Loss of heterozygosity of CDKN2A (p16INK4a) and RB1 tumor suppressor genes in testicular germ cell tumors

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Background. Testicular germ cell tumors (TGCTs) are the most frequent malignancies in young adult men. The two main histological forms, seminomas and nonseminomas, differ biologically and clinically. pRB protein and its immediate upstream regulator p16INK4a are involved in the RB pathway which is deregulated in most TGCTs. The objective of this study was to evaluate the occurrence of loss of heterozygosity (LOH) of the CDKN2A (p16INK4a) and RB1 tumor suppressor genes in TGCTs.

Materials and methods. Forty TGCTs (18 seminomas and 22 nonseminomas) were analyzed by polymerase chain reaction using the restriction fragment length polymorphism or the nucleotide repeat polymorphism method.

Results. LOH of the CDKN2A was found in two (6%) out of 34 (85%) informative cases of our total TGCT sample. The observed changes were assigned to two (11%) nonseminomas out of 18 (82%) informative samples. Furthermore, LOH of the RB1 was detected in two (6%) out of 34 (85%) informative cases of our total TGCT sample. Once again, the observed changes were assigned to two (10.5%) nonseminomas out of 19 (86%) informative samples. Both LOHs of the CDKN2A were found in nonseminomas with a yolk sac tumor component, and both LOHs of the RB1 were found in nonseminomas with an embryonal carcinoma component.

Conclusions. The higher incidence of observed LOH in nonseminomas may provide a clue to their invasive behavior.

Key words: loss of heterozygosity; CDKN2A; RB1; seminomas; nonseminomas

Introduction

Testicular germ cell tumor (TGCT) is diagnosed mainly after puberty and is the most frequent malignancy in young adult men, however, it is also not rare in childhood. The two main histological forms, seminomas and nonseminomas, differ biologically and clinically. About 50% of TGCTs are pure seminomas and 40% pure or mixed nonseminomas. The remaining 10% containing both seminoma and nonseminoma components are classified as being nonseminoma according to the World Health Organization (WHO) classification system. The genetic alterations underlying the development of these neoplasms have not been understood fully, although much has been done to elucidate them.

The cell cycle regulatory pathway deregulated in almost all human tumors appears to be the G1 phase-controlling mechanism centered around the pRB protein. Different cancers seem to have altered different key components of that mecha-
nism, which may be connected with gene activity patterns in different target cells. The mechanism involves pRb and its immediate upstream regulators, the cyclin dependent kinases (CDK4 and CDK6), their catalytic partners (cyclin D1, cyclin D2 and cyclin D3), and the members of the INK4 family of CDK inhibitors (p16INK4a, p15INK4b, p18INK4c and p19INK4d). This mechanism seems to be a common point for various signaling pathways, serving as a growth factor dependent cell cycle switch. Deregulation of the RB pathway may be an obligatory step in oncogenesis, making tumor cells less dependent on growth stimuli.

The pRB is essential in cell cycle regulation and its function is regulated by phosphorylation. In Go and the early G1 phase, hypophosphorylated pRB is complexed with the transcription factor E2F. In late G1, a significant hyperphosphorylation of the pRB by CDK4 and CDK6 in complex with D cyclins (D1, D2, D3) occurs.

The CDKN2 locus at chromosomal region 9p21 encodes p16INK4a tumor suppressor protein involved in the RB cell cycle control pathway. p16INK4a functions as a regulator of G1/S phase transition by inhibiting the activity of CDK4 and CDK6. Thus, by inhibiting pRB phosphorylation, p16INK4a can promote the formation of a pRB-E2F repressive transcriptional complex, which blocks cell cycle progression past G1/S restriction point.

In diverse types of cancer the RB pathway becomes deregulated through alterations in one or more of its components. The most common defects of the RB pathway are mutations or deletions of RB1 and inactivating mutations or promoter methylation of the CDKN2A (p16INK4a) tumor suppressor gene, as well as the overexpression of the cyclin D2/CDK4 complex.

The objective of this study was to evaluate the occurrence of the loss of heterozygosity (LOH) of the CDKN2A and RB1 tumor suppressor genes in TGCTs.

**Materials and methods**

**Patients and tumor material**

Fouiry TGCT samples (18 seminomas and 22 non-seminomas) were collected from Sisters of Mercy University Hospital and University Hospital Center, Zagreb, Croatia. The samples were formalin-fixed and paraffin-embedded. Clinical and pathological data for 40 TGCTs according to the WHO 2004 classification are shown in Table 1.

**DNA extraction**

For each specimen, 20 µm paraffin-embedded section was prepared for DNA extraction. In addition, 4 µm section was stained with hematoxylin-eosin to identify the tumor and normal tissue areas which were removed separately from the microscopic slide, transferred to microtubes and extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

**LOH analysis of CDKN2A gene**

A previously described polymorphic microsatellite marker hMp16α-I1 consisting of a mononucleotide tract of (A)23 located close to intron 1 of the CDKN2A gene was analyzed in this study. Primers used for polymerase chain reaction (PCR) amplifications were 5’-CAATTACCACATTCTGCCTTTTGC-3’ and 5’-CAGGCAGAGAGCACTGTGAG-3’, which produced 190-210 bp fragments. PCR amplifications were performed in 25 µl reaction volume with a final concentration 0.2 mM of each dNTP, 3 mM MgCl2, 0.2 µM of each primer (Sigma-Aldrich, St. Louis, MI, USA), 1x Flexi buffer (Promega, Madison, WI, USA) and 0.5 U of GoTaq® Hot Start Polymerase (Promega, Madison, WI, USA). One hundred nanograms of DNA were used in each PCR reaction. PCR amplifications were carried out in a Eppendorf Mastercycler Personal (Hamburg, Germany), with cycling times of 96°C for 5 min (one cycle), then 45 cycles of 96°C for 30 s, 57°C for 45 s, and 72°C for 30 + 1 s. The final step was incubation at 72°C for 10 min. Amplified DNA fragments were analyzed on silver-stained 15% polyacrylamide gels. LOH of CDKN2A was considered to have occurred if one out of two alleles (heterozygous samples) of a gene marker was missing or significantly reduced in comparison to alleles from adjacent normal tissue.

**LOH analysis of RB1 gene**

LOH of RB1 was detected using polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP). Amplification with RB1 primers 5’-TCCCACCTCAGCCTCCTTAG-3’ and 5’-GTAGGCCAAAGAGTGGGCAG-3’ used in our study produced a 190 bp segment of intron 17. PCR amplifications were performed under conditions mentioned above. To generate the RFLP pattern for LOH analysis, 10 µl of PCR product was digested with 5 U of XbaI restriction enzyme (Fermentas, Vilnius, Lithuania) in a total volume of
TABLE 1. Clinical and pathological data for 40 testicular germ cell tumor cases

| Patient no.* | Age | pTNM | Histology       |
|--------------|-----|------|----------------|
| 1            | 26  | pT1NXMX | ITGCN, S      |
| 2            | 26  | pT1NXMX | ITGCN, S      |
| 3            | 37  | pT1NXMX | S             |
| 4            | 33  | pT1NXMX | ITGCN, S      |
| 5            | 31  | pT1NXMX | ITGCN, S      |
| 6            | 29  | pT1NXMX | ITGCN, S      |
| 7            | 39  | pT1NXMX | ITGCN, S      |
| 8            | 27  | pT1NXMX | S             |
| 9            | 41  | pT1NXMX | ITGCN, S      |
| 10           | 48  | pT1NXMX | S             |
| 11           | 48  | pT1NXMX | S             |
| 12           | 34  | pT1NXMX | ITGCN, S      |
| 13           | 60  | pT1NXMX | ITGCN, S      |
| 14           | 29  | pT1NXMX | ITGCN, S      |
| 15           | 60  | pT1NXMX | S             |
| 16           | 29  | pT1NXMX | ITGCN, S      |
| 17           | 28  | pT1NXMX | ITGCN, S      |
| 18           | 32  | pT1NXMX | ITGCN, S      |
| 19           | 37  | pT1NXMX | EC            |
| 20           | 18  | pT1NXMX | EC, IT, MT, S |
| 21           | 24  | pT1NXMX | EC, ITGCN, S  |
| 22           | 22  | pT1NXMX | EC, YST       |
| 23           | 37  | pT1NXMX | EC, ITGCN, S  |
| 24           | 28  | pT1NXMX | C, EC, IT, MT |
| 25           | 17  | pT1NXMX | EC, MT        |
| 26           | 34  | pT1NXMX | EC            |
| 27           | 19  | pT1NXMX | EC, ITGCN, MT, YST |
| 28           | 39  | pT1NXMX | MT, YST       |
| 29           | 21  | pT1NXMX | EC, MT, YST   |
| 30           | 23  | pT1NXMX | EC, IT, MT    |
| 31           | 22  | pT1NXMX | MT, YST       |
| 32           | 25  | pT1NXMX | EC            |
| 33           | 45  | pT1NXMX | EC, ITGCN, S, YST |
| 34           | NK  | pT1NXMX | C, EC, ITGCN, S, YST |
| 35           | 23  | pT1NXMX | EC, IT, ITGCN, MT, YST |
| 36           | 39  | pT1NXMX | EC, ITGCN, S, YST |
| 37           | 24  | pT1NXMX | EC, ITGCN, YST |
| 38           | 30  | pT1NXMX | EC, ITGCN, YST |
| 39           | 36  | pT1NXMX | EC, ITGCN, MT, YST |
| 40           | 58  | pT1NXMX | EC, ITGCN, YST |

*seminomas, patients no. 1-18; nonseminomas, patients no. 19-40

C = choriocarcinoma; EC = embryonal carcinoma; IT = immature teratoma; ITGCN = intratubular germ cell neoplasia; MT = mature teratoma; S = seminoma; YST = yolk sac tumor; NK = not known
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25 µl for 12 h. The restriction digestion resulted in fragments of 75 and 115 bp. DNA fragments were analyzed on silver-stained 15% polyacrylamide gels. LOH was recognized as a partial or complete loss of either the uncleaved (190 bp) or the cleaved (75 + 115 bp) allele.

Results

In this study 40 TGCTs, 18 seminomas and 22 nonseminomas, were analyzed. First, we searched for LOH of the intragenic polymorphic microsatellite marker hMp16α-I1 in the CDKN2A gene. From 40 TGCTs, 34 (85%) tumors were informative for this polymorphism, 16 (89%) seminomas and 18 (82%) nonseminomas. Our analysis revealed that two (6%) samples showed LOH of hMp16α-I1 marker. The observed changes were assigned to two nonseminomas (11%, patients no. 31 and 34, Table 2). In both tumor cases, one out of two alleles of gene marker was missing in comparison to alleles from the adjacent normal tissue (Figure 1). In addition, both LOHs of the CDKN2A were found in nonseminomas with a yolk sac tumor component. LOH of the CDKN2A gene was not observed among seminomas.

The analysis of intragenic polymorphic restriction marker of the RB1 gene showed that 34 (85%) of total TGCTs were heterozygous for this polymorphism; 15 (83%) seminomas and 19 (86%) nonseminomas. LOH was observed in two (6%) samples when looking at the total TGCTs analyzed. Once again the observed allelic losses were assigned to nonseminomas: two samples (10.5%, patients no. 20 and 25, Table 2) had one of the alleles missing in comparison to bands from the adjacent normal testis tissue. These nonseminoma samples showed loss of the cleaved allele (75- and 115-bp fragments), as the single uncleaved allele (190-bp fragment) appeared on the silver stained 15% polyacrylamide gel (Figure 2). Furthermore, both LOHs of the RB1 were found in nonseminomas with an embryonal carcinoma component. None of the seminomas demonstrated LOH of the RB1 gene.

No statistically relevant correlation between the occurrence of LOH, form of TGCT, histological type of contained components and tumor stage according to TNM classification could be determined by Fisher’s exact test.

Discussion

TGCT is associated with characteristic abnormalities in the RB pathway including upregulation of cyclin D2, and downregulation of pRB and the CDK inhibitors such as p16INK4a.7

The inactivation of the CDKN2A gene, which encodes an inhibitor of CDK4 and CDK6, is one of the most common molecular events in human neoplasms. The major mechanisms contributing to CDKN2A silencing are promoter methylation, gene mutations and hemizygous or homozygous deletions. When one CDKN2A allele is mutated or methylated, the second allele is often deleted.16

The analysis of the expression of INK4 family has pointed to a down-regulation of CDKN2A in testicular neoplasms.7,12 Honorio et al.17 demonstrated that promoter hypermethylation of that gene is not involved in the decrease of p16INK4a protein expression. In contrast, some studies have found promoter mutation, a half of analyzed TGCTs had de novo promoter methylation and approximately half of TGCTs showed hypermethylation of CDKN2A exon 1α. All that correlated with a decreased level of CDKN2A mRNA expression.1,18 However, Chaubert et al.18 have not detected any CDKN2A mutations and observed LOH of the CDKN2A
In the context of testicular germ cell tumors (TGCTs), the inactivation of tumor suppressor genes plays a crucial role in the development and progression of these tumors. One of the key genes involved in this process is the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene, which is often deleted or mutated in various forms of TGCTs. This gene is crucial in regulating the cell cycle and apoptosis, and its inactivation may contribute to the aggressiveness of these tumors.

The involvement of CDKN2A in TGCTs is well-documented, with studies demonstrating that it is often deleted in nonseminomatous germ cell tumors (NSGCTs) and in yolk sac tumor components. Loosely observed loss of heterozygosity (LOH) and allelic imbalance at this region have been reported in various TGCTs, including seminomas and nonseminomas. The location of the CDKN2A gene (9p21) is frequently affected by LOH in TGCTs, with a reported frequency ranging from 5% to 42%.

The role of TP53, another important tumor suppressor gene, is also significant in TGCTs. TP53 mutations are common in TGCTs, and they contribute to the development of stage II metastatic testicular cancer. The TP53 gene is known to be involved in the regulation of apoptosis and the cell cycle, and its inactivation may result in the loss of pRB function.

In our study, we observed LOH of TP53 in nonseminomatous tumors with an embryonal carcinoma component, along with deletions of the RB1 gene. This pattern of LOH is not observed in seminomas, suggesting that TP53 inactivation is a late event in the tumorigenesis of these tumors. The LOH of TP53 gene was also demonstrated in the LOH of the TP53 gene, further supporting the role of TP53 in the development of these tumors.

The RB1 gene, located on chromosome 13q14, is another important tumor suppressor gene in TGCTs. LOH of RB1 is common in TGCTs, with a frequency of up to 50% in choriocarcinomas. In our study, we found LOH of RB1 in 11% of nonseminomas, which may reflect its deregulation by normal mechanisms in testicular germ cells. The loss of pRB function, due to the inactivation of RB1, may contribute to the development of a more aggressive tumor phenotype.

In summary, the inactivation of tumor suppressor genes such as CDKN2A, TP53, and RB1 is a hallmark of TGCTs, and it plays a critical role in the tumorigenic potential of these tumors. Further studies are needed to better understand the mechanisms underlying the inactivation of these genes and their role in the development and progression of TGCTs.
a synergistic effect, which imposes a stronger selective pressure for the cellular transformation. This may also help to explain the high proliferation rate and/or invasiveness of TGCTs with embryonal carcinoma and yolk sac tumor component. A higher incidence of LOH in nonseminomas may provide a clue to their invasive behavior, because for some of the nonseminoma types there seem to be a region of preferential loss (3q27–3q28 in embryonal carcinoma), and all of the TGCTs show gain of 12p11–12p12 sequences. Knowing the exact nature of genetic alterations associated with these tumors may provide novel treatment strategies.

However, the low frequency of observed LOHs in this study could be a consequence of genomic instability in above mentioned nonseminomas, rather than the main cause of CDKN2A and RBP1 inactivation.

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