017). Rs3746444 and rs197414 were genotyped using PCR–RFLP method, while genotyping of rs11614913, rs784567 and rs3742330 was performed by High Resolution Melting Analysis (HRMA) (NIKOLIĆ *et al*., 2014; NIKOLIĆ *et al*., 2015; NIKOLIĆ *et al*., 2017). Allele-specific PCR was optimized for assessing genotypes of rs895819, while genotyping of rs4961280 was done by using custom-designed real-time PCR-based genotyping assay with specific probes (PrimerDesign Ltd, Southampton, UK) (NIKOLIĆ *et al*., 2015; NIKOLIĆ *et al*., 2017). Raw data on genotyping results were available for epistasis analysis, with missing genotype call being the exclusion criterion. After the exclusion of subjects with missing genotype data, control groups were reanalyzed for Hardy-Weinberg equilibrium using exact test implemented in SNPStats software (SOLÉ *et al*., 2006).

The nonparametric MDR method implemented in open-source MDR software (v.3.0.2) (MDR) was used for assessing potential statistical epistatic interactions (MOORE *et al*., 2006). 10-fold cross validation and 10 times repeated analysis with different seeds were applied in order to reduce the possibility of biased results. Mean prediction error percentage and cross-validation consistency (CVC) frequency were used to choose the best model. In order to evaluate the prediction error and the CVC frequency, permutation test was performed with 1000 permutations using MDR permutation testing module MDRpt v.1.0 beta 2 (MOORE *et al*., 2006). This test provided *P* values associated with each prediction error and CVC. Interaction entropy

graph was constructed in order to visually inspect the potential epistasis and quantify the synergistic and non-synergistic interactions (MOORE *et al*., 2006). Information gain values in the nodes of the graph refer to main effect of genetic variants, while those located on the connecting lines between nodes indicate independence or epistatic interaction. Information gain (value > 0) suggests synergistic interaction, while information loss (value < 0) indicates redundancy (MOORE *et al*., 2006).

RESULTS

The number of cases and controls included in MDR analysis was 344 and 293, respectively. For all genetic variants included in the analysis, recalculation conducted after the exclusion of subjects with missing data showed no significant deviations from HWE (results not shown). When patients with PCa were selected based on the risk of PCa progression, 139 were found to have low risk, while 178 were selected in the group with the high risk for PCa progression.

The results of the exhaustive MDR analysis that evaluated combinations of these 4 polymorphisms in microRNA genes with those located in genes encoding RISC components are presented in Tables 1 and 2. All MDR tests on PCa risk yielded statistically insignificant results, based on permutation *P* values (Table 1).

Table 1. The MDR results for potential association of interaction between genetic variants in microRNA and RISC genes with PCa risk.

MicroRNA genetic variant	Model	Training BA	Testing BA	CVC	*P* value
rs2910164	rs784567	0.5396	0.5396	10/10	0.97
	rs4961280,rs784567	0.5647	0.4864	6/10	0.99-1.00
	rs2910164,rs4961280,rs784567	0.5962	0.5248	9/10	0.977-0.978
	rs2910164,rs7813,rs4961280,rs784567	0.6331	0.5419	10/10	0.238
	rs2910164,rs7813,rs197414,rs4961280,rs784567	0.6761	0.5368	10/10	0.972-0.973
rs3746444	rs784567	0.5403	0.5205	9/10	0.880-0.881
	rs4961280,rs784567	0.5625	0.5097	7/10	0.969
	rs7813,rs3746444,rs784567	0.5839	0.4841	6/10	0.99-1.00
	rs7813,rs197414,rs4961280,rs784567	0.6191	0.4864	5/10	0.99-1.00
	rs7813,rs3746444,rs197414,rs4961280,rs784567	0.6656	0.5222	10/10	0.852
rs11614913	rs784567	0.5396	0.5396	10/10	0.928
	rs4961280,rs784567	0.5613	0.5364	10/10	0.928-0.929
	rs11614913,rs4961280,rs784567	0.5859	0.5298	10/10	0.94
	rs7813,rs197414,rs4961280,rs784567	0.6156	0.4814	6/10	0.99-1.00
	rs7813, rs11614913,rs197414,rs4961280,rs784567	0.663	0.4811	10/10	0.99-1.00
rs895819	rs895819	0.546	0.5108	7/10	0.919
	rs895819,rs784567	0.5639	0.5284	5/10	0.625-0.626
	rs895819,rs4961280,rs784567	0.5936	0.5413	10/10	0.383
	rs7813, rs895819,rs4961280,rs784567	0.6233	0.4673	5/10	0.99-1.00
	rs7813, rs895819,rs197414,rs4961280,rs784567	0.664	0.4621	9/10	0.99-1.00

The MDR analysis that included genetic variants in RISC genes and rs2910164 yielded the best 4-locus model (rs2910164, rs7813, rs4961280 and rs784567) with testing balance accuracy of 0.5419 and CVC of 10/10, but without statistical significance (P = 0.238). The same CVC was detected for 1-locus model (rs784567) and for 5-locus model involving all genetic variants included in this analysis (Table 1). When the analysis included rs3746444, the best model was found to be 5-locus model of interaction with testing balance accuracy of 0.5222 and permutated P value of 0.852. The CVC obtained for this model was found to be 10/10. In the analysis of interaction between rs11614913 and RISC genetic variants, the best model comprised only rs784567. The testing balance accuracy for this model was 0.5396, with permutated P value of 0.928 and CVC 10/10. Results obtained in the analysis involving rs895819 suggested that the best model comprises rs895819, rs4961280 and rs784567, with P = 0.383, testing balance accuracy of 0.5413 and CVC of 10/10.

Table 2. The MDR results for comparison of PCa patients with high and low cancer aggressiveness that included rs11614913 and genetic variants in RISC genes.

Model	Training BA	Testing BA	CVC	*P* value
rs7813	0.5445	0.4737	7/10	0.953-0.954
rs7813,rs784567	0.5986	0.54	6/10	0.362
rs11614913,rs7813,rs784567	0.6419	0.6054	10/10	0.026-0.027
rs11614913,rs7813,rs197414,rs784567	0.6808	0.5417	9/10	0.376-0.377
rs11614913,rs7813,rs197414,rs4961280,rs784567	0.7206	0.5387	10/10	0.413

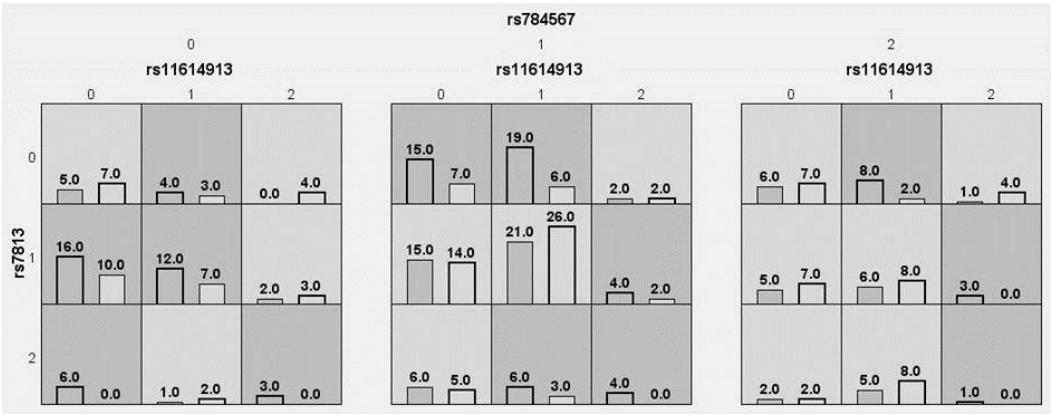

Figure 1. Graphical representation of the interaction between rs11614913, rs7813 and rs784567 in PCa case-control comparison. Summary of genotype combinations.

MDR results for the comparison of PCa patients with high and low cancer progression risk were statistically significant only for the analysis that included rs11614913 (Table 2). Graphical representation of this model is presented in Figure 1. The entropy based Fruchterman-Rheingold chart is presented in Figure 2. These results suggested that the best model was 3-locus model which included rs11614913, rs7813 and rs784567. The obtained testing balance accuracy was 0.6054 with P = 0.026-0.027 and CVC of 10/10. Other model with the same CVC and lower testing balance accuracy (0.5387) was the 5-locus model involving all genetic variants tested. These results were further confirmed by the data obtained in MDR analysis involving only genetic variants located in RISC genes, which yielded the marginally significant 2-locus model consisting of rs7813 and rs784567. The testing balance accuracy for this model was 0.5914, with P value of 0.046 and CVC 10/10 (results not shown).

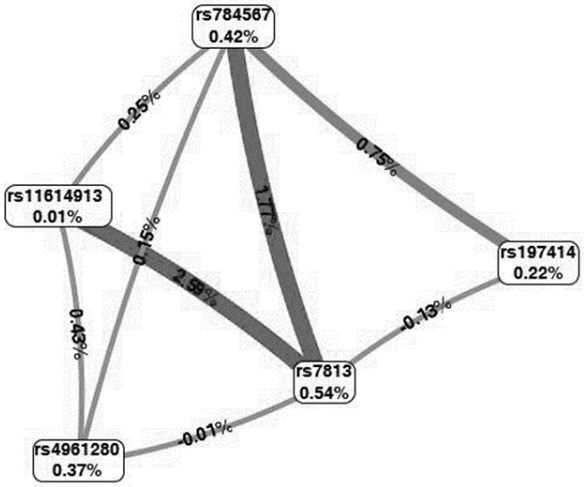

Figure 2. Fruchterman-Rheingold chart of the interaction between rs11614913, rs7813 and rs784567 in PCa case-control comparison. The chart summarizes the results of the entropy-based information gain analysis

DISCUSSION

Various aspects of deregulation of microRNA-based regulatory mechanisms have been implicated in PCa pathogenesis (KHANMI *et al*., 2015). Numerous microRNAs, as well as components of RNA interference machinery, were found to be overexpressed or downregulated in malignant tissue, compared to the surrounding normal prostatic glandular epithelium (PATIL *et al*., 2015; WANG *et al*., 2015). Most of the studies on relation between microRNAs and PCa involved leader strands of microRNA molecules. Nevertheless, in recent years, microRNA passenger strands are also in the focus of this type of research, mainly because of the accumulating evidence of their functional significance (YANG *et al*., 2011; YANG *et al*., 2013;

CANNISTRACI *et al*., 2014). Accordingly, deregulation in expression of both strands of mature microRNAs was found to be related to PCa. Also, forced or silenced expression of various microRNAs was shown to be correlated with PCa progression (CANNISTRACI *et al*., 2014; JANSSON *et al*., 2012). Based on these experimental data, various microRNAs were classified as potentially oncogenic and/or tumor-suppressive in PCa (JANSSON *et al*., 2012). Therefore, genetic variants which may affect the biogenesis or functional properties of these microRNAs emerged as candidates for case-control studies on prostate adenocarcinoma. Several were conducted to date, providing unique or conflicting data (XU *et al*., 2010; GEORGE *et al*., 2011; LIU *et al*., 2012; NIKOLIĆ *et al*., 2014; NIKOLIĆ *et al*., 2015; STEGEMAN *et al*., 2015; CHU *et al*., 2016; HASHEMI *et al*., 2016; NIKOLIĆ *et al*., 2017; KOTARAC *et al*., 2019; KOTARAC *et al*., 2020; DAMODARAN *et al*., 2020).

Genetic variant rs2910164, potentially affecting the biogenesis of mature miR-146a molecules and target specificity of the "passenger" strand, was identified as PCa susceptibility variant in Han Chinese (XU *et al*., 2010). Conversely, data obtained in a case-control study in Northern Indian population did not support the supposed association between rs2910164 and the PCa risk (GEORGE *et al*., 2011). Also, our previously published results on this issues conducted on the same research cohort included in the present study suggested the association between rs2910164 and PCa aggressiveness, but not with susceptibility to this malignancy (NIKOLIĆ *et al*., 2014). Other microRNA genetic variants which we previously analyzed in the present study population are rs3746444, rs11614913 and rs895819 (NIKOLIĆ *et al*., 2015). Based on these results, rs3746444 was found to be associated with PCa aggressiveness. This genetic variant, which potentially affects miR-499 biogenesis and target specificity of the "passenger" strand, was not found to be a PCa susceptibility variant in Serbian population, which contrasts the results previously obtained in North Indians (GEORGE *et al*., 2011; NIKOLIĆ *et al*., 2015). Similar discordance between the results of these two studies was obtained for genetic variant rs11614913, which was hypothesized to affect the interaction between miR-196a2 "passenger" strand and target mRNAs (GEORGE *et al*., 2011; NIKOLIĆ *et al*., 2015). For genetic variant rs895819, evidence was obtained in Serbian population suggesting its association with both PCa risk and the risk of developing distant metastases (NIKOLIĆ *et al*., 2015). Even though this genetic variant does not affect the sequence of mature microRNAs, it was speculated to potentially influence the biogenesis process. The obtained data are unique, since the effect of rs895819 on PCa risk was not analyzed in any other population.

Our previous results on genetic variants rs3742330 in *DICER1*, rs4961280 in *AGO2*, rs784567 in *TARBP2*, rs7813 in *GEMIN4* and rs197414 in *GEMIN3*, obtained in a study involving the same group of subjects, showed no evidence of association between these genetic variants and PCa susceptibility (NIKOLIĆ *et al*., 2017). These data contrasted the previously obtained results in Han Chinese which suggested association between rs7813 and PCa risk (LIU *et a*l., 2012). Nevertheless, rs3742330, rs4961280 and rs7813 demonstrated the protective effect against PCa progression (NIKOLIĆ *et al*., 2017). Based on these findings and the results from a study in Han Chinese, rs7813 allele G qualifies for a protective allelic variant against disease aggressiveness (LIU *et a*l., 2012; NIKOLIĆ *et al*., 2017).

This study aimed to identify a potential effect of epistatic interactions between genetic variants in genes encoding microRNAs and RISC components on PCa risk, as well as on PCa

aggressiveness. Undertaking these statistical tests holds its importance since traditional approaches for data analysis in case-control studies might not detect the associations between genetic variants and binary outcomes since they only assess marginal main effects of these potential risk factors (MOORE *et al*., 2006; WEI *et al*., 2014). Therefore, the non-additive effect of genetic variants on PCa risk may not be detected by standard tests of association which were employed in our previous study involving the same group of subjects. Also, the MDR analysis used to assess statistical epistases is a data mining method which does not require the assumption of a specific genetic model (MOORE *et al*., 2006).

The results obtained in this study did not support the effect of epistatic interactions between genetic variants in microRNA and RISC genes on PCa risk. In contrast to these results, epistatic interaction between rs11614913, rs7813 and rs784567 was found to be associated with PCa aggressiveness. Other MDR tests on PCa aggressiveness yielded statistically insignificant results. Interpretation of the best model for the MDR analysis which included rs11614913 and genetic variants in RISC genes indicated a synergistic interaction. Neither rs11614913 nor rs784567 showed significant main effect in our previous study, while a statistical trend of association was shown for rs7813 (LIU *et a*l., 2012; NIKOLIĆ *et al*., 2017). Therefore, this model is unlikely to be biased by the significant main effect of a certain genetic variant. This suggests that the MDR analysis yielded evidence of association of genetic variant in *hsa-miR-196a2* and those located in *GEMIN4* and *TARBP2* genes with the risk of PCa progression which is based on epistatic interactions rather than on independent main effects.

The statistical interaction detected in the present analysis could potentially reflect the biological epistatic interaction between miR-196a2 and the components of RISC, TARBP2 and GEMIN4. For rs11614913 in *hsa-miR-196a2* gene, it was hypothesized that it represents a functional genetic variant potentially affecting the interaction between the mature microRNA and its target mRNAs, while it also induces secondary structure changes relevant to biogenesis process (GEORGE *et al*., 2011; NIKOLIĆ *et al*., 2015). Considering the fact that silencing complex comprises both TARBP2 and GEMIN4, and that its functional properties are related to microRNA biogenesis and to their regulatory activities, both hypothesized functional consequences of rs11614913 could provide biological explanation for detected epistases.

In order to make further conclusions, additional analyses of the effect of potential epistatic interactions between microRNA and RISC variants are needed. Taking into account potential ethnic differences, these analyses should be conducted in other European and non-European populations, together with the assessments of the main effect of genetic variants included in this study.

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EFEKAT EPISTATIČKIH INTERAKCIJA IZMEĐU VARIJANTI U GENIMA ZA MIKRORNK I GENIMA ZA PROTEINE UTIŠAVAJUĆEG KOMPLEKSA NA RIZIK ZA RAZVOJ I PROGRESIJU KARCINOMA PROSTATE

Zorana DOBRIJEVIĆ[1,2], Jelena KARANOVIĆ[1], Dušanka SAVIĆ-PAVIĆEVIĆ[1], Goran BRAJUŠKOVIĆ[1]

[1]Univerzitet u Beogradu – Biološki fakultet, Centar za humanu molekularnu genetiku, Beograd, Srbija
[2]Trenutna ustanova: Univerzitet u Beogradu – Institut za primenu nuklearne energije, Odeljenje za metabolizam, Beograd, Srbija

Izvod

Ranije studije u evropskim i azijskim populacijama ukazale su na značajnu asocijaciju genetičkih varijanti sa efektom na funkciju mikroRNK sa rizikom za razvoj i/ili progresiju karcinoma prostate (KP). Međutim, rezultati navedenih studija su nepodudarni, za šta je jedan od mogućih razloga nemogućnost pristupa baziranih na proceni pojedinačnih efekata genetičkih varijanti da detektuju značajne zajedničke efekte više varijanti, a koji se reflektuju u statističke epistaze. Iz tog razloga, cilj naše studije bila je analiza potencijalnih epistatičkih interakcija između varijanti lociranih u genima za mikroRNK molekule i u genima za komponente utišavajućeg kompleksa indukovanog sa RNK (RISC) kao faktora rizika za razvoj i/ili progresiju KP. Rezultati genotipizacije dobijeni tokom sprovođenja naših ranijih studija, a koji uključuju podatke za četiri varijante u genima za mikroRNK i pet u genima za komponente RICS, podvrgnuti su inicijalnoj obradi podataka u smislu isključivanja uzoraka sa nedostajućim rezultatima, nakon čega je procenjeno odstupanje od Hardi-Vajnbergove ravnoteže. Rezultati su zatim analizirani metodom redukcije dimenzionalnosti višestrukih faktora (Multifactor dimensionality reduction - MDR analysis). Permutacioni testovi su sprovedeni sa ciljem procene statističke značajnosti najboljih modela iz MDR analize. Dobijeni rezultati MDR testova koji su se odnosili na rizik za razvoj KP nisu bili statistički značajni. S druge strane, MDR rezultati koji se odnose na rizik za progresiju KP bili su značajni za model koji uključuje tri lokusa: rs11614913, rs7813 and rs784567. Stoga, zaključci naše studije ukazuju na značaj epistatičkih interakcija između varijanti rs11614913 u *hsa-miR-196a2*, rs7813 u *GEMIN4* i rs784567 u *TARBP2* kao faktora koji ispoljavaju efekat na invazivnost KP.

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