

Association Between *RASSF1A* Promoter Methylation and Testicular Germ Cell Tumor: A Meta-analysis and a Cohort Study

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Abstract. *Background:* The RAS association domain family protein 1a (*RASSF1A*) is a prominent tumor suppressor gene showing altered promoter methylation in testicular germ cell tumors (TGCT). *RASSF1A* promoter hypermethylation might represent an early event in TGCT tumorigenesis. We investigated whether the *RASSF1A* promoter methylation in peripheral blood of TGCT patients can be associated with testicular cancer risk. *Materials and Methods:* Following a meta-analysis, we performed a cohort study including 32 testicular cancer patients and 32 healthy controls. Promoter methylation of the *RASSF1A* and *O*⁶-methylguanine-DNA-methyltransferase (*MGMT*) genes was analyzed using bisulfite pyrosequencing of DNA from peripheral blood. *Results:* Meta-analysis showed an odds ratio (OR) of 7.69 for *RASSF1A* promoter methylation as a risk factor for TGCT. Cohort study found altered methylation of the *RASSF1A* promoter in blood of TGCT patients. Methylation was higher in TGCT patients before BEP chemotherapy. *Conclusion:* The meta-analysis indicates a role of the *RASSF1A* promoter hypermethylation from peripheral blood

in TCGT. We confirmed that finding in our cohort study, which represents the first report of changed *RASSF1A* promoter methylation in peripheral blood TGCT.

Testicular germ cell tumors (TGCTs) account for most testicular cancers. Incidence of 55 new cases per 100,000 males per year world-wide, and increase in number of diagnosed men with TC in US and other Western countries, makes it an important healthcare issue (1, 2). The most affected subjects are young Caucasians (between age of 25 and 29 years) and some of the most rapid trends are observed in men of European descent, especially in Scandinavian countries (3). About 23,000 new cases are predicted to occur in Europe by 2025 (4). Today, more than 90% of patients with TGCT are cured if diagnosed early, *i.e.* only 10-20% of patients with metastatic tumors at the time of diagnosis do not become permanently cured. The therapy depends on the type and the stage of the disease. The cure usually consists of surgery followed by chemotherapy and/or radiotherapy (5). The most commonly used chemotherapy regimens are based on the treatment protocol, which contains several cycles of antineoplastic drugs cisplatin, bleomycin and etoposide (BEP chemotherapy). Except self-inspection, there is no appropriate diagnostics that can be used in routine screening of a population under the risk for TC. The European Association of Urology recommends biopsy in high-risk patients, but non-invasive diagnostic methods, such as immunocytochemistry of semen smears are not sufficiently sensitive (6). This is why introduction of new diagnostics, that would enable recognition of TGCT at early stages, as well as estimation of therapy efficiency at the molecular level, is of special interest.

Histologically, TGCTs are characterized by substantial heterogeneity. The new updated WHO classification system

This article is freely accessible online.

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Key Words: DNA methylation, *RASSF1*, *MGMT*, testicular cancer, BEP chemotherapy.

divides testicular germ cell tumors into two major groups: those that are derived from germ cell neoplasia *in situ* (GCNIS) and those that are not (7). Germ cell neoplasia *in situ* cells precedes both seminoma and non-seminoma (NSGCT) in the tumors of young adults. While seminomas are morphologically homogenous (they account for around 60% of TGCT cases), non-seminomas are morphologically very heterogeneous and can be composed of one or more components (such as embryonal carcinoma, teratoma, yolk sac tumor, and choriocarcinoma and other trophoblastic tumors). Experimental evidence suggest that seminomas and non-seminomas differ in the underlying genetic lesions (8).

Besides genetic component, the familial risk of TGCT is one of the highest among cancers (9), TGCT has a strong environmental component. Genome wide association studies (GWAS) have uncovered some candidate genetic loci involved in testicular cancer susceptibility (10-13) and study of single nucleotide polymorphisms (SNPs) in the same loci showed the risk variants which predispose to both familial and bilateral TGCT, which accounted for only 11-16% of the risk for the disease (14). Various external stressors, including diagnostic procedures, contribute to TGCT pathogenesis (15). A pilot study suggests a possible familial TGCT susceptibility that may be associated with promoter methylation in previously identified TGCT risk genes in GWA studies (16).

Aberrant promoter hypermethylation of candidate tumor suppressor genes (TSG) or tumor-related genes seems not to be a frequent event in TGCT as it is the case in many other human cancers (17). Nevertheless, the RAS association domain-containing protein 1A (*RASSF1A*), a tumor suppressor gene located on the chromosome region 3p21, was identified as one of the main TSGs that change promoter methylation status in TGCT. This gene is functionally relevant for cell-cycle control, microtubule stabilization, cellular adhesion, motility and apoptosis, and depletion of *RASSF1A* is shown to be associated with higher risk of chromosome rearrangements, accelerated mitotic progression and enhanced cellular motility (18-20). Epigenetic inactivation of the *RASSF1A* gene by CpG methylation is recorded in many cancers (for a review see (21)), and hypermethylation of *RASSF1A* promoter has a prognostic value for some tumor types (22-24). The *RASSF1A* promoter is also found hypermethylated in both seminomas and non-seminomas, while three additional candidate genes, *MGMT*, *APC* and *FHIT*, were frequently found hypermethylated only in NSTGCT (25, 26). Therefore, it seems that *RASSF1A* promoter hypermethylation might occur as the first epigenetic event in TGCT tumorigenesis and additional specific epigenetic events characterize morphologically heterogeneous non-seminoma TGCT.

Identification of sensitive and specific indicators, and possibly non-invasive biomarkers that would predict early-

onset TGC tumors, are still in research focus. Peripheral whole blood is the most convenient sample for non-invasive biomarker analyses. Even though the tissue specificity of DNA methylation pattern could be a problem in such approach, there is an increasing number of publications showing that aberrant DNA methylation of candidate genes can be detected in peripheral blood of patients with cancer, even for solid tumors (27-29).

In this study, we conducted a meta-analysis of the association of the *RASSF1A* gene and TGCT and estimated its prognostic value for TGCT. We examined the odds ratio (OR) derived from eligible studies in order to establish a link between *RASSF1A* promoter methylation and risk for testicular cancer. Additionally, we validated the findings of the meta-analysis in our cohort of TGCT patients with seminoma and non-seminoma testicular cancer in order to ascertain if the *RASSF1A* promoter hypermethylation could be useful as diagnostic factor from whole blood. We also analyzed methylation status of the O⁶-methylguanine-DNA-methyltransferase (*MGMT*) gene in our cohort in order to compare diagnostic potential of the *RASSF1A* promoter hypermethylation (already established TSG which show promoter hypermethylation in both seminoma and non-seminoma tumor tissue), with that of the *MGMT* gene, which shows aberrant promoter methylation only in non-seminoma tissue (25, 26).

Materials and Methods

Meta-analysis of association using odds ratio. Meta-analysis was conducted according to the PRISMA guidelines (30, 31). Eligible case-control studies included investigations of the role of *RASSF1A* methylation in the pathogenesis and/or progression of testicular cancer. No publication date or publication status restrictions were imposed; however, only studies reported in English were included. Both seminoma and non-seminoma samples were included in the meta-analysis. Patient data, such as age, were not always available. We used relatively permissive criteria for study eligibility due to the small overall number of available reports. Eligibility assessment was performed independently by two reviewers (IS and AV) and the final decision was reached by consensus.

The PubMed database was searched for studies published up to March 1st, 2017. The following search strategy was used: (testicular OR testis) AND (cancer OR carcinoma OR tumor) AND (*RASSF1A* methylation). The following information was extracted from the studies: first author's name, year of publication, material for methylation analysis, control type, the measuring method of methylation, the number of individuals in the case and control groups.

To assess heterogeneity of the included studies, we calculated Cochran's Q with the corresponding *p*-value and the associated I² value (measure of inconsistency) for the meta analysis. As a visual aid for assessing bias across studies, we constructed a "funnel plot" of the effect (natural logarithm of odds ratio) by its standard error in order to intuitively demonstrate symmetry; to formally assess the degree of asymmetry, we used Egger's test.

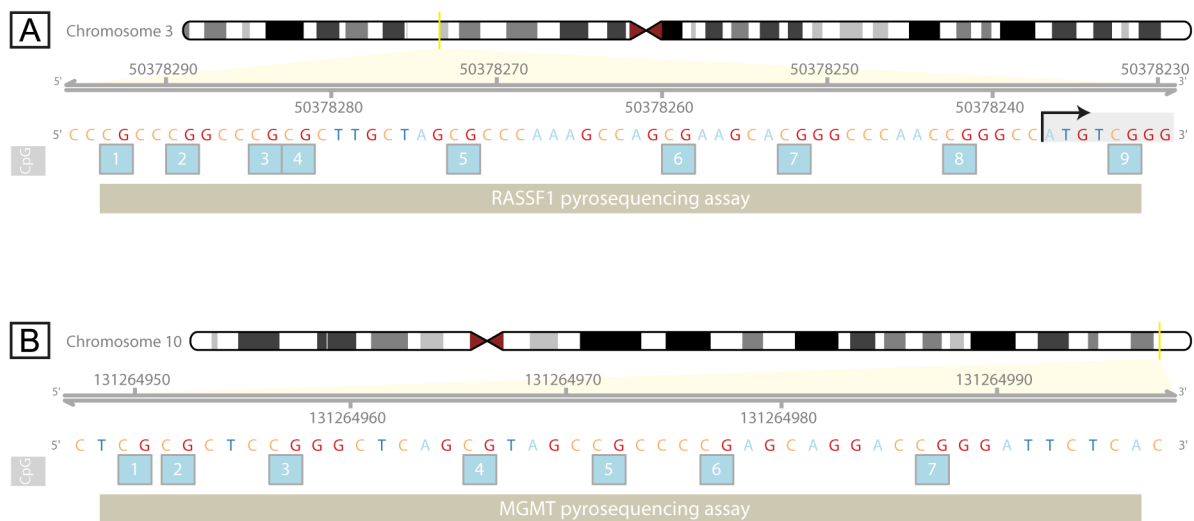


Figure 1. Location of the pyrosequencing assay within the *RASSF1A* (A) and *MGMT* (B) genes used for analysis of CpG methylation status. Position of the analyzed CpG sites is shown with absolute genomic coordinates according to the human genome GRCh37/hg19 assembly. Positions of the pyrosequencing assays on the chromosome 3 (A) and chromosome 10 (B) are indicated by the yellow bar. Translation start is indicated with the arrow above the ATG start codon (A).

Meta-analysis of prognostic value of the *RASSF1A* gene expression. Association between the *RASSF1A* gene expression levels and prognosis in different types of cancer was analyzed using the PrognScan database, which employs the minimum *p*-value approach for grouping patients for survival analysis, thus enabling systematic analysis of multiple datasets (32). Briefly, patients were ordered by expression levels of a gene of interest and then divided into high- and low-expression groups at all possible cut-off points. The risk differences of all groups were then estimated by the log-rank test. Finally, the grouping that gave the most significant *p*-value was selected.

Cohort study. To verify results from the meta analysis, we performed our own cohort study. We collected all available Croatian TC samples and matching controls.

Patients and sample collection. Our cohort included 32 testicular cancer patients and the same number of healthy controls from the general population matched according to age (± 4 years) and smoking status, since it is well known that aging and smoking alter DNA methylation status in general, and particularly of the *RASSF1A* gene promoter (33, 34). Control subjects were not occupationally exposed to ionizing radiation or known chemical mutagens/carcinogens and were not diagnosed or treated for any neoplastic disease. According to the histological type of tumor 11 patients had seminoma and 21 had non-seminoma tumor (7 out of 21 had combined seminoma and non-seminoma tumors which is considered by pathologist as non-seminoma).

All patients were, following the surgical procedure, subjected to two rounds of chemotherapy. Both rounds included etoposide and cisplatin, while the first round also included antibiotic treatment with bleomycin (BEP). The time interval between the two rounds of chemotherapy was three weeks. The first blood sample was obtained from patients after the surgical procedure (*i.e.*

orchiectomy), but before the use of chemotherapy. The second blood sample was taken after two rounds of chemotherapy. Sampling took place in the years 2012 and 2013. The research has been approved by the appropriate ethics committee.

DNA methylation analysis. DNA was extracted from 200 μ L of blood using the QIAamp DNA Blood Mini Kit (Qiagen) in accordance with the manufacturer's protocol. For DNA methylation analysis, 500 ng of DNA was subjected to bisulfite conversion, which was performed according to manufacturer's instructions (EZ DNA Methylation-Gold Kit, Zymo Research).

Polymerase chain reaction (PCR) was performed using PyroMark PCR Kit (Qiagen) according to the manufacturer's instructions. Specific regions rich in CpG dinucleotides of the genes *RASSF1A* and *MGMT* were amplified using the primers specific for bisulfite modified DNA. The *RASSF1A* fragment encompassed by the pyrosequencing assay was mostly within the UTR, located 57 nucleotides upstream to 6 nucleotides downstream from the translation initiation site (TIS), within a CpG island spanning the promoter, the first exon and a part of the first intron (Figure 1A). We chose this fragment because first CpG site (cg04743654) from the assay was found differentially methylated on Illumina 450K for different cell lines including human testicular embryonic carcinoma cell line NT2/D1 in which this CpG site was moderately methylated. The *MGMT* fragment encompassed by pyrosequencing assay was located 531-484 nucleotides upstream from the TIS, within a CpG island (Figure 1B). Previously described primers were used for *RASSF1A* pyrosequencing (35). Primers used for *MGMT* were: 5'-GGT GAT TGT AGT TTT TGG A-3' and 5'-TCC TAT CAC AAA AAT AAT CC-3' (biotinylated at the 5' end). PCR was initiated by pre-denaturation for 15 min at 95°C followed by 50 cycles of 94°C (*MGMT*, *RASSF1A*) for 30 s, 51°C (*MGMT*) or 65°C (*RASSF1A*) for 30 s and 72°C for 30 s, with final extension at 72°C for 10 min.

Table I. The studies selected for meta analysis of association between the RASSF1A promoter methylation and testicular cancer risk.

Author	Year	Material	Control (comparison) source	Method	Case				Control	
					Seminoma		Non-seminoma		Met	Non-met
					Met	Non-met	Met	Non-met		
Koul	2002	Tissue	Healthy	MSP	1	28	19	44	0	4
Honorio	2003	Tissue	Tumor-free epididymal tissue	MSP	4	6	15	3	4	17
Kawakami	2003	Tissue	Testes from prostate cancer patients	MSP	0	15	0	10	0	4
Lind	2006	Tissue	Intratubular germ cell neoplasias	MSP	0	19	10	25	0	7
Ellinger	2009	cfDNA	Healthy	RE	15	21	19	18	0	35
Tian	2011	Tissue	Healthy	MSP	5	0	2	0	0	1
Total					25	89	65	100	4	68

PCR products were separated by agarose gel electrophoresis to ascertain correct amplification and sufficient product quality.

For quantitative measurement of DNA methylation levels in the genomic fragments of interest, bisulfite converted and PCR amplified DNA was sequenced using pyrosequencing technology (PyroMark Q24 Advanced, Qiagen) according to manufacturer’s instructions. Sequencing primers for *MGMT* and *RASSF1A* were: 5’-GGT ATT AGG AGG GGA GAG ATT-3’ and 5’-GTT TTG TGG TTT-3’, respectively. The pyrosequencing assay comprised 7 CpG sites for *MGMT* and 9 CpG sites for *RASSF1A*.

Statistical analysis. Differences in methylation level of the *RASSF1A* and *MGMT* genes were assessed separately for each tested CpG site. Differences between the patients before and after therapy, as well as corresponding controls, were tested using the Mann-Whitney *U*-test. For matched samples before and after therapy, the Wilcoxon signed-rank test was used. Data processing and visualization was done using the R statistical environment (36). Meta analysis was done using the “metafor” package (37).

Results

Meta-analysis. Study selection and characteristics. To identify the association between the *RASSF1A* promoter methylation and TGCT, we conducted a meta-analysis of 6 eligible studies (25, 38-42) involving a total of 279 TGCT cases and 72 controls to calculate the odds ratio (OR) and the corresponding 95% confidence intervals where the *RASSF1A* promoter methylation was modelled as a risk factor for TGCT.

Details of the eligible studies are summarized in Table I. Five studies analyzed testicular cancer tissue and one used cell-free circulating DNA. For the analysis, all studies used methylation specific PCR (MSP), except for one that used restriction endonuclease (RE) based assay (Table I). As a control source, three studies used healthy testicular tissue of different types: tumor-free epididymal tissue, testes from prostate cancer patients and intra-tubular germ cell neoplasia which are considered a precursor lesion for many types of TGCT.

Results of individual studies and their synthesis. Odds ratio (OR) for TGCT – considering the *RASSF1A* promoter methylation – was taken as the outcome, which is shown in the forest plot (Figure 2). Only the study by Kawakami (39) showed negative log OR, contradicting the results of other studies; yet, this value was accompanied by wide confidence limits. Other studies supported increased odds for TGCT with increased *RASSF1A* promoter methylation, as seen in Figure 2. Summarized OR under random effects model was 7.69 (95% CI=2.86-20.70). Heterogeneity based on Cochran’s Q for the six studies was 5.34 (*p*=0.225), giving an *I*² value of 0.00%.

When patients were stratified according to histology type (seminoma vs. non-seminoma), we found a difference in the association between DNA methylation and TGCT, with OR 2.89 (95% CI=0.54-15.33; *I*²=41.05%) for seminoma and 12.55 (95% CI 4.22-37.71; *I*²=0.00%) for non-seminoma. Large heterogeneity and wide confidence intervals for the seminoma group are due to the reduced sample size of that group after stratification (Table I).

Risk of bias across studies. We found no bias in the included studies, which can be seen from the symmetry of the funnel plot in Figure 3. Egger’s test gave a *p*-value of 0.679, which formally confirms the absence of significant bias across included publications. When cases were stratified according to histology type into seminoma and non-seminoma group, evidence against bias was even stronger in the seminoma group (*p*=0.795), although not as strong for non-seminoma group (*p*=0.306).

Prognostic value of RASSF1A promoter methylation. In the PrognScan database, we found 25 statistically significant (Cox *p*-value <0.05) probes indicating a possible prognostic value of the *RASSF1* gene in various types of cancers, 20 of which (80%) were associated with better survival in cases of

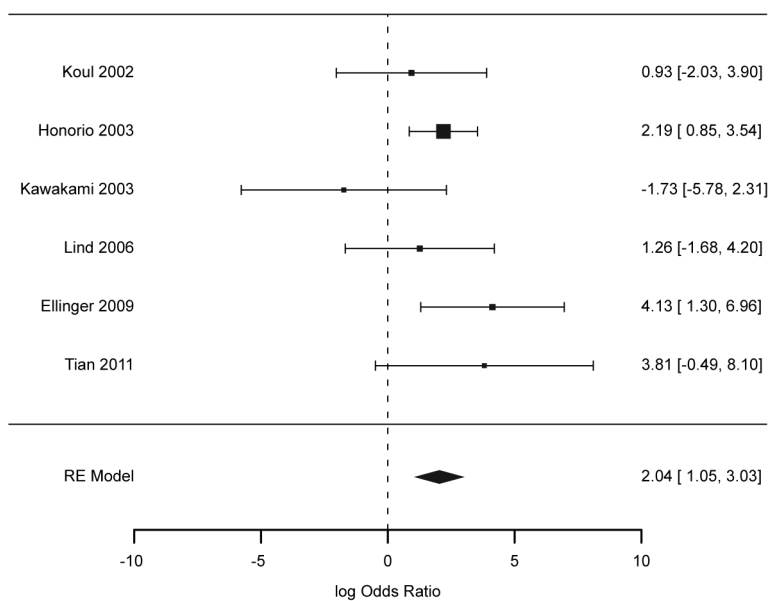


Figure 2. Forest plot of the logarithm of odds ratio (OR) for the association between the *RASSF1A* promoter methylation and testicular cancer. Selected publications are listed on the Y-axis with the summary (random effects model) at the bottom. Error bars represent 95% confidence intervals.

high expression. Therefore, high expression of *RASSF1* gene in cancer seems to indicate better prognosis.

Cohort study. In our cohort study, we wanted to verify if *RASSF1A* hypermethylation could be diagnosed from blood of TC patients. The *MGMT* gene was chosen as a “control gene” for methylation difference between patients with histologically-different testicular tumors and healthy controls since *RASSF1A* hypermethylation was signature for both seminomas and NSTGCT tissue and the *MGMT* gene was found hypermethylated only in tissue of NSTGCT (25, 26). Additionally, we were interested on the effects of BEP chemotherapy on *RASSF1A* promoter methylation.

RASSF1A and *MGMT* promoter methylation was analyzed from DNA of peripheral blood nucleated cells (PBNC) using the pyrosequencing method after bisulfite conversion of DNA. The pyrosequencing assay for *RASSF1A* comprised 9 CpG sites and the methylation levels were generally low (less than 3%) at this fragment. However, the methylation levels at 5 out of 9 CpG sites in the *RASSF1A* fragment were significantly higher in the TC patients before BEP chemotherapy compared to the healthy controls (CpG sites number 2, 3, 4, 5 and 7; Figure 4A). In addition, CpG site number 5 (CpG5) showed statistically significant difference ($p=0.0115$) in the methylation level between patients before and after the BEP chemotherapy. The general trend was the that in CpG methylation in patients, which had a tendency to return to the methylation level found in healthy controls

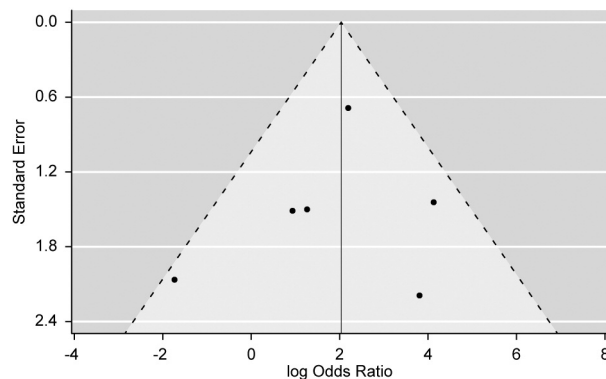


Figure 3. Funnel plot of the logarithm of the odds ratio with its corresponding standard error (Y-axis), where each black dot represents one of the six studies included in the meta-analysis, shows no apparent asymmetry would have indicated bias across studies. The absence of bias was formally confirmed using Egger's test ($p=0.679$).

after the BEP therapy, as illustrated in Figure 4A). No significant difference in CpG methylation was found after stratification of patients into the seminoma and non-seminoma, which is probably due to too small number of individuals in each group.

The pyrosequencing assay for the *MGMT* gene comprised of 7 CpG sites (Figure 4B). The highest methylation level was recorded at the CpG site number 3 (CpG3; around 70%),

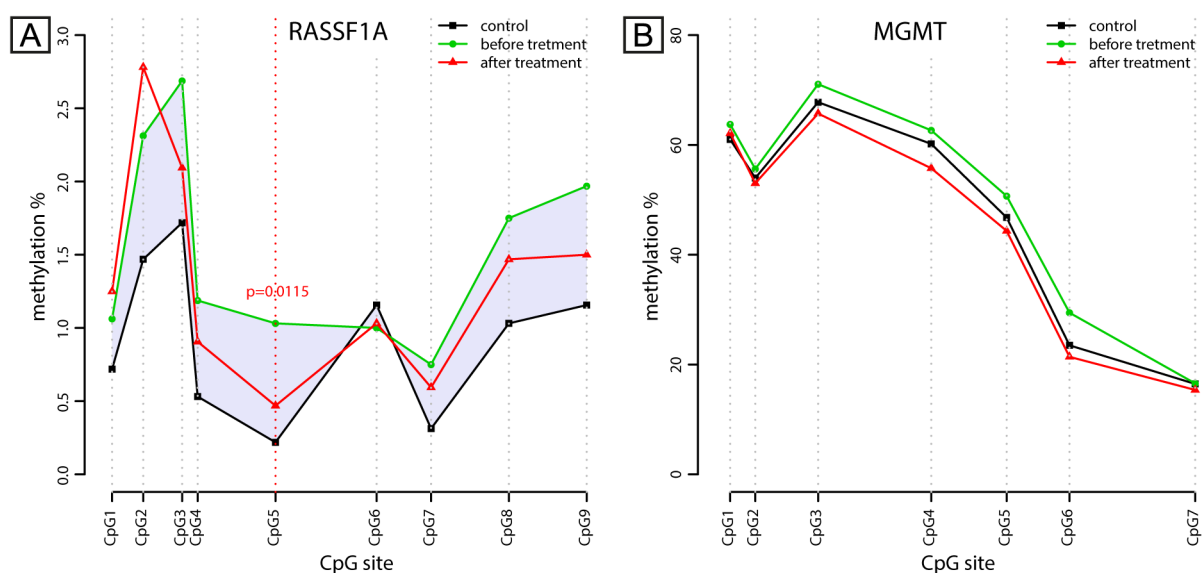


Figure 4. Methylation levels of the individual CpG sites within the *RASSF1A* (A) and *MGMT* (B) gene promoters. Methylation level is determined by pyrosequencing and is expressed here as a percentage. The distance between the CpG sites corresponds to their relative position within the analyzed DNA fragment. Points (triangle, circle and square) represent the mean methylation level for each group. Lines are drawn as a visual aid for illustrating the spatial organization of methylation levels along the analyzed region. A) The CpG sites 2, 3, 4, 5 and 7 in the analyzed fragment within the *RASSF1A* promoter show statistically significant difference between patients (before chemotherapy) and healthy controls. Methylation at the site CpG5 shows statistically significant difference ($p=0.0115$) between the patients before and after BEP chemotherapy (indicated by vertical dashed line). Shaded area emphasizes CpG methylation difference between healthy controls and testicular cancer patients. B) No significant difference was found in the *MGMT* promoter methylation.

while other CpG sites had a lower methylation level in all studied groups. We could not find any statistically significant difference in CpG methylation status in the blood of the TGCT patients and healthy controls.

Discussion

Aberrant gene-specific promoter methylation occurs in virtually every type of tumor. Potential for measuring such gene-specific methylation in different tumors and its diagnostic and predictive value have already been demonstrated for many cancers. Previous studies have identified associations between methylation levels of several genes with TGCT, which could be a molecular mechanism for aberrant activation/inactivation of these genes in this cancer (16, 25). Even though seminomas and non-seminomas demonstrate (cyto)genetic similarities, it appears that the two types of tumors differ significantly at the epigenetic level (16, 25, 43, 44). The *RASSF1A* gene has been identified as the main tumor-suppressor gene silenced in testicular cancer tissue by aberrant hypermethylation and the first epigenetic event in the TGCT tumorigenesis (25). Moreover, the methylation status of the *RASSF1A* promoter appears to play a role in cisplatin chemotherapy resistance and sensitivity

(45) and correlates with clinical parameters (46) suggesting that this gene might be profoundly implicated in molecular mechanisms of TGCT.

Several meta-analysis studies showed an association between *RASSF1A* promoter hypermethylation and different types of tumors as well as biomarker value of the *RASSF1A* methylation status (47-49). This study represents the first meta-analysis of published studies to evaluate the association of *RASSF1A* promoter hypermethylation with testicular cancer. The association and the odds ratio (OR) with TGCT were indeed different when patients were stratified according to histology type, with stronger association appearing for NSTGCT. This is in accordance with the finding of Honorio (25) who detected *RASSF1A* hypermethylation in 83% of non-seminoma, and only in 40% of seminoma components. In addition, seminomas are more highly hypomethylated than non-seminoma throughout the genome, with almost unmethylated CpG islands in seminomas in contrast to hypermethylated CpG islands in non-seminomas (43).

This study has some limitations such as the relatively small total number of patients with TC included in the meta-analysis. However, there was no apparent risk of bias in the included studies. The only exception was the non-seminoma group after stratification, where the additional reduction in

sample size introduced by stratification decreased the statistical power in analysis of the non-seminoma group, thus making conclusions about this type of tumor less reliable.

Heterogeneity among studies was expected at least to a certain degree, due to typically small sample size of each individual study (which is the result of the rarity of TCGC) and a relatively small overall number of studies that matched our inclusion criteria. However, two complementary measures of inconsistency across studies, Cochran's Q and I^2 , both indicated that the included studies were in agreement with each other and as such could provide material for a valid meta-analysis. In order to accommodate for variation between study outcomes, we opted for the less statistically powerful random effects model and still obtained a significant value for the combined odds ratio.

Even though TCGC is the most common type of testicular cancer, it is still a relatively rare disease. Therefore, the total number of included studies and the total sample size, both in the meta-analysis and the cohort study, was limited by this fact. It should also be noted that the methods for methylation analysis were different – MSP and RE based assays were used in studies included in the meta-analysis, while bisulfite pyrosequencing was applied in our cohort analysis. Even though sequencing techniques are generally superior to PCR based methodologies or those based on restriction with methylation sensitive enzymes, pyrosequencing method has a limitation in the length of the analyzed sequence (approx. 120 bp) and in “fishing” the functionally relevant CpG sites. The CpG islands within gene promoters are large and contain many CpG dinucleotides potentially involved in transcriptional regulation; however, they are unidentified in most of the gene promoters.

The results of methylation level difference in promoter of the *MGMT* gene between TGCT patients and healthy controls in our cohort study showed no statistically significant differences, and this finding could be interpreted in two ways. Either the difference in the methylation level for this gene, found in testicular tissue of the NSTGCT patients (25, 26) is not strong enough to be detectable in PBNC (in our cohort study), or different methods used in our study and other studies led to different results. Namely, in two studies that report differences in the methylation level in the *MGMT* promoter of TC tissue and in whole blood of patients with non-seminomas (but not seminoma), the method of methylation-specific PCR was used (25, 26, 44). Our pyrosequencing assay covered seven CpG sites located within CpG island of the *MGMT* gene, and it is possible that these sites do not change methylation level in TGCT. It is documented that either solitary CpGs can change methylation level in cancer or many CpGs within large fragments change methylation level and this would depend on specific TSG and the specific cancer (50-52). Therefore, some other, unidentified regions within the CpG island of the *MGMT*

gene could be involved in transcriptional regulation of this gene and might contain differentially methylated CpG sites.

Diverse sample types employed in the studies used for meta-analysis, which includes testicular cancer tissue, whole blood and cell free circulating DNA, highlight the requirement for careful interpretation. In our cohort study, we chose to analyze methylation from whole blood since this type of analysis could be useful in non-invasive early diagnostics. It is known that any tumor has its tissue specific methylomes and even if they can give insights on the molecular pathology of a particular cancer, they cannot serve as prognostic and/or diagnostic biomarker due to unavailability of target tissue, and invasiveness of methods that would provide such tissue. Recent studies suggest that DNA methylation markers from peripheral blood might serve as diagnostic, early detection/risk or prognostic and recurrence cancer markers and a number of publications show such good correlation in different cancers (53-56). In our cohort study, we confirmed that *RASSF1A* promoter hypermethylation could be diagnosed from blood of TGCT patients. The level of *RASSF1A* methylation at several CpG sites, located within the CpG island, was significantly increased in patients before BEP chemotherapy compared to healthy controls, suggesting that hypermethylation of *RASSF1A* play role in the disease. Even though absolute differences in CpG methylation levels between cases and controls were small, they were statistically significant. Since we analyzed methylation from whole blood, one should in principle account for the lack of discrimination between different cell types within PBMCs. However, our necessary assumption was that every tissue reflects a degree of global *RASSF1A* methylation, including methylation of the target (testicular) cells that are candidates for malignant transformation in the context of our study.

Other authors (45) found that, among other genes, promoter hypermethylation of the *RASSF1A* gene plays a role in resistance of TGCT to chemotherapy. In our study, we analyzed DNA methylation status in peripheral blood of drug naïve and cisplatin treated TC patients. We investigated whether cisplatin treatment also affects the methylation of this gene in the non-target tissue (*i.e.* blood) and whether this effect can be a marker of successful treatment. The methylation level that we recorded returned to background levels, further suggesting a role of *RASSF1A* methylation in TGCT pathogenesis (57, 58). The *MGMT* promoter methylation did not show this effect. Even if there might be a potential of *RASSF1A* promoter methylation as prognostic factor after chemotherapy, the study needs to be extended to include a larger sample in order to obtain conclusive information within a narrow confidence limit.

To our knowledge, this is the first report of changed *RASSF1A* promoter methylation in peripheral blood in any TGCT cohort. Due to the scarcity of adequate samples and

the relatively low incidence of the disease in Croatia, our cohort was too small for statistically powerful results when we performed stratification of the patients into subgroups according to tumor type (seminomas and non-seminomas). However, the pooling of the similar results and conducting a meta-analysis of different TC datasets (279 TGCT patients from eligible studies) helped us to strengthen the case for potential role (or at least a significant association) of *RASSF1A* gene promoter hypermethylation found in peripheral blood of both seminoma and non-seminoma TC patients. This is further strengthened by the results of our “control gene”, the *MGMT*, which did not show any methylation differences between the TGCT patients and healthy controls. The *MGMT* promoter hypermethylation was found only in non-seminomas, therefore, it is considered to constitute additional epigenetic alteration subsequent to hypermethylation of the *RASSF1A* gene, which was suggested to represent an early epigenetic event in the pathway of NSTGCT development (25, 26). Since we could not obtain any conclusive results when we performed stratification of our TGCT patients into the subgroups, it was not possible to replicate the results reported by others who found differences in the methylation level in the *MGMT* promoter in whole blood of NSTGCT patients (44). Larger studies, both observational cohorts and clinical trials are required to test whether *RASSF1A* promoter hypermethylation can be useful diagnostic and prognostic factors for testicular cancer.

Acknowledgements

The research presented here was supported by European Commission (FP7) project RegPot Integra-Life (Grant Agreement № 315997).

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Received July 6, 2017
Revised August 5, 2017
Accepted August 8, 2017