Apoptosis Regulation and Spontaneous Apoptosis Index of Testicular Germ Cell Tumors are Associated with Differentiation and Resistance to Systemic Treatment

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Abstract. *Background: Cisplatin-based chemotherapy can cure more than 80% of metastatic germ cell testicular tumors (GCT). Germ cells are particularly susceptible to apoptosis and it is reasonable to presume that GCTs are curable because of an intact and effective apoptotic pathway. Patients and Methods: The expression of p53 and p21 was investigated in conjunction with the spontaneous apoptotic index in 20 refractory and 50 chemosensitive GCTs, with a complete follow-up. To detect a differentiation-dependent alteration in the apoptotic pathway, all of the histological tumor types were examined separately. Results: Embryonal carcinoma components showed significantly higher p53 expression compared to other histological subtypes of GCTs. p21 was barely detectable in the majority of tumors. Seminomatous components showed no p21 expression. Mature teratomas and syncytiotrophoblasts showed significantly higher p21 expression than other tumor subtypes. Embryonal carcinomas showed significantly higher apoptotic indices than other non-seminomatous components. On the other hand, choriocarcinomas and mature teratomas showed the lowest spontaneous apoptotic potential. The apoptotic index correlated with the fraction of p53-positive cells, but not with the p21 expression rate. The refractory group showed significantly lower p53 expression, higher p21 expression and a higher apoptosis index than the sensitive group. Conclusion: Our results suggest that the p53 and p21 expression levels and the apoptosis index seem to be important factors in the issue of the chemosensitivity of GCTs. The protein expression pattern reflects a differentiation-dependent preference for G_1/S-phase arrest in terminally differentiated syncytiotrophoblasts and mature teratoma cells, while p53 mediated apoptosis induction is meaning to less differentiated tumor types.*

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In Western countries, testicular germ-cell tumors (GCTs) account for up to 60% of all malignancies diagnosed in male patients between 20 and 40 years of age (1). GCTs are derived from totipotential germ cells. Seminoma evolves from a common neoplastic precursor lesion, while embryonal carcinoma arises from seminoma. The other differentiated forms of GCT, representing somatic (teratomatous) and trophoblastic differentiation most commonly arise from embryonal carcinoma. This differentiation recapitulates the embryogenesis. Both seminomas and nonseminomatous GCTs (NSGCTs) account approximately for half of the GCTs.

One of the greatest successes of cancer chemotherapy is the incorporation of *cis*-diamino-dichloride-platinum (cisplatin; CDDP) into the treatment of GCTs. Even in patients with metastatic disease, cure rates of 80% can be achieved with multiagent, cisplatin based combination chemotherapy followed by secondary resection in the case of residual tumor lesions (2). However, mature teratoma elements do not share the general chemosensitivity of less differentiated histological subtypes of GCTs. Due to intrinsic chemoresistance, mature teratoma can be found in 30-40% of residual lesions after chemotherapy for NSGCT (3). More differentiated GCT cells therefore seem to be more resistant to chemotherapy. Regardless of their benign behaviour, complete resection of residual mature teratoma is mandatory to prevent transformation into secondary non-germ-cell malignancies. However, the biological basis of the chemosensitivity of GCTs in general and the extreme chemoresistance of mature histological subtypes have not been elucidated yet.

It is generally accepted that the cytotoxic activity of cisplatin results from its interactions with DNA. DNA-platinum covalent adducts inhibit fundamental cellular processes, including replication, transcription, translation and DNA repair (4). Though the cellular basis for chemotherapy response in GCTs seems multifactorial, it is almost certain that an intact apoptotic pathway is crucial for an effective CDDP-mediated cell death (5).

Germ cells are particularly susceptible to apoptosis (6). Presumably, the apoptotic elimination of a high fraction of potential sperm is the result of a quality control mechanism that is designed to reduce the risk of passing on genetic defects to offspring. The hypothesis that most GCTs are curable because an apoptotic pathway is up-regulated is attractive, and might explain the general sensitivity to CDDP. However, the studies published on this matter so far have yielded contradictory results.

A major role for p53 in the response to chemotherapeutic drugs and the execution of apoptosis has been described (7). In response to genotoxic damage, p53 is up-regulated, resulting in cell cycle arrest of cells in the G_1/S-phase, and participates in DNA repair or drives cells toward apoptosis (8). The p53 gene is the most frequently mutated gene in human cancers, however, in human GCTs almost no p53 mutations have been detected, while the p53 protein is expressed at high levels in the majority of these tumors (9). A cell line expressing mutant p53 was derived from a cisplatin-resistant human GCT and exhibited relative resistance to cisplatin and reduced apoptotic cell death compared with a cell line expressing wild-type p53 that was derived from a sensitive GCT (10). These data indicate that the presence of wild-type p53 seems to be important in determining the sensitivity of GCT cells to chemotherapy. So far, however, clinicopathological studies have not proved strong correlation between p53 level and chemosensitivity of GCTs (11).

p53-dependent cell cycle arrest is in part a consequence of the up-regulation of $p21^{waf1/Cip1}$, which binds to and inhibits cyclin-dependent kinases, which causes hypophosphorylation of retinoblastoma protein, thereby preventing the release of the transcription factor E2F. This leads to repression of E2F-dependent transcription and G_1/S cell cycle arrest to repair the DNA damage (12). p21 is not only involved in cell cycle arrest, but also in suppressing apoptosis (13). Cytoplasmic p21 can associate with ASK1 and thereby inhibit ASK1-mediated apoptosis (14). Bartkova *et al*. (15) revealed that although p21 was rarely detectable in the seminoma and embryonal carcinoma components of combined GCT, a rather abundant nuclear accumulation was observed in differentiated structures in teratomas and teratomatous components of combined tumors. In addition, retinoic acid treatment of human Ntera2/D2 teratocarcinoma cells resulted in induced p21 levels, which correlated positively with retinoic acid-induced differentiation into neurons. These observations suggest a possible correlation between p21 expression upon differentiation and resistance of GCTs to cisplatin.

Our primary aim was to detect the change of expression of some major apoptosis regulator proteins (p53 and p21) and the spontaneous apoptosis rate of GCTs upon tumor differentiation. We decided to investigate the relationship between the expression of the aforementioned proteins, the spontaneous apoptosis index, phenotypic change and resistance to systemic treatment.

Patients and Methods

Patients. Specimens from 70 patients diagnosed between 1990 and 2003 were obtained by semicastration, performed in the National Institute of Oncology, Budapest, Hungary. Prior to surgery the patients received neither radio- nor chemotherapy. Histological examination was performed on haematoxylin and eosin-stained tissue sections. The tumors were histopathologically classified according to WHO criteria (16). For clinical staging, physical examination, serum tumor markers (β subunit of hCG and AFP) (17), chest X-ray, abdominal ultrasound and CT scans were routinely used. Additional CT or MRI was performed if clinically needed. Staging was based on the UICC classification (18). Early stage was defined as stage I or stage IIA. Late stage was defined as stage IIB, IIC or stage III. Therapy was performed according to the protocol of the National Institute of Oncology, Budapest, Hungary (19). Clinical response was measured in accordance with generally used criteria in testicular tumor (20).

Refractory group (20 patients): Patients were considered refractory when progression or relapse occurred despite adequate initial or salvage treatment.

Chemosensitive group (50 patients): Only patients with a complete remission and relapse-free follow-up more than 1 year were included.

Relevant clinicopathological data are shown in Table I.

Immunohistochemical detection of p21 protein expression. The 4 μm formalin-fixed paraffin embedded tumor sections were deparaffinized in two changes of xylene for 30 minutes each and then hydrated in decreasing concentration of ethanol and washed in two changes of distilled water. Endogenous peroxidase blocking was performed with 3% H_2O_2 for 10 minutes. Slides were washed in two changes of distilled water and were rinsed in citrate buffer (pH 6). Heat-induced epitope retrieval was performed in water bath (97˚C, 35 minutes). After cooling for 5 minutes, the slides were removed to /phosphate-buffered saline (PBS). Primary antibody [$p21^{WAF1/Cip1}$: SX118 (DAKO, Denmark)] was applied at a dilution of 1:50, for 1 hour at room temperature. The sections were washed 3×5 minutes with PBS, and were incubated with polymer-horseradish peroxidase (EnVision+ System; DakoCytomation, CA, USA) for 30 minutes. The sections were washed twice with TBS, and were incubated with DAB Substrate-Chromogen (Dako CA, USA), slightly counterstained with Mayer's haematoxylin and mounted (21).

Immunohistochemical detection of p53 protein expression. Because of the high false positivity rate of p53 expression with the aforementioned method, the immunohistochemical detection of p53

Table I. *Clinical and pathological characteristics of the patients.*

	Number of cases %
Number of patients	70 (100%)
Mean age (years)	32 (17-60)
5-year survival	91.4%
Median follow-up (months)	51 (2-135)
Histology of the primary tumor	
S	16 (22.3%)
EC	4 (5.7%)
CC	1 (1.4%)
T	10 (14.3%)
S+EC	7 (10%)
S+T	4 (5.7%)
S+EC+T	4 (5.7%)
EC+YS	4 (5.7%)
EC+T	8 (12.1%)
EC+YS+T	4 (5.7%)
EC+CC+T	7 (10%)
CC+T	1 (1.4%)
Clinical stage	
Early stage (I-IIA)	33 (47.1%)
Late stage (IIB, IIC, III)	37 (52.9%)
Therapy after semicastration	
Chemotherapy	47 (67.2%)
Chemotherapy+salvage surgery	1 (1.4%)
RLA+chemotherapy	17 (14.3%)
Chemotherapy+radiotherapy	5 (7.1%)
Clinical outcome	
No evidence of disease	10 (14.3%)
Complete response	37 (52.9%)
Partial response	10 (14.3%)
Stable disease	4 (5.7%)
Progressive disease	4 (5.7%)
Died	5 (7.1%)

T, teratoma; S, seminoma; EC, embryonal carcinoma; CC, choriocarcinoma; YS, Yolk sac tumor; RLA, retroperitoneal lymphadenectomy.

was performed using the Ventana Medical Systems' *i*VIEW™ DAB Detection Kit with the Ventana Automated ES Slide Staining System according to the manufacturer's protocol.

For positive controls, paraffin-embedded human colon adenomatous polyp, colon cancer tissues, human tonsil, as well as normal mouse testis were used. The primary antibody was replaced with 3% bovine albumin solution in PBS as a negative control. The fraction of positive tumor cells (with strong nuclear signal) was expressed as a percentage of the total number of cells counted. These percentages were used in the statistical analysis.

Apoptosis detection. Specimens were fixed in 4% buffered formalin. After paraffin embedding, 4 μm sections were cut. The sections were washed in PBS for 10 minutes. Permeabilization was performed with Proteinase K Working Solution in a humidity chamber for 15 minutes at room temperature. After washing in DNase-free water for 2×2 minutes, samples were immersed in 5% H_2O_2 to block endogenous peroxidase. Samples were washed with DNase-free water for 1 minute at room temperature than TdT labeling buffer was applied for 5 minutes at room temperature. Samples were treated with labeling reaction mix (1× TdT dNTP Mix, 1× Md^{2+}, 1× TdT Enzyme, 50× TdT labeling buffer) in humidity chamber in a 37˚C incubator for 1 hour. Reaction was stopped by immersing the samples in TdT Stop Buffer for 5 minutes at room temperature. The sections were washed with PBS for 2×5 minutes. Streptavidin-horseradish peroxidase complex was applied for 10 minutes at room temperature. The sections were washed twice with PBS, and were incubated with the DAB working solution, slightly counterstained with 1% methyl green and mounted. The fraction of strongly positive, characteristically condensated, or ring nuclei was determined as a percentage of the total number of cells counted. These percentages were used in the statistical analysis.

Two different methods were used for the evaluation of the results. The protein expression and apoptosis index were determined each tumor component (for example the expression rate of a protein in embryonal carcinomatous and volk sac components of a mixed tumor) and the average expression rate for the whole tumor also determined (in the cases of mixed GCTs). For more specific results, the syncytiotrophoblasts and cytotrophoblasts of choriocarcinomas, and the epithelial and stromal components of immature and mature teratomas were determined separately, with special regard to scattered syncytiotrophoblasts – a particularly differentiated cell type in many kind of GCTs.

Statistical analysis. The comparison of two unpaired groups was analysed by the Mann-Whitney test. Association between two variables was quantified with Spearman's correlation. A probability of ≥95% ($p \leq 0.05$) was considered to present statistical significance. SPSS 11.0 for Windows® (SPSS Inc. IL, USA) was used for calculations.

Results

Expression of p53. In this series, 52.9% of the tumors showed no p53 expression at all. Considering all tumor components, an average of 2.0% of tumor cells showed intense intranuclear positivity. The highest positivity rate was 40% in an embryonal carcinoma component of a mixed tumor (Figure 1). Embryonal carcinoma components showed significantly higher p53 expression compared to other histological subtypes of GCTs (3.95% *vs*. 1.39%; p=0.0006) (Figure 3A).

Expression of p21. P21 was barely detectable in the majority (71.7%) of the tumor samples (Figure 2). Seminomatous components showed no p21 expression. Mature teratomas and syncytiotrophoblasts showed significantly higher p21 expression (p=0.0036) than other tumor subtypes (Figure 2 and 3 C). More than 80% of the syncytiotrophoblasts found in any components were positive (Figure 3 B). No correlation was found between the percentage of p21 positive tumor cells and p53 expression (p=0.8725).

Apoptosis detection in correlation with p53, and p21 expression. The apoptotic index varied between 0 and 10% (mean: 1.4%) (Figure 4). Embryonal carcinomas showed

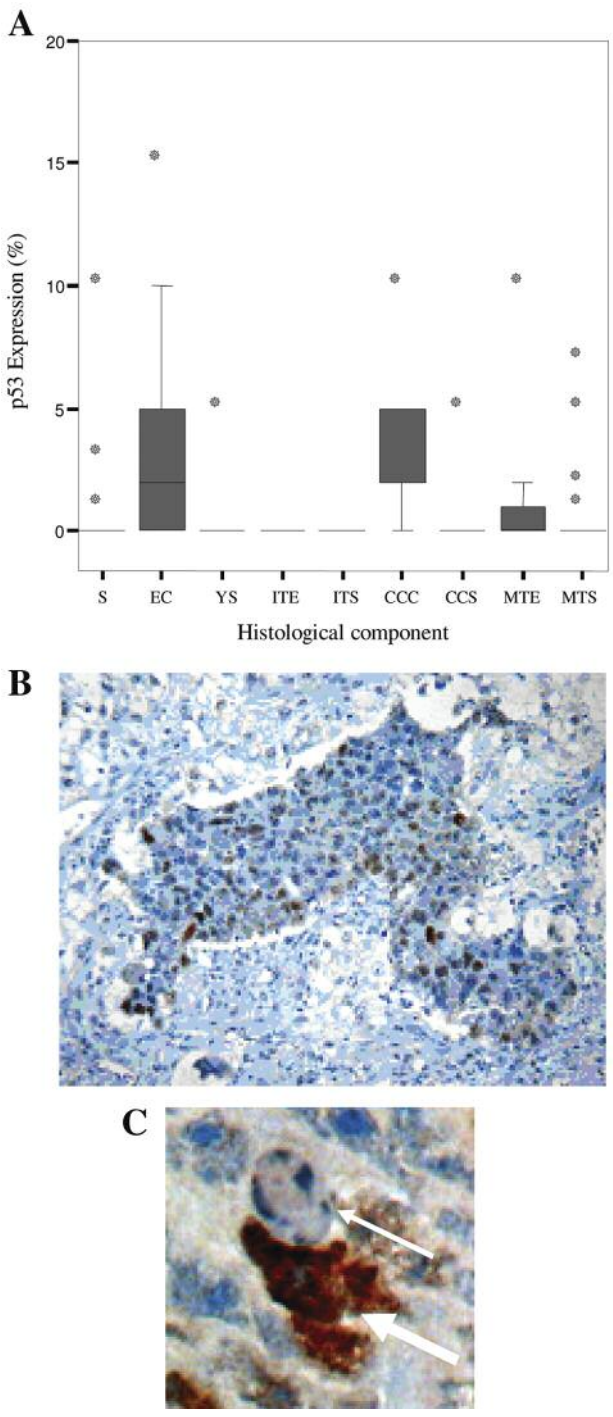


Figure 1. *A, p53 expression in different histological components. Significant difference between embryonal carcinoma and other components (p=0.0006); B, Embryonal carcinoma. Intense intranuclear p53 positivity in approximate 25% of tumor cells (IH, ×40); C, Close relation of an apoptotic figure (thin arrow) and a p53-positive nucleus (thick arrow) (IH, ×600). Abbreviations: S: seminoma; EC: embryonal carcinoma; YS: yolk sac tumor; ITE: immature teratoma epithelium; ITS: immature teratoma stroma; CCC: choriocarcinoma-cytotrophoblasts; CCS: choriocarcinoma syncytiotrophoblasts; MTE: mature teratoma epithelium; MTS: mature teratoma stroma.*

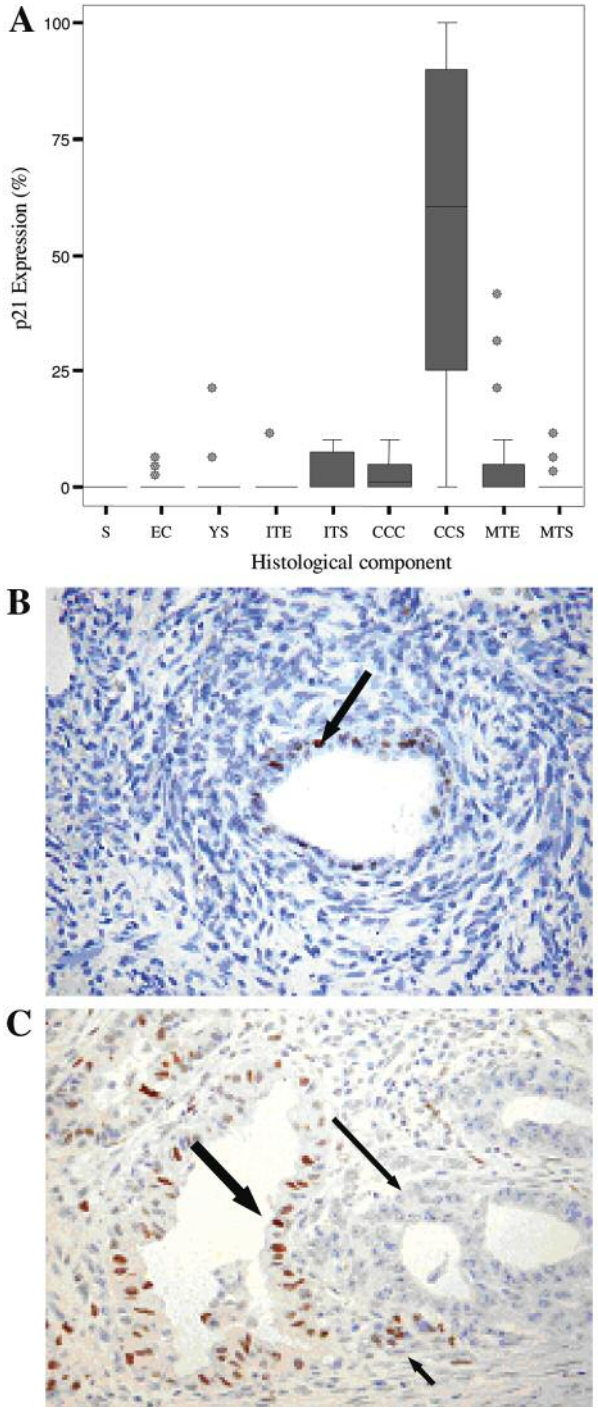


Figure 2. *A, Expression of p21 in different histological components. Significant difference between differentiated components and other components (p=0.0013). Syncytiotrophoblasts were consistently positive; B, Immature teratoma. Only scattered p21 positivity was observed in the glandular epithelial component (arrow). The stromal component was negative (IH, ×100); C, Mixed tumor. Approximately 40% of epithelial cells of the mature teratoma component showed intense intranuclear p21 positivity (thick arrow), whereas staining embryonal carcinoma was completely absent (thin arrow). A p21 positive multinucleated syncytiotrophoblast is also represented (short arrow) (IH, ×100).*

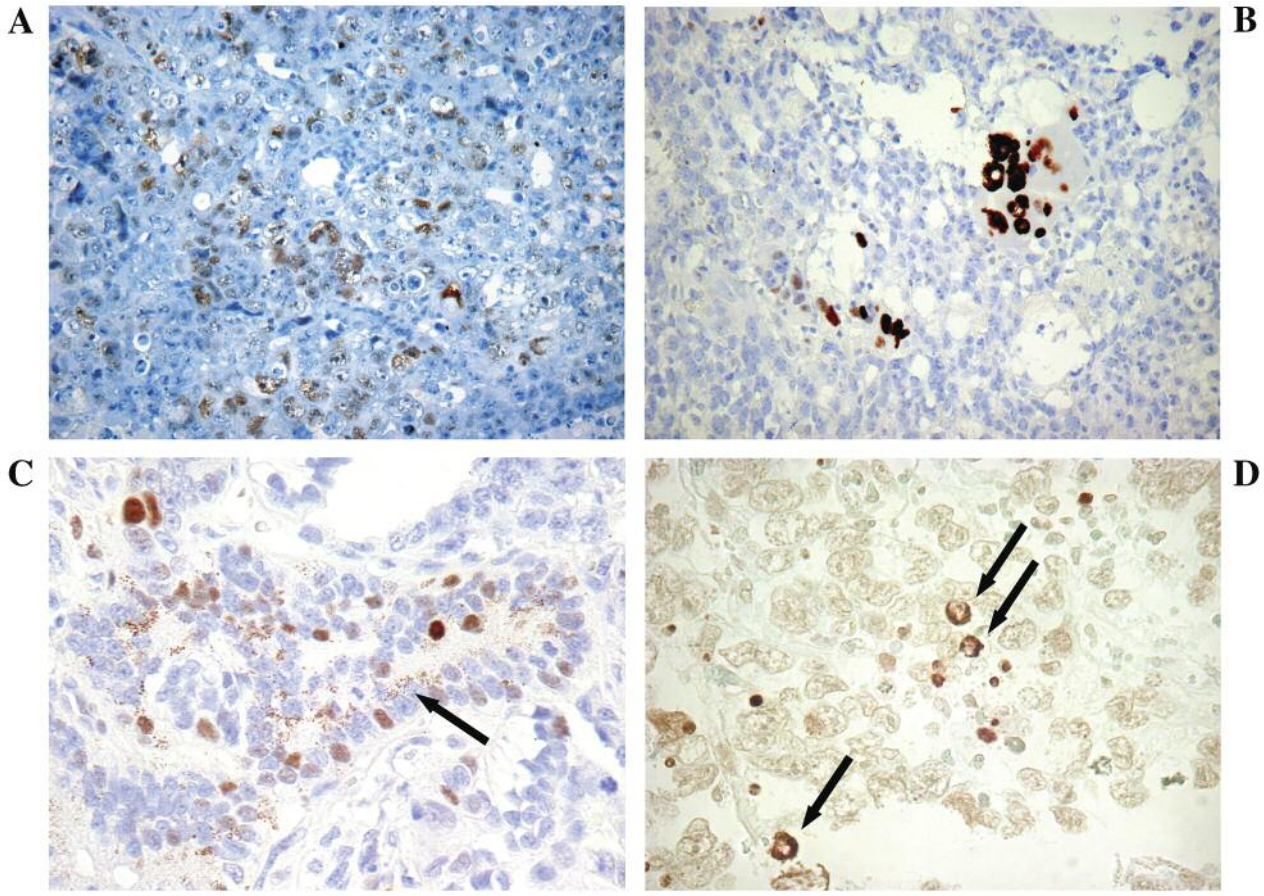


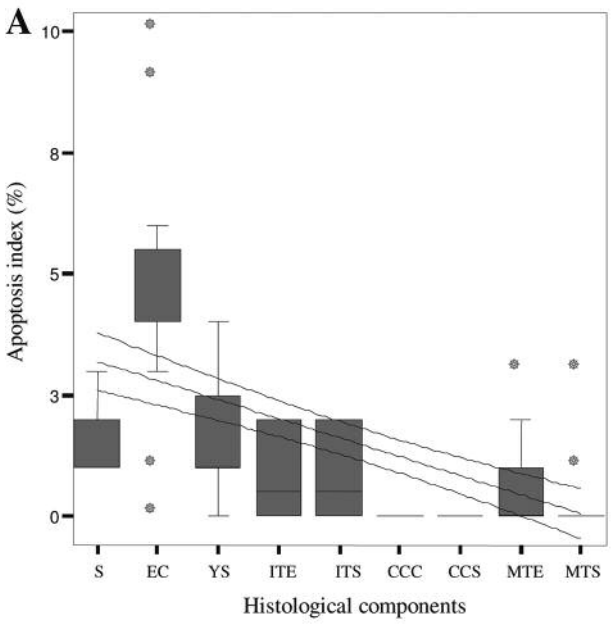
Figure 3. *A, Embryonal carcinoma. Intense intranuclear p53 positivity in approximately 25% of tumor cells (IH, ×100); B, No p21 expression in tumor cells of embryonal carcinoma, however, the scattered multinucleated syncytiotrophoblasts showed strong intranuclear positivity for p21 (IH, ×100); C, Immature teratoma, melanotic neuroectodermal component (retinal anlage). Tubules are lined by p21 positive cells with finely granular intracytoplasmic melanin pigmentation (arrow) (IH, ×200); D, Embryonal carcinoma. Apoptotic figures. Arrows indicate the characteristic ring formations (in situ apoptosis detection, ×200).*

significantly higher apoptosis indices than other nonseminomatous components (p=0.0008) (Figure 3 D and 4 B). On the other hand, choriocarcinomas and mature teratomas showed the lowest spontaneous apoptotic potential compared with other elements (p=0.0006) (Figure 4 C). The apoptosis index was correlated with the fraction of p53-positive cells (p=0.0001) (Figure 5) but not with the p21 expression rate (p=0.2583) (Figure 6). Detailed values for protein expressions and apoptosis index in the various histological components are shown in Table II.

Correlation of p53, p21 expression, and spontaneous apoptosis index with resistance to systemic treatment. To shed light on the role of p53 in chemosensitivity and resistance, the p53 expression of the refractory group was compared with that of the sensitive group (Figure 7). The p53 expression proved significantly lower in the refractory group than in the sensitive group (0.69% *vs*. 2.84%; p=0.0110). Refractory tumors showed significantly higher p21 expression level than sensitive ones (7.77% *vs*. 5.37%, p=0.0459). The mean percent of apoptotic figures in the chemosensitive and refractory groups were 1.67 and 1.19, respectively. A significant negative correlation was found between apoptotic index and resistance to systemic treatment (p=0.0313).

Discussion

Analysis of potentially relevant parameters in CDDP sensitivity, including cellular detoxification mechanisms (*e.g*. the glutathione and the metallothionein systems), platinum accumulation and expression of Bcl-2 family proteins, has not been able to elucidate the enigma of the inherent sensitivity of testicular GCTs. The rapid time course of apoptosis induction after exposure to chemotherapeutic drugs suggests that GCT cells may



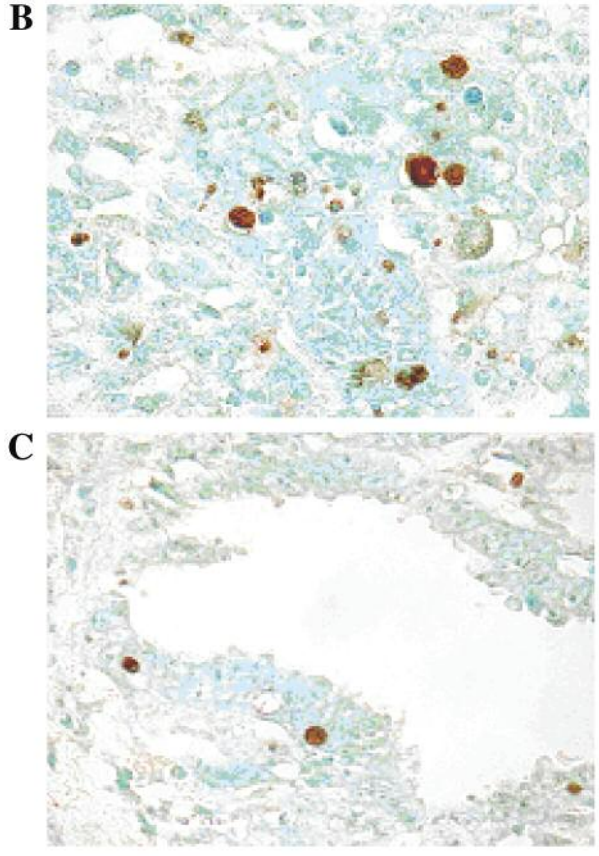


Figure 4. *A, Apoptosis index in different histological components. Significant difference between embryonal carcinoma and other non seminomatous components (p=0.0008). Linear regression with 95% mean prediction interval (positivity gradually decreases with differentiation). B, Embryonal carcinoma. Apoptotic figures (in situ apoptosis detection, ×200). C, Mature teratoma. Only scattered apoptotic bodies in a glandular structure (in situ apoptosis detection, ×200).*

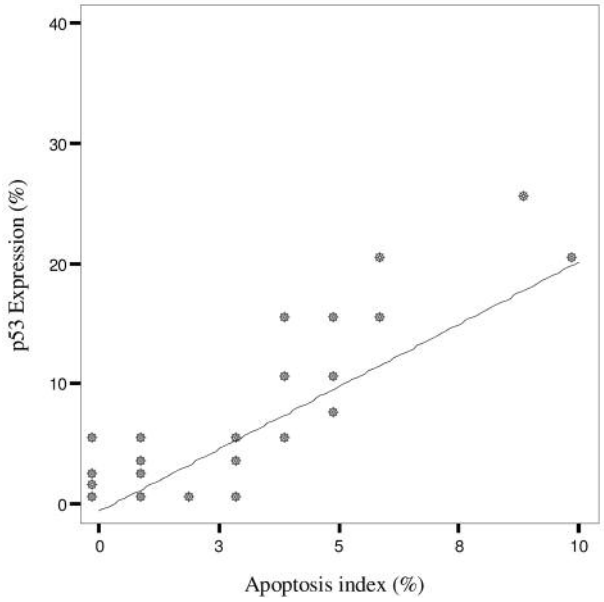


Figure 5. *Correlation of p53 expression and apoptosis index (p=0.0001; CC=0.547; R-square: 0.71).*

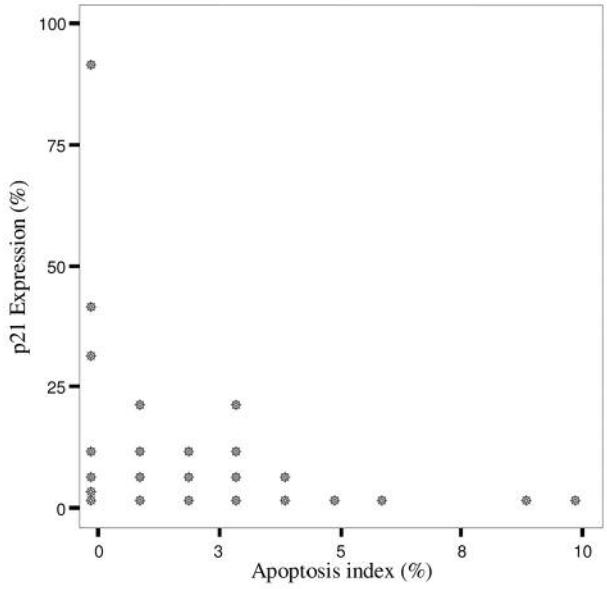


Figure 6. *No correlation between p21 expression and apoptosis index (p=0.2583).*

already be primed to undergo programmed cell death. Sensitivity to apoptotic stimuli may well be an inherent property of the cell of origin. In addition, during normal spermatogenesis, apoptosis occurs in the testis as an important physiological mechanism to adjust germ cell numbers to that of the supporting Sertoli cells and to ensure quality control of the gametes produced (22).

Table II. *Expression of p53, p21 protein and apoptosis index in different histological components.*

Histological component	p53	p21	Apoptosis index
Seminoma			
Mean	0.69	0.00	1.90
Minimum	0	0	1
Maximum	10	0	3
Std. Error of Mean	0.40	0.00	0.23
Std. Deviation	2.07	0.00	0.73
Embryonal carcinoma			
Mean	4.17	0.41	4.62
Minimum	0	0	0
Maximum	25	5	10
Std. Error of Mean	1.09	0.21	0.61
Std. Deviation	6.48	1.28	2.47
Yolk sac tumor			
Mean	1.11	2.77	1.62
Minimum	0	0	0
Maximum	5	20	4
Std. Error of Mean	0.73	2.22	0.46
Std. Deviation	2.20	6.66	1.30
Immature teratoma			
Epithel			
Mean	0.00	1.25	0.83
Minimum	0	0	0
Maximum	0	10	2
Std. Error of Mean	0.00	1.25	0.40
Std. Deviation	0.00	3.53	0.98
Stroma			
Mean	0.00	1.25	0.83
Minimum	0	0	0
Maximum	0	10	2
Std. Error of Mean	0.00	1.25	0.40
Std. Deviation	0.00	3.53	0.98
Cytotrophoblasts			
Mean	3.80	2.83	0.00
Minimum	0	0	0
Maximum	10	10	0
Std. Error of Mean	1.74	1.64	0.00
Std. Deviation	3.89	4.02	0.00
Syncytiotrophoblasts			
Mean	1.00	60.00	0.00
Minimum	0	0	0
Maximum	5	100	0
Std. Error of Mean	1.00	10.87	0.00
Std. Deviation	2.23	37.65	0.00
Mature teratoma			
Epithel			
Mean	3.00	5.23	0.47
Minimum	0	0	0
Maximum	40	40	3
Std. Error of Mean	1.52	1.79	0.19
Std. Deviation	8.48	9.83	0.87
Stroma			
Mean	0.83	1.23	0.28
Minimum	0	0	0
Maximum	7	10	3
Std. Error of Mean	0.34	0.57	0.15
Std. Deviation	1.89	3.12	0.71

p53 is a tumor suppressor gene and its activity is crucial for cells to be appropriately monitored during the cell cycle. Following p53 activation in the result of DNA damage, a number of gene expressions are stimulated that coordinately force the cells into either cell cycle arrest or apoptosis. *p53* is mutated to an inactive form in at least 50% of all known human cancers (23). However, p53 is present in its wild-type in most GCTs with a mutation rate of about 5-7% (11). In contrast to the low mutation rate, most studies reported a high level of p53 protein based on immunohistochemistry (24). Kersemaekers *et al*. (11) give an overview of selected immunohistochemical studies on p53 in GCTs. A high percentage of positive tumor cells are reported generally. We had also reached a relatively high overall positivity rate (data not shown) with a non-controlled, conventional immunohistochemical method. However, the false-positivity rate in definitely benign, G_0 phase cells (*i.e*. endothelium, smooth muscle cells, or fibrocytes) was unacceptable. Therefore we repeated the investigations with a highly specific automated staining system (Ventana Medical Systems' *i*VIEW™). The relatively low mean p53 positivity rate, with the a maximum of 40% was comparable with the results of others (11). They demonstrated convincingly that the actual protein levels of p53 of GCTs are far lower than that found in colon cancer- and breast cancer-derived cell lines, all known to have a high level of mutant p53. In our recent study, p53 expression correlated with the apoptosis index (p=0.0001). Thus, the relatively low, but functional p53 can induce an effective apoptotic cascade. Characteristic apoptotic figures can be recognized morphologically. In a number of cases the close relation of apoptotic figures and p53-positive cells attracted our attention (Figure 1 C). This phenomenon may be the indication of a physiological reaction of these cells to apoptotic stimuli. Nevertheless, the local increase of extracellular proapoptotic signals (*e.g*. Fas ligand) is also possible. p53 expression correlated with the chemosensitivity of tumors (p=0.0110). This finding confirms the results of Kersemaekers *et al*. authoritative paper (11). They found a trend toward a lower number of completely p53-negative samples in the refractory group, however this difference did not reach the significance level. In contrast to the aforementioned authors, we established a significant correlation between p21 expression and resistance to systemic treatment (p=0.0459). Although p53 is the most important activator of p21 – the most effective downstream effector of cell cycle arrest and apoptosis inhibition – p53 levels did not correlate with p21 expression in our study. This indicates that in GCTs, p53 induces proapoptotic signals, rather than cell-cycle arrest. Since we found no correlation between p21 and p53 values in the mature teratoma group (data not shown), the high p21 expression and the chemoresistance of mature teratoma components seem, at least partly, independent of p53 activity. Indeed, *p21*

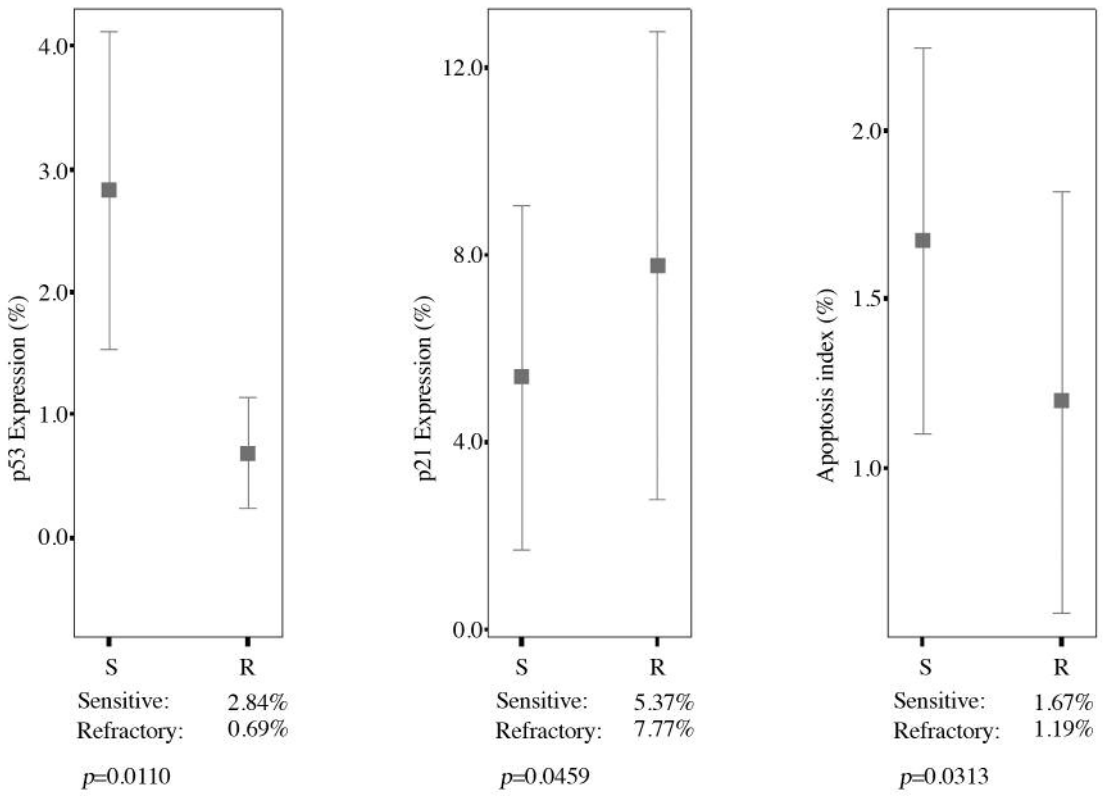


Figure 7. *Correlation of p53, PARP-1, p21 expression and apoptosis index with resistance to systemic therapy. Error bars show a 95.0% confidence interval of the mean. S: sensitive cases; R: refractory cases.*

gene transcription can be initiated by several other transcription factors such as E2F, C/EBP, AP2 or BECA1 (25). However, no relationship was found between p21 expression levels and apoptosis index (an inverse correlation was expected). Nevertheless, it is possible that the low p21 level in GCTs is not only the result of reduced transcription of the *p21* gene, and a p53-independent activation of proteasome-dependent degradation pathway is presumable (25). In our series, the apoptosis index showed a relationship with chemosensitivity of GCTs (p=0.0313). Surprisingly, we have found only one paper in the literature on this topic. Mayer *et al.* (26) found that the apoptosis index did not differ between unselected, responding, and nonresponding patients (p=0.08). However, in their study, the fraction of p53-positive cells was correlated with the apoptosis index, but not with the percentage of p21-positive cells. These latter findings are similar to our results. The relatively higher number of refractory teratomas and teratomatous components in our series can explain the slight differences between the results of the aforementioned studies and those of our recent work. Nevertheless, we found no relationship between sensitivity to systemic treatment and the existence of differentiated tumor components. The comparison of our work with others leads us to conclude that although the exact significance values are different, a trend toward a common conclusion (*i.e.* the p53 and p21 expression levels and the apoptosis index seem to be important factors in the issue of the chemosensitivity of GCTs) is noticeable. The protein expression pattern reflects a differentiation-dependent preference for G_1/S-phase arrest in terminally differentiated syncytiotrophoblasts and mature teratoma cells, while p53-mediated apoptosis induction is peculiar to less differentiated tumor types.

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