PILOT STUDY OF THE ASSOCIATION BETWEEN THE HLA REGION AND TESTICULAR CARCINOMA AMONG CROATIAN PATIENTS

Authors: Kristina Gotovac[1], Zorana Grubic[1], Zeljko Kastelan[2], Katarina Stingl[1], Tomislav Kulis[2], Ivan Krhen[2], Tvrtko Hudolin[2], Maja Kastelan[2], Renata Zunec[1]

[1]Tissue Typing Centre, [2]Department of Urology, University Hospital Centre Zagreb, Croatia

ABSTRACT

OBJECTIVES To analyze the distribution of HLA alleles and HLA microsatellite alleles in Croatian patients with testicular carcinoma, compare it with that of healthy controls and investigate whether the polymorphism within the HLA region could be associated with the development of testicular cancer.

METHODS Genomic DNA was isolated from the peripheral blood of 24 patients with testicular germ cell tumors (TGCT). Patients and controls were typed for HLA class I and class II polymorphism by PCR-SSO method. Nine HLA microsatellites were analyzed by PCR and electrophoresis in an automated sequencer.

RESULTS No significant deviation in the distribution of frequencies at HLA class I alleles was observed between patients and controls. Among HLA class II alleles, a statistically significant increase in frequency of HLA-DPB1*1701 allele was found among patients. The frequency of the HLA-DRB1*07-DQA1*0201-DQB1*0202 haplotype was increased in patients in comparison to the controls. Analysis of HLA microsatellites showed an increased frequency of D6S291-3 allele (p_{corr}=0.0455, OR=3.05) among patients.

CONCLUSIONS The observed association of the disease and DPB1*1701 allele as well as with the D6S291-3 allele suggests that this part of the HLA region might be involved in the pathogenesis of TGCT. Our data provide a basis for further studies about the correlation between HLA region and testicular cancer.

INTRODUCTION

Testicular germ cell tumors (TGCT) constitute one of the most common malignancies in men 15–40 years of age and account for 2% of all malignancies in men.[1] The incidence is 3-10 in 100,000 males per year in Western society, while the number of diagnosed males/patients in Croatia was 145 in 2008 (crude rate 6.8 in 100,000 male population)[2]. This represents an increase since the incidence in 2001 was 5.7 per 100,000. The predominant histological type of testicular cancer is germ cell carcinoma (90-95%). TGCTs are divided into two major subgroups based on histological findings: seminoma (30-60%) and non-seminoma germ cell tumor. Seminoma cases account for approximately half of all TGCTs and most frequently appear in the fourth decade of life. The remaining cases of TGCTs are composed of nonseminoma histology (embryonal cell carcinoma, yolk sac tumor, choriocarcinoma and teratoma) and frequently present in the third decade of life. Most nonseminomatous tumors are mixed (composed of two or more cell types of which seminoma may be a component). Seminoma tumors are also more sensitive to chemotherapy and radiotherapy than nonseminomatous TGCTs.[3] The etiology of TGCT is still poorly understood. In addition to possible environmental predisposing factors such as radiation, smoking and drug use, the role of genetic factors in the development of TGCT has also been suggested. This claim is supported by the fact that familial and bilateral testicular carcinoma cases occur more frequently than expected by chance with the odds risk (OR) for brothers of TGCT patients ranging from 3 to 13.[4-6] Genetic susceptibility is also reflected by an increased incidence of TGCT in persons with certain rare malformations of the urogenital system such as Down and Klinefelter syndromes.[7] Furthermore, the age distribution of TGCT might suggest a genetic origin of the disease: the young age at onset of testicular neoplasms indicates a

role of important etiologic factors operating early in life, either in utero or shortly after birth. Finally, ethnic differences in the incidence of TGCT may point to a genetic component in the etiology of the disease. The highest incidence is observed in Caucasians from Northern Europe, while Africans have a universally low incidence of TGCT.[8]

Although only a few studies have documented the association between the TGCT and HLA region, a difference in distribution of HLA antigens in patients compared to healthy controls has been found. Studies report higher frequencies of antigens HLA-DR1 and HLA-DR5 antigens in patients with seminoma, and increased frequencies of antigens HLA-DR5 and HLA-DR7 antigens in patients with nonseminoma.[9] These studies indicate a connection between HLA-DR antigens and TGCT.

Microsatellites (Msats) are repeats of a DNA base motif (1-6bp, mostly CA repeats) up to 100 times; they are distributed throughout the genome. Many of them display a high polymorphism which is based upon a variable number of repeats. Such markers have also been described in the HLA region[10] and a growing interest in their potential applications, particularly in disease association studies, is being expressed. Microsatellite instability has been observed in different carcinomas among which is testicular carcinoma where such an instability has a pathogenetic significance.[11]

In the present study we analyzed the distribution of HLA class I (A, B, C) and HLA class II (DRB1, DQA1, DQB1, DPB1) alleles in Croatian patients with testicular carcinoma and healthy controls. The second aim of this pilot study was to investigate whether the polymorphism of HLA microsatellites in the HLA central region could be associated with the development of testicular carcinoma.

MATERIAL AND METHODS

Patients and controls

A total of 24 unrelated patients treated from March 2004 to April 2005 at the Department of Urology, University Hospital Zagreb were included in the analysis. Patient's characteristics are presented in table 1. Preoperative diagnosis was assessed by clinical examination, testicular ultrasound and preoperative level of tumor markers (alpha-fetoprotein, beta human chorionic gonadotropin, lactate dehydrogenase). After orchidectomy, histologic diagnosis was established by pathologists experienced in urological pathology.

One hundred unrelated healthy individuals without any signs of the disease were analyzed as a control group for HLA class I and HLA class II polymorphisms while 170 unrelated subjects comprised the control group for Msats analysis. They originate from different regions of Croatia and form a representative sample of the Croatian population.

Samples of 5 ml peripheral blood with EDTA were taken from each patient as well as from the control subjects.

DNA isolation

Genomic DNA was isolated using NucleoSpin kit following the manufacturer's protocol (NucleoSpin, Macherey-Nagel, Düren, Germany).

HLA class I and class II typing

Patients and controls were typed for HLA class I (A, B, C) and class II (DRB1, DQA1, DQB1, DPB1) polymorphism by the Polymorphism Chain Reaction – Sequence Specific Oligos (PCR-SSO) method using Innolipa kit (Innogenetics, Gent, Belgium). HLA class I

polymorphisms were typed at low resolution level (two digits), while HLA class II polymorphisms were determined at high resolution level except for the DRB1 locus. With this protocol we were able to determine 18 specificities at HLA-A, 41 specificities at HLA-B, 15 specificities at HLA-C, 13 specificities at HLA-DRB1, 86 alleles at HLA-DQB1, 34 alleles at HLA-DQA1 and 98 alleles at DPB1.

HLA microsatellite typing

Nine HLA microsatellites (D6S291, D6S1014, D6S273, TNFa, TNFd, TNFb, D6S2793, STR_MICA, and D6S2927) were analyzed. The Msats were amplified using primers labeled with Cy5. The primer sequences were as previously published.[12] Amplifications were performed in a volume of 15µl containing 50ng DNA, 2.5mM dNTPs, $MgCl_2$ (concentration varied for each Msat), 5U of Taq polymerase, 1.0µl PCR buffer and 10pmol of each primer. The cycling parameters were as previously reported.[13] After amplification, PCR products were run on a 6% polyacrylamide gel in an automated sequencer (ALFexpress, Amersham Pharmacia, Uppsala, Sweden). The assignment of alleles was performed using AlleleLocator software (Amersham Pharmacia), which calculates the length of fragments by comparison, to the internal size markers. In addition, ALFexpress sizer 200 bp was also used as standard. To further improve the precision of fragment length determination, homozygous cell lines from the 10th International Histocompatibility Workshop (IHW) (which were previously typed for tested Msats) were also amplified for investigated Msats and run on each gel as external controls. The following cell lines were used: AMALA, BTB, KAS011, KAS016, TAB089 and VAVY.

Statistics

The frequency of HLA specificities as well as Msat alleles were determined by direct counting. The χ^2 test was used to compare the expected and observed value of genotype sequences in order to confirm whether it satisfies the Hardy–Weinberg law. p values were corrected by Yates' correction and the number of HLA or Msat alleles under investigation (p_{corr}). The OR was determined according to Woolf.[14]

RESULTS AND DISCUSSION

The presence of HLA class I alleles in 24 patients with testicular carcinoma was determined. No significant deviation in the distribution of frequencies at HLA-A, HLA-B and HLA-C loci was found between TGCT patients and controls (data not shown).

The significance of differences in allele frequencies between two tested groups for HLA class II alleles was also evaluated (table 2). The frequency of HLA-DRB1*07, DRB1*11 and DQA1*0201 specificities was increased among patients compared to controls, but without statistical significance. Conversely, a significant increase in occurence of HLA-DQB1*0202 (P=0.0313, OR=3.56) among patients was observed, but only before correction. This is not in concordance with the published data which indicated an increase in HLA-DQ4 antigen frequency as well as the increased frequency of HLA-DRB1*0405 and HLA-DQB1*0401 alleles among patients.[15] Recently, a high-resolution genotyping study in TGCT patients indicated HLA-DRB1*0410 (OR=3.26) as a susceptibility allele and HLA-DQB1*0602 as a candidate protective allele (OR=0.26) for TGCT.[16] We did not find an association between these alleles and TGCT in our group of patients. It is also important to mention that difference in alleles associated with TGCT could be explained by a variation of DRB1*0405, DRB1*0410 as well as DQB1*0401 allele frequencies in the Japanese and Croatian populations. On the other hand, there was a statistically significant increase in the frequency of the HLA-DPB1*1701 allele among patients (P=0.0140, OR=17.9) in comparison to controls. To the best of our knowledge, this is the first report about association between DPB1 alleles and TGCT. Analysis of haplotypic associations was also performed and 13 different haplotypic associations were observed among patients. The most frequent alleles in both the patient and the control groups were HLA-DRB1*11, HLA -

DQA1*0505 and HLA-DQB1*0301. Haplotype HLA-DRB1*07-DQA1*0201-DQB1*0202 was increased in patients in comparison to that obtained for the control group (P=0.0313, OR=3.56). It should be taken into consideration that the DRB1*0701 allele is one of the frequent alleles of the HLA-DR53 group in the Croatian population and that it belongs to the same serological haplotype group as DRB1*04 alleles (mentioned before) associated with TGCT among Japanese. It can therefore be speculated that the same configuration of these HLA haplotypes can be a predisposition for TGCT. The very first studies performed two decades ago on this subject reported about the association between TGCT and DRB4 gene but not with any particular allele at that locus. However, in order to reach any definitive conclusion, it is necessary to perform a large population study with the aim of analyzing a possible existence of linkage disequilibrium between DRB1 alleles and DPB1 alleles. This would ensure determining which of the mentioned HLA loci (DRB1 or DPB1) is primarily associated with TGCT since there are currently only a few studies available about the extended HLA haplotypes that include HLA-DPB1 alleles.

The next part of the study involved the analysis of the nine HLA microsatellites among TGCT patients and the controls. The differences in frequencies between these two tested groups were observed for 3 alleles. The increase in the frequency which remained statistically significant even after the correction of p value, was observed for the D6S291-3 allele (p_{corr}=0.0455) (table 3).

Relative risk values for microsatellite alleles which demonstrated a significant association with TGCT were calculated. The highest OR values were observed for the D6S2927-6 and D6S291-3 alleles (OR=8.30 and OR=3.05, respectively). A reasonable explanation for such a high value of OR for the D6S2927-6 allele with TGCT might be found in the fact that this D6S2927 allele is in linkage disequilibrium with some HLA-B alleles which are slightly more

present among patients. Study on the analysis of microsatellites in children with acute leukemia and Down's syndrome also reported about a potential role of the D6S291 microsatellite in the susceptibility to disease. The authors of this study observed a significantly increased frequency of two alleles at the D6S291 locus in patients.[17] This may suggest that the D6S291 locus might be a susceptibility locus in certain carcinomas. Research of head and neck squamous cell carcinomas also show that the D6S291 marker appears to be an informative disease marker since a high frequency of allelic loss was found on this locus.[18]

On the base of our data, we can propose that the DPB1 locus or region around that HLA class II gene plays an important role in the genetic predisposition to TGCT. The confirmation for such a theory might also be the association with the D6S291-6 allele. Namely, the D6S291 microsatellite locus is located near the DPB1 locus. However, it is essential to point out the need foe a DPB1-D6S191 linkage analysis to elucidate the existence of linkage disequilibrium between the alleles of these two neighboring loci within the HLA region.

In general, a in the HLA region may provide tumor cells with an immune escape tumor phenotype. At the same time, microsatellite instability seems to be a late event associated with tumor progression. Most studies show that invasive carcinomas manifest more microsatellite instability than precursor lesions, suggesting progressive accumulation of microsatellite instability during tumor development. Because of those observations, it would be interesting to analyze not only peripheral blood from patients with TGCT but also the carcinoma tissue to check for the presence of microsatellite instability as well as loss of heterozygosity in this type of carcinoma. The D6S2927-1 allele (previous name of locus was C1_4_1) was observed as a potential risk factor for spondyloarthropathies in South India population, while authors from Iran reported about the association between D6S2927-2 allele and Behcet's disease. Recently,

D6S2927 has also been implicated in the pathogenesis of Crohn's disease and ulcerative colitis in different ethnic populations.

The observed significantly higher frequency (before correction) of the STR_MICA-A5 allele among patients with TGCT could be explained with linkage disequilibrium of this MICA_STR allele with different HLA-B alleles which have shown slightly higher frequencies among patients. At the same time, this microsatellite is involved in pathogenesis of different diseases such as psoriatic arthritis, ulcerative colitis, breast cancer and colorectal cancer.[19, 20]

In the end, it is of interest to note that, although this microsatellite has shown association with numerous diseases in studies reported so far, we have not observed any correlation between D6S273 and TGCT. [20]

The possible role of the HLA region in TGCT development could result from the effects of the HLA variation on the immune response to carcinogenic factors, e.g. viruses that may be etiologically associated with the development of TGCT. The importance of the HLA system in regulating susceptibility to a growing number of neoplastic conditions as well as tumor development is becoming increasingly clear. An impaired immune system, genetically or acquired, favours carcinogenic factors.[21]

CONCLUSION

This is, to our knowledge, the first study which has shown an association between HLA-DPB1 allele as well as HLA microsatellites and TGCT. Although the results of this pilot study need confirmation on a larger number of patients, they indicated the direction which future studies should undertake in the clarification of the role of these polymorphisms in the etiology of TGCT.

ACKNOWLEDGMENT

This study was supported by a grant (214-0000000-3354) from the Ministry of Science, Sport and Education, Republic of Croatia.

Table 1. Patient's characteristics (n=24)

Median age at diagnosis (range), years	30 (21-60)
Histologic type, %	
Pure seminoma	50.0
Nonseminoma	50.0
Bilateral testicular carcinoma, %	8.0
TNM stage, %	
Low stage*	70.8
High stage**	29.2

TNM (tumor, node, metastasis) classification 2002; * N0, N1; **N2, N3, M1

Table 2. HLA alleles and HLA haplotypes with significantly different frequencies between patients with testicular carcinoma (n=24) and controls (n=100)

HLA ALLELE / HAPLOTYPE	PATIENTS	CONTROLS	p	p_{corr}	OR
DQB1*0202	10 (41.67%)	6 (6.00%)	0.0313	n.s.	3.56
DPB1*1701	4 (16.67%)	0	0.0140	n.s.	17.90
DRB1*07-DQA1*0201-DQB1*0202	10 (41.67%)	6 (6.00%)	0.0313	n.s.	3.56

P_{corr} - corrected p value; OR- odds ratio; ns- not significant.

Table 3. Msat alleles with significant differences between patients with testicular carcinoma (n=24) and healthy controls (n=170)

Msat	ALLELE	PATIENTS	CONTROLS	p	p_{corr}	OR
D6S291	3 (204bp)	12 (50.00%)	42 (24.71%)	0.0065	0.0455	3.05
STR_MICA	A5 (182bp)	10 (41.67%)	33 (19.41%)	0.0282	n.s.	2.97
D6S2927	6 (233bp)	4 (16.67%)	4 (2.35%)	0.0091	n.s.	8.30

p_{corr} - corrected P value; OR- odds ratio; n.s.- not significant

LITERATURE

1. Lin K, Sharangpani R.,Screening for Testicular Cancer: An Evidence Review for the U.S. Preventive Services Task Force. Ann Intern Med. 2010; 153: 396-399.
2. Croatian National Cancer registry, Croatian National Institute of Public Health, Cancer Incidence in Croatia 2008, Bulletin No. 33 (edition by Croatian National Institute of Public Health 2010)
3. Oliver RTD, Hope-Stone HF, Blandy JP: Possible new approaches to the management of seminoma of the testis. British Journal of Urology 1984; 56: 729-733.
4. Dieckmann KP, Pichlmeier U: The prevalence of familial testicular cancer: an analysis of two patient populations and a review of the literature. Cancer 1997; 80: 1954−1960.
5. Dong C, Lonnstedt I, Hemminki K: Familial testicular cancer and second primary cancers in testicular cancer patients by histological type. Eur J Cancer 2001; 37**:** 1878–1885.
6. Sonneveld DJ, Holzik MF, Nolte IM, et al: Testicular carcinoma and HLA Class II genes. Cancer 2002; 95: 1857-1863.
7. Holzik L, Sijmons RH, Sleijfer DT, et al: Syndromic aspects of testicular carcinoma. Cancer 2003; 97: 984-992.
8. Bosl GJ, Motzer RJ: Testicular germ-cell cancer. N Engl J Med 1997; 337: 242-253.

9. Oliver RTD, Stephenson CA, Parkinson MC, et al: Germ cell tumors of the testicle. A model of MHC influence on human malignancy. J Immunogenet 1986;13: 85-92.
10. Nedospasov SA, Udalova IA, Kuprash DV, et al: DNA sequence polymorphism at the human tumor necrosis factor (TNF) locus. J Immunol 1991; 147: 1053-1059.
11. Faulkner SW, Friedlander ML: Microsatellite instability in germ cell tumors of the testis and ovary. Gynecol. Oncol 2000; 79: 38-43.
12. Gourraud PA, Mano S, Barnetche T, et al: Integration of microsatellite characteristics in the MHC region:a literature and sequence based analysis. Tissue Antigens 2004; 64: 543-555.
13. Grubić Z, Štingl K, Žunec R: Linkage disequilibrium between human leucocyte antigen-B and closely linked microsatellites in the Croatian population. Tissue Antigens 2007; 69: 86-94.
14. Woolf B: On estimating the relation between blood group and disease. Ann Hum Genet 1955; 19: 251-253.
15. Nishimura K, Miura H, Yasunaga Y: HLA antigens in patients with testicular germ cell tumors. Hinyokika Kiyo 1996; 42: 95-99.
16. Özdemir E, Kakehi Y, Mishina M: High-resolution HLA-DRB1 and DQB1 genotyping in Japanese patients with testicular germ cell carcinoma. Br J Cancer 1997; 76: 1348-1352.
17. Flores H, Alaez C, Manzanares F, et al: Analysis of MHC microsatellites in children with acute leukemia and Down syndrome. Humman Imunology 2003; 64: S141-S141.

18. Feenstra M, Verdaasdonk K, Van der Zwan AW, et al: Microsatellite analysis of microdissected tumor cells and 6p high density microsatellite analysis in head and neck squamous cell carcinomas with down-regulated human leukocyte antigen class I expression. Lab Invest 2000; 80: 405-414.
19. Fang M., Chen R, Cai Q, et al: Association of HLA genes with ankylosing spondylitis in Han population of eastern china. Scandinavian Journal of Immunology 2007; 65: 559-566.
20. Dharam PS, Jianping L, Kewu L: Genetics of rheumatoid arthritis: two separate regions in the major histocompatibility complex contribute to susceptibility to RA. Immunology Letters 1999; 69: 301-306.
21. Bateman AC, Howell WM: Human leukocyte antigens and cancer: is it in our genes? J Pathol 1999; 188: 231-236.