p53, Fas (APO 1) and Ki-67 immunostaining in testicular germ-cell tumors and correlation with pathologic stage, tumor size, necrosis, and vascular invasion

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ABSTRACT

The objective of this study was to analyze the relationships among immunohistochemical expression of p53, Ki-67, Fas antigens (CD 95) and tumor size, pathological stage, and the presence of vascular invasion and necrosis in testicular germ-cell tumors. Representative archival tissues from 36 testicular germ cell tumors were studied for p53, Ki-67 and Fas antigens by immunohistochemistry. All of the tumors’ size, pathological stage, and the presence of vascular invasion and necrosis within tumors were recorded. Significant correlation with pathological phase was found for Ki-67 and Fas expressions. No correlation was determined between any antibody and maximum tumor diameter or necrosis. While a positive correlation (p<0.05) with vascular invasion in mixed germ-cell tumors was found with Ki-67 staining intensity, no significant correlation (p>0.05) was found with Fas and p53. In the previous and current studies, the percentage of embryonal carcinoma and the presence or absence of vascular invasion in NSGCT appear to be reliable prognostic factors to identify patients at high risk and low risk for occult retroperitoneal disease. Additionally, our results, suggest that Ki-67 plays a role at all stages of tumorigenesis and that it could be used as an auxiliary to other parameters in tumor prognosis. It was concluded that Ki-67 and Fas expressions may be important prognostic parameters in the staging of germ-cell tumors. [Turk J Cancer 2003;33(3):137-143]

KEY WORDS:
Germ cell tumor, testis, p53, Ki-67, CD 95

INTRODUCTION

Germ-cell tumors (GCTs) are the most common form of testicular tumors (1). Cryptorchidism, family history, gonadal dysgenesis, and oligospermic infertility are the most widely recognized risk factors for GCTs. Histologically, GCT comprises two main entities, seminomas and nonseminomas (NSGCT), such as embryonal carcinoma (EC), mature or immature teratoma, choriocarcinoma (CC), and yolk-sac tumor (YST) (1). There is no consensus on the staging of germ-cell tumors, which account for 90-95% of testicular tumors. Various staging systems exist for GCTs, most of which are based on the localization of the tumor in the testis, lymph-node involvement, and distant metastasis (1-3).

The p53 gene is a tumor suppressor capable of negative regulation of neoplastic cell proliferation. While normally
present p53 has a too short half-life to be detected immuno-
histochemically, mutant p53 that occurs during tumor-
ogenesis is immunohistochemically detectable (2-10). The
Ki-67 (MIB), a nuclear antigen expressed in all phases
except G0, is essential to cell proliferation. Like p53, Ki-
67 monoclonal antibody (mAb) has been used in assessing
proliferative activity in human tumors, and the Ki-67 values
have generally been in conformance with tumor prognosis
(10-17).

The Fas receptor and the ligand system, on the other
hand, are the best-known components of the system that
regulates apoptosis. The Fas receptor (Apo-1, CD 95) is a
transmembrane glycoprotein belonging to the nerve growth
factor/tumor necrosis factor (NGF/TNF) family of receptors.
Apoptosis is triggered by the binding of the natural ligand
(Fas L) or the diagonal binding of antibodies against the
Fas receptor (18,19).

In the present study, we investigated p53, Ki-67, and
Fas antigen (CD 95) expression in clinical stage I (CSI),
testicular germ-cell tumors (14 seminomas, 22 NSGCTs).
Additionally we studied for the presence of vascular invasion
(VI) and necrosis. We compared all parameters to patho-
logical tumor stage, tumor size and the percentage of
embryonal carcinoma (EC%) in NSGCT.

**MATERIALS AND METHODS**

Formalin-fixed, paraffin-embedded testicular germ cell
tumor orchiectomy materials obtained between 1998 and
2002 was selected from the archives of Başkent University
Medical Faculty and Mersin University Medical Faculty
Hospital. Primer tumor histopathology slides stained with
hematoxylin and eosin (H&E) were available for 22 clinical
stage I NSGCT, and 14 seminomas. CSI conformed to
negative radiographic staging of the chest, pelvis and
abdomen, and appropriate tumor marker decrease following
orchiectomy. From the review of all available H&E stained
slides, the presence or absence of VI and necrosis, tumor
size, histologic cell type of each tumor were recorded for
all cases. The percentage of embryonal carcinoma (EC%) in
cases was estimated for each section under low-power
magnification, based on the method used by Fung et al.
(20), that include a semiquantitative analysis by scoring
tumors on the basis of whether they contained 50% or more
or less than 50% of EC. Two pathologists who were unaware
of the clinical findings examined the sections independently.
After the independent reviews, each case was evaluated
jointly and disagreements were resolved by consensus. The
WHO system was used for pathologic classification, and
the T system for pathologic staging (T1, tumor localized
in the testis; T2, tumor extending through the tunica albug-
inea; T3, tumor extending through the epididymis or the
rete testis; T4, metastasis to the spermatic chord or the
scrotal wall) (21). The distribution of stages in these tumors
is shown at table 1.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Seminoma N (%)</th>
<th>NSGCT N (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>8 57.1</td>
<td>10 45.5</td>
</tr>
<tr>
<td>2</td>
<td>4 28.6</td>
<td>6 27.3</td>
</tr>
<tr>
<td>3</td>
<td>2 14.3</td>
<td>3 13.6</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>3 13.6</td>
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<tr>
<td>Total</td>
<td>14 100</td>
<td>22 100</td>
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The chosen one or two paraffin block for each case was cut into 5 mm sections. The sections were deparaffinized and endogenous peroxidase was blocked with H₂O₂ in methanol, the sections were heated in 0.01 mol/l citrate buffer in a microwave pressure cooker for 20 minutes. The slides were allowed to cool to room temperature, and nonspecific binding was blocked with normal horse serum for 20 minutes at room temperature. The MIB-1 monoclonal antibody was used for detection of nuclear Ki-67, a marker of proliferating cells (code no: M7187, dilution 1:40, DAKO). Monoclonal antibody which recognizes both wild type and mutant p53 was used (code no: M7001, dilution 1:50, DAKO) for p53 detection. Fas antigen (APO-1) monoclonal antibody used with 1/10 dilution (code no: M3553, DAKO). The antibodies were incubated overnight at 4°C in a humidified chamber. The sections were stained using the avidin-biotin complex (ABC) immunoperoxidase technique employing commercially available reagent (ABC kit, DAKO). The sections were counterstained with Mayer’s hematoxylin and mounted with paramount. Human tonsil sections served as positive controls for MIB-1, p53. Ductal carcinoma of breast sections served as a positive control for Fas antigen.

In evaluation of each marker calculating the percentage of positively stained cells in the total number of tumor cells under x40 magnification. The degree of immunopositivity was evaluated semiquantitatively. Only nuclear staining was considered positive for p53, Ki-67 and membranous staining for Fas antigen. The scoring system for p53, Ki-67 antibodies tested was as follows: 0-5%, negative; 5-25%, low positivity [1]; 25-50%, moderate positivity [2]; 50-75%, high positivity [3], >75% higher positivity [4]. The scoring system for Fas antigen tested was as follows: 0%, negative; 5-10, low positivity [1]; 10-20%, moderate positivity [2]; %25-50%, high positivity [3], and >50% higher positivity [4].

Statistical analyses were performed with the SPSS 9.00 package program for windows. The categorical variables (pathologic stage of tumor, necrosis, VI, EC% and expression of p53, Fas antigen, and Ki-67) were analysed with the chi-square test. The nonparametric Kruskal-Wallis test, and Mann-Whitney U test were used to compare the expression of p53, Fas antigen, and Ki-67. P values <0.05 were considered to be statistically significant.
RESULTS

In the study group, the average age of GCT patients was 31.5±0.93 (range 19–42). The average ages of patients with seminomas and with NSGCTs were 32.5±1.2 (range 25-42) and 30.1±1.3 (range 19-40), respectively. Of the seminomas, 57.2% were located in the right testis (8 patients), 35.7% in the left testis (5 patients), and 7.1% in both testes (1 patient). Of the NSGCTs, 59.1% were located in the right testis (14 patients), and 40.9% in the left testis (9 patients). Although both tumor types were located more frequently in the right testis, the difference was not statistically significant (p>0.05). The maximum diameter of tumors located in the right testis was 3.31±0.46 and 6.23±0.65, and that of tumors located in the left testis was 4.41±1.03 and 4.0±0.55 for seminomas and NSGCTs, respectively. There was no significant correlation between tumor diameter and location in either GCT.

While Ki-67 expressions were observed in all patients (100%), Fas antigen expression was observed in 8 of seminomas (57.1%) and 15 of NSGCTs (77.4%). P53, Fas antigen, and Ki-67 staining intensities in seminomas are as follows: low positivity in 5 patients, moderate positivity in 8 patients and high positivity in 1 patient with p53, negative staining in 5 patients and low positivity in 9 patients with Fas antigen, low positivity in 7 patients, moderate positivity in 6 patients, high positivity in 1 patient with Ki-67. No significant difference was determined between staining intensities with p53, Fas antigen, and Ki-67 expressions in seminomas. Staining intensities in NSGCT are as follows: negative staining in 1 patient, moderate positivity in 5 patients and high positivity in 16 patients with p53, negative staining in 9 patients, low positivity in 12 patients, and moderate positivity in 1 patient with Fas antigen, low positivity in 2 patients, moderate positivity in 12 patients, high positivity in 8 patients with Ki-67 (Table 2). Significant difference was determined between staining intensities with p53, Fas, and Ki-67 expressions in NSGCTs (p<0.01). The strongest expression with p53 and Ki-67 in NSGCTs was determined in the EC, CC and YST components. No expression was observed in mature teratoma components, except a few scattered positive stained cells with p53 and Fas antigen (Figures 1-5).

In the comparison of pathologic tumor stage and p53, Fas antigen and Ki-67 expressions, statistically significant correlations were found between pathologic stage and both Ki-67 and Fas antigen expressions in both seminomas and NSGCTs (p<0.05), while the correlation with p53 expression was not significant in both tumor types (p>0.05). No significant difference was determined between necrosis and Ki-67, Fas antigen, and p53 expressions in either tumor (p>0.05). In NSGCTs, a positive correlation was found between vascular invasion and Ki-67 staining intensity (p<0.05), and a negative correlation between VI and Fas

### Table 2

<table>
<thead>
<tr>
<th>Intensity of immunreactivity in 36 germ cell tumors for p53, Ki-67 and Fas</th>
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<tbody>
<tr>
<td><strong>Intensity of immunreactivity, mean ± SD</strong></td>
</tr>
<tr>
<td><strong>Total</strong></td>
</tr>
<tr>
<td>p53</td>
</tr>
<tr>
<td>Fas</td>
</tr>
<tr>
<td>Ki-67</td>
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<tr>
<td>Total</td>
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antigen and p53 staining intensity (p>0.05). The correlation between tumor diameter and the expressions of the three antigens was not found to be significant (p>0.05).

We found that in total 21 NSGCT, EC was <50% in 8 cases and >50% in 13 cases. The percentage of EC were the significant correlation with pathological stage of tumor, VI and necrosis (p<0.05, p<0.001, p<0.05, respectively). There was no significant correlation between EC% and p53, Ki-67 and Fas expression (Table 3).

In seminomas, Ki-67 expression was significantly different between stages 1 and 2 and between stages 1 and 3. In NSGCTs, Fas expression was significantly different between stages 1 and 4 and between stages 2 and 4, while Ki-67 expression was significantly different between all stages (p<0.05). In the analysis of the correlation between pathologic stage and p53, Ki-67, and Fas expressions, the correlation between Ki-67 and pathologic stage was determined to be significant in all patients (p=0.013 and p=0.004, respectively). p53 and Fas had no correlation with pathologic stage at all stages, and Ki-67 had no correlation with pathologic stage at T2 and T3. The only significant result according to tumor stage was found in Ki-67 staining (p<0.05).

**Table 3**

<table>
<thead>
<tr>
<th>Intensity of immunreactivity, mean ± SD</th>
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<tr>
<td><strong>N</strong></td>
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<tr>
<td>&lt;EC%</td>
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<tr>
<td>&gt;EC%</td>
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**DISCUSSION**

The most important prognostic factor for germ-cell tumors of the testis is the tumor stage. The presence of lymphoid infiltration suggests a good prognosis in clear granulomatous reaction seminomas (1). Mitotic activity is not a valid measure in seminoma prognosis. In NSGCTs, on the other hand, prognostic factors include vascular invasion and extratesticular pathology, lymph-node involvement, tumor components, the presence of EC and CC (particularly within the tumor), and high S-phase and proliferation indicators in flow cytometry (1,4-8,10, 22-24). Pathology reports in testicular tumors must include the status of the tumor in the testis, extratesticular pathology, the presence of lymphovascular invasion, and the ratios of tumor subtypes.

p53 gene mutation has been demonstrated in a number of human tumors, including testicular tumors (2-9,15). Lewis et al. (4) demonstrated p53 protein expression at a rate of 90% in seminomas, and of 94% in NSGCTs. In this study, p53 mutation was shown in 100% of CSI seminomas, and 98% of CSI NSGCTs, and as the clinical stage progressed, the p53 overexpression percentage decreased, more markedly in seminomas (50% in clinical stage III seminomas, 88% in clinical stage III NSGCTs) (4). p53 expression was observed in all components in NSGCTs, but a strong correlation with clinical stage was observed in ECs. In this study, it was asserted that VI and the intratumor EC ratio were more useful clinical parameters for prognosis (4). In a study by Eid et al. (8), p53 expression was observed in all tumors except differentiated teratomas in 77 untreated GCTs, and the strongest p53 expression was determined in seminomas and ECs. Moreover, a negative correlation was observed between clinical stage and p53 expression. In this study, a positive correlation was determined between p53 expression and response to treatment; nonetheless, in another study by Eid et al. (9), no correlation was determined by multidrug-resistance-associated protein (MRP) between metastatic behavior and p53 expressions. In contrast, p53 and Ki-67 expression were not found to be of prognostic significance in Heidenreich et al.’s (22) 149 NSGCT cases. According to our results, p53 is not a useful marker to discriminate pathologic stages. Similar to our results, DeRiese et al. (24) in their study found similar p53 expression in both stage I and stage II tumors. On the other hand, p53 protein overexpres-
sion is shown in most testicular tumors but mutations are rare (25). In our study, no significant correlation was determined between p53 expression and necrosis or VI.

In a study on 76 stage-A germ-cell tumors without lymph-node metastasis, Albers et al. (17) found that Ki-67 was a good prognostic parameter, and that it could be used even to classify patients with low metastatic risk. However, in patients with a risk so high as to be clinically demonstrable, no valid classification has been achieved by assessment of tumor-cell proliferation (17). In Heidenreich et al.’s studies (22,23), Ki-67 expression did not correlate with pathological stage in CSI, NSGCTs. In these studies, the presence of VI and the intratumor EC ratio were determined as significant risk factors for occult nodal disease and prognosis (22,23).

In our study, the correlation between pathologic stage and Ki-67 expression was found to be significant (p<0.05). While the Ki-67 correlation was stronger between stages 1 and 2 and stages 1 and 3 in seminomas, significant differences in Ki-67 expression were observed at all stages in NSGCTs. These results suggest that Ki-67 is not useful in discriminating between advanced stages in seminomas. Nonetheless, Ki-67 expression appears to be an important indicator in discriminating between stages in NSGCTs, while p53 expression is not a good indicator in advanced stages. In NSGCTs, the positive correlation between vascular invasion and Ki-67 staining intensity suggests that Ki-67’s correlation in pathologic staging is no coincidence.

Fas receptor and Fas ligand can be shown immunohistochemically in normal and tumoral tissues, including the testes (18,19). Fas-Fas ligand expression has been determined in Leydig, Sertoli, and germ cells in the testis. Sugihara et al. (18) obtained positive staining immunohistochemically in 23 seminomas, 8 ECS and 3 YSTs with the Fas-Fas ligand, and they found a single major band heavier than 47.5 kilodaltons, to which antibodies were bound for Fas and Fas ligand. These researchers asserted that the Fas system had a role other than signal producing for apoptosis in testicular tumors. Braenstrup et al. (19) did not determine Fas-fas ligand secretion in invasive testicular tumors, but they did demonstrate its presence in normal seminiferous tubules and occasionally in carcinoma in situ (CIS) areas. We observed Fas expression in intratubular lymphocytes in normal seminiferous tubules, Leydig cells, and teratomas, and ECs. We observed that immunoreactivity with Fas is generally weak and there is a low number of cells. In NSGCTs, although a significant correlation was determined between stage and Fas expression in tumor cells, the scattered and weak Fas expression was not considered to be a good indicator in the staging of testicular tumors.

Wishnow et al (24) were first to do a quantitative analysis of the percentage of EC and in their study, it was reported that percentage of EC was an important prognostic factor for relapse. The studies of Lewis et al. (4) and Heidenreich et al. (10,22,23) have also suggested this result. In contrast, in one of the largest studies of prognostic factors in CSI, NSGCT, Klepp et al. (27) did not confirm the importance of EC. In our study, significant correlations were found between EC% and pathological stage, VI and necrosis. But, we could not determine the correlation between EC% and p53, Ki-67 and Fas expressions.

In conclusion, the high p53 expression in germ-cell tumors, especially in CIS and at low pathologic stages, supports the idea that p53 mutation in these tumors occurs at the early stages. Correlation between pathologic stage, VI and Ki-67 in NSGCTs suggest that Ki-67 plays a role at all stages of tumorogenesis and that it could be used as an auxiliary to other parameters in tumor prognosis. However, in the evaluation of apoptosis in germ-cell tumors, we think that, besides immunohistochemical findings, more advanced techniques would be more specific and enlightening. We recommend that in orchiectomy specimens of all patients with CSI, NSGCT evaluated for EC% and the presence of VI to determine their risk for occult retroperitoneal metastasis.
References


