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Is p53 Expression, Detected by Immunohistochemistry, an Important Parameter of Response to Treatment in Testis Cancer?*

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Abstract. *Background: Several prior studies revealed positive p53 expression via immunohistochemistry (IHC) in a large percentage of germ cell testicular cancers (GCTTs). However, the predicting and prognostic value of this protein remains to be defined. Therefore, the aim of our study was to further clarify the role of p53 protein in GCTTs and to look for correlations between its gene expression and other disease parameters, including histological subtype, stage and clinical resistance/ sensitivity. Furthermore, we correlated p53 protein expression with that of MDRl gene product protein (Pgp) in order to examine the interrelationship between these two markers. Patients and methods: 77 untreated patients with GCTTs were investigated for their p53 expression using monoclonal antibody and immunohistochemistry in paraffin-embedded specimens. There were 34 patients with stage I, 16 with stage II, 27 with stage III disease. Results: All tumor types, except differentiated teratomas, were immunoreactive for p53 to a various extent ranging from scarcely positive to homogeneously stained tumor cells. Seminomas (S) and embryonal carcinoma (EC) components showed the most positive nuclear staining. p53 expression showed a significant inverse correlation with the stage of disease (P<0.0003). There was a significant positive relationship between p53 immunoreactivity and response to treatment (P = 0,0012), i.e. high levels of p53 expression correlated with clinical sensitivity of the tumors to chemotherapy. We could demonstrate a statistically signiflcant opposite relationship between p53 and Pgp immunoreactivity (P<0,0005). Conclusion: Our results show that p53 status in tumor cells may be a strong determinate of susceptibility to chemotherapy and that p53 overexpression has a favorable prognosis in terms of response to treatment in GCTTs. Moreover, the findings provide clinical evidence for the presense of significant relationship between p53 and MDR1/Pgp immunoreactivity. They also suggest that patients resistant to chemotherapy and lacking p53 expression might benefit from an alternative appropriately designed chemotherapeutic regimen to achieve further successful treatment in GCTTs.*

One of the most common cellular genes which negatively regulates the cell cycle, thus functioning as tumor suppressor gene, is the p53 gene (1). Mutations in this gene can result in a loss of tumor suppressor activity and lead to the initiation and/or progression of the neoplastic event (1,2). This mutatic event seems to be the most common genetic change in several solid and infusion human malignancies with a frequency varying from 15 % to 60 %. The half life of the mutated protein is increased by a factor of 10-20 as compared with the wild type (wt) protein. This interesting characteristic of the mutated p53 leads to its accumulation in the cell nucleus, which makes it detectable by standard immunohistochemical procedures (2). However, immunohistochemical positivity may result from mechanisms other than p53 gene mutation and in some tumors it may reflect wild-type p53 accumulation(3.)

In vitro and human model studies show that cells with loss of wt p53 function are more sensitive to DNA-damaging agents, as wt p53 facilitates DNA damage repair (4,5). Therefore, theoretically, the presence of p53 abnormalities could be a possible positive prognostic factor in malignant tumors as a result of increased sensitivity to chemotherapy of malignant tumors after loss of wt p53. In contrast, a number of recent studies have indicated that the presence of p53 mutation in a particular tumor indicates a poor prognosis for response to treatment and survival (6,7).

Several prior studies revealed positive p53 expression *via* IHC in a large percentage of GCTTs (8,9,10), Although, the predicting and prognostic value for this protein remains to be defined.

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Regarding p53 gene mutations in testicular tumors, 2 independent groups have not found mutations among 62 testicular tumors (11,12), which is stricking considering the immunohistochemical results obtained by previous studies. One study, however, did report mutations in seminomas (13). This unique and intriguing situation, *i.e.* the great majority of GCTTs overexpress p53 in the absence of accompanied mutations along with the lack in data concerning the clinical relevance of p53 expression led us to further investigate the role of p53 expression in GCTTs.

Patients and Methods

Specimens were obtained by semicastration of 77 patients with testis cancer biopsied for diagnostic evaluation and treatment for disease. Prior to surgery the patients received neither radio- nor chemotherapy. The mean follow-up period of these patients was 32 months (range from 6 to 72 months). The mean age was 30 years (ranges from 15 to 70 years). All primary tumors were removed surgically, and a portion of each resected tumor was snap frozen in liquid nitrogen and stored at -80°C or fixed in formalin for paraffin embedding for analysis. Histological examination was performed on haematoxylin and eosin-stained tissue sections. Clinical staging was done on the basis the TNM classification of malignant tumors UICC, 1987 (14) as well as marker investigation (Alpha fetoprotein, Beta-human choriogonadotropin) and other clinical features (15). There were 34 patients with stage I (localized without nodal involvement), 16 with stage II (retroperitoneal or regional) and 27 with stage III (distant) disease.

The histological classification of the tumors was made according to WHO, Mostofi (16) and comprised 27 seminomas (SGCTT) and 50 nonseminomatous germ cell testicular tumors (NSGCTT). The latter group contained 10 embryonal carcinomas (EC), 1 with yolk sac differentiation (Y.s), 7 teratomas (T), 1 choriocarcinioma (CC). The remaining 31 patients had mixed tumors.

Immunohistochemistry. The p53 antigen was detected using the streptavidin-biotin-alkaline phosphatase immunostaining method. The paraffin sections were routinely deparaffinized and pretreated, as described by Cattoretti *et al* (17), by microwaving at 750 W in 10 mM citrate buffer DAKO (pH 6.0). After incubation for 20 minutes at room temperature with 1% BSA (Bovin Serum Albunin-Serva/Heidelberg), a carrier protein to block nonspecific binding of reagents to the sections, the monoclonal mouse anti-human p53 antibody/DAKO-clone-DO-7/ was applied at a dilution of 1:25 for 2 hours at 37 °C. This antibody reacts with both wild and mutant types of p53 protein. Slides were washed three times in Tris buffer (0,05 M Tris/HCl, pH 7.6, 0.15 M NaCl) and incubated with a second antibody (biotinylated anti-mouse antibody from Amersham) for 30 minutes at room temperature and washed twice with Tris buffer. Thereafter, they were incubated for 45 minutes at room temperature with the streptavidin-biotin-alkaline phosphatase. The enzyme activity was detected by incubation with its substrate (New fuchsin - DAKO). Endogenous alkaline phosphatase (AP) was inhibited by the addition of levamisol (DAKO) to the substrate. Specimens were slightly counterstained with Mayer's haematoxylin, and mounted with glycerol gelatin. Sections of ovarian carcinoma known to contain mutant p53 protein were used as a positive control. The same slides, after omitting the primary antibody, were used as a negative control. In addition, 4 testes removed for nonneoplastic disease and 10 uninvolved normal tissues, not adjacent to tumor location, were used as additional controls to examine p53 expression in normal testes. All controls gave satisfactory results. Three slides of each tumor were evaluated by two of the authors without knowledge of the clinical data, and the average value was considered.

Table I. *Correlation between P53 expression and histology.*

Histology	-	±	+	++	All cases
SGCT	0	0	7	20	27
NSGCT					50
TT	5	1	1	0	7
EC	1	2	2	5	10
Y.s	0	0	1	0	1
CC				1	1
Mixed	1	5	11	14	31

- = No obvious positive tumor cells, ± Rare positive = up to 5 % positive tumor cells
+ = between 5 and 50 % positive tumor cells, ++ = more then 50 % positive tumor cells.

p53 scoring. Since the number of cells labelled may be more meaningful than the intensity of staining *per se* (18) and since the latter differed from one slide to another and sometimes within different areas of the same slide, we were encouraged to exclude the intensity of staining from the interpretation of our results. The extent of staining was evaluated as the percentage of positively stained tumor cells of the total number of tumor cells in five adjacent high power fields at a magnification of (x 400) as recently described by Lipponen *et al* (19). First the entire section was screened carefully and counts were performed in representative areas, *i.e.* regions with the maximum fraction of positively stained cells, well fixed and free of background. Stromal components were avoided by comparing the section with the haematoxylin and eosin-stained counterpart. Tumors according to their extent of staining were classified as: (-) signifying no obvious positive staining at all, ± indicates that 5% or less of tumor cells were positive, + indicating that 5-50% of tumor cells were positive, ++ signifies that over 50% of tumor cells were positive.

When the tumors were divided into the above-mentioned four groups no significant difference in response to treatment could be established. However, if the tumors were divided into only two groups- low or focal (-/±, showing 0-5% positive cells) and high (+/++, showing more than 5% positive cells), a statistically significant difference in response to treatment was observed.

Chemotherapeutic schedule and evaluation of response to treatment *i.e.,* definition of objective response were performed according to UICC, 1981 criteria (20).

Statistical methods. The two-sided Fischer's exact probability test was used for statistical evaluation. The cases were first divided into sensitive (complete remission, CR) and resistant (stationer disease SD and died) subgroups and then dichotomied based on the expression of p53 (≤ 5 %, > 5 %). A difference was regarded as significant if the P value was $\leq$ 0,05.

Results

p53 expression and histology. The results of this immuno-histochemical analysis are summarized in Table I, and

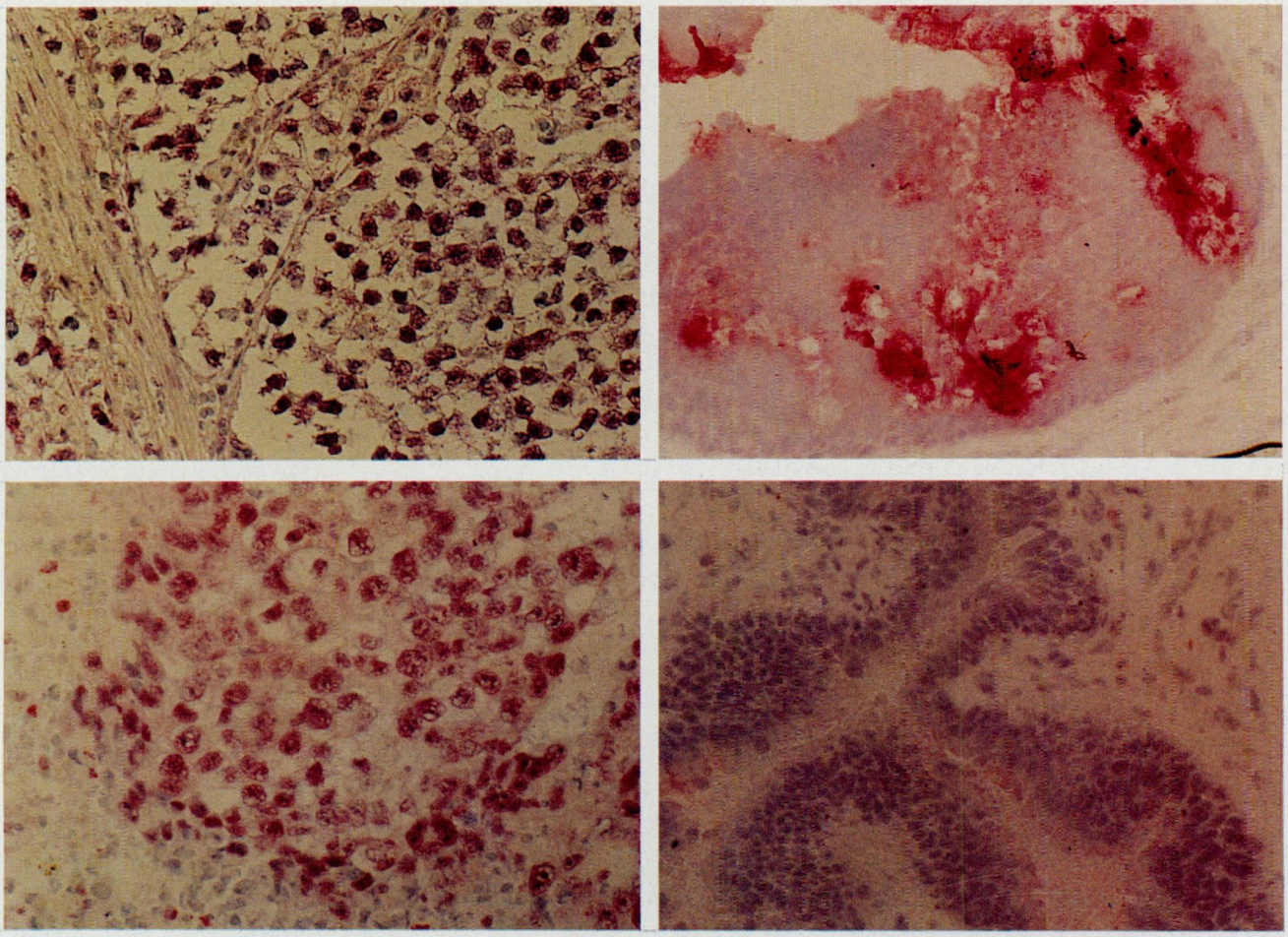

Figure 1. *p53 immunoreactivity in early stage seminoma. Most of the tumor cells strongly reacted /++/ with the anti-p53 DO-7 Mab. Immunoalkaline-phosphatase /x125/.*

Figure 2. *Positive nuclear p53 reaction /++/ of sensitive embryonal carcinoma. Immunoalkaline-phosphatase /x125/.*

Figure 3a. *Negative immunoreactivity of p53 in resistant teratoma.*

Figure 3b. *Positive P-glycoprotein reactivity of the same case /C219 Mab/. Immunoalkaline-phosphatase /x125/.*

representative examples of the staining patterns observed are shown in Figure 1, 2, 3, and 4.

Neither the 10 uninvolved normal tissues nor the 4 testes which were removed for non neoplastic disease showed obvious positive staining. This confirms the results of Bartkova *et al* (9) and Lewis *et al* (10) and suggests the presence of wild-type p53 protein in normal testes. Out of 77 cases 70 were positive (91 %) and 7 were negative (9%), 5 T, 1 EC and 1 mixed tumor. Out of 27 seminoma components 20 (74 %) were ++, while out of 50 NSGCTs 20 (40 %) were ++ indicating a higher incidence of the highest p53 expression in seminomas (Table I).

Relationship between p53 expression and the clinical stage. The distribution of patients according to the stage of tumors and the immunoreactivity of p53 is shown in Table II. In this series of GCTTs we could establish a statistically significant inverse relationship (P=0.0003) between p53 and stages of disease *i.e.,* p53 expression was predominant in the early stage testicular tumors (I, IIA). (Figure 1).

Correlation between p53 expression and responce to treatment. The correlation between p53 expression and clinical resistance/sensitivity is shown in Tables III and IV. In IA, IB and IIA stages, where the prognosis is excellent, all 43 patients but two showed high (+/++) p53 immunoreactivity. Three patients with stage II/B, II/C displayed resistant tumors (two died, one stationer disease SD). All three patients showed no or low p53 expression (-/±). Of 24 patients with stage III, 6 expressed no to low protein level. Of these 6 patients 5 did not respond (4 died, 1 SD) and one achieved complete remission. Indeed this study identifies the

Table II. *The correlation between p53 expression and the stage of tumors.*

Clinical stage	p53 expression		All cases
	Low -/±	High+/++	
Early-stage groups I, IIA	2	41	43
Later-stage groups IIB,III	13	21	34
	15	62	77

P* = 0.0003 (Fisher's exact test).

Table III. *Correlation between p53 expression and response to treatment in stage I, II and III patients.*

Response	p53 expression		All cases
	Low -/±	High +/++	
Sensitive	7	57	64
Resistance	8	1	9

P=0.0000 (Fischer's exact test).

Table IV. *Correlation between p53 expression and chemoresistance/ sensitivity in stage II/B and III patients.*

*Response	p53 expression		All cases
	Low -/±	High +/++	
Sensitive	5	17	22
Resistance	8	1	9

P = 0.0012 (Fischer's exact test). *MR, NR, SD and died were considered to be resistant tumors. In case of mixed tumor the component with highest expression has been considered representative for the whole tumor.

Table V. *Relationship between p53 and Pgp expression of GCTTs.*

	p53 (high)	p53 (low)	P-value*
Pgp neg.	46	4	P<0.0005
Pgp pos.	14	11	

P* Fisher's exact test.

overexpression of this protein as a marker for prediction of sensitivity of GCTTs to anti-cancer therapy. Table III shows significant relationship between p53 overexpression and response to the treatment modalities used for stage I, II and III patients (P = 0,0000). (Figure 2). However, the relationship between expression and response to chemotherapy as curative treatment should be evaluated on patients with disseminated disease *i.e.,* stage II/B, II/C and III patients. To meet that, we examined the relationship between p53 and response to chemotherapy in advanced stage patients.

Of 31 patients receiving curative cisplatin-based combination chemotherapy 9 showed resistant disease (7 died, 2 SD). Eight of 9 resistant tumors (89 %) displayed no to low p53 expression. This relationship was again proved to be significant (Table IV, P= 0.0012) proving that p53 is a marker of chemosensitivity and that its absence? when occuring, indicates a germ cell tumor of poor prognosis. (Figure 3a)

Pgp and p53 expression. A statistically significant inverse association could be seen between p53 and Pgp expression *i.e.,* high p53 levels were associated with negative Pgp expression while low levels of p53 assosiated with positive Pgp staining (two-tailed P=0.0005, Table V), (Figure 3b).

Discussion

Overall, in the present study p53 immunoreactivity was detectable in 70 (91%) of 77 GCTTs. All tumor types, except differentiated teratomas, overexpressed this protein. Although this finding is in agreement with the previous studies, the difference, however, occurs in seminomas. The total positivity of seminomas was 77% in the study of Bartkova *et al* (1991). It increased to 90 % in the Lewis *et al* (1994) study and reached 100 % in our present study. The explanation for these differences lies in the different antibodies used, or perhaps more importantly, in increasing sensitivity of the methods used. Bartkova *et al* (1991) performed IHC on paraffin blocks using mouse monoclonal antibody BP53-11 raised and characterized in their own laboratory, in parallel with a polyclonal rabbit antiserum CM-1. Lewis *et al* (1994) performed IHC on paraffin sections using a commercially available anti-p53 rabbit polyclonal antibody. Most importantly, the previous authors did not employ the antigene retrieval technique, which can significantly decrease the threshold of the detection system, thus allowing otherwise undetectable trace expression to appear (21).

Eighty six per cent of teratomas showed very little if any nuclear staining. One may speculate that in teratomas the p53 gene has a limited role, and oncogenes or anti-oncogenes other than p53 may be overexprexsed and contribute to tumorigeneses. Shuin *et al* (22) reported on the presence of a

high level of C-erbB-1 and C-erbB2 in teratomas. These and other cellular oncogenes or proteins, for instance mdm2 or bcl-2 may directly or indirectly interact with and alter the function of p53 in teratomas.

Experimental studies have suggested a relationship between the presence of mutant p53 in tumor cells and upregulation of MDR1 transcription. In various independent studies, transfection of mutant but not of wild-type p53 was found to transactivate the MDR1 promoter (23,24,25). However, Wosikowski *et al* (1995) found wild type but not mutant p53 to stimulate MDR1 expression (26). Furthermore, Goldsmith *et al* (1995) in a panel of drug-resistant human breast cancer cell lines, demonstrated that six cell lines overexpressed MDR1 while containing wild-type p53 (27).

In a recent study by our group the immunoreactivity of these tumors for the MDR1 gene product protein Pgp was examined (28). In the present work a statistically significant inverse association between p53 and Pgp expression could be seen *i.e.,* high p53 levels were associated with negative Pgp staining while low levels of p53 were associated with positive Pgp staining (two-tailed P=0.0005).

Since high levels of p53 immunoreactivity represent, in general, altered wt-p53 function, yet do not necessarily indicate mutant p53 (3,29), we preferred not to use any of the terms used in the previous experimental studies namely wild or mutant p53 protein. On this basis, we proposed it to be more reasonable to interpret our latter finding *i.e.,* high but not low p53 levels were associated with negative staining of Pgp, so that inactive or altered function of p53 suppreses Pgp expression wherease native or intact p53 stimulates Pgp expression. As mutant p53 protein is inactive our finding seems to support that of Wosikowski *et al* (1995) and Goldsmith *et al* (1995).

In clinical studies in B-cell chronic lymphocytic leukaemia (30), myelodysplastic syndromes (31), gynocological (32) and colon (33) cancers, no correlation has been found between the presence of mutant p53 and overexpression of MDR1/Pgp. However, in 29 tumors of colorectal origin Kant *et al* (34) found a significant positive correlation between p53 and MDR1 expression in p53-mutated tumors (P=0.005; r=0.596), but not in tumors without a p53 mutation. p53 expression in this study was determined by differenlial PCR using the β_2-microglobulin gene as a reference. This contrast with our finding, and might be due to the particular and intriguing situation of GCTTS *i.e.,* the high p53 protein level in the absence of accompanied mutations as well as differences in the techniques used. To the authors knowledge, in GCTTs this is the first clinical study which supports the experimental finding speaking in favour of an *in vivo* relationship between p53 and MDR1/Pgp expression. However, the functional status of p53 should be determined to precisely clarify the nature of the interrelationship between these two markers.

In this study of p53 expression the tumors, on the basis their clinical stage, were divided into two main subgroups: The early-stage groups (I and IIA) and the later-stage groups (IIB and III). The difference in the pattern of relapse and survival curves between the two subgroups in the Royal Marsden series was significant (22). Ninety five % of patients with early stage disease *versus* 60% of later-stage expressed high (+/++) p53 levels. This difference was statistically significant (two-tailed P= 0.0003). The prevalence of p53 overexpression in the early-stage tumors suggests that the overexpression of this protein is an early event in GCTTs progression. This is consistent with previous studies reporting that p53 staining is frequently found at an early preinvasive stage of intratubular germ cell neoplasia known to be associated with a high risk of developing invasive testicular cancer (9,35).

The most interesting immunohistochemical finding of p53 expression in GCTTs was that the aberrant p53 expression, unlike that shown in several other neoplasms, was not indicative for clinical resistance and consequently poor prognosis, but rather it significantly correlated with clinical sensitivity and favorable prognosis (Table IV). In this context, although not the focus of this work, the question of whether the detectable amounts of p53 in GCTTs result from overexpression of wild or mutant p53 protein needs to be addressed.

p53 protein overexpression is the end point of the action of many different stabilizing mechanisms which ullimately lead to p53 accumulation. IHC detects this overexpressed or accumulated p53, without indicating the mechanisms by which accumulation occurs. Thus, IHC is unable to differentiate between wild and mutant p53 protein and it is now evident that p53 immunopositivity in some twmors may reflect wild-type p53 accumulation (3). However, despite these limitations, IHC may be a useful marker of an altered wild-type p53 function. The above-mentioned scenario suggests that, in this study, the used MAb Do7 may have detected the wild-type rather than the mutant p53 protein. This is supported by our previous PCR-LOH study reporting a low allelic loss of TP53 (36) and by sequencing studies obtained by olhers (11, 12). Peng *et al* (1993) found no mutation in 23 GCTTs and stated that although the presence of p53 protein may correlate with mutations of the gene in most common epithelial tumors, its appearance in testis cancer as well as some childhood tumors may reflect an abundance of wild type protein due to an increased half-life and/or increased transcription. However, arguing against the concept of "abundance of wild-typ p53, Wei *et al* (1993) found p53 mutation in 23,5 % of seminomas. These mutations lead to an amino acid change but a normal (wild-type) sequence in the remaining allele. This encouraged them to suggest that mutation of p53 gene is involved in the development of seminomas as is the case in several other types of human cancers. Therefore, the answer to the question whether the detectable amounts of p53 in GCTTs are the result of overexpression of wild or mutant p53 protein

is still not clear cut and needs further and larger studies using methods other than IHC.

When arguing that immunohistochemical detection of p53 indicates an altered wild-type p53 function, the question of how inactive p53 protein sensitizes tumor cells to chemotherapy becomes crucial.

Recent investigations of Smith *et al* and Fan *et al* (1995) have obtained evidence that disruption of p53 function sensitizes colon cancer RKO cells and human breast cancer MCF-7 to UV light (37) and cisplatin (38) and may do so by: (a) preventing the Gl checkpoint response to DNA damage; and/or (b) impairing nucleotide excision repair of cisplatin-induced DNA lesions or, in another word, blocking p53-dependent DNA repair pathway. Most recently, Hawkins *et al* (1996), using a model of functional p53 deficiency resulting from the expression of HPV 16E6 in primary human fibroblast, demonstrated that cells lacking p53 are more sensitive to a variety of chemotherapeutic agents including alkylating agents and paclitaxel in addition to cisplatin (39). For cisplatin, enhanced sensitivity is associated with delay in progression through S phase. p53 protein levels are induced in a time course coincident with delay in progression through S phase, suggesting a role for p53 in DNA repair of cisplatin-induced damage. Taken together these studies along with the fact that the patients after surgery had cisplatin based combination chemotherapy, an anticancer agent that inflicts DNA damage of the type repaired primarily through nucleotide excision, it seems logical that cells lacking p53 function might be more sensitive to therapy.

The findings presented here further support the unexpected favourable prognosis for patients with positive p53 immunostaining that hase been observed recently for nonsmall-cell lung cancers (40, 41, 42) and for gliomas (43). However, the organ and the tissue of origin of the tumor seems to have a crucial role in determining overall p53 function (38). Moreover, our results suggest that p53 may be an important determinant in the regulation of MDR1 expression in these tumors and that the absence of protein immunoreactivity, when occuring, may identify patient subgroups with less susceptibility to anti-cancer treatment and worse prognosis. These patients may benefit from a yet to be defined alternative chemotherapeutic schedule.

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Drug Resistance and Sensitivity of Germ Cell Testicular Tumors: Evaluation of Clinical Relevance of MDR1/Pgp, p53, and Metallothionein (MT) Proteins*,**

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Abstract. *Backround: Although in vitro and clinical studies indicate that overexpression of P- glycoprotein (Pgp), p53, or metallothionein (MT) is involved in modulating drug resistance/sensitivity of cancer cells, the clinical relevance of the overexpression remains to be elucidated. Materials and Methods: In this paper the expression and clinical value of Pgp, p53, and MT were evaluated immunohistochemically in 77 specimens of germ cell testicular tumors (GCT). We also studied the interrelationship(s) between the investigated markers. Results: Pgp positivity correlated with cancers of advanced stages (P = 0.000). p53 and MT immunostaining does not predict a poor response to chemotherapy, but rather is correlated to a favorable clinical outcome (P = 0.001, P = 0.00006 respectively). We obtained an inverse association between Pgp and p53 (P = 0.0005), and positive strong association between p53 and MT immunoreactivity (P = 0.0002). Conclusions: Based on our results in patients with germ cell testicular tumors we assume that the poor clinical outcome seen in certain Pgp positive tumors is the consequence of Pgp association with a more progressive malignant phenotype, rather than its role in multidrug resistance (MDR). p53 and MT immunoreactivity predicts a better response rate to chemotherapy, whereas tumors lacking or demonstrating low MT and or p53 expression show a worse prognosis.*

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Testicular tumors account for 1-2 % of all male malignancies. Over 90% of testis cancers are a germ cell malignancies two thirds of which are of non-seminomatous type (NSGCT). Germ cell testicular tumors (GCT) are one of the most sensitive tumors to cytostatic therapy, since in disseminated cases a remission rate of 70-80% can be achieved with cisplatin based treatment modality. However, some patients ultimately die of their disease due to tumor resistance (1). Drug resistance (DR) is a major cause of treatment failure in cancer. It is evident now that DR is rather complicated and heterogeneous.

P-glycoprotein (Pgp) which functions as an energy-dependent multidrug transporter has been shown to form the genetic basis for classical MDR (2). Strong data, however, are now available demonstrating that Pgp positivity indeed might be a marker for more aggressive tumor behavior and thus poor treatment outcome, independent of its effect on chemosensitivity (3, 4, 5).

Metallothioneins (MT) are a family of metal-binding proteins. The proteins bind a number of trace metals and clearly protect cells and tissues against heavy-metal toxicity (6). In addition, MT may facilitate metal exchange with zinc-dependent enzymes and thus assist indirectly in the repair of the lethal DNA damage and regulate important cellular. activities (7). Cell lines expressing high levels of MT have been reported to be resistant to DNA-damaging agents like cisplatin and alkylating agents (8). Nevertheless an increase in MT does not always result in a phenotype that is less sensitive to the toxic effects of electrophilic antineoplastic agents (9, 10) and in fact it leads to increased sensitivity (11).

A novel concept of DR implies alterations of tumor suppressor genes (p53 protein) and/or apoptotic pathways. Wild type (wt) p53 facilitates DNA damage repair (12). Therefore, theoretically, the presence of p53 abnormalities could be a positive prognostic factor in malignant tumors as a result of increased sensitivity to chemotherapy of malignant tumors after loss of wt p53. In contrast. a number of recent

studies have indicated that the presence of p53 mutation in a particular tumor indicates a poor prognosis for response to treatment and survival (13, 14).

In this study, immunostaining of Pgp, p53, and MT was related to conventional prognostic parameters in GCT such as tumor histology, clinical stage, as well as to response to treatment.

Patients and Methods

Specimens were obtained by semicastration of 77 patients with testis cancer. Prior to surgery the patients received neither radio - nor chemotherapy. The mean follow-up period of these patients was 32 months (range 6-72 months). The mean age was 30 years (range 15-70 years). Histological examination was performed on haematoxylin/eosin-stained tissue sections. Clinical staging was done on the base of the TNM classification of malignant tumors UICC 1987 (15) as well as marker investigation (Alpha fetoprotein, Beta-human choriogonadotropin) and other clinical features (16). There were 34 patients with stage I (localized without nodal involvement), 16 with stage II (retroperitoneal or regional) and 27 with stage m (distant) disease. The histological classification of the tumors was according to WHO, Mostofi *et al* (1986)(17) and identified 27 seminomatous (S) and 50 nonseminomatous (NSGCT) tumors. The latter group contained 10 embryonal carcinomas (EC), 1 with Yolk sac differentiation (Ys), 7 teratomas (T), 1 Choriocarcinoma (CC). The remaining 31 patients had mixed tumors. The chemotherapeutic schedule and evaluation of response to treatment, *i.e.* definition of objective response, were evaluated according to the UICC 1981 criteria (18).

Antibodies. The monoclonal antibodies C 219 (Centocor USA, dilution 1/20) binds the cytoplasmic epitope of Pgp; p53 (Dako clone DO-7, dilution 1/25) recognizes both wt and mutant p53 protein; and MT (Dako MT E-9, dilution 1/40) combines with the single and highly conserved epitope shared by the I and II isoforms of human, rat and horse were used.

Immunohistochemistry (IHC). With C 219 cryostat, with p53 and MT antibodies paraffin sections were used. The highly sensitive streptavidin-biotin-alkaline phosphatase immunostaining method was applied. Briefly, the paraffin sections were routinely deparaffinized and pretreated by microwaving at 750 W in 10 mM Citrate buffer (DAKO, pH 6.0) twice for 5 minutes as described previously by Cattoretti *et al* (1992)(19). They were then incubated for 20 minute at room temperature with 1% Bovine Serum Albumin (Serva/Heidelberg) a carrier protein to block non-specific binding of reagents to the sections. The monoclonal mouse antibodies were applied. Slides were washed three times in TRIS buffer (0,05 M TRIS/HCI, pH 7.6, 0.15 M NaCl) and incubated with a second antibody (biotinylated anti-mouse antibody from Amersham) for 30 minutes at room temperature and washed twice with TRIS buffer. Thereafter, they were incubated for 45 minutes at room temperature with the streptavidin-biotin alkaline phosphatase complex (Dako). The enzvme activity was detected by incubation with its substrate (New fuchsin-Dako). Endogenous alkaline phosphatase (AP) was inhibited by the addition of levamisol (Dako) to the substrate. Specimens were counterstained with Mayer's haematoxylin, and mounted with glycerol gelatin. The MDR KB 8-5 cell lines, sections of ovarian carcinoma and invasive breast ductal carcinoma known to express Pgp, p53, and MT proteins respectively were used as a positive control. The same slides, with the primaly antibody omitted, were used as a negative control. All controls gave satisfactory results. Three slides of each tumor were evaluated by two of the authors without knowledge of the clinical data, and the average value was considered.

Table I. *Relationship between response to chemotherapy and Pgp. p53, or MT immunoreactivity of germ cell testicular tumors.*

	Early stage (I, II/A)	Later stage (II/B-III)	P-value*
Pgp (-)	37	13	P = 0.0000
pgp (+)	4	21	
p53 (low)	2	13	P = 0.0003
p53 (high)	41	21	
MT (low).	4	10	P = 0.24
MT (high).	39	24	

* Fisher's exact test.

Scoring of positive immunoreactivity

a) Pgp scoring: All slides were evaluated on a 2 points basis: - = no obvious positive staining, including granular and occasional cytoplasmic staining on certain individual cells, I = obvious diffuse positive staining (20).

b) p53 and MT scoring: Although it may not apply to other organs, previous studies on GCT, show, that the intensity of staining and the percentage of stained cells are closely related (21), and the number of cells labeled may be more meaningful than the intensity of staining *per se* (22). In this study, we were encouraged to exclude the intensity of staining from the interpretation of our results and to rank only the extent of staining. The extent of staining was evaluated as the percentage of positively stained tumor cells of the total number of tumor cells in five adjacent high power fields at a magnification of (x 400). This scoring system has been validated in other disease before as recently described by Lipponen *et al* (1993)(23). First the entire section was screened carefully and counts were performed in representative areas, *i.e.* the region with the maximum fraction of positively stained cells, well fixed and free of background. Stromal components were avoided by comparing the section with the haematoxylin and eosin-stained counterpart. Tumors, according to their extent of staining, were classified as: (-) signifying no obvious positive staining at all, + indicating that less than 5% of tumor cells are positive, t signifying that 5-50% of tumor cells are positive, ++ signifying that more than 50% of tumor cells are positive. When the tumors were divided into only two groups *i.e.* no to low (-/±)(showing 0-5% positive cells) and high staining (+/++) (showing more than 5% positive cells), a statistically significant difference in response to treatment was observed.

Statistical methods. The two-sided Fischer's exact probability test was used for statistical evaluation. The lcases were first divided into sensitive (complete remission, CR) and resistant stable disease, SD, and died) subgroups and then dichotomied based on the expression of MT (≤ 5 5%, > 5%). A difference was regarded as significant if the P value was≤ 0,05.

Results

Pgp. Pgp positive reaction was detected in two (8%) of the 25 seminomatous germ cell testicular tumors (SGCT) and 23

Table II. *Relationship between response to chemotherapy and Pgp, p53, or MT immunoreactivity of stage II/B, II/C and III germ cell testicular tumors.*

	Response to treatment		P-value*
	sensitive	resistant	
Pgp (-)	8	1	P = 0.167
Pgp (+)	14	8	
p53 (low)	5	8	P = 0.0012
p53 (high)	17	1	
MT (low).	3	7	P = 0.0013
MT (high).	19	2	

* Fisher's exact test.

(50%) of the 50 NSGCT. The incidence of Pgp expression was significantly higher (P = 0.0000, Table I) in the later stage or advanced cancer groups (II/B-III). There was a tendency towards poor response rate in tumors which are positive for Pgp staining. However, this association between Pgp expression and clinical chemoresistance did not reached statistical significance (P = 0.16, Table II).

p53. Out of 77 tumors 70 (91%) were immunoreactive for p53 to a various extent ranging from scarcely positive to homogeneously stained tumor cells. 7 (9%) were negative, 5 teratoma, 1 embryonal carcinoma and 1 mixed tumor. p53 expression showed significant inverse correlation with the stage of disease (P = 0.0003, Table I). High levels of p53 expression correlated with better response of tumors to chemotherapy (P = 0.0012, Table II). A statistically significant opposite relationship was demonstrated between p53 and Pgp immunoreactivity (P = 0.0005).

MT. Tumor, seminomas and nonseminomas all expressed MT irrespective of their histological subtype. There was no significant difference between these two major subtypes. No relationship could be demonstrated between MT expression and stage (0.24, Table I). The immunoreactivity of MT showed a significant positive association with response rate to chemotherapy (0.0013, Table II). Significant association between p53 and MT immunostaining could be established (P = 0.0002).

Discussion

Overall, in the present study our immunohistochemical investigations revealed Pgp positivity in 25 (33%) of 75 tumors. In a previous immunohistochemical study of Pgp expression of 26 GCT Katagali *et al* (1993) found a positive reaction in 9 of 26 cryostat sections (24). All of 7 T, 3 S and 2 EC components showed elevated Pgp levels. This findings are in agreement with ours. A difference occurs, however, in cases showing Yolk sac (Ys) differentiation. Katagari *et al* found Ys parts in 9 of 13 NSGCT and the 5 cases under study were Pgp negative. In our series two tumors contained Ys parts and one showed marked Pgp expression. This difference may be the result of using different anti-Pgp monoclonal antibodies and/or subjective differences in interpretation of immunostaining results. As described by other authors, teratomas comprised of columnar cells and lumen forming squamous epithelium show a positive staining pattern (25). The possibility that Pgp expression of teratomas may reflect the degree of tumor differentiation, as reported elsewhere in the literature should be considered (26, 27). Therefore, it is presumable that the resistance mechanism is mediated by Pgp results, at least in part, from its association with well-differentiated tumors.

The frequency of Pgp expression was significantly higher in NSGCT than in seminomas. As compared to seminomas, NSGCT are biologically more aggressive and in general have a poorer prognosis. The significantly higher incidence of Pgp expression in NSGCT might be one simple explanation for the differences existing between these two broad categories.

p53 immunostaining was demonstrated in 70 (91%) of 77 GCT. All tumor types, except differentiated teratomas, overexpressed this protein. Interestingly, Bartkova *et al* (28) found positivity in 77 ‰ of seminomas (28). Lewis *et al* (29) detected p53 immunoreactivity in 90% of seminomas. In our study all seminomas stained for p53 (30). The explanation for these differences lies in the different antibodies and perhaps more importantly, in increasing sensitivity of the methods used. The previous authors did not employ the antigen retrieval technique, which can significantly decrease the threshold of the detection system, thus allowing otherwise undetectable trace expression to appear (31). Eighty six percent of teratomas showed very little if any nuclear staining, indicating that in teratomas other oncogenes or anti-oncogenes may be overexpressed and contribute to tumorigeneses. Shuin *et al* (32) reported on the presence of a high level of C-erbB-1 and C-erbB2 in teratomas. These and other cellular oncogenes or proteins, for instance mdm2 or bcl-2, may override and alter the tumor suppressive function of pS3 in teratomas.

In the literature concerning MT expression in GCT, there are two studies for almost the same group (21, 33). Kontozoglou *et al* (33) studied tissue sections of nine human embryonal carcinomas and suggested that MT may be considered an oncodevelopmental product which could be useful as a tumor marker. Chin *et al* (21) assessed immunohistochemically the presence of MT in 33 untreated primary testicular germ cell tumor. Regardless of the clinical stage, seminomas stained weakly or not at all, most nonseminomas stained strongly for MT. In comparison with our results, the sharp difference lies apparently in seminomas.

Twenty three out of 27 (85%) seminomas expressed MT with 21 (77.7 %) showing MT expression in 50 % or more of tumor cells. As we have mentioned earlier, differences in the antibodies, methods and/or interpretation of the immunostaining results may be behind the controversial results (34).

In this study of Pgp, p53 and MT expression, the tumors, on the base of their clinical stage, were divided into two main subgroups (Table I): the early-stage groups (I and IIA) and the later-stage groups (IIB-III). We could establish a statistically significant relationship between the two main subgroups as to the expression of Pgp or p53 and not of MT. Pgp overexpression was prevalent in the later-stage or advanced tumors. This finding is consistent with Pgp overexpression being associated with an advanced and more aggressive tumor phenotype (3, 4, 5).

Ninety five per cent of patients with early stage disease *versus* 60% of later-stage expressed high (+/++) p53 levels. This difference was statistically significant (two-tailed P= 0.0003). The prevalence of p53 overexpression in the early-stage tumors suggests that the overexpression of this protein is an early event in GCTTs progression. This is in agreement with previous studies reporting that p53 staining is frequently found at an early pre-invasive stage of intratubular germ cell neoplasia known to be associated with a high risk of developing invasive testicular cancer (28, 35).

In our work we examined the relationship between Pgp, p53 or MT staining and response to chemotherapy for stage IIB, IIC, and III patients, where the chemotherapy is curative (Table II). With Pgp we found a strong tendency towards resistance in tumors showing Pgp positivity. This relationship approached, but did not reach, statistical significance (P = 0.16). This is, of course, not a surprising result because it is now clear that although Pgp positivity is a factor indicating poor prognosis in a variety of malignancies, its presence, does not necessarily mean that chemoresistance is present, since Pgp could simply be as we found in this series, a marker of a more aggressive tumor phenotype.

The most interesting immunohistochemical finding was that the aberrant expression of p53 and MT in the tumor cells, unlike what has been shown in several other neoplasms, was associated with better response to the treatment modality used. In particular the p53 and MT group, which expressed these proteins in more than 5% of tumor cells, responded significantly better than the others (P = 0.0001, P = 0.0006 respectively).

Regarding p53 gene mutation in testicular tumors, 3 studies have not found mutations (36, 37, 38), while 1 has found mutations in 23% of seminomas (39). Thus, p53 immunopositivity of GCT, as in the case with some other tumors (40), may reflect wild-type p53 accumulation. It is important to note, however, that the absence of mutation, *i.e.* presence of wild type p53 protein does not necessarily mean that this protein is active. Clearly, other inactivating conditions can also render non-mutated, wt p53 inactive *in vivo.* Methods that investigate the functional status of p53 in GCT are needed to answer the substantial question of whether accumulated p53 represents functional/active or inactive protein. The following evidence, however, indicates that this accumulated p53, although it may be wild type, is the inactive protein (1) Immunohistochemical detection of p53 indicates an altered wild-type p53 function; (2) MT, acting as zinc chelator (41). may be one mechanism of wt p53 inactivation *in vivo.* In this study MT was significantly (P = 0.0002) co-overexpressed with p53. This finding clearly supports the presence of a regulating relationship between MT and p53 and implicates MT as another way to inactivate the p53 pathway in tumors, without mutating p53 itself. From the above-mentioned scenario we may state that p53 is inactive wt protein. If p53 is inactive, then the question of how inactive p53 protein sensitizes tumor cells to chemotherapy becomes crucial.

Recent investigations of Smith *et al* and Fan *et al* (1995) have obtained evidence that disruption of p53 function sensitizes colon cancer RKO cells and human breast cancer MCF-7 to UV light (42) and cisplatin (43) and may do so by a) preventing the Gl checkpoint response to DNA damage; and/or b) impairing nucleotide excision repair of cisplatin-induced DNA lesions or, in other words, blocking p53-dependent DNA repair pathway. More recently, Hawkins *et al* (1996) supported these data by demonstrating that cells lacking p53 are more sensitive to a variety of chemotherapeutic agents including alkylating agents and paclitaxel in addition to cisplatin (44). Considering these studies, along with the fact that the patients after surgery had cisplatin based combination chemotherapy (an anticancer agent that inflicts DNA damage of the type repaired primarily through nucleotide excision) it seems logical that cells lacking p53 function might be more sensitive to therapy. This result further supports the unexpected favorable prognosis for patients with positive p53 immunostaining that has been observed recently for non-small-cell lung cancers (45, 46) and for gliomas (47). However, the organ and the tissue of origin of the tumor seems to have a crucial role in determining overall p53 function.

MT expression, like p53, was found to predict a better response rate to treatment. Although Chin *et al* (21) could not establish a direct correlation between cisplatin resistance and MT content, they suggested such a relationship from their and other studies. Interestingly, in their study, 4 patients died and 3 of them had no MT staining. The staining was also absent from the only patient who had recurrent disease. From our data it appears that 78% of poor responders (all with non-seminomas of advanced stage) indeed show no or little staining for MT. This finding clearly argues against the putative role of MT expression in developing drug resistance and suggests that the absence of MT expression from NSGCT indicates a resistant tumor. The explanations for this contrast may lie in the following facts. (1) Immunohistochemical detection does not necessalily imply functional capability. Thus, the MT expression detected in this study does not

necessarily reflect a working protein but rather a mutated or inactive one. (2) Almost all the studies that have involved MT in the development of drug resistance are *in vitro* and may not mirror the situation *in vivo* or human tumors. (3) The physiological and clinical role of MT is not fully understood and it still remains a fertile area of research.

In vitro studies have shown that Pgp gene may he activated by mutant p53 (48). In sharp contrast, however, Wosikowski *et al* (1995) found wild type but not mutant p53 to stimulate MDR1 expression (49). In the present work a statistically significant inverse association between p53 and Pgp expression could be seen *i.e.,* high p53 levels associated with negative Pgp staining while low levels of p53 associated with positive Pgp staining (P = 0.0005). Since high levels of p53 immunoreactivity represent, in general, altered wt-p53 function, yet do not necessarily indicate mutant p53 (40), we stated that inactive or altered function of p53 suppresses Pgp expression whereas native or intact p53 stimulates Pgp expression. As mutant p53 protein is inactive, our findings seemt to support those of Wosikowski *et al* (49). In 29 tumors of colorectal origin Kant *et al* (1996) found a significant positive correlation between p53 and MDR1 expression in p53-mutated tumors (P = 0.005: r=0.596), but not in tumors without a p53 mutation (50). Taking into consideration that germ cell testicular tumor occur without p53 mutation, the findings of Kant *et al* do not seem to contrast with our's.

The findings reported here suggest that in germ cell tumors Pgp immunoreactivity is associated with a more progressive malignant phenotype, apart from its role in MDR. p53 and MT immunoreactivity predicts a better response to chemotherapy. Moreover, they suggest that negative p53 or MT and positive Pgp immunoreactivity may identify patient subgroups with less susceptibility to anti-cancer treatment and worse prognosis. May these tumors benefit from alternative chemotherapy? The answer would be very interesting and needs larger clinical studies.

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Expression of *bcl*-2 in Testicular Carcinoma

Correlation with Tumor Progression and MDR1/Pgp

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BACKGROUND. The expression of *bcl*-2 has been studied extensively in a variety of human tumors. However, there is a lack of clinical data regarding its expression in germ cell testicular tumors (GCTTs).

METHODS. In this study, the authors screened 70 patients with GCTTs for *bcl*-2 expression using immunohistochemistry (IHC) and the streptavidin-biotin–alkaline phosphatase method. This expression was also correlated with the metastatic behavior, clinical stages, and multidrug resistance gene product protein (MDR1/Pgp) immunostaining of GCTTs.

RESULTS. Overall, 41 carcinomas (58%) stained positively with anti-*bcl*-2 monoclonal antibody. According to histologic type, these lesions with positive staining included 11 of 26 seminomas (42.3%) and 30 of 44 nonseminomatous germ cell testicular tumors (NSGCTs) (68%). The incidence of *bcl*-2 immunostaining was higher ($P = 0.05$, two-tailed Fisher's exact test) among NSGCTs than among seminomas. The expression of *bcl*-2 was more prevalent among tumors from patients with metastases than among tumors from metastasis free patients ($P = 0.000$). There was a significant difference between the three stages of disease in the expression of *bcl*-2 ($\chi^2 = 0.000$), i.e., *bcl*-2 expression was clearly dominant among tumors at advanced stages. A significant association between *bcl*-2 and Pgp immunostaining was established ($P = 0.004$).

CONCLUSIONS. These findings revealed that *bcl*-2 expression occurs in GCTTs. Furthermore, they suggest that *bcl*-2 is associated with a more advanced malignant phenotype of this tumor. ***Cancer* 1998;83:331–6.**

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KEYWORDS: *bcl*-2 expression, testicular carcinoma, immunohistochemistry, tumor progression.

The *bcl*-2 gene was first identified in 1984 during a study of the t(14;18) chromosome translocations that occur frequently in B-cell leukemia and non-Hodgkin's follicular lymphomas.[1,2] The gene is located at chromosome 18q21, but the t(14;18) translocation juxtaposes the gene to the immunoglobulin heavy chain (IGH) loci at chromosome 14. This creates a so-called *bcl*-2/IGH fusion gene, resulting in high levels of *bcl*-2 protein. Expression of *bcl*-2 was initially thought to be restricted to B-cell malignancies with translocation, but subsequent studies have reported its expression in a wide range of lymphoproliferative diseases that lack this chromosomal abnormality as well as in normal lymphoid cells.[3–5] Ectopic *bcl*-2 expression has been reported to block apoptosis induced by a number of agents, including treatments with calcium ionophores, ethanol, irradiation, corticosteroids, and heat shock.[6–8] *bcl*-2 expression, by inhibiting apoptosis, prolongs the life span of cells and increases the risk that they will develop other changes, such as chromosomal abnormalities

or viral infections, that will result in malignant transformation or overt tumor progression.[9–11]

Rearrangement of the *bcl*-2 gene with its protein expression may induce resistance to multiple cancer agents, resulting in unfavorable prognoses for patients with certain types of cancers, according to clinical correlative studies.[12–15] The association of *bcl*-2 with tumor progression and resistance to treatment has been well established in cases of prostate carcinoma. In such cases, *bcl*-2 expression in prostate carcinoma was associated with tumor progression after hormone therapy, suggesting that *bcl*-2 may cause resistance to androgen ablation treatment.[15] Furthermore, using immunohistochemistry in a series of human and rodent prostate carcinomas, Furunya et al. demonstrated that there was a statistically significant ($P < 0.05$) association between the expression of *bcl*-2 and the progression of human prostate carcinoma cells to metastatic phenotype.[16] In contrast to the situation seen in prostate carcinoma, *bcl*-2 immunoreactivity in breast carcinoma and in nonsmall cell lung carcinoma has been found to be associated with markers of better prognosis.[17–19] To our knowledge, no previous study has investigated *bcl*-2 expression and its clinical significance in GCTTs.

MATERIALS AND METHODS

Patients

Specimens were obtained by the semicastration of 70 patients with testicular carcinoma and biopsied at the National Institute of Oncology, Péterfy S. and ORFI Hospital, Budapest, Hungary, for diagnostic evaluation and treatment of disease. Prior to surgery the patients received neither radiotherapy nor chemotherapy. The mean follow-up period of these patients was 32 months (range, 6–72 months). The mean age was 30 years (range, 15–70 years). A portion of each resected tumor was snap frozen in liquid nitrogen and stored at −80°C until analysis, or fixed in buffered formalin for paraffin embedding.

Histologic examination was performed on hematoxylin and eosin–stained tissue sections. Clinical staging was done on the basis of the International Union Against Cancer's *TNM Classification of Malignant Tumours*,[20] as well as marker investigation (α-fetoprotein and β-human choriogonadotropin) and other clinical features.[21] There were 32 patients with Stage I (localized without lymph node involvement), 14 with Stage II (retroperitoneal or regional) and 24 with Stage III (distant) disease. The tumors were classified histologically according to the World Health Organization criteria described by Mostofi et al.[22] and included 26 seminomas and 44 nonseminomatous germ cell testicular tumors (NSGCTs). The latter group contained 9 embryonal carcinomas (ECs), 1 with yolk sac differentiation, 5 teratomas, and 1 choriocarcinioma (CC). The remaining 28 patients had mixed tumors.

Immunohistochemical Detection of *bcl*-2 Protein

The 4 μm formalin-fixed paraffin tumor sections were deparaffinized in ? changes of xylene for 10 minutes each and then hydrated in decreasing concentrations of ethanol and rinsed in Tris-buffered saline (0.05 M Tris/HCl, pH 7.6, 0.15 M NaCl) (TBS). An antigen retrieval procedure was then performed by immersion of the slides in 10 mm (pH 6.0) citrate buffer and exposure to microwave irradiation twice for 5 minutes each time with a cooling period of 3 minutes each time.[23] In between the sessions, the fluid level in the Coplin jar was topped off with water. After cooling to room temperature, slides were removed to TBS and incubated with 1% bovine serum albumin to reduce nonspecific bindings. Slides were incubated overnight at +4°C with a 1:20 dilution of the primary mouse antihuman *bcl*-2 monoclonal antibody (clone 124, isotype immunoglobulin [Ig]G1; DAKO). The streptavidin-biotin detection system was employed with new fuchsin as the chromogen. Sections were washed twice for 5 minutes each time with TBS and incubated for 30 minutes with biotinylated secondary antibody (Amersham) diluted at 1:50 in TBS. As a third step, streptavidin biotinylated alkaline phosphatase complex (DAKO) was applied for 30 minutes. Sections were washed twice with TBS, developed with new fuchsin substrate (DAKO), slightly counterstained with Mayer's hematoxylin, dehydrated, cleared in xylene, and mounted. As a positive control we used paraffin-embedded sections from a normal human tonsil. At the same time, positive staining of small lymphocytes provided a further internal control. Staining without anti-*bcl*-2 monoclonal antibody was performed as a negative control. Positive and negative controls were included in each staining run, and all gave satisfactory results.

Assessment of *bcl*-2 Immunoreactivity

Immunoreactive *bcl*-2 expression was analyzed independently by two observers (M.B. and H.E.). Only cells that stained well above the background level were considered positive, and reaction product was registered as either present or absent. The samples, as described previously by Zhao et al., were categorized into two categories, negative (−) in cases with complete lack of immunoreactivity and positive (+) if there were occasional scattered positive cells or clusters of positive cells. As an internal control, lymphoid cells that exhibited *bcl*-2 immunoreactivity were assessed in all specimens.[24]

TABLE 1
Correlations between *bcl*-2 Expression and Histologic Subtype, Metastatic Potential and Pgp Expression in GCTTs

	bcl-2 (−)	*bcl*-2 (+)	*P* value[a]
Seminomas	15	11	0.05
NSGCTs	14	30	
No metastasis	23	9	0.000
Metastatic tumors	6	32	
Pgp (−)	24	4	0.004
Pgp (+)	21	19	

GCTTs: germ cell testicular tumors; NSGCTs: nonseminomatous germ cell testicular tumors.
[a] Fisher's exact test.

TABLE 2
Correlation between *bcl*-2 Immunostaining and Clinical Staging of Patients with GCTTs[a]

Stage	*bcl*-2 (+)	*bcl*-2 (−)	All cases
I	10	22	32
II	10	4	14
III	22	2	24
	42	28	70

[a] Chi-square test, $\chi^2 = 0.000$.

Immunohistochemical detection and scoring of Pgp immunoreactivity was published elsewhere.[25]

Statistical Methods

The two-sided Fisher's exact probability test was used for statistical evaluation of the correlations between *bcl*-2 immunoreactivity and histologic subtype, metastatic potential, and Pgp expression (Table 1). The cases were first divided into two subgroups (seminomas vs. nonseminomas; no metastasis vs. metastatic tumors; Pgp negative vs. positive) and then dichotomized based on the expression of *bcl*-2 (negative vs. positive). The chi-square test (χ^2) was performed for statistical evaluation of the correlation between *bcl*-2 expression and tumor stage (Table 2). Variables of interest were clinical stage (Stage I, II, or III) and the expression of *bcl*-2 (positive vs. negative). A *P* value of ≤ 0.05 was considered statistically significant.

RESULTS

bcl-2 Expression and Histologic Tumor Type

Overall, 41 carcinomas (58%) stained with anti-*bcl*-2 (Table 1). By histologic type, these lesions included 11 of 26 seminomas (42%) and 30 of 44 NSGCTs (68%). The latter included 3 teratomas (50%), 7 ECs (78%), 1 CC (100%), and 19 mixed tumors (68%). Cytoplasmic staining was observed in the majority of cases (Figs. 1–4). In some tumor cells, however, some degree of intranuclear staining was also observed (Fig. 5). The correlations between *bcl*-2 expression and the two major subtypes of GCTTs, namely, seminoma and NSGCT, are shown in Table 1. The incidence of *bcl*-2 immunostaining was higher among NSGCTs than among seminomas; this difference was of borderline significance ($P = 0.05$).

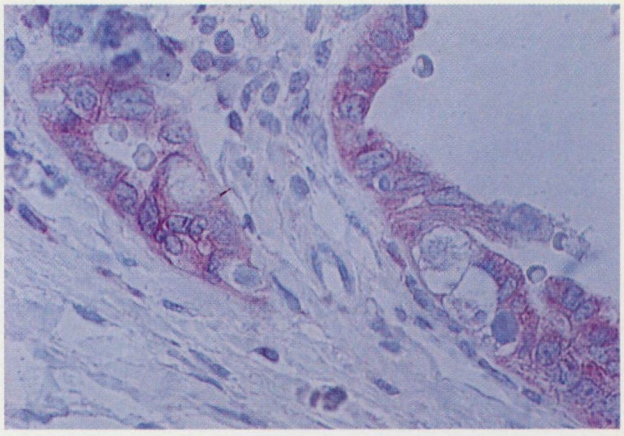

FIGURE 1. Cytoplasmic staining of Stage III teratoma for *bcl*-2 protein, using antihuman *bcl*-2 monoclonal antibody (clone 124, DAKO), is shown (AP, original magnification ×200).

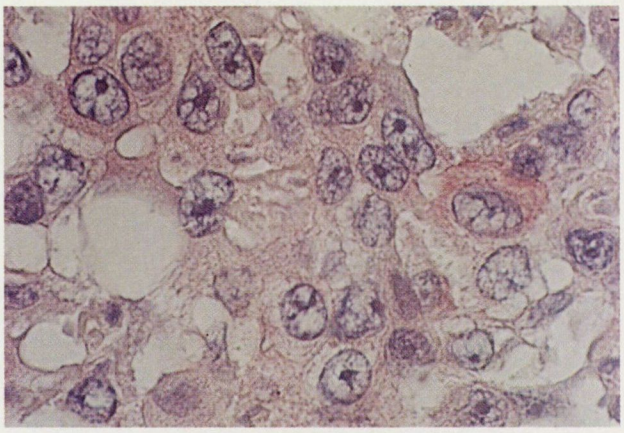

FIGURE 2. Cytoplasmic *bcl*-2 immunoreactivity of Stage III embryonal carcinoma for *bcl*-2 protein, demonstrated with antihuman *bcl*-2 monoclonal antibody (clone 124, DAKO), is shown (AP, original magnification ×200).

bcl-2 Immunoreactivity in Relation to Metastasis

bcl-2 expression was analyzed in tumors from patients with and without metastasis. Nine of 32 metastasis free patients (28%) had *bcl*-2 expression, whereas 32 of 36 patients with metastases (89%) had expression of *bcl*-2 protein (Table 1). Furthermore, we found that the mean incidence of cells that expressed *bcl*-2 increased with an increase in stage.

The incidence of *bcl*-2 expression was signifi-

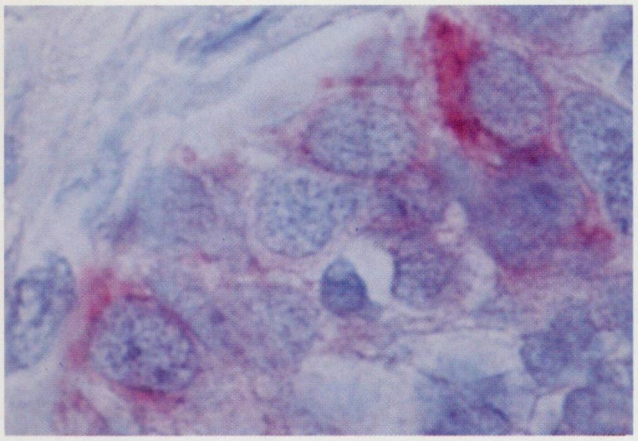

FIGURE 3. Cytoplasmic and paranuclear staining of Stage IIC teratoma for *bcl*-2 protein, using anti-human *bcl*-2 monoclonal antibody (clone 124, DAKO), is shown (AP, original magnification ×400).

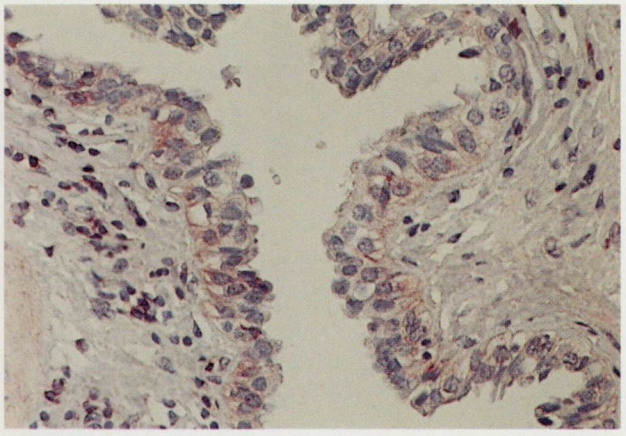

FIGURE 4. Delicate cytoplasmic staining of Stage III teratoma for *bcl*-2 protein, using antihuman *bcl*-2 monoclonal antibody (clone 124, DAKO), is shown (AP, original magnification ×200). Note that the infiltrating lymphocytes are positive.

cantly higher among tumors from patients with metastases than among tumors from metastasis free patients ($P = 0.000$). This suggests that *bcl*-2 influenced the aggressiveness of GCTTs.

The correlation between *bcl*-2 expression and clinical stage is summarized in Table 2. There was a significant difference between the three stages of disease regarding the expression of *bcl*-2 ($\chi^2 = 0$). In this series of GCTTs, *bcl*-2 expression was clearly dominant in tumors of advanced stages.

The Correlation between *bcl*-2 and Pgp Immunoreactivity

A significant association was demonstrated between these two parameters ($P = 0.004$, Table 1), suggesting that Pgp and *bcl*-2 were probably not independent of each other.

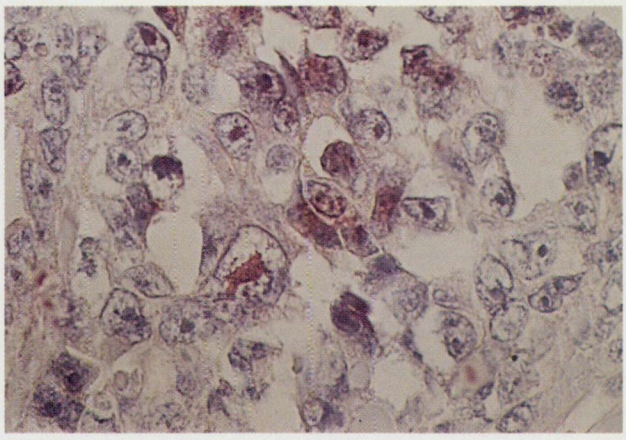

FIGURE 5. Nuclear staining of Stage III embryonal carcioma for *bcl*-2 protein, using anti-human *bcl*-2 monoclonal antibody (clone 124, DAKO), is shown (AP, original magnification ×100).

DISCUSSION

bcl-2 expression has previously been investigated extensively in a variety of human cancers, but not in GCTTs. We therefore investigated *bcl*-2 expression and its association with histologic subtype, metastatic potential, and clinical stage of GCTT.

In this immunohistochemical study, cytoplasmic staining was observed in the majority of cases. In some tumor cells, some degree of intranuclear immunoreactivity was also observed. However, it is important to note that, unless other methods (such as the double immmunostaining technique and confocal microscopy) are used, the distribution of *bcl*-2 in different cell compartments is difficult to determine. The physiologic and pathologic significance of different staining patterns of *bcl*-2 in different cell compartments are of interest and remain to be clarified in other studies.

bcl-2 protein was detectable by immunohistochemical analysis in 41 of 70 GCTTs (58%). Seminomas and NSGCTs expressed *bcl*-2 as well, although the incidence of this expression in the latter subtype was higher ($P = 0.05$). In general, the intensity of staining in seminomas was weak to moderate and lower than that of infiltrating lymphocytes. In NSGCTs the staining was stronger and similar to that of infiltrating lymphocytes.

Seminomas and NSGCTs have somewhat distinctive clinical features. More importantly, they also differ with respect to optimal therapy and prognosis.

Compared with seminomas, NSGCTs are biologically more aggressive and generally associated with a poorer prognosis. The higher incidence of *bcl*-2 expression among NSGCTs, which was of borderline significance, might be one simple explanation for the

differences that exist between these two broad categories.

In a very recent study, Furunya et al. presented data demonstrating that there was a statistically significant ($P < 0.05$) association between the expression of *bcl*-2 and the progression of human prostate carcinoma cells to a metastatic phenotype.[16] However, the *bcl*-2 expression was not absolutely essential to either androgen independence or metastatic potential in human prostate carcinoma cells. To determine whether the alterations in the *bcl*-2 protein might be an indicator of more aggressive GCTT, we analyzed *bcl*-2 expression in tumors from patients with and without metastases. We found that *bcl*-2 expression was significantly higher in tumors from patients with metastases than in tumors from metastasis free patients ($P < 0.000$). Furthermore, the mean percentage of cells that expressed *bcl*-2 increased with increasing clinical stage. In concordance with that result, there was a significant difference between the three stages of disease regarding the expression of *bcl*-2 ($\chi^2 = 0$). In this series of GCTTs, *bcl*-2 expression was clearly dominant in tumors of advanced stages. Six of 36 metastatic tumors (16.6%) did not express *bcl*-2 protein, suggesting that *bcl*-2 expression in GCTTs is not an absolute requirement for their metastatic potential. One may speculate that oncogenes or proteins other than *bcl*-2, either alone or in association with it, influence the metastatic behavior and aggressiveness of GCTTs.

Various clinical studies have suggested that the presence of the MDR1 gene product protein Pgp is associated with more aggressive tumor behavior.[26–28] In a recent immunohistochemical study, we examined the correlation between Pgp immunoreactivity and tumor stage, using cryostat sections and C219 monoclonal antibody. We found that Pgp expression was significantly correlated with advanced tumor stage and worse prognosis.[25] Because the same specimens were screened for their *bcl*-2 immunoreactivity, we were encouraged to examine the possible correlation between Pgp and *bcl*-2 expression. As expected, a significant correlation between these two markers was demonstrated ($P = 0.004$, Table 1), suggesting that Pgp and *bcl*-2 are probably not independent of each other. This correlation between Pgp and *bcl*-2 expression provides further indirect evidence of the role of *bcl*-2 in tumor progression and prognosis.

It would be more informative to demonstrate a correlation between *bcl*-2 immunohistochemistry and Western blot analysis in selected samples. This would provide with some in-depth analysis of *bcl*-2 function in GCTTs. Unfortunately, the tumorous tissues of the patients included in this work were no longer available to us for such an investigation.

In conclusion, the current findings revealed that *bcl*-2 expression occurs in GCTTs. Furthermore, they suggest that *bcl*-2 is associated with a more advanced malignant phenotype of this tumor. The clinical significance of the above-mentioned findings in terms of response to treatment and survival is the focus of a further investigation that is now being conducted in our laboratory.

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ORIGINAL PAPER

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Do metallothioneins affect the response to treatment in testis cancers?

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Abstract *Purpose*: Data on the involvement of elevated metallothionein (MT) expression in resistance to some of the commonly used anticancer treatments are scattered and conflicting. This encouraged us to examine further the contribution of metallothionein expression to the development of this resistance phenotype. *Patients and methods*: Formalin-fixed, paraffin-embedded blocks of primary untreated germ cell testicular tumor specimens, obtained from 77 patients following radical orchiectomy, were examined for their MT expression using monoclonal antibody and immunohistochemistry. Clinical staging, the chemotherapeutic schedule and evaluation of response to treatment (defining objective response) were performed according to UICC criteria. *Results*: All tumor types, including seminomas and nonseminomas, expressed MT, regardless of their histology and clinical stage. The immunoreactivity of MT showed a significant positive correlation with the clinical sensitivity of cancer to antitumor therapy ($P = 0.0001$). *Conclusion*: In patients with germ cell testicular tumors, high MT expression, as detected by immunohistochemistry, predicts a better response rate to chemotherapy whereas tumors lacking or demonstrating low MT expression show a worse prognosis. These data do not support the hypothesis that MT overexpression contributes to cisplatinum resistance, at least in this tumor type.

Key words Metallothionein · Immunohistochemistry · Testicular germ cell tumors · Response to treatment

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Introduction

Metallothioneins (MT) are a family of low-molecular mass (6–7 kDa) cysteine-rich metal-binding proteins. The protein is highly nuclophilic and has no sulfhydryl bonds. The physiological function of MT is not yet fully understood. The proteins bind a number of trace metals including cadmium, platinum and silver, and clearly protect cells and tissues against heavy-metal toxicity (Hamer 1986). The transcription of MT genes is induced by cadmium, glucocorticoid hormones (Sadhu and Gredamu 1988), ultraviolet irradiation, mitomycin C (Angel et al. 1986) and heat shock (Leyshon-Sorland et al. 1993).

In addition, MT may facilitate metal exchange with zinc-dependent enzymes and thus assist indirectly in the repair of lethal DNA damage and regulate important cellular activities (Zeng et al. 1991; Li et al. 1980). Cell lines expressing high levels of MT have been reported to be resistant to DNA-damaging agents like cisplatin, alkylating agents (Kelley et al. 1988) and doxorubicin (Webber et al. 1988). In addition, MT overexpression has been found to confer resistance to radiotherapy (Renan and Dowman 1989). Nevertheless, an increase in MT does not always result in a phenotype that is less sensitive to the toxic effects of electrophilic antineoplastic agents (Schilder et al. 1990; Murphy et al. 1991) and in fact it leads to increased sensitivity (Robson et al. 1992). Further, there remains uncertainty about whether data describing mechanisms of resistance in vitro are related to human tumors. Thus the impact of MT on the response to many commonly used therapeutic agents remains controversial. This stimulated us to examine further the role of this protein in modulation of cellular sensitivity and in predicting the clinical response in patients with testis carcinomas.

Patients and methods

Specimens were obtained by semicastration of 77 patients with testis cancer biopsied at the National Institute of Oncology and in

Table 1 Histology, stage, treatment and response to treatment of germ cell testicular tumors. *NSGCT* non-seminomatous germ cell tumor, *RLA* retroperitoneal lymphadenoctomy, *Ch* chemotherapy, *Rad* radiation, *W&S* wait and see, *CR* complete response, *PR* partial response, *NR* no response, *D* died

Histology Stage	No.	Treatment				Response rate
		RLA	Ch	Rad	W&S	
Seminomas	27					
IA	6				6	6 CR
IB	14		13[*1]	1		14 CR
IIA	4	1	4[*2]			4 CR
IIIB	3		3[*3]	1		3 CR
NSGCT	47					
IA	4	2			2	4 CR
IB	10	5	10[*4]			10 CR
IIA	5	2	4[*5]			4 CR
IIB	5	3 salvage	5[*6]			3 CR, 2 D
IIC	2	2 salvage	2[*7]			2 CR
IIIA	4	1 salvage	4[*8]			4 CR
IIIB	17	3 salvage	20[*9]	1		11 CR, 1 NR, 5 D

[*1–*3] Chemotherapy of seminoma: [*1] 2× vinblastine/cisplatin/bleomycin (VPB) (11); 2× bleomycin/etoposide/cisplatin (BEP) (2). [*2] 2,4× VPB (1,1); 2× BEP (2), [*3] 4–6 BEP (1,1), 3× BEP, 3× vinblastine/ifosfomide/cisplatin (VIP)

[*4–*9] Chemotherapy of NSGCTT: [*4] 2× VPB (10). [*5] 4× VPB (2); 2× VPB (2). [*6] 4–6× BEP (3); VPB, BEP, VIP, vinblastine/Adramycin D/cisplatin (VAP) (2). [*7] 6× BEP (2). [*8] 6× BEP (4). [*9] 4–8 BEP/etoposide/cisplatin (EP) (11); 6× VPB (3) and variable BEP/VIP, VIP, VAP/VIP etc. (6)

other municipal hospitals in Budapest, Hungary, for diagnostic evaluation and treatment for disease. Prior to surgery the patients received neither radio- nor chemotherapy. The mean follow-up period for these patients was 32 months (range 6–72 months). Their mean age was 30 years (range 15–70 years). All primary tumors were removed surgically, and a portion of each resected tumor was snap-frozen in liquid nitrogen and stored at –80°C or fixed in buffered formalin for paraffin embedding and analysis.

A summary of the histology, stage, treatment and response-rate to treatment is given in Table 1.

Histological examination was performed on hematoxylin/eosin-stained tissue sections. Histological classification of the tumors was according to WHO (Mostofi et al. 1986) and identified 27 seminomatous and 50 nonseminomatous tumors. The latter group contained 10 embryonal carcinomas, 1 with yolk sac differentiation, 7 teratomas, and 1 choriocarcinoma. The remaining 31 patients had mixed tumors.

For staging and measurements of the results, a physical examination, hemogram, 12-channel screening profile, serum human chorionic gonadotrophin β subunit (HCG), α-fetoprotein (Javadpour 1980), chest X-ray, abdominal ultrasound and/or computed tomography (CT) scan were used. Brain, bone and liver isotopic or CT scans were performed when clinically warranted. There were 34 patients with stage I (localized without nodal involvement), 16 with stage II (retroperitoneal or regional lymph node metastases) and 27 with stage III (distant) disease.

The chemotherapeutic schedule and evaluation of response to treatment, i.e. definition of objective response were performed according to UICC criteria (Monfardini et al. 1981). Complete response means that the clinical symptoms, the radiological, abdominal ultrasound, CT, brain, liver and bone isotopic- or CT-scan lesions had completely regressed, and the increased levels of radioimmunoassay, α-fetoprotein and β-HCG serum observed prior to the start of treatment fully normalized. A partial response was considered to be a 50% or more decrease in the diameter of measurable lesions. A 25% or greater increase in the diameter of measurable lesions was regarded as progression. Between partial response and progression there were patients with no response.

Immunohistochemistry

The MT antigen was detected by the streptavidin-biotin-alkaline-phosphatase immunostaining method. Briefly, the paraffin sections were routinely deparaffinized and pretreated in a microwave oven at 750 W in 10 mM citrate buffer (Dako, pH 6.0) twice for 5 min as described previously by Cattoretti et al. (1992). They were then incubated for 20 min at room temperature with 1% bovine serum albumin (Serva/Heidelberg) a carrier protein to block non-specific binding of reagents to the sections. The monoclonal mouse anti-metallothionein (Dako-MT, E9) was applied at a dilution of 1:40 for 2 h at 37°C. This antibody is directed against a single and highly conserved epitope shared by isoforms I and II of the human, rat and horse proteins. Slides were washed three times in TRIS buffer (0.05 M TRIS/HCl, pH 7.6, 0.15 M NaCl), incubated with a second antibody [biotinylated anti-(mouse Ig) antibody from Amersham] for 30 min at room temperature and washed twice with TRIS buffer. Thereafter they were incubated for 45 min at room temperature with the streptavidin/biotin/alkaline-phosphatase complex (Dako). The enzyme activity was detected by incubation with its substrate (new fuchsin; Dako). Endogenous alkaline phosphatase was inhibited by the addition of levamisol (Dako) to the substrate. Specimens were counterstained with Mayer's hematoxylin, and mounted with glycerol gelatin. Sections of invasive breast ductal carcinoma, known to express MT, were used as a positive control. The same slides, with the primary antibody omitted, were used as a negative control. All controls gave satisfactory results. Three slides of each tumor were evaluated by two of the authors without knowledge of the clinical data, and the average value was considered.

Metallothionein scoring

Although it may not apply to other organs, previous studies on germ cell testicular tumors (GCTT), show that the intensity of staining and the percentage of stained cells are closely related (Chin et al. 1993), and the number of cells labelled may be more meaningful than the intensity of staining per se (Hall and Lane 1994). In this study, we were encouraged to exclude the intensity of staining from the interpretation of our results and to rank only the extent of staining. The extent of staining was evaluated as the percentage of positively stained tumor cells relative to the total number of tumor cells in five adjacent high power-fields at a magnification of (×400). This scoring system has been validated in other diseases, as recently described by Lipponen (1993).

First the entire section was screened carefully and counts were performed in representative areas, i.e. the region with the maximum fraction of positively stained cells, well fixed and free from background. Stromal components were avoided by comparing the section with the hematoxylin/eosin-stained counterpart. Tumors, according to their extent of staining, were classified as – signifying

no obvious positive staining at all, ± indicating that fewer than 5% of tumor cells were positive, + signifying that 5%–50% of tumor cells were positive, ++ signifying that more than 50% of tumor cells were positive.

When the tumors were divided into only two groups – no to low (−/±; showing 0–5% positive cells) and high staining (+/++; showing more than 5% positive cells), a statistically significant difference in response to treatment was observed.

Statistical methods

The two-sided Fischer's exact probability test was used for statistical evaluation. The cases were first divided into sensitive (complete remission) and resistant (no response, progression and death) subgroups and then divided on the basis of the expression of MT (≤5%, > 5%). A difference was regarded as significant if P was 0.05 or less.

Results

All tumor types expressed MT protein. The extent of staining on the section was estimated to range from (0), signifying no staining at all in a few cases, to more than 90% in a few others. The majority of cases were distributed between these two extremes. Tumors, seminomas and nonseminomas all expressed MT irrespective of their histological subtype. There was no significant difference between these two major subtypes. Cancers of 54 patients (70%) showed more than 50% of tumor cells to be positive. In general, the staining was easy to interpret and MT could be demonstrated in the cytoplasm, nucleus or both. When the staining was confined only to the cytoplasm or the nucleus it tended to be less intensive than when it occupied both. The localization of MT was variable among individual cells and groups of cells. Stained and unstained single cells or cell groups were localized side by side (Fig. 1).

The distribution of patients according to the stage of their tumors and the extent of MT staining is listed in Table 2. Ninety per cent of patients with early-stage disease, compared to 70% at a later-stage, expressed high MT staining. This may represent a tendency towards decreasing MT expression with increasing stage in GCTT (Figs. 2, 3). However this difference did not reach statistical significance (two-tailed $P = 0.24$).

In this study 73 patients were evaluated for response to the treatment used; 64 achieved complete remission and have been judged as sensitive (Table 3). Seven patients died and 2 showed no response, and these 9 patients belonging to the advanced-stage group, were judged to be resistant. Of 64 sensitive tumors, 57 (89%) showed more than 5% positive tumor cells and 7 (11%) showed no to low staining, i.e. staining in less than 5% of tumor cells. Of the resistant tumors, 2 (22%) showed staining in more than 5% of tumor cells while no or low staining has been observed in the remaining 7 (78%) tumors. The expression of MT showed a statistically significant (two-sided Fisher's exact test, $P = 0.0001$) correlation with rate of response to the treatment modalities used in this group of patients with cancers at all three stages (Fig. 4). However, the relationship between MT expression and response to chemotherapy as a curative treatment should be evaluated on patients with advanced disease, i.e. stages II/B, II/C and III patients, where all resistant tumors were found. (Table 4). To meet this criterion we examined the relationship between MT expression and rate of response to chemotherapy in advanced-stage patients. Of 31 patients who received curative cisplatin-based combination chemotherapy, 9 showed resistant disease (7 died, 2 had stable disease) and 7 of them (86%) expressed no to low MT expression. In contrast, 19 of 22 sensitive tumors (86%) expressed high MT levels. This relationship proved again to be significant ($P = 0.0013$).

Table 2 The correlation between metallothionein (*MT*) expression and stages of disease. $P = 0.241$ (Fischer's exact test)

Clinical stage	MT expression		
	Low −/+	High +/++	All cases
Early-stage group I, IIA	4	39	43
Later-stage groups IIB, III	10	24	34
Total	14	63	77

Table 3 The correlation between MT content and rate of response to treatment in stage I, II and III patients. $P = 0.0001$ (Fischer's exact test)

Response to treatment	MT expression		
	Low −/+	High +/++	All cases
Resistant	7	2	9
Sensitive	7	57	64
Total	14	59	73

Table 4 The correlation between MT content and response to chemotherapy of advanced germ cell testicular tumors. $P = 0.0013$ (Fischer's exact test)

Response treatment	MT expression		
	Low −/+	High +/++	All cases
Resistant	7	2	9
Sensitive	3	19	22
Total	10	21	31

Discussion

Testicular neoplasms span an amazing gamut of anatomical types. Approximately 95% arise from germ cells, and most of these germinal tumors are highly aggressive cancers capable of rapid and wide dissemination; however, with current cisplatin-based combination chemotherapy the outlook for these patients has improved considerably. The overall cure rate of dissemi-

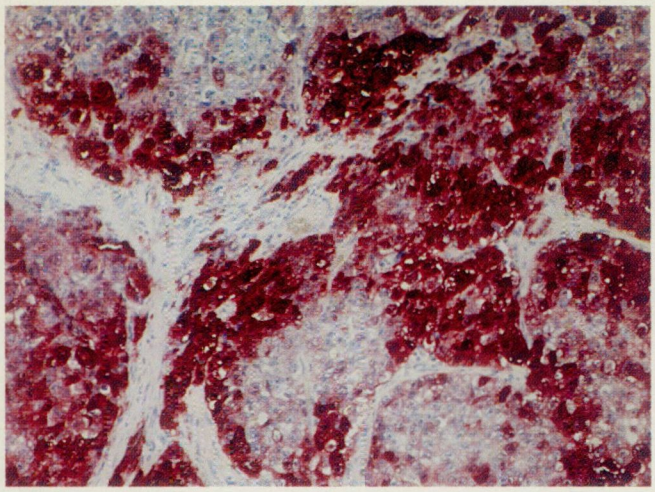

Fig. 1 Embryonal carcinoma. Metallothionein (MT) immunostaining represents ++ staining index. Immunoalkaline phosphatase (Reduced from ×30)

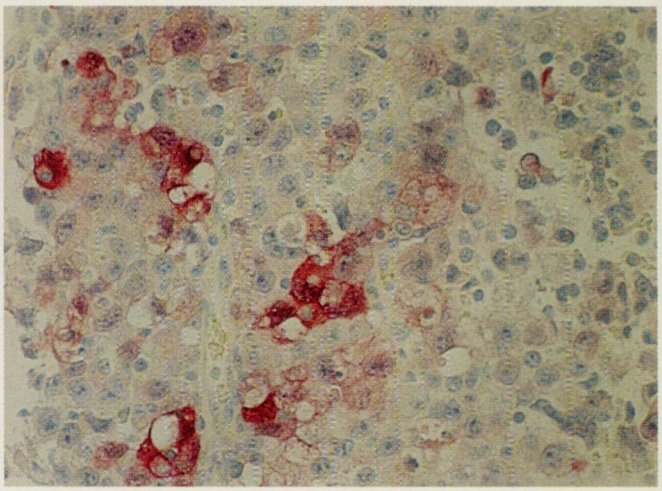

Fig. 3 Seminoma stage III. Positive MT staining could be detected only in few cells (+). Immunoalkaline phosphatase. (Reduced from ×100)

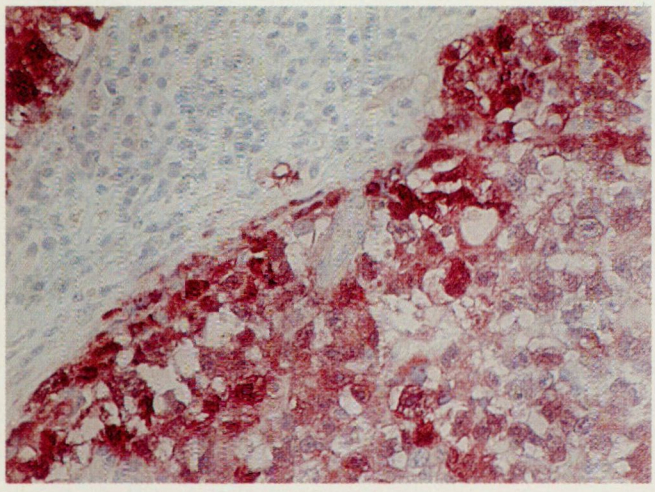

Fig. 2 Seminoma stage IA. Most of the tumor cells strongly reacted with the MT antibody (++). Immunoalkaline phosphatase. (Reduced from ×100)

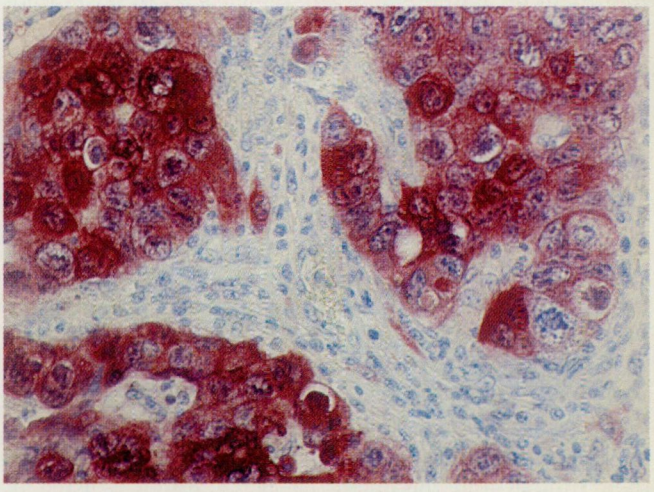

Fig. 4 Marked positive MT staining (++) of a sensitive embryonal carcinoma. Immunoalkaline phosphatase (Reduced from ×100)

nated testis cancer following chemotherapy is as high as 70%–80% (Williams et al. 1987). However, some patients ultimately die of their disease because of tumor resistance to chemotherapy. What are the causes of this remarkable chemosensitivity and what are the underlying mechanisms of drug resistance that ultimately lead to death?

In literature concerning MT expression in GCTT, there are two studies of almost the same group (Kontozoglou et al. 1989; Chin et al. 1993). Both used their own polyclonal rabbit antibody to rat liver MT, which shows cross-reactivity with human MT. Kontozoglou et al. (1989) studied tissue sections of 9 human embryonal carcinomas and suggested that MT may be considered an oncodevelopmental product that could be useful as a tumor marker. Chin et al. (1993) assessed immunohistochemically the presence of MT in 33 untreated primary testicular germ cell tumors. Regardless of the clinical stage, seminomas stained weakly or not at all, and most nonseminomas stained strongly for MT. Although a direct correlation between cisplatin resistance and MT content has not been established, Chin et al. suggested such a relationship from their and other studies. Interestingly, in their study, 4 patients died and 3 of them had no MT staining. The staining was also absent from the only patient who had recurrent disease.

In our present work we also used immunohistochemistry to detect MT expression. This technique has a major advantage over other methods – either indirect (e.g. chromatographic separation) or direct (e.g. radioimmunoassay) – of allowing MT localisation within the tissue examined. Since the MT gene is conserved, a similar phenotypic expression in primary and metastatic

lesions is expected. However, possible differences should be considered. In 3 teratocarcinomas, 2 teratomas and 1 seminoma, MT in primary and secondary lesions showed similar immunoreactivity.

However, the striking difference, compared with the previous studies, appears to lie in the seminomas. Out of 27 (85%) seminomas, 23 expressed MT, while 21 (77.7%) showed MT expression in 50% or more of tumor cells. However, this discrepancy may be simply explained by the fact that Chin et al. (1993) used their own polyclonal antibody and evaluated the intensity but not the extent of the staining. Further, and more importantly, they did not employ the antigen retrieval technique, which can significantly decrease the threshold of the detection system, thus allowing otherwise undetectable trace expressions to appear (Lambkin et al. 1994).

In this immunohistochemical study of MT expression, the tumors, on the basis of their clinical stage, were divided into two main subgroups: the early-stage groups (I and IIA) and the later-stage groups (IIB–III), the difference in the pattern of relapse and survival curves between the two subgroups in the Royal Marsden series was significant (Peckham 1979). In this series of GCTT we could not establish any significant relationship between MT expression and tumor stage. No significant relationship between MT expression and the clinical stage could be found by Murphy et al. (1991) in ovarian tumors or by Chin et al. (1993) in GCTT.

An important finding, in patients with advanced disease was that high expression of MT in the tumor cells was associated with favorable clinical outcomes in terms of response-rate to chemotherapy. In particular the MT group, which expressed MT in more than 5% of tumor cells, responded significantly better than the others ($P = 0.0013$). This suggests that MT expression may be a marker of chemosensitivity, and its absence, when it occurs, indicates a germ cell tumor of poor prognosis. The latter finding raises a question of crucial clinical importance: can these tumors with low levels of MT benefit from alternative chemotherapy? The answer would be very interesting and needs further and larger clinical studies.

Seminomas stained strongly for MT and this high expression correlated well with the clinical outcome. However, irrespective of the MT staining, seminomas respond well to both radio and chemotherapy. Therefore, the high expression of MT in seminomas may or may not be related to their sensitivity. The question of whether or not MT expression can predict outcome in nonseminomas becomes important here.

From our data it appears that 78% of poor responders (all with non-seminomas of advanced stage) indeed show no or little staining for MT. This finding strongly argues against the putative role of MT expression in developing drug resistance and suggests that the absence of MT expression from nonseminomatous germ cell testicular tumors indicates resistant tumor. In contrast to our findings, expression of MT has been reported to be associated with increased resistance to chemotherapy. The explanations for this contrast may lie in the following facts. (1) The physiological and clinical role of MT is not fully understood and still remains a fertile area of research. (2) Almost all the studies that have involved MT in the development of drug resistance are in vitro and may not mirror the situation in vivo or in human tumors. (3) Immunohistochemical detection does not necessarily imply functional capability. Thus, the MT expression detected in this study does not necessarily reflect a working protein but rather a mutated or inactive one.

One explanation of how an elevated MT level can modulate cellular sensitivity and sensitize tumor cells to cancer therapy may be that these modulating effects are a function of a still not fully understood connection to other identified or not fully identified factors, for instance the *p53* tumor suppressor gene.

Many of the stresses and drugs that have been found to induce MT synthesis such as, ultraviolet irradiation, mitomycin C, glucocorticoids, heat shock, heavy-metals such as cadmium and many others, were also found to lead to the accumulation of the wild-type *p53* (Fritsche et al. 1993; Sugano et al. 1995). Furthermore, MT has been involved in DNA repair (Kaina et al. 1990) and very recently in programmed cell death (Murphy et al. 1994). Thus one may speculate that there is an interplay between the *p53* gene and MT causing MT expression to influence the response to chemotherapy. In an attempt to support the presence of a relationship between these two proteins, we correlated our immunohistochemical results on p53 protein expression, reported elsewhere (Eid et al. 1997), with the MT immunostaining results obtained here for the same specimens. We found a very strong positive relationship between MT and p53 immunoreactivity ($P = 0.0002$, Table 5), suggesting that these two gene products are probably not independent of each other. Similar simultaneous expression of p53 and MT was obtained in irradiated skin (Jasani et al. 1993). The possible relationship between MT and p53 and its impact on tumor treatment should be the focus of other clinical studies.

This report clearly shows that there is no causal relationship between MT overexpression and cisplatin resistance. Furthermore, MT overexpression being one possible factor correlated to chemotherapy sensitivity, stresses the need for a larger prospective clinical investigation in order to see whether it can be of multivariate prognostic relevance.

Table 5 Relationship between p53 expression and MT of germ cell testicular tumors. *P* was estimated by Fisher's exact test

	P53 expression		
MT expression	High	Low	*P*
High	57	5	< 0.0002
Low	5	7	

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mdm-2 Expression in Human Testicular Germ-Cell Tumors and its Clinical Value

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Abstract. *Background. In germ cell testicular tumors (GCTT) mdm-2 gene was analyzed for amplification and transcripts but not for protein. The purpose of this study is to determine whether mdm-2 protein level is aberrant in GCTT and if so, what is the relationship between mdm-2 overexpression and other disease parameters including histologic subtypes, p53 status, metastatic potential and clinical stage. Methods. 81 testicular germ-cell tumors were screened for their mdm-2 expression at the protein levels using immunohistochemistry (IHC) and Western blot (WB) analysis. Results. Of 81 GCTTs 45 (55.55 %) showed mdm-2 nuclear immunoreactivity, 34 (41,97%) of which were strongly positive. The incidence of mdm-2 immunostaining was significantly higher (P=0.0007) in non-seminomas (NSGCT) than in seminomas (SGCT). The frequency of positive tumor was higher in tumors from metastatic patients than in tumors from metastatic-free patients (P = 0.011). mdm-2 expression was detected significantly more frequently in tumors of advanced stages, i.e. IIB, IIC and III versus tumors of early stages (I and II/A) (P = 0.0098). A significant difference between the three stages of disease as to the expression of mdm-2 (χ^2 = 0.0386) could be established, namely the incidence of mdm-2 expression increased with an increase in stage. Using Westem blotting 22 (68.75 %) out of 32 tumors overexpressed the mdm-2 oncoprotein of 90 kd (p90). Conclusions. mdm-2 expression as detected by immunohistochemistry may provide a reliable prognostic tool to isolate subgroups of patients with more aggressive GCTT.*

The mdm-2 (murine double minute) gene was originally identified in a spontaneously arising tumorigenic murine Balb/c 3T3 fibroblast cell line (1). When stably transfected and amplified in normal NIH3T3 or Rat-2 cell lines, mdm-2 gene converts these cells into a tumorigenic cell line in nude mice (2), suggesting that mdm-2 is a cellular proto-oncogene.

Subsequent studies have demonstrated that mdm-2 may promote the tumorigenicity of the cell by abrogating the tumor suppressor function of wild-type p53 (3). Abrogation of wild-type p53 tumor suppressor function by mdm-2 may be a consequence of inhibition of wild-type p53 trans-activation function by concealing the acidic domain of p53 (4) or by preventing p53 from binding DNA (5), or a consequence of inhibition of p53-mediated apoptosis. (6). The latter finding indicates that tumors expressing high levels of mdm-2 may be less susceptible to apoptosis during chemotherapy and therefore, more prone to drug resistance. Clinical studies have shown that chromosomal position of mdm-2 is altered in human sarcomas (4) and in other tumor types including human malignant gliomas (7), breast cancers (8,9) and non-small cell lung carcinomas (10). Importantly, in human osteosarcoma mdm-2 gene amplification was more frequently observed in metastatic or recurrent than in primary tumors (11), implicating a role for mdm-2 in late stage tumor progression. Bueso-Ramos *et al* (1996) found that mdm-2 overexpression correlates with more aggressive breast carcinomas and with metastatic disease (9). Moreover, Cordon-Cardo *et al* (1993) reported on a good correlation between mdm-2 overexpression and poor prognosis in soft tissue tumors (12). To the best of our knowledge, in GCTT mdm-2 gene was analyzed for amplification and transcripts but not for protein. Therefore, in this study we screened a series of 81 GCTT for their aberrations of mdm-2 expression at the protein levels using standard IHC and WB analysis. The immunoreactivity of the tumors was correlatcd with histology, p53 status, metastatic potential, and clinical stage.

Materials and Methods

Patients. Specimens were obtained by semicastration of 81 untreated patients with testis cancer operated at cooperating hospitals and Semmelweis Medical University, Clinic of Urology in Budapest, Hungary. The mean follow-up period of these patients was 30 months (range from 6 to 72 months). The mean age was 27 years (ranges from

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Key Words: mdm-2 expression, testicular tumors, immunohistochemistly, Western blotting, tumor progression.

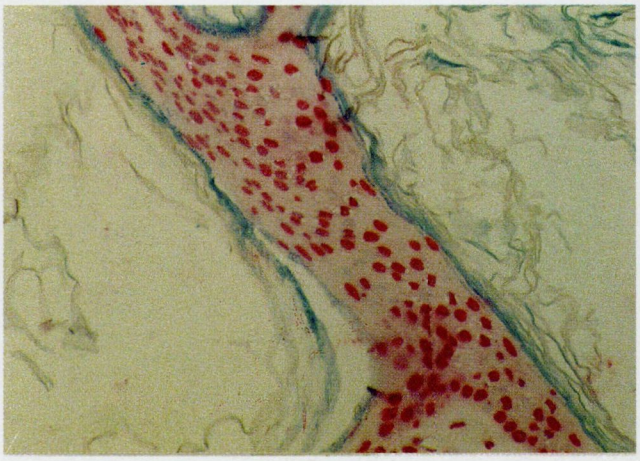

Figure 1. *Nuclear immunoreactivity of teratoma (stage III) for mdm-2 protein (AP, × 100).*

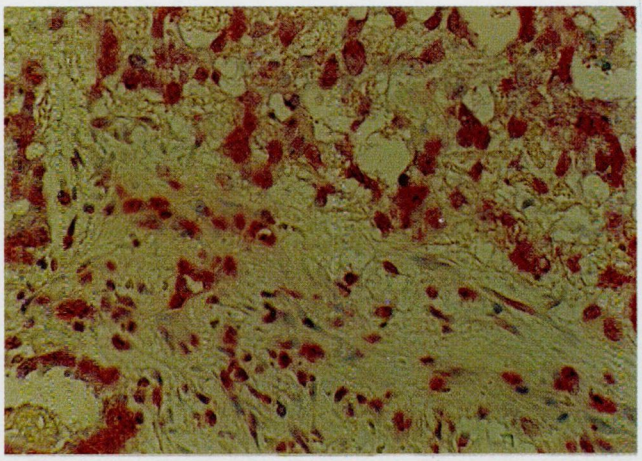

Figure 3. *mdm-2 immunoreactivity of embryonal carcinoma (stage II/C) (AP, × 200).*

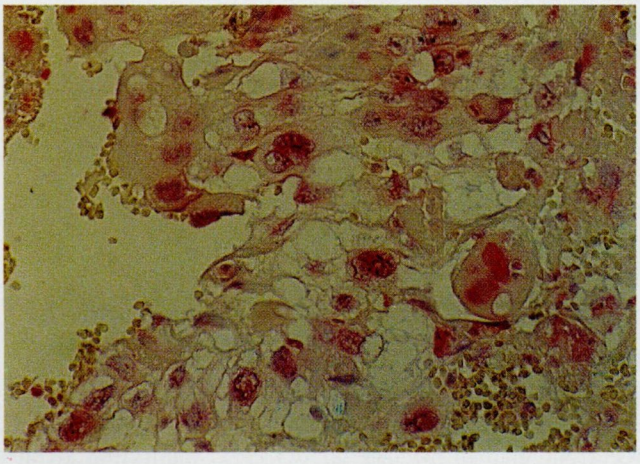

Figure 2. *Expression of mdm-2 oncoprotein of choriocarcinoma (stage II/B)(AP, × 200).*

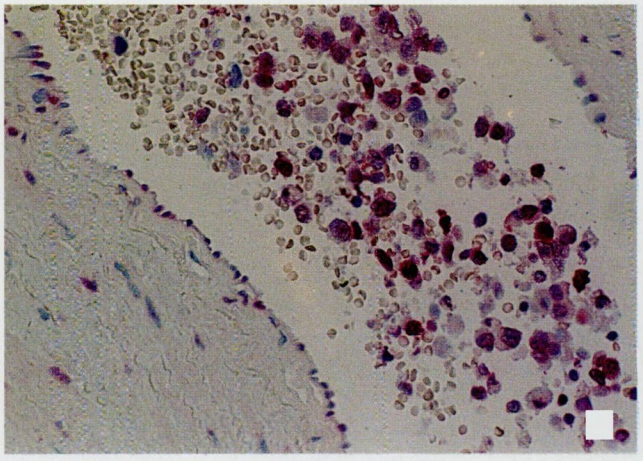

Figure 4. *blood-vessel invasion of mdm-2 positive tumor cells in stage I/B seminoma, (AP, × 100).*

15 to 45 years). A portion of each resected tumor was snap frozen in liquid nitrogen and stored at -80°C until analysis, or fixed in buffered formalin for paraffin embedding and analysis.

Histological examination was performed on haematoxylin and eosin-stained tissue sections. Histological classification of the tumors was according to WHO (13) and identified 26 seminomas (S) and 55 NSGCT. The latter group contained 9 embryonal carcinomas, 2 with yolk sac differentiation, and 6 teratomas. The remaining 38 patients had mixed tumors.

For clinical staging physical examination, haemogram, 12-channel screening profile, serum human chorionic gonadotrophin beta subunit (HCG), alpha-fetoprotein (AFP) (14), chest X-ray, abdominal ultrasound and computed tomography (CT) scan were routinely used. Brain or other organs CT or MRI were performed when clinically warranted. All patients were staged according to UICC classification. (15) There were 37 patients with stage I (localized without nodal involvement), 18 with stage II (regional) and 26 with stage m (distant) disease.

Definition of clinical stage I/A: Negative tumor markers AFP and B-HCG after orchidectomy or marker decay in keeping with the biologic marker half-lives and normal x-rays and CT scans were defined as the criteria of clinical stage I.

Definition of clinical stage IIB: The same criteria of clinical stage I/A with the presence of one or more of the following signs: a) blood or lymphatic-vessels invasion (VI); b) invasion of the rete testis or into tunica albuginea or into epididymis (pT2); c) tumor infiltration into spermatic cord (pT3); and d) tumor volume of > 3 cm for seminomas and > 2 cm for NSGCT.

Immunohistochemical detection of mdm-2 protein. The mdm-2 antigen was detected by the streptavidin-biotin-alkaline-phosphatase immunostaining method as described previously (16,17). Briefly, paraffin embedded tumor sections were routinely deparaffinized and pretreated by immersion of the slides in 1 mM EDTA (pH 8) buffer and exposure to microwave irradiation at 750 W for 5 min. They were then incubated with 1 % bovine serum albumin (Serva/Heidelberg).

Slides were incubated overnight at 4°C with a 1:75 dilution of the primary NCL- MDM2 mouse monoclonal antibody (clone IB10, Novocastra, UK). Sections were washed twicc for 5 min with TBS (0.05M TRIS/HCl pH 7.6, 0.15 M NaCl), incubated with a second antibody [biotinylated anti-(mouse Ig) antibody from Amersham] for 30 min at room temperature and washed twice with TRIS buffer. As a third step the highly sensitive streptavidin biotinylated alkaline-phosphatase complex (Dako) was applied for 40 min. Section were washed twice with TBS, developed with new fuchsin substrate (Dako). Endogenous alkaline phosphatase was inhibited by the addition of levamisol (Dako) to the substrate. Specimens were slightly counterstained with Mayer's haematoxylin, and mounted with glycerol gelatin. As a positive control we used a paraffin-embedded sections from a myxofibrosarcoma known to overexpress mdm-2. Staining without anti-mdm-2 monoclonal antibody was performed as a negative control. Positive and negative controls were included in each staining run and all gave satisfactory results.

Assessment of mdm-2 immunoreactivity. The immunoreactive mdm2 expression was analyzed independently by two observers (M.B. and H.E.). Only cells staining well above the background level were considered to be positive, and reaction product was registered as either present or absent in tumorous nuclei. The samples were scored as - in case of complete lack or equivocal (no obvious) staining, + indicating that 5% or fewer of tumor cell were positive, ++ if there werc more than 5% of tumorous cells positive. For slatistical analysis tumors containing 5% or fewer of positive tumorous cells were considered negative [mdm-2 (-)] while tumors containing more than 5 % of positive tumor cells were considered positive [mdm2 (+)].

Western blot analysis. Tissue samples were homogenized by ultra-turrax at 4 °C in RIPA buffer (50 mM TRIS, pH 7.5, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150mM NaCl) supplemented with 5mM EDTA and I mM PMSF (phenylmethyl-sulfonyl fluoride) Extracts of tissues were clarified by'centrifugation for 15 min. at 12.000 *g* at 4°C and small aliquot was removed for determination of protein (Bio-Rad DC protein assay). Additional protease inhibitors were added to a final concentration of 2 µg/ml leupeptin, 10 µg/ml aprotinin. Equal quantities of protein were electrophoresed on SDS-7.5% polyacrylamide gel (18) and then blotted onto nitrocellulose. After blocking, the membrane was hybridized with the same mdm-2 mouse monoclonal antibody (Novocastra, UK) for overnight at 4°C. After washing the membrane was transferred to the Vectastain ABC-AP reagent according to the instruction of Vector Labs., CA 94010 USA. Visualization was carried out by BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate salt/ nitro blue tetrazolium).

Statistical methods. The two-sided Fisher's exact probability test was used for statistical evaluation of the relationship between mdm-2 immunoreactivity and histological subtype, metastatic potential and clinical stage. The cases were first divided into two subgroups (seminomas *vs.* non-seminomas; no metastasis *vs.* metastatic tumors; early-stage groups, I-II/A, *vs.* later-stage groups, II/B-III) and then dichotomized based on the expression of mdm-2 (negative ≤ 5 % *vs.* positive > 5%). The Chi-square test (χ^2) was performed for statistical evaluation of the relationship between mdm-2 expression and tumor stage. Variables of interest were categorized as: clinical stage (Stage I, II, III), and the expression of mdm-2 (positive *vs.* negative). A probability ≤ 0.05 was considered to represent statistical significance.

Results

mdm-2 expression as detected by Immunohistochemistry. mdm-2 expression and tumor histology: Of the 81 primary testicular tumors studied, 36 (44.44 %), 11 (13.58 %) and 34 (41.97 %) tumors showed -, +, ++ mdm-2 nuclear staining index respectively in a variable fraction of malignant cells (Figures 2 and 3). In general, the distribution of staining was almost exclusively nuclear with only rare cells showing trace of cytoplasmic staining. Histologicaly, all mature teratomas, yolk sac parts, and choriocarcinomas were strongly positive for mdm-2. Positive tumors, *i.e.* tumors expressing mdm-2 in more than 5 % of tumor cells included 4 (11.7 %) of 26 seminomas and 30 (54.45 %) of 55 NSGCT. The incidence of mdm-2 immunostaining was significantly higher (P = 0.0007) in NSGCT than in seminomas (Table I).

mdm-2 expression and metastatic status: Of 37 stage I tumors from metastatic-free patients 10 (27 %) were mdm-2 positive. All 10 tumors were stage I/B and 8 of them showed frank blood-vessel invasion (Figure 4). Of 44 metastatical tumors 24 (54.54%) stained positively for anti mdm-2 antibody *i.e.* mdm-2 expression was significantly more frequent in tumors from metastatic patients than in tumors from metastasis-free patients (P=0.011) (Table II).

mdm-2 expression in relation to clinical stage: The frequency of positive tumors increased from 27 % in stage I to 50 % in stage II and to 57 % in stage III. The difference between the three stages of disease as to the expression of mdm-2 proved to be significant (Chi Square test χ^2 = 0.0386), *i.e.* the incidence of mdm-2 expression increased with an increase in stage (Table III). Tumors of early stage (I, II/A) showed mdm-2 staining in 15 of 49 (30.6 %) cases while tumors of advanced stage *i.e.* II/B, II/C and III expressed mdm-2 in 19 of 32 (59.37%) cases. The difference is also significant (P = 0.0098) (Table IV).

mdm-2 protein overexpression as detected by Western blot analysis. 22 (68.75 %) out of 32 tumors overexpressed the mdm-2 proteins. mdm-2 of p90 was detected in all 22 cases (Figure 5). Of 22 tumors expressing mdm-2 by WB 14 (63.63 %) expressed mdm-2 protein by IHC also. 8 tumors assessed to have negative mdm-2 immunostaining were found to be positive by WB. 5 tumors were negative by WB and positive by IHC. 5 carcinomas did not show mdm-2 expression by both methods.

Discussion

Status of the mdm-2 gene in GCTT was investigated in two studies. Strohmeyer *et al* (1993) found no evidence of mdm-2 gene amplification using Dot blot test (18). Riou *et al* (1995) analyzed mdm-2 gene for amplification and transcripts in a small series of 25 GCTT. Interestingly, mdm-2 gene was found to be amplified in 12 % of the tumors while mdm-2 transcript (mRNA levels) of lhe expected size of 5.5 kb was observed in all 24 tumor specimens and normal testicular tissues that could be analyzed (19). Riou *et al* (1995) raised the question of

Table I. *Relationship betweeen mdm-2 expression and histological subtype.*

Histology	mdm-2 expression		
	mdm-2 (-)	mdm-2 (+)	All cases
Seminomas	22	4	26
Non-seminomas	25	30	55
All cases	47	34	81

Fisher's exact test P = 0.0007

Table II. *Relationship between mdm-2 expression and metastatic potential.*

	mdm-2 expression		
	mdm-2 (-)	mdm-2 (+)	
No metastasis	27	10	37
Metastatic tumors	20	24	44
All cases	47	34	81

Fisher's exact test P = 0.011

Table III. *Relationship between mdm-2 immunoreactivity and clinical stage of germ cell tumors.*

Stage	mdm-2 expression		All cases
	mdm-2 (-)	mdm-2 (+)	
Stage I	27	10	37
Stage II	9	9	18
Stage III	11	15	26
All cases	47	34	81

Chi square test $\chi^2 = 0.0386$

Table IV. *Relationship between mdm-2 expression and clinical stage.*

Stage	mdm-2 expression		All cases
	mdm-2 (-)	mdm-2 (+)	
Early-stage group (I/A and I/B)	34	15	49
Later-stage group (II/B, II/C and III)	13	19	32
All cases	47	34	81

Fisher's exact test P = 0.0098

whether tumors with high mdm-2 mRNA levels also overexpress mdm-2 protein, which could then in turn inactivate the p53 wild type function. Our results indicate that 55.55 % of GCTTs express mdm-2 protein with 42 % expressing mdm-2 in more than 5 % of their tumor cells. In our work however, less mdm-2 oncoprotein expression was detected at the protein levels, both by IHC and WB, than at mRNA levels in the study of Riou *et al* (1995). The explanations for this difference may lie in the following facts. (1) Contamination of RNAs extracts with mdm-2-expressing normal cells, thus leading to some degree of false positivity. (2) mRNA analysis is more sensitive than IHC and WB. (3) Alternatively, certain mdm-2 mRNA can be coded truncated forms of mdm-2 proteins that cannot be recognized by the antibody used. To confirm the immunohistochemical results, we studied mdm-2 protein expression in 32 samples by immunoblotting There was no complete agreement between WB and IHC. 8 tumors interpretated to have negative mdm-2 immunostaining were found to be positive by WB. Of these 8 tumors 3 with diffuse and very week staining showed definite immunoreactivity by immunoblotting. The remaining 5 tumors were presumably contaminated with some normal cells or tissues expressing mdm-2, thus conferring positivity on tumors frec of mdm-2 protein. 5 tumors overexpressing mdm-2 by IHC were negative by WB analysis. IHC seems to be more sensitive than WB analysis since it is able to detect even a single positive cell or subpopulation of positive tumor cells among numerous negative cells. Therefore, we may say that IHC is the method of first choice in detecting mdm-2 protein. Nevertheless, since by Western blot analysis, a positive identification of the molecular weight of mdm-2 can be obtained, which may help in the elucidation of crossreactions in suspected cases, it may help in confirming the presence and quantification of these proteins.

p53 gene mutation is a rare event in GCTT (19,20), indicating that other mechanisms than mutations could be implicated in p53 wt inactivation. In our previous study, we found a highly significant positive relationship between metallothioneins (MT) and p53 proteins, *i.e.* MT was co-overexpressed with p53 in the same tumor cells (17). In the present study a significant proportion (41,97 %) of the same GCT Ts expressed mdm-2. Taking together these data one may speculate that MT, as zinc chelators (21), and mdm-2 oncogene expression may enable GCTT to escape from p53-regulated growth control and explain why these tumors do not need to have mutations or losses of the p53 gene for functional inactivation of this tumor suppressor gene. This result parallels that of Oliner *et al* (1992) in

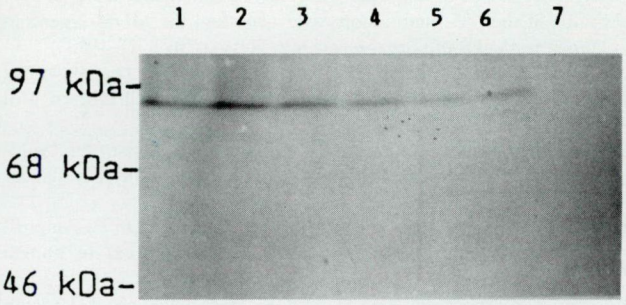

Figure 5. *mdm-2 p90 protein as detected by Western blot in some selected samples of testicular tumors. Lanes contain: 1. myxofibrosarcoma used as positive control; 2. mixed tumor (EC, T, CC); 3. mixed tumor (S, EC, T); 4. embryonal carcinoma with yolk sac differentiation; 5. seminoma; 6. teratocarcinoma; 7. human placenta as negative control.*

sarcomas (22) and Reifenberger *et al* (1993) in malignant gliomas (7).

In previous study we analyzed the same series of GCTT for aberrations in the p53 gene by IHC. In this work 40 (49.38 %) of 81 tumors coexpressed both mdm-2 and p53 proteins, demonstrating that mdm-2 overexpression was not limited to those tumors without p53 alterations, *i.e.* aberrations of p53 and mdm-2 in the same tumor are, in some cases, not biologically redundant. This notice is in agreement with that of Cordon-Cardo *et al* (1994) in soft tissue sarcomas (12).

A direct role of mdm-2 in the terminal differentiation of some adult tissue was envisioned. This role, however, is still not fully elucidated and seems to differ in different tissues. mdm-2 expression was decreased in muscle (23), and increased in skin differentiation (24). In this series of GCTT all mature teratomas, yolk sack parts, and choriocarcinomas were strongly positive for mdm-2, suggesting a role for mdm-2 expression in the differentiation of GCTT. The impact of mdm-2 expression on GCTT differentiation is of interest and remains to be clarified in studies focusing on the relationship between mdm-2 expression and differentiation status of GCTT.

In our present work out of 37 stage I tumors 10 were positive for mdm-2 antibody. All of 10 patients were stage I/B, as defined earlier in this study, with 8 showing signes of blood-vessel invasion (VI) by the primary tumor (Figure 4). There is general consensus that patients with clinical stage I/B, especially who show VI, carry a high risk of relapse and developing disseminated disease. These patients may benefit from adjuvant short-term chemotherapy(25). More prognostic factors, however, are needed to justify the application of chemotherapy. On the base of our results mdm-2 may be such a biological predictor that may help in isolating patients with clinical stage I who may be subjected to the adjuvant chemotherapy.

In the present study, most importantly, the frequency of positive tumor was significantly higher in tumors from metastatic patients than in tumors from metastatic-free patients (Table II) and the incidence of mdm-2 exnression increased significantly with an increase in stage (Tables III and IV), implicating a role for mdm-2 in tumor aggressiveness and progression. This is in keeping with the results of Ladanyi *et al* (1993) in human osteosarcoma (11) and Bueso-Ramos *et al* (1996) in breast carcinoma (9).

In conclusion, our results show that mdm-2 immunoreactivity may provide a reliable prognostic tool to isolate patient subgroups with more aggressive tumors. This tool may be of special importance in identifying patients with clinical stage I testicular germ cell tumor who carry a high risk of developing disseminated disease and may benefit from adjuvant chemotherapy. To validate this hypothesis it is essential however, to perform larger studies in the future.

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Clinical Study

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Three-Year Results of the First Educational and Early Detection Program for Testicular Cancer in Hungary

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Key Words
Testicular cancer · Screening · Early detection · Self-examination · Ultrasound examination · Germ-cell cancer

Abstract
Objective: To determine whether a testicular self-examination-based early-detection program may help in the early diagnosis of testicular cancer. ***Methods:*** Advertisements were placed in the media describing the early signs of testicular cancer, the risk factors, the correct method of self-examination and the importance of early detection. Between April 1995 and April 1998, 5,056 men underwent physical and ultrasound examination of the testicles, and in case of suspicious findings tumor markers were checked. ***Results:*** Testicular tumors were found in 1.28% of the men with symptoms. No tumors were found in men without symptoms or in men with pain, sensitivity to palpation, or complaints unrelated to the testicle. Of those with a palpable lump or swollen testicle, 4.5 and 3.9% were found to have a tumor. In total, 28 testicular cancers (15 seminomas and 13 nonseminomas) in 26 volunteers and 4 benign tumors were detected. The occurrence of cancer was most frequent in the age group of 15–40 years (1.6%). ***Conclusion:*** The rate of cancer detection and the detected seminoma rate in the program are not sufficient to justify a widespread early detection program for testicular cancer (examination of men who reveal testicular abnormalities by self-examination) despite the increased tumor incidence. Early diagnosis should be based on an educational program for the population at risk, the training of staff engaged in the health care of the young, and the use of early ultrasound examination in men with palpable lumps and swollen testicles, especially in young men.

Introduction

Testicular tumor accounts for approximately 1% of all male neoplasms and is the most common solid tumor between 15 and 35 years of age. The incidence of germ-cell testicular tumors has been increasing, and the increase in incidence has been described mainly in younger men [1]. The prognosis of germ cell tumors has improved markedly following the introduction of cisplatin-based chemotherapy [2]. Approximately 48–93% of the patients with testicular cancer are cured following therapy in

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Europe [3]. The chance of cure depends on histology, and patients with pure seminoma have the best prognosis [4]. The prognosis is less favorable in eastern European countries [5], and the mortality rate of testicular cancer in these countries may be explained by a delay in the institution of cisplatin-based chemotherapy [3]. The differences in the cure rate of cancer might also be due to national characteristics, variance in the quality of care and in the incidence of advanced stages at diagnosis due to diagnostic delay [6].

Many patients discover their tumors following the onset of symptoms, and only seek medical advice after a delay of several months. Another cause of delay is the failure of physicians to make the correct diagnosis at the time of the first medical consultation [7]. A third potential problem is the use of inappropriate treatment, which can be avoided by treatment in specialized centers [8]. It has been shown that a delay of more than 3 months is correlated with a decreased 5-year survival [9]. Since the introduction of cisplatin-based chemotherapy in 1979, our department in the National Institute of Oncology in Hungary has been the main center for chemotherapy of germ cell tumors. We register about 230 new testicular cancer patients annually, which represent about 95–98% of the patients receiving chemotherapy for testicular cancer in our country. The chance of cure for patients with a poor prognosis, and for those with relapse or resistance to cisplatin, is poor because of the lack of treatment modalities such as intensified or high-dose chemotherapy, and the limited availability of new drugs such as paclitaxel, gemcitabine and oxaliplatin [10, 11]. The incidence of potential resistance to cisplatin-based chemotherapy may be relatively higher because of the more advanced stages and the resultant diagnostic delay. Most of our patients are not familiar with the early signs of testicular tumor and have not received any information regarding testicular cancer and testicular self-examination before the time of diagnosis. For this reason, we started an educational and early detection program for testicular cancer in cooperation with the Hungarian Cancer League in 1995.

Patients and Methods

Information describing the early signs of testicular cancer, the risk factors, the correct method of self-examination and the importance of early detection were provided in the media (television, radio, widely read papers, youth magazines and at all events organized by the League). Men who responded were given an appointment to have a medical examination. For educational reasons, recruitment was not limited to age groups or complaint categories as we wanted to analyze the distribution of volunteers by demographic characteristics and the presence or absence of complaints. The medical examination consisted of physical and ultrasound examination of the testicles. The volunteers were given a demonstration of the correct method of testicular self-examination and were provided with additional informative material. In any case of suspicious findings, tumor markers (AFP, β-hCG) were also checked.

An Acuson 128 PX ultrasound device with a 7.5-MHz linear transducer was used for the testicular ultrasound examination (TUS). Images of each testis were obtained in traverse and sagittal planes individually. Additional views and techniques were occasionally performed according to the clinical condition. A single type of nonmalignant pathological finding in a volunteer was considered as one pathological event regardless of bilateral or multiple appearances, for example testicular cysts or hydroceles. The sonographic criterion of discovered abnormalities was based on three clinically experienced authors using the same diagnostic guideline [12].

The clinical staging of testicular tumors was carried out using the TNM classification of malignant tumors proposed by UICC in 1997 [13]. The histological classification of the tumors was done according to the WHO criteria [14].

The complaints and pathological findings of all volunteers were analyzed and compared. The volunteers were divided into two main groups based on the presence or absence of complaints. The first group was subdivided by the nature of the complaint observed through testicular self-examination: pain, sensitivity to palpation of the testicle, palpable lump, swelling of the testicle or a complaint unrelated to the testicle. If multiple complaints were present at the time of the examination, we classified the patients according to their most important complaint. The clinical details of the 30 patients with a testicular tumor are also presented.

The delay in the diagnosis of patients treated with chemotherapy in the chemotherapy department at the National Institute of Oncology, Budapest, in 1994 and in 1998 was also retrospectively analyzed and compared to measure the educational impact of the program. All details were entered into the institutional computer database at the time of the first observation. Diagnostic delay was defined as the time period between the first appearance of any symptoms and the date of the diagnosis of testicular cancer. Medical delay was defined as the time period between the first medical consultation following symptoms and the date of the diagnosis. The mortality rate of testicular cancer in Hungary was also analyzed between 1994 and 1998.

The proportions of the findings between patients with and without complications were compared using the χ^2 test. The diagnostic and medical delays between 1994 and 1998 were analyzed by Student's t test. A difference was regarded as significant if the p value was ≤ 0.05. According to the institutional protocol based on the TNM [15] classification (1993), the treatment of advanced germ cell tumors (stage II/C, III) was primary chemotherapy with bleomycin, etoposide and cisplatin. In stage I seminoma, radiation was given if there was an involvement of the epididymis, funicular spermatic cord, capsule or rete testis, if the primary tumor was bigger than 3 cm, and for stages II/A/B with para-aortic and ipsilateral iliac lymph node involvement. For primary nonseminomas larger than 2 cm and having the above-detailed criteria, retroperitoneal lymphadenectomy (RLA) was performed and followed by adjuvant chemotherapy: two to four cycles of the cisplatin, vinblastine and bleomycin combination were given depending on patient histology and tumor stage [16]. For tumors with vascular invasion, systemic chemotherapy was applied. A wait-and-see policy was used for patients with stage I tumors (without the above-mentioned criteria) if the pri-

Table 1. Distribution of findings in the complaint-free population and in the population with complaints

Findings	Complaint-free population (2,714 volunteers)		Population with complaints (2,342 volunteers)		p value	Complaint-free population 1,599 findings %	Population with complaints 2,194 findings %
	n	%	n	%			
Epididymal and testicular cyst	526	19.4	676	28.9	<0.001	32.9	30.8
Testicular atrophy	124	4.6	136	5.8	0.06	7.8	6.2
Hydrocele	480	17.7	585	25.0	<0.001	30.0	26.7
Epididymitis	39	1.4	232	9.9	<0.001	2.4	10.6
Varicocele	399	14.7	497	21.2	0.10	25.0	22.7
Tumor	0	0.0	30[a]	1.3	<0.001	0.0	1.5
Microcalcification	11	0.4	11	0.5	0.73	0.7	0.5
Other	20	0.7	25	1.1	0.22	1.3	1.1

[a] 30 patients with 32 tumors.

mary tumor was localized only in the testis. Nevertheless, chemotherapy was applied if the patient did not accept the wait-and-see policy. The main goal of this paper was to determine, by an analysis of the results of the first 3 years, the efficacy of such a program on the early detection of testicular cancer.

Results

Between April 1995 and April 1998, 5,056 volunteers participated in the program. The median age was 42 years (range 16–76 years), and 32 tumors were diagnosed in 30 patients (0.6%). Among the 5,056 volunteers, 2,714 were complaint free and 2,342 patients presented different complaints. In the complaint-free population 1,323 men had no physical or radiology findings (49%), but in the remaining 1,391 men, 1,599 different findings were detected by physical examination and/or TUS. No tumors were found in the complaint-free population. In the 2,342 men with different complaints, 532 (23%) had no detectable findings, but in the remaining 1,810 men, 2,194 findings were discovered. The incidence of patients with tumors in this group was 1.66%, representing 1.5% of the findings detected (table 1). The incidence of men having tumor in the group of 2,342 volunteers with complaints was 1.28%. In the group of volunteers with complaints, more patients with abnormal findings were detected (77 versus 51%, $p < 0.001$) than in the complaint-free population. Cysts ($p < 0.001$), hydroceles ($p < 0.001$) and epididymitis ($p < 0.001$) occurred more frequently in the group with complaints. A history of cryptorchidism was noted in 1.9% of the men with complaints and in 0.8% of the complaint-free population ($p < 0.001$). Testicular hypoplasia and microcalcification did not differ significantly between the two groups.

The volunteers with complaints were subdivided according to the main symptoms as follows: 464 patients (20%) had a palpable lump, 228 (10%) had a swollen testicle, 472 (20%) had testicular pain, 897 (38%) had sensitivity to palpation of the testicle and 281 patients (12%) had symptoms unrelated to the testicles, such as dysuria, scrotal pruritus or impotence.

No tumors were found in the group with pain, sensitivity, or complaints unrelated to the testicle. The percentage of men with various abnormalities in these groups were 79 (373), 71 (363) and 67% (186), respectively, and consisted mainly of cysts, hydroceles and varicoceles (table 2). Hydrocele was the most frequent finding in men (56%) with a swollen testicle. In men with a palpable lump, cysts and varicoceles were observed most frequently.

Patients with clinically detected significant abnormalities were referred to an urologist (3.9 versus 0.9% in the complaint and complaint-free group). The remaining patients were informed on their abnormalities and were directed to a general practitioner, with suggestions for treatment if appropriate. Among the 464 men who palpated a lump, 64 (14%) had no abnormal findings detected but in the remaining 400 men 477 abnormalities were discovered, among them 22 tumors. The incidence of men with tumors in this group was 4.5%, representing 4.6% of all abnormalities. Among the 228 men whose main complaint was a swollen testicle, 13 (5.4%) had no abnormalities detected, but in the remaining 215 men there were 249 findings, and 10 tumors were detected. The incidence of patients with tumor was 3.9% in this

Table 2. Findings according to the main complaints of the volunteers

Findings	Pain 373 men 457 findings		Sensitivity 636 men 782 findings		Palpable lump 400 men 477 findings		Swollen testicle 215 men 249 findings		Unrelated compla 186 men 229 findings	
	n	%	n	%	n	%	n	%	n	%
Epididymal and testicular cyst	125	27.4	246	31.5	207	43.4	46	18.5	52	22.7
Testicular atrophy	20	4.4	63	8.1	11	2.3	10	4.0	32	14.0
Hydrocele	103	22.5	209	26.7	75	15.7	139	55.9	59	25.8
Epididymitis	62	13.6	88	11.2	50	10.5	19	7.6	13	5.7
Varicocele	141	30.9	169	21.6	98	20.6	21	8.4	68	29.7
Tumor	0	0.0	0	0.0	22	4.6	10	4.0	0	0.0
Microcalcification	2	0.4	4	0.5	2	0.4	0	0.0	3	1.3
Other	4	0.8	3	0.4	12	2.5	4	1.6	2	0.8

group, representing 4% of all detected abnormalities (table 2). During the 3-year period, 4 benign testicular tumors were discovered among 5,056 volunteers (0.08%). The histological findings were: cavernous hemangioma, dermoid cyst, Leydig cell tumor and adenomatoid tumor. Only 1 additional volunteer had a testicular exploration because of suspicion of a tumor, but the histology showed granulomatous orchitis. Among the 26 men with testicular cancer, 2 patients with bilateral synchronous seminomas were detected.

In the group with complaints, the occurrence of testicular cancer was most frequent (1.6%) in the age group of 15–40 years. Only 3 testicular cancers were detected in men over the age of 45 (0.3%), including 2 with seminomatous tumors (table 3). Among the 26 men with germ cell testicular tumors, 19 stage I tumors were detected and 16 were diagnosed within 12 weeks after the onset of the symptoms. The medium age was 33 years (range of 20–48 years) and the overall median duration of complaints was less than 12 weeks (range 1–48 weeks). Fifteen (2 bilateral) seminomas, and 13 non-seminoma tumors were diagnosed. The clinical stages were: 9 I/A, 9 I/B, 1 I/S, 3 II/A, 1 II/B, and 2 III/B (UICC 1997). One patient refused further treatment and was lost to follow-up. Tumor markers were elevated in 8 patients, 7 with increased β-hCG and 4 with increased AFP.

According to the classification of the International Germ Cell Cancer Collaborative Group classification [17], all patients belonged to the good prognostic group, except for the patient who was lost to follow-up after orchidectomy. Selected treatments following orchidectomy were in line with institutional policies. All of our treated patients are in complete remission and are probably cured of their disease.

Six out of 7 RLA were nerve sparing, and the 7th wa: partial RLA. Two patients developed retrograde ejacul tion. In 1 patient with clinical symptoms, radiological e dence of pulmonary fibrosis was observed after prima chemotherapy with bleomycin. The median follow-up the time of the writing of this paper was 36 months (16–4 months).

The median duration of complaints in the unilater seminoma and in non-seminoma testicular cancer pa tients was identical: less than 12 weeks. The seminom group was composed only of stage I tumors while in th other group 50% of the cases had more advanced stages Five patients with seminoma received chemotherapy be cause of vascular invasion, and all but 1 patient received chemotherapy in the non-seminoma group.

A retrospective analysis of the diagnostic delay of germ cell testicular cancer patients treated with chemotherapy in our department in 1994 and in 1998 (excluding the patients detected in the program) did not reveal a significant difference ($p = 0.58$). There is a non-significant tendency for a decrease in the duration of medical delay in favor of 1998 especially in stage III patients (table 4). The changes in the mortality rate of testicular cancer in Hungary between 1994 and 1998 are demonstrated in table 5.

Conclusions

During the 3-year study period, 732 new testicular cancer patients (26% seminoma) were treated with chemotherapy in our department and annually 60–75 patients with radiotherapy in the National Institute of Oncology. It implies about 300–310 new testicular cancer patients annually in our Institute. The estimated number of new

3. Testicular germ cell tumors found in 5,056 volunteers between April 1995 and April 1998

Age years	Side	Symptom duration weeks	Size of tumor by US cm	Palpation of the testicle	Tumor (TNM) 1997	Stage (UICC) 1997	His-tology	Treatment: orchidectomy +	AFP	β-hCG	LDH
39	L	24	3.0×3.5×2.2	lump	T/2	II/A	NS	nRLA + 4 VPB	77	<4	371
48	R	8	3.6×4.8×3.0	swelling	T/2	I/B	S	2 VPB	<5	<4	452
36	D	16	2.4×1.1×1.2	lump	T/1	I/A	S	wait and see	<5	<4	620
			0.8×0.9×10	lump	T/1						
34	R	1	2.6×2.5×1.4	hard surface	T/2	I/B	S	2 VPB	<5	26	376
20	R	12	4.2×4.5×4.4	swelling	T/1	II/B	NS	RLA + 4 VPB	<5	27	401
26	L	3	4.5×3.6×2.5	swelling	T/1	I/A	S	irradiation	<5	<4	534
48	R	24	5.5×3.5×4.5	swelling	T/1	I/A	S	irradiation	<5	<4	481
39	R	12	6.5×4.5×5.0	swelling	T/1	I/S	S	2 VPB	<5	<4	2,746
28	L	24	2.5×1.9×1.5	lump	T/1	I/A	S	wait and see	<5	<4	412
33	R	16	1.0×0.8×1.5	lump	T/1	II/A	NS	nRLA + 4 VPB	<5	<4	312
35	D	16	1.1×1.9×1.7	lump	T/1	I/A	S	irradiation	<5	<4	397
			0.8×1.6×1.5	non-palpable	T/1						
24	R	48	3.2×3.3×2.7	lump	T/1	I/A	NS	nRLA + 2 VPB	<5	<4	433
45	R	2	3.2×2.3×2.8	lump	T/2	III/B	NS	RLA + 6 BEP	<5	<4	509
23	R	2	3×2×2	lump	T/2	I/B	NS	2 VPB	686	689	359
26	R	2	2×1.5×1	lump	T/2	I/B	NS	2 VPB	<5	<4	424
45	R	4	5.1×4×4.5	swelling	T/2	I/B	S	2 VPB	<5	<4	590
20	R	8	1.7×1.5×1.2	swelling	T/2	I/B	NS	2 VPB	<5	<4	307
33	L	24	2×2.1×1.9	lump	T/2	I/B	S	2 VPB	5	<4	386
36	L	4	2×1.1×0.9	lump	T/1	I/A	S	irradiation	<5	<4	452
29	R	20	3.5×2.8×2.9	lump	T/2	I/B	S	2 VPB	<5	<4	526
34		1	2.5×2×2.2	lump	T/1	I/A	S	irradiation	<5	<4	570
26	R	1	3.8×4×3.3	swelling	T/2	I/B	NS	2 VPB	302	163	243
27	R	1	2.5×2×1.8	lump	T/2	III/B	NS	nRLA + 6 BEP	<5	25	491
47	L	24	2.7×2.5×2.2	lump	T/1	II/A	NS	nRLA + 4 VPB	6	336	401
23	R	4	0.9×0.8×0.9	lump	T/1	I/A	NS	wait and see	<5	<4	283
41	R	4	3.5×4×3.6	swelling	T/1	I/S (?)	NS	refused	1,272	375	508

LA = Nerve-spearing RLA; VPB = vinblastine, cisplatin, bleomycin; BEP = bleomycin, etoposide, cisplatin;
S = ultrasound; L = left; R = right; D = duplex; S = seminoma; NS = non-seminoma.

4. Diagnostic and medical delay in germ cell testicular cancer patients treated with chemotherapy in 1994 and 1998[a] in the chemother-partment at the National Institute of Oncology, Budapest

ostic delay[b]							Medical delay[c]				
n	1994		n	1998		p value	1994		1998		p value
	mean	range		mean	range		mean	range	mean	range	
87	14	1–48	86	13	1–96	0.66	3	0–32	3	0–64	0.78
95	18	2–96	76	16	1–72	0.33	4	0–40	5	0–48	0.77
48	26	2–72	52	27	1–112	0.78	11	0–48	7	0–44	0.07
230	18	1–96	214	17	1–112	0.58	6	0–48	4	0–54	0.33

cludes men diagnosed in the current program.
me between the first appearance of any symptoms and the date of diagnosis.
me between the first medical consultation related to the symptoms and the time of diagnosis.

Table 5. Testicular cancer mortality rate in Hungary between 1994 and 1998

Year	Deaths	Death rate/ 100,000 men
1994	45	0.92
1995	53	1.08
1996	62	1.27
1997	40	0.82
1998	41	0.84

testicular cancer patients in Hungary is about 320–330/ year. These data reflect the centralized treatment strategy in our country.

In the 3-year study period, 5,056 volunteers were examined and 32 tumors were found in 30 patients, all among the 2,342 volunteers with complaints. These data confirm that screening of asymptomatic patients does not necessarily lead to the detection of tumors [18], and the incidence of detected tumors is low even in volunteers with complaints in spite of the increasing incidence of testicular cancer. Surprisingly, about 50% of the volunteers without complaints had malformations detected mainly by TUS, but only 0.9% required further urological treatment. The probability of detecting coexisting pathology was much higher in the population with complaints than in volunteers without complaints, mainly hydrocele, cysts, and epididymitis. However, the prevalence of pathology which required further urological health care was still low. No tumors were detected among men with pain, with sensitivity to palpation and with symptoms unrelated to the testicle. In such patients physical examination appears to be sufficient at the first medical consultation. If the onset of palpable abnormality had been more than 2–3 months prior to the time of the first consultation or if it persisted in spite of the treatment, TUS is indicated. In men with a lump or a swollen testicle, about 90% of patients were found to have various abnormalities and the incidence of tumor was 4.5 and 3.9%, respectively. In the case of a palpable lump and/or a swollen testicle, TUS is obligatory to aid in the physical examination at the time of the first consultation, especially in young men.

Among the 26 germ cell tumor patients, 13 had seminomas and all had stage I tumors. Among the 12 nonseminoma patients, 4 had clinically detected regional metastases, and 2 had hematogenous dissemination. These data suggest that a seminoma is detected more frequently and earlier in an early detection program. Because of the early stages and the high percentage of seminoma, the tumor markers aided in cancer diagnosis in only 8 cases. In most cases, the diagnosis was based on the physical and ultrasound examination, confirmed by histology. Tumor markers had a limited role in an early detection program.

Testicular cancers that were detected following the program were restricted largely to those in the age range of 15–40 years (1.6%), however the low incidence of detection and the seminoma rate detected in our patient population (48%) do not justify an early detection program even in this age group [19, 20], in spite of the increasing testicular cancer incidence.

Among the 32 tumors detected, 4 were benign. Testicular exploration helped to identify benign lesions, and allowed testicular preservation in 2 cases [21]. In 2 cases orchidectomy was carried out because of the large size of the tumor. The detected frequency (7%) of bilateral germ cell tumors in the program is probably a statistical artifact, the incidence of bilateral synchronous testicular tumors quoted in the literature is about 0.5% [22].

During the 3-year period, only 3–4% of the estimated testicular tumors were discovered by the program in Hungary. Although early detection might help in the identification of some testicular cancers, the efficiency of the program is limited. The effect of the program on outcome is uncertain since the contribution of early detection to the probable 100% cure rate cannot be estimated. The majority of testicular cancers diagnosed were stage I tumors, and all of the treated patients belonged to the good prognostic group: this fact made it possible to apply less aggressive treatment and improve the patients' quality of life.

Concerning the educational aspect of the program, we did not observe a significant decrease in the diagnostic and medical delay in the patient population treated with chemotherapy in our department between 1994 and 1998. A tendency for the medical delay to decrease may be promising, suggesting greater awareness of the need for treatment, especially among stage III patients. The impact of this educational and early detection program for testicular cancer mortality cannot be justified, but the 3-year study interval may be too short.

In conclusion, despite the increasing incidence of testicular cancer, the widespread use of an early detection program, the examination of patients who reveal symptoms through testicular self-examination cannot generally be recommended [23]. Although early detection is fundamental to treatment strategies that aim to cure patients with minimum side effects and smallest possible costs [24], the overall impact of our program was small. Early

diagnosis should be based on an educational program for the population at risk, the appropriate training of doctors and staff engaged in the health care of the young, and the use of early ultrasound examination for men with palpable lumps and swollen testicles, especially in young men.

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ORIGINAL PAPER

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The incidence, prognosis, clinical and histological characteristics, treatment, and outcome of patients with bilateral germ cell testicular cancer in Hungary

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Abstract *Purpose*: To examine the incidence, prognosis, clinical and histological characteristics, treatment, and outcome of patients with bilateral testicular cancer in the referral center in Hungary, to determine which parameters might predict a second testicular tumor. *Methods*: Clinical parameters—such as time of original surgery, histology of primary tumor, extent of the disease, serum marker concentrations, history of testicular abnormalities, treatment, response to treatment, follow-up period, data on second carcinoma—of bilateral testicular tumors among the 2,386 patients with testicular cancer treated between November 1988 and November 1998 were analyzed. *Results*: The incidence of patients with synchronous testicular tumor was 0.8% (19 of 2,386 patients). The clinical stages were 8 I/A, 5 I/B, 1 II/A, 2 II/B, 1 III/A, and 2 III/B. Median follow-up time was 93 months and the 5-year overall survival was 84%. The incidence of patients with metachronous testicular cancer (median age 28 years and 35 years at first and second tumor diagnosis) was 2.2% (53 of 2,386 patients) and the median time to second tumor was 76 months (range 18–203 months). The clinical stages at the first and second tumor diagnosis were: 14 I/A, 21 I/B, 15 II/A, 2 II/B, and 1 III/B, and 26 I/A, 16 I/B, 3 II/A, 1 II/B, 7 III/B, respectively. The median follow-up time was 42 months and the 5-year overall survival was 93%. In thirteen patients with metachronous cancers, two family histories of testicular cancer, five cases of undescended testicles, seven cases of testicular atrophy, and one case of azoospermia were detected. There was a non-significant trend to a longer cancer interval after chemotherapy and radiotherapy and a tendency to a greater incidence of asynchronous seminoma after chemotherapy. Clinical stage I tumors were more frequent in the surveyed group than among patients not followed up according to the institutional protocol ($P = 0.01$), but the survival rate was good in both groups. Seminoma as a second tumor was diagnosed in an older age group (median 38 years, range 25–49 years) than nonseminoma (median 32 years, range 21–51 years, $P < 0.045$). The interval till the appearance of a metachronous testicular cancer depended on tumor histology: in seminoma cases it was longer than in nonseminoma cases (median time: 121 months versus 50 months, $P = 0.002$). *Conclusions*: The overall incidence of bilateral testicular cancer in the referral center in Hungary was 3%. We could not identify clinical factors which predicted a higher risk for metachronous testicular cancer. With regular follow-up the early diagnosis of second testicular tumors is probable; therefore education, self-examination of the remaining testicle, and long-term follow-up are important in early detection.

Keywords Early detection · Bilateral germ cell tumor · Testicular cancer · Metachronous tumor · Self-examination

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Introduction

Testicular cancer is one of the most common cancers among men of 15–35 years of age, and the incidence is increasing (Bergstrom 1996). In developed countries the overall survival rate of newly diagnosed patients with testicular germ cell cancer (GCTT) is good, about 90–95% (Einhorn 1997; Hartman 1999). In this population the risk of second primary testicular cancer is 2.5–5%. (Dieckmann 1996). The risk is higher for patients with nonseminomatous cancer and for patients younger than 30 years of age at the time of diagnosis (Osterlind 1991; Wanderas 1997). Most second testicular tumors are discovered by the patients following the onset of symptoms. Regular follow-up may allow earlier diagnosis of a second tumor (Sonneweld 1997).

In this study we analyze the incidence, prognosis, clinical and histological characteristics, treatment, and outcome of patients with bilateral GCTT, who have been treated in the referral center at the National Institute of Oncology in Hungary. We investigate which clinical parameters might predict a metachronous testicular tumor, and whether regular follow-up may help in the early diagnosis of second testicular cancers and have an influence on patient outcome.

Patients and methods

Between November 1988 and November 1998, 2,386 testicular tumor patients were registered, and 72 bilateral germ cell cancer patients were retrospectively explored.

Detailed information on patient characteristics—such as time of original surgery, location and histology of primary tumor, extent of the disease, serum concentrations of human β-chorionic gonadotropin (β-hCG), α-fetoprotein (AFP), and lactate dehydrogenase (LDH), history of testicular abnormalities, treatment, response to treatment, follow-up period, and data on second carcinoma—were obtained from the patients' hospital record.

The clinical staging of testicular tumors was by the TNM classification of malignant tumors proposed by UICC (Sobin 1997). The histological classification of the tumors was in line with WHO criteria (Mostofi 1986). Before treatment all pathological specimens of 72 patients were reviewed by experts in our institute to verify the histological subtypes of both the primary and the secondary tumors.

The patients were divided into two main groups based on the synchronous or metachronous appearance of bilateral testicular cancer. The interval between first and second testicular tumors was defined as the time period between surgical removal of the tumors. Metachronous testicular tumor was defined if this interval was longer than 6 months, and testicular ultrasound examination did not show contralateral testicular mass at the time of the first operation.

Men with metachronous tumors were further subdivided into two subgroups. Patients who were followed in the institutional surveillance program after treatment of the first cancer were enrolled in group A. Those who were lost to follow up or who did not participate in the last two scheduled follow-up appointments before diagnosis of second testicular cancer were registered in group B.

Our current follow-up policy for patients who achieve complete remission after treatment of testicular cancer involves a history and physical exam, including palpation of contralateral testis, laboratory tests with tumor markers, chest X-ray every month in the first year, and every 2 months, 3 months, 4 months, and 5 months in the second, third, fourth, and fifth year, respectively, every 6 months in years 6–10, and then annually. CT-scans are done at least every 3 months in the first year, every 6 months in the second and third year, and annually in years 4–5. Thereafter, annual US is used, but if there are suspicious findings or signs, a CT scan is undertaken.

The treatment policy for first testicular cancers have been published elsewhere (Géczi 2001). Radical inguinal orchidectomy was standard treatment for testicular cancer. Chemotherapy was used for treating metachronous GCTT that had spread beyond the testis in patients who received retroperitoneal irradiation or lymphadenectomy for the first cancer. A wait-and-see policy (introduced in 1995) was used for patients with tumor localized to the testis, but chemotherapy was applied in the case of vascular invasion. Oral testosterone replacement was used in all cases. Evaluation of response to treatment was in line with generally used criteria for testicular cancer.

We analyzed the distribution of main histological subtypes, clinical stages, treatments, survival, and risk factors (cryptorchidism, infertility, atrophic testis, familial history of testicular tumor) in the groups with synchronous and metachronous testicular cancers. We investigated the histological characteristics, the proportion of pure seminoma, the presence of vascular invasion, and the presence of embryonal components in the first and second metachronous cancers, and the effects of previously applied treatment on the incidence of the secondary tumors. The interval between tumors was analyzed in relation to the patient's age, previously applied treatment and histological subtypes. Statistical analyses were performed using SPSS for Windows (version 10.07; SPSS, Chicago, Ill., USA). Categorial variables were compared by the Chi-square test or Fisher's exact test, as appropriate. Continuous variables were compared by the Wilcoxon Mann Whitney test. Binomial-related variables were compared by McNemar's test. The Kaplan-Meier method was used to evaluate survival. The closing date for our collection of data relative to living patients is November 2000.

Results

Incidence of bilateral germ cell testicular cancer

Of 2,386 patients with testicular tumor, 72 patients were retrospectively found to have bilateral testicular carcinoma (3%) (95% CI 2.3–3.7); 19 cases (0.8%) (95% CI 0.4–1.2) with synchronous tumors; and 53 cases (2.2%) (95% CI 1.6–2.8) with metachronous tumors.

Synchronous testicular germ cell cancer

The median age of the 19 synchronous tumor patients was 38 years (range: 19–71 years) at the time of orchidectomy. The clinical details of patients is presented in Table 1. Thirteen patients (68%) with bilateral seminoma were diagnosed. Three patients (16%) had a history of cryptorchidism and hypoplasia, and three patients were younger than 30 years at the time of operation. No patients had a familial history of testicular cancer. Stage I tumors were diagnosed in 13 (68%) cases. Chemotherapy was applied in five I/B stage tumors and also in five advance cases. Six patients were offered radiotherapy, but one refused it and received chemotherapy. Another patient of the six, who had anaplastic seminoma, received both radiotherapy and chemotherapy. Retroperitoneal lymphadenectomia was used only in two cases. In two cases a wait-and-see policy was applied. After primary treatment, 17 (90%) CR and one PR was obtained. One patient was lost to follow-up after castration. Fifteen patients (79%) are alive with no evidence of disease. The 5-year overall survival was 84% (Fig. 1); three patients died, two due to tumor progression. The median follow-up time was 93 months.

Metachronous testicular germ cell cancer

Incidence, risk factors, and appearance

Fifty-three of 72 patients (74%) had metachronous GCTT. The median time to the development of second

Table 1 Clinical characteristics of synchronous germ cell testicular cancer in the National Institute of Oncology in Hungary between 1988 and 1998 (n = 19)

Median age (years)	38
Range (years)	19–71
Histology, no. of patients (%)	
Bilateral seminoma	13 (68%)
Bilateral nonseminoma	3 (16%)
Seminoma and nonseminoma	3 (16%)
Clinical stages (WHO 1997), no. of patients (%)	
I/A	8 (42%)
I/B	5 (26%)
II/A	1 (5%)
II/B	2 (11%)
III/A	1 (5%)
III/B	2 (11%)
Type of treatment (orchidectomy +)	
Retroperitoneal lymphadenectomy	2 (11%)
Chemotherapy	12 (63%)
Radiotherapy	5 (26%)
Wait-and-see	2 (11%)
Lost	1 (5%)
Follow-up status, no. of patients (%)	
Alive	15 (79%)
Dead of disease	2 (11%)
Dead of other cause	1 (5%)
Recurrence or progression	2 (11%)
Lost to follow-up	1 (5%)
Estimated survival, % (95% CI)	
At 5 years	84% (61–94)
Median follow-up time, range (months)	93 (38–150)

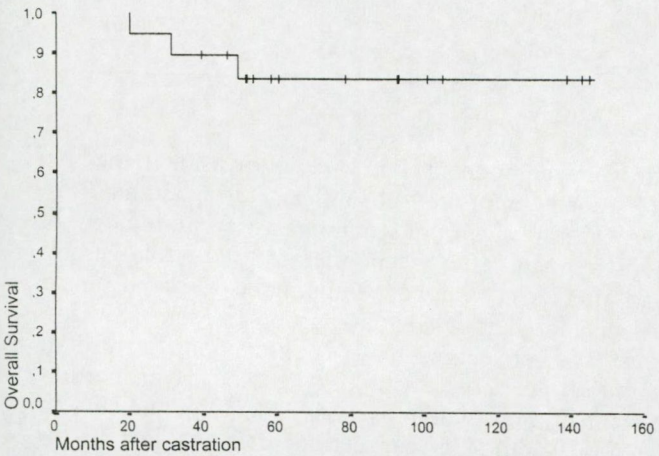

Fig. 1 Median follow-up = 93 months (range: 38–150 months). At 5 years, the rate of overall survival is 84%. The survival calculation were performed from the time point of orchidectomy to the last follow-up visit, or to the time of patient's death

tumor varied from 18 months to 203 months (median 76 months). The median age at the time of first and second tumor diagnosis was 28 years (range 16–41 years), and 35 years (range 21–51 years), respectively. Among the 53 patients with metachronous GCTT, two patients (4%) had a family history of GCTT, five (9%) testicular maldescent, seven (13%) testicular atrophy, and one (2%) azoospermia. Thirty patients (57%) were younger than 30 years at the first tumor diagnosis. The primary tumor was located in the right testis in 29 patients (55%). After the first operation 21 (40%), 16 (30%), and 15 (28%) of metachronous GCTT were diagnosed in the first, second, and third 5-year periods, respectively. Only one (stage I) seminoma was found 15 years after the first orchidectomy.

Table 2 Clinical characteristics of patients with metachronous germ cell testicular cancer at the National Institute of Oncology in Hungary between 1988 and 1998. (*AFP* alfa-fetoprotein, *β-hCG* beta human chorionic gonadotropin, *RLA* retroperitoneal lymphadenectomy, *ChT* chemotherapy, *Rt* radiotherapy)

	First tumor (n = 53)	Second tumor (n = 53)
Age (years)		
Median (years)	28	35
Range (years)	16–41	21–52
Clinical stages, no. of patients (%)		
I/A	14 (26%)	26 (49%)
I/B	21 (40%)	16 (30%)
II/A	15 (28%)	3 (6%)
II/B	2 (4%)	1 (2%)
III/B	1 (2%)	7 (13%)
Type of treatment (orchidectomy +)		
RLA	31 (59%)	4 (8%)
ChT	25 (47%)	38 (72%)
Rt	17 (32%)	2 (4%)
Wait-and-see	3 (6%)	14 (26%)
Serum tumor marker at diagnosis, no. of patients (%)		
AFP (elevated, > 10)		
Elevated	9 (18%)	15 (28%)
Normal	22 (41%)	38 (72%)
Unknown	22 (41%)	0 (0%)
β-hCG (elevated, > 10)		
Elevated	4 (8%)	9 (17%)
Normal	26 (49%)	44 (83%)
Unknown	23 (43%)	0 (0%)
Follow-up status, no. of patients (%)		
Alive	51	
Dead of disease	1	
Dead of other cause	1	
Recurrence	1	
Second nongerminal malignancies	1	
Estimated survival, % (95% CI)		
At 5 years	93% (78–98)	
Median follow-up time, range, (months)	42 (27–121)	

Clinical stages and marker values (AFP, β-hCG)

The clinical details of metachronous tumors are presented in Table 2 and Table 3. Clinical stage I tumors were more frequent as second tumors than first tumors (P = 0.23, not significant). The percentages of stage I tumor in the 5-, 10-, and 15-year period after the first orchidectomy were 71%, 81%, and 87%. Clinical stage I tumors were statistically more frequent in those followed according to our surveillance policy than in group B,

Table 3 Clinical characteristics of patients with metachronous testicular tumor by survey at the National Institute of Oncology In Hungary between 1988 and 1998. Group A: patients with metachronous tumor surveyed by the institutional follow-up program, group B: patients with metachronous tumor, not followed or lost for surveillance. (*AFP* alfa-fetoprotein, *ß-hCG* beta human chorionic gonadotropin, *RLA* retroperitoneal lymphadenectomy, *ChT* chemotherapy, *Rt* radiotherapy)

	Group A ($n=33$)	Group B ($n=20$)
Age (years)		
Median (years)	33	36
Range (years)	(21–47)	(21–52)
Clinical stages, no. of patients (%)		
I/A	16 (49%)	10 (50%)
I/B	14 (42%)	2 (10%)
II/A	2 (6%)	1 (5%)
II/B	0 (0%)	1 (5%)
III/B	1 (3%)	6 (30%)
Type of treatment (orchidectomy +)		
RLA	2 (6%)	2 (10%)
ChT	25 (76%)	13 (65%)
Rt	0 (0%)	2 (10%)
Wait-and-see	7 (21%)	7 (35%)
Serum tumor marker at diagnosis, no. of patients (%)		
AFP (elevated, > 10)		
Elevated	7 (21%)	8 (40%)
Normal	25 (76%)	12 (60%)
Unknown	1	0
ß-hCG (elevated, > 10)		
Elevated	5 (15%)	4 (20%)
Normal	27 (82%)	16 (80%)
Unknown	1	0
Follow-up status, no. of patients (%)		
Alive	32	19
Dead of disease	0	1
Dead of other cause	1	0
Recurrence	1	0
Second nongerminal malignancies	1	0

$P < 0.01$. Marker determinations were useful in establishing the first and second tumor diagnosis in 10 (32%) and in 18 patients (34%), respectively. The diagnostic value of markers did not differ for primary and second tumors, P (AFP) = 1, P (ß-hCG) = 0.55, and in group A and group B at the time of second tumor diagnosis, P (AFP) = 0.14, P (ß-hCG) = 0.72.

Histological characteristics of metachronous testicular cancers

Table 4 shows the histological characteristics of metachronous tumors. The anaplastic seminoma was included in the seminoma group. The distribution of the two main histological subtypes (i.e., seminoma and nonseminoma) did not differ between the first and second tumor, $P = 0.48$. In nine cases both tumors were seminoma. In 37 patients with initial nonseminoma, nine developed a second nonseminomatous tumor with identical histological subtypes. The prevalence of

Table 4 Histological characteristics of metachronous tumors ($n=53$). (*Surgery* castration +/- retroperitoneal lymphadenectomy, *ChT* chemotherapy, *Rt* radiotherapy)

	First tumor	Second tumor
Histology, no. of patients (%)		
Seminoma	16 (30%)	20 (38%)
Nonseminoma	37 (70%)	33 (62%)
Presence of embryonal carcinoma, no. of patients (%)		
Yes	33 (62%)	29 (55%)
No	20 (38%)	24 (45%)
Vascular invasion, no. of patients (%)		
Yes	10 (19%)	21 (40%)
No	27 (51%)	28 (53%)
Unknown	16 (30%)	4 (7%)
Occurrence of seminoma according to first tumor treatment		
Surgery	1	1
Surgery and ChT	1	9
Surgery and Rt	14	8
Surgery, Rt and ChT	0	1
Wait-and-see	0	1

Table 5 Cancer intervals between metachronous tumors according to primary tumor treatments (in months). (*Surgery* castration +/- retroperitoneal lymphadenectomy, *ChT* chemotherapy, *Rt* radiotherapy)

First tumor treatment	No. of patients (%)	Median interval (range)
Surgery	13 (25%)	56 (18–140)
Surgery and ChT	23 (43%)	76 (21–180)
Surgery and Rt	15 (28%)	87 (31–170)
Surgery, ChT, and Rt	2 (4%)	123 (42–203)

embryonal components and vascular invasion in the first and second tumors did not differ ($P = 0.52$ and $P = 0.18$). The prevalence of seminoma as second tumor was more frequent after chemotherapy and radiotherapy than after surgery alone. Seminoma as a second tumor was diagnosed in a later age group (median 38 years, range 25–49 years) than nonseminoma (median 32 years, range 21–51 years), $P < 0.045$. The proportion of metachronous seminoma was 19%, 37%, and 60% in the 5-, 10-, and 15-year period after the first orchidectomy, respectively. The clinical stage of the second tumor in cases of seminoma and nonseminoma were the following: two stage III, 18 stage I, and five stage III, three stage II, and 24 stage I, respectively.

Cancer interval between metachronous tumors

The interval between metachronous tumors did not depend on the first treatment (Table 5). There is a tendency for a longer interval after chemotherapy and especially radiotherapy, but the number of patients is too low to provide statistical evidence. The age of patients at the time of diagnosis of first and second tumors did not have an influence on the interval (Pearson's

Table 6 Cancer intervals between metachronous tumors according to the main histological subgroups (in months)

No. of patients (%)	Primary tumor	Second tumor	Median interval (range)
9 (17%)	Nonseminoma	Nonseminoma[a]	45 (21–180)
17 (32%)	Nonseminoma	Nonseminoma[b]	50 (21–140)
7 (13%)	Seminoma	Nonseminoma	86 (31–183)
9 (17%)	Seminoma	Seminoma	120 (39–170)
11 (20%)	Nonseminoma	Seminoma	121 (18–203)

[a]concordant
[b]discordant

correlation coefficient = 0.104). The interval did not depend on the presence (median 78 months, range 21–122 months) or absence (median 83 months, range 18–203 months) of testicular hypoplasia in the patients' history (data not shown). The relation of tumor histology with cancer interval is shown in Table 6. The first testicular cancer was followed by a statistically significant longer interval (median 121 months, range 18–203 months) in case of seminoma than in patients with nonseminoma (median 50 months, range 21–183 months), $P < 0.002$. The second tumor was diagnosed earlier in the surveillance group (median time 72 months, range 18–203 months) than in group B (median time 77 months, range 21–170 months); the difference did not have significant influence on the interval.

Treatment, and survival after metachronous tumor

The treatment of first testicular cancer (Table 2) revealed CR in 52 patients (98%) and PR in one. Chemotherapy was applied in 25 out of 53 patients (47%).

The primary treatment of the second testicular cancer resulted in CR for 52 patients. Chemotherapy was used in 38 (72%), and a wait-and-see policy in 14 of 53 patients (26%) in line with institutional policy. Among the 53 patients 25 (76%) were treated by chemotherapy in the surveillance group, and 13 (65%) in group B (Table 3). The 5-year median overall survival (Fig. 2) was 93%. During the 42 months median follow-up time one patient died due to testicular cancer 30 months after diagnosis of the second testicular tumor. One patient relapsed 6 months after CR following primary treatment and was saved by second-line chemotherapy and surgical resection of the residual tumor. One more patient died due to treatment-related (radiotherapy) pancreatic cancer 50 months after diagnosis of the second testicular tumor, which amounts to 1.9% of nongerminal malignancies among patients with metachronous GCTT.

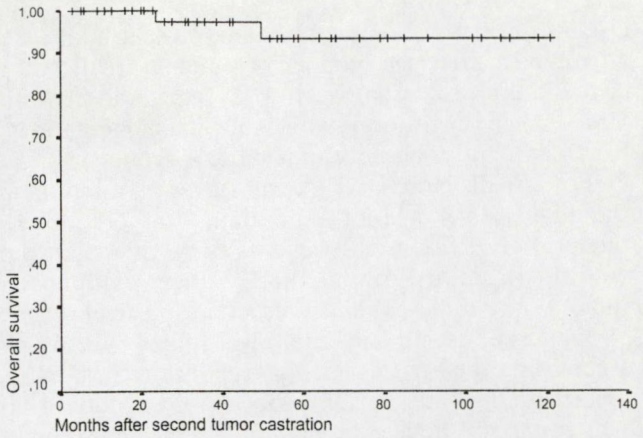

Fig. 2 Median follow-up = 42 months (range: 27–121 months). At 5 years, the rate of overall survival is 93% after second tumor. The survival calculation were performed from the time point of second orchidectomy to the last follow-up visit, or to the time of patient's death

Discussion

There is a reported increase in the incidence of bilateral testicular cancer following the improvement in survival of testicular cancer (Sokal 1980; Tekin 2000). The prevalence of bilateral GCTT reported by Alberts et al. (Alberts et al. 1999) in a review of papers published from 1990 to 1999 was 2.8% (range 1.0–4.0%), which corresponds to our data (3.0%). An even higher incidence is reported in the Scandinavian population.

The cohort of synchronous GCTT in our series (0.8%) is larger than reported, 0.18–0.67% (Osterlind 1991; Wanderas 1997; Coogan 1998; Bokemayer 1993; Colls 1996; Reinberg 1991). This can be explained by national characteristics, but our centralized treatment strategy allows more frequent reporting, because patients with synchronous tumors are likely to be referred to our center. The real incidence of synchronous testicular tumor is difficult to estimate because of spontaneous tumor regression, and regression of an undetected second tumor due to chemotherapy, if the diagnosis was based on testicular palpation. The widespread use of high-resolution testicular ultrasound examination helps in the differentiation of testicle abnormalities and in the diagnosis of synchronous and metachronous testicular cancers (Géczi 2001).

The incidence of metachronous GCTT in the referral center in Hungary was 2.2%. However, patients not requiring further treatment after orchidectomy for a second tumor may not have been referred to the center. The national characteristics and the long follow-up time may also have an impact on the observed incidence (Osterlind 1991; Wanderas 1997; Bokemayer 1993; Colls 1996). In the two papers with the largest number of patients the cumulative risk of metachronous testicular cancer was 3.2% at 10 years, 3.9% at 15 years, and 5.2% at 25 years after first tumor diagnosis (Osterlind 1991; Wanderas 1997). The authors reported patients with metachronous GCTT in the period 1953–1990 and 1960–1979 with a prevalence of 2.7% and 2.6%, respectively. The population in these studies was not homogenous because they included patients who had been treated before and after the introduction of

cisplatin-based chemotherapy. The present study takes advantage of a large number of patients treated in the cisplatin era and is based on a 10-year data-set from a large single institution with good documentation of treatment, and a homogenous patient group.

Because of the relatively early stages distribution and the high percentage of seminoma, the prognosis of bilateral GCTT in our series was not worse than those with single tumors. Among the 72 patients with bilateral tumors only three patients died due to testicular cancer (4.2%). The first-line treatment was highly effective, and second-line therapy revealed a response rate of 50% in metachronous tumors. The prognosis was good for both seminoma and nonseminoma, and in metachronous tumors which did not depend on the patients' age at first tumor diagnosis. However, nonseminoma occurred more frequently in younger men. In our series the aggressiveness of the first and second metachronous tumors did not differ significantly, which corresponds to the observation of Albers et al. (Alberts 1999), although they used other parameters beside vascular invasion (MIB-I proliferation rate, E-cadherin expression) to evaluate aggressiveness. Some authors have observed worse outcome for synchronous than metachronous cancers (Tekin 2000).

We could not identify clinical factors which predicted patients at risk for metachronous GCTT even though a higher risk have been reported by others (Harland 1993; Loy 1993) in cases of chriptorchidism, atrophic testis, infertility, and familiar testicular cancer. Forty among the 53 patients with metachronous cancers had none of these risk factors. This finding underlines that every patient with unilateral testicular cancer has a risk of developing a contralateral tumor in the remaining testis (Tekin 2000).

The estimated incidence of the so-called precursor lesion for testicular cancer in the contralateral testis (carcinoma in situ, CIS) is approximately 5% in the literature (Dieckmann 1996). As a routine, contralateral biopsy at the time of first tumor diagnosis is not undertaken in Hungary, which is why the corresponding data are not published. The pathological reports of the revised specimens investigated between 1988 and 1998, apart from a few cases, did not describe the presence of CIS in the surrounding tumor tissue, therefore we were not able to produce data on the prevalence of CIS in our series. The low incidence of metachronous testicular cancer, the high percentage of early stage at tumor diagnosis, and the good outcome of patients with metachronous tumors do not support the introduction of a biopsy unless a suspicious finding is detected by ultrasound examination.

Publications have suggested a decreased incidence as well as a delay in the appearance of metachronous GCTT after chemotherapy (Van Basten 1997) and an increased incidence of asynchronous seminoma (Sokal 1980; Thompson 1988); however, the data are controversial. The given explanation is that the results depend on selection bias in studies based on data from large single institutions. The exact documentation of treatment data, the standardized and homogenous patients group, and the adequate follow-up also have an influence on the reported incidence.

We found a non-significant trend to a longer interval after chemotherapy and radiotherapy. We also observed a tendency to a greater incidence of asynchronous seminoma after chemotherapy. Further follow-up and a greater number of patients is needed to evaluate the impact of cisplatin-based chemotherapy on the interval and on the incidence of metachronous seminoma. The suppressive effect of chemotherapy might also explain a lower incidence of metachronous GCTT in a chemotherapy unit.

Our results showed that regular follow-up might improve the early diagnosis of metachronous GCTT, but we could not find a difference in survival between patients who underwent strict follow-up and those who did not. We can conclude, therefore, that such a strict follow-up as used in group A does not result in a better prognosis and does not have influence on patient outcome. Our findings showed that the cancer interval of a metachronous GCTT depended on tumor histology and patient age, and the probability of an early stage seminoma increased with follow-up time. Our data support the favorable clinical behavior of most asynchronous GCTT with slow development and late onset of distant metastasis and symptoms. This finding underlines the importance of patient education and self-examination of the remaining testis and long-term follow-up.

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10. ANNEX

Annex I. Histological classification of testicular cancers

GERM CELL TUMORS

Precursor lesions:

- Intratubular germ cell neoplasia (CIS), unclassified, specific types

Germ cell tumors of one histologic type:

- Seminoma (variant: with syncytiotrophoblast cells)
- Spermatocytic seminoma (variant: with sarcomatous component)
- Embryonal carcinoma
- Yolk sac tumor (endodermalis sinus tumor)
- Trophoblastic tumor
  - Choriocarcinoma
  - Placental site trophoblastic tumor
  - Unclassfied
- Teratoma
  - Mature teratoma (variant: dermoid cyst)
  - Immature teratoma
  - Teratoma with a secondary malignant component
- Monodermal teratoma
  - Carcinoid
  - Primitve neuroectodermal tumor
  - Others

Germ cell tumors of more than one histologic type

- Mixed germ cell tumors (specify individual components)
- Polyembryoma
- Diffuse embryoma

Regressed, ("burnt-out") germ cell tumors (scar, scar with CIS, or scar with residual germ cell tumor, teratoma, seminoma, or others)

GERM CELL -SEX CORD-STROMAL TUMORS:

- Gonadoblastoma
- Unclassified

SEX CORD - STOMAL TUMOR:

- Sertoli-stomal cell tumors
  - Sertoli cell tumor (variants: large cell calcifying, sclerosing)
  - Sertoli and Leydig cell tumor
  - Leydig cell tumor
- Granulosa-stomal cell tumors
  - Granulosa cell tumor (variants: adult, juvenile)
  - Tumor in the fibroma-thecoma group
  - Mixed
  - Unclassified

TUMORS OF THE RETE TESTIS

- Carcinoma

PARATESTICULARIS TUMORS (sarcoma, mesothelioma, etc.)

HEMATOPOIETIC TUMORS (plasmocytoma, lymphoma etc.)

SECONDARY TUMORS

Annex II. TNM clinical classification of testicular cancer (UICC 1997)

PT- Primary tumor			
pTX Primary tumor cannot be assessed (if no radical orchiectomy has been performed, TX is used.			
pT0	No evidence of primary tumor (e.g. histological scar in testis)		
pTis	Intratubular germ cell neoplasia (carcinoma in situ, CIS)		
pT1	Tumor limited to the testis and epididymis without vascular/lymphatic invasion; tumor may invade the tunica albuginea but not the tunica vaginalis		
pT2	Tumor limited to the testis and epididymis with vascular/lymphatic invasion, or tumor extending through the tunica albuginea with involvement of the tunica vaginalis		
pT3	Tumor invades spermatic cord with or without vascular/lymphatic invasion		
pT4	Tumor invades scrotum with or without vascular/lymphatic invasion		
N – REGIONAL LYMPH NODES			
NX	Regional lymph nodes cannot assessed		
N0	No regional lymph node metastasis		
N1	Metastasis with a lymph node mass 2 cm or less in greatest dimension or multiple lymph nodes, none more than 2 cm in greatest dimension		
N2	Metastasis with a lymph node mass more than 2 cm but not more than 5 cm in greatest dimension, or multiple lymph nodes, any one mass greater than 2 cm but not more than 5 cm in greatest dimension		
N3	Metastasis with lymph node mass more than 5 cm in greatest dimension		
M – DISTANT METASTASIS			
MX	Distant metastasis cannot be assessed		
M0	No distant metastasis		
M1	Distant metastasis		
M1a	Non-regional nodal or pulmonary metastasis		
M1b	Distant metastasis other than non-regional lymph nodes and lungs		
S – SERUM TUMOR MARKERS			
SX	Marker studies not available or not performed		
S0	Marker study levels within normal limits		
	LDH	**hCG (mIU/ml)**	**AFP (ng/ml)**
S1	< 1,5 x normal	< 5 000	< 1 000
S2	1,5-10 x normal	5 000-50 000	1 000-10 000
S3	> 10 x normal	> 50 000	> 10 000

Annex III. Stage grouping of testicular cancer

This stage grouping uses the TNM classification shown in Annex 2.

Stage 0	pTis	N0	M0	S0
Stage I	PT1-4	N0	M0	SX
Stage IA	PT1	N0	M0	S0
Stage IB	PT2-3-4	N0	M0	S0
Stage IS	Any pT/TX	N0	M0	S1-3
Stage II	Any pT/TX	N1-3	M0	SX
Stage IIA	Any pT/TX	N1	M0	S0-1
Stage IIB	Any pT/TX	N2	M0	S0-1
Stage IIC	Any pT/XT	N3	M0	S0-1
Stage III	Any pT/TX	Any N	M1	SX
Stage IIIA	Any pT/TX	Any N	M1a	S0-1
Stage IIIB	Any pT/TX	N1-3	M0	S2
	Any pT/Tx	Any N	M1a	S2
Stage IIIC	Any pT/TX	N1-3	M0	S3
	Any pT/Tx	Any N	M1a	S3
	Any pT/TX	Any N	M1b	Any S

Annex IV. International Germ Cell Cancer Collaborative Group (IGCCCG) classification of testicular cancer

	Non-seminoma	Seminoma
Good prognosis	Testis/retroperitoneal primary and No nonpulmonary visceral metastases and Good markers: AFP < 1000 ng/ml, hCG < 5 000 IU/L and LDH < 1,5 x upper limit of normal 56% of nonseminoma 5-yr PFS = 89% 5-yr survival = 92%	Any primary site and No nonpulmonary visceral metastases and Normal AFP, any hCG and any LDH 90% of seminoma 5-yr PFS = 82% 5-yr survival = 86%
Intermediate prognosis	Testis/retroperitoneal primary and No nonpulmonary visceral metastases and Intermediate markers: any of AFP 1000 - 10 000 ng/ml, or hCG, 5 000-50 000 IU/L, or LDH 1,5 – 10 x normal 28% of nonseminoma 5-yr PFS = 75% 5-yr survival = 80 %	Any primary site Nonpulmonary visceral metastases (liver, brain, os etc.) Normal AFP, any hCG and any LDH 10% of seminoma 5-yr PFS = 67% 5-yr survival: 72%
Poor prognosis	Mediastinal primary or Nonpulmonary visceral metastases (liver, brain, os etc.) or Poor markers: Any of AFP > 10 000 ng/ml, hCG > 50 000 mIU/ml, LDH ➢ 10 x normál ➢ 16% of nonseminoma 5-yr PFS = 41% 5-yr survival = 48%	No patients classified as poor prognosis

Annex V. Chemotherapy protocols of germ cell tumors

VPB	vinblastine 6 mg/m^2	d 1-2
	cisplatin 20 mg/m^2	d 1-5
	bleomycin 30 mg	d 1, 9, 16
EP	etoposide 100 mg/m^2	d 1-5
	cisplatin 20 mg/m^2	d 1-5
BEP	bleomycin 30 mg	d 1, 9, 16
	etoposide 100 mg/m^2	d 1-5
	cisplatin 20 mg/m^2	d 1-5
VeIP	vinblastine 0,11 mg/kg	d 1-2
	ifoszfamide 1,2 gr/m^2 (mesna)	d 1-5
	cisplatin 20 mg/m^2	d 1-5
VIP	etoposide 100 mg/m^2	d 1-5
	ifoszfamide 1,2 gr/m^2 (mesna)	d 1-5
	cisplatin 20 mg/m^2	d 1-5

d=day / treatment interval is 21d