

Molecular Determinants of Treatment Response in Human Germ Cell Tumors¹

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ABSTRACT

Purpose: Germ cell tumors (GCTs) are highly sensitive to cisplatin-based chemotherapy. This feature is unexplained, as is the intrinsic chemotherapy resistance of mature teratomas and the resistant phenotype of a minority of refractory GCTs. Various cellular pathways may influence the efficacy of chemotherapy. Their impact has not been investigated in a comprehensive study of tumor samples from clinically defined subgroups of GCT patients.

Experimental Design: We investigated proteins involved in regulation of apoptosis (p53, BAX, BCL-2, and BCL-X_L), cell cycle control [p21 and retinoblastoma protein (RB)], and drug export and inactivation [P-glycoprotein, multidrug resistance-associated protein (MRP) 1, MRP2, breast cancer resistance protein, lung resistance protein, metallothionein, and glutathione S-transferase π] immunohistochemically in samples of unselected GCT patients ($n = 20$), patients with advanced metastatic disease in continuous remission after first-line chemotherapy ($n = 12$), and chemotherapy-refractory patients ($n = 24$). Mature teratoma components ($n = 10$) within tumor samples from all groups were analyzed separately. The apoptotic index was studied by terminal deoxynucleotidyl transferase-mediated nick end labeling assay.

Results: Invasive GCTs of all groups showed a correlation between wild-type p53 and apoptotic index ($r_s = 0.66$;

$P < 0.001$). The levels of the antiapoptotic proteins BCL-2 and BCL-X_L were generally low. p21 was hardly detectable and did not correlate with p53 ($r_s = 0.29$; $P = 0.07$). No significant differences among the three patient groups were identified regarding any of the investigated parameters (all P s were >0.08), even though only individual samples from chemotherapy-resistant cases showed a strong staining for MRP2 and GST π . In contrast to other components, mature teratomas showed an intense p21 and RB staining and were mostly positive for MRP2, lung resistance protein, and GST π .

Conclusions: Our results indicate a multifactorial basis for the chemosensitivity of GCTs with lack of transporters for cisplatin, of antiapoptotic BCL-2 family members, of p21 induction by p53, and of RB and an intact apoptotic cascade downstream of p53. These findings suggest a preference for apoptosis over cell cycle arrest after up-regulation of p53. None of the examined parameters offers a general explanation for the chemotherapy-resistant phenotype of refractory tumors. The up-regulation of various factors interfering with chemotherapy efficacy and ability for a p21-induced cell cycle arrest may explain the intrinsic chemotherapy resistance of mature teratomas.

INTRODUCTION

GCTs,⁴ in particular those of the testis, are the most frequent malignancy in males between 20 and 45 years of age (1). Based on histological, biological, and clinical differences, GCTs are divided into seminomas and nonseminomas. The nonseminomas can be composed of one or more of the following elements: embryonal carcinoma; yolk sac tumor; choriocarcinoma; mature teratoma; and immature teratoma. They can also contain a seminoma component (2). Compared with the vast majority of solid tumors of adults, GCTs are highly sensitive to cytotoxic treatment. Even in metastatic stages, 80% of patients can be cured by a CDDP-based multiagent chemotherapy followed by secondary resection in case of residual tumor lesions, which can contain pure necrosis, viable malignant cells, or mature teratoma (3, 4). In contrast to the other histological components, mature teratomas show a benign clinical behavior but are unresponsive to chemotherapy. Mature teratomas need to be surgically removed to avoid growing teratoma (5) or transformation into a secondary non-germ cell malignancy (6).

About 10% of patients diagnosed with a GCT will be

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⁴The abbreviations used are: GCT, germ cell tumor; CDDP, cisdiamino-dichloroplatinum; ABC, ATP binding cassette; GST, glutathione S-transferase; LRP, lung resistance protein; RB, retinoblastoma protein; Pgp, P-glycoprotein; MRP, multidrug resistance-associated protein; BCRP, breast cancer resistance protein; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

unresponsive to CDDP-based chemotherapy or will relapse and subsequently develop progressive disease despite further treatment. Even though several new drugs including paclitaxel, oxaliplatin, and gemcitabine have shown some promise in this setting (7–10), there is hardly a chance to cure patients suffering from chemotherapy-resistant disease (11). The biological basis for the differential behavior of GCTs to chemotherapy is unclear. It is generally assumed that the high curability reflects the characteristics of primordial germ cells, which are the presumed cells of origin (12), and the embryonal cell types derived from them. These cells undergo apoptosis readily upon exposure to external stress (13). We have recently demonstrated that the mere level of wild-type p53 does not explain the chemosensitivity of these tumors (14).

CDDP is considered the most important drug in the systemic treatment of GCTs. CDDP exerts its action via induction of apoptosis (15). The crucial damage inflicted by CDDP is supposed to be covalent binding to DNA. The DNA damage has to be detected to activate an apoptotic cascade leading to cell death. Theoretically, resistance can occur at various levels in this process (16): (a) the drug can be inactivated by reduction with glutathione or metallothionein or exported out of the cell by various transporter proteins, e.g., members of the ABC transporters, before it can interact with DNA (17); (b) the generated DNA damage may be repaired before it results in activation of an apoptotic cascade, predominantly by the nuclear excision repair pathway (18); and (c) the damage recognition or execution of an apoptotic program can be impaired or blocked by mutations/deletions of apoptotic effectors or overexpression of antiapoptotic proteins (for example, members of the BCL-2 family). The common result of these different scenarios is failure to undergo programmed cell death (16). Several explanations for the chemosensitivity of GCTs such as impaired up-regulation of GST π (19), low DNA repair capacity (20), or lack of antiapoptotic members of the BCL-2 family (21) have been suggested. These models are predominantly based on *in vitro* analyses of cell lines and lack confirmation of their relevance in clinical material (22). We therefore investigated a variety of putative resistance mechanisms in such clinical material to seek an explanation for the exquisite chemosensitivity of GCTs in general, the intrinsic resistance of mature teratomas, and the potential occurrence of chemotherapy resistance in GCTs.

MATERIALS AND METHODS

Patients and Tissue Samples. Tumor tissue from three patient groups was investigated: (a) unselected patients with GCTs containing all histological subtypes ($n = 20$); (b) patients with advanced metastatic nonseminomas achieving long-term remissions after first-line CDDP-based chemotherapy ($n = 12$); and (c) clinically defined refractory patients ($n = 24$). Due to the difference in clinical behavior of mature teratomas, components of this histology occurring in all of these groups ($n = 10$) were analyzed separately.

Formalin-fixed paraffin-embedded tissue blocks from the 20 unselected cases (8 seminomas and 12 nonseminomas containing various histological subtypes) were collected between 1998 and 2001 in close collaboration with urologists and pa-

thologists in the southwestern part of the Netherlands. They were retrieved from the archive of the Laboratory for Experimental Patho-Oncology, Department of Pathology, Erasmus Medical Center/Daniel. No data on the clinical course were available for these patients. The 12 patients with advanced metastatic disease were treated within a Phase I/II study of the German Testicular Cancer Study Group evaluating a dose-intensified first-line treatment strategy for patients with poor prognostic features (23). All patients remain in complete remission or marker-negative partial remission for a minimum follow-up of 1 year (range, 1–6 years). The chemotherapy-resistant series consisted of 24 patients diagnosed between 1986 and 1998 and treated within various experimental chemotherapy trials led by Tübingen University (Tübingen, Germany). Patients were considered refractory when progression or relapse of the disease occurred despite adequate CDDP-based initial and salvage treatment. The tumor samples were obtained either at initial diagnosis (*i.e.*, before chemotherapy; $n = 16$) or by resection of a metastatic lesion in relapse ($n = 8$).

Baseline characteristics of the patients are given in Table 1.

Immunohistochemistry. Paraffin sections of 3 μm were mounted on adhesive slides, deparaffinized, and rehydrated. Whenever necessary, antigen retrieval was performed by autoclaving [120°C; 1.2 bar] in different buffers 0.01 M sodium citrate (pH 6) or 0.001 M EDTA (pH 8). Biotin-labeled rabbit antimouse, swine antirabbit, or rabbit antirat immunoglobulins and a biotinylated horseradish peroxidase-streptavidin complex (both from DAKO, Glostrup, Denmark) were subsequently applied for 30 min at room temperature. Diaminobenzidine (Sigma, Zwijndrecht, the Netherlands) was used as chromogen. The antibodies for the ABC transporters were raised by R. S. and G. L. S. and were previously shown to be specific (24, 25). For the remaining targets, commercially available antibodies were used. Table 2 specifies the primary antibodies, incubation conditions, and the positive controls for each antibody. Omitting the primary antibody was used as a negative control in each case.

For ABC transporters, LRP, metallothionein, GST π , RB, BCL-2, and BCL-X_L, a staining unequivocally visible under low-power magnification in the correct localization in at least 10% of tumor cells was considered positive. For p53 and p21, the fraction of tumor cells with an intense nuclear staining was counted in five randomly selected high-power fields for each case; for further analysis, the mean of positive cells from all fields counted was used. Differences in the range of p53-positive cells compared with a previous publication (14) are caused by the use of a different detection system.

TUNEL Assay to Detect Apoptotic Cells. Paraffin sections of 3 μm were mounted on adhesive slides, deparaffinized, rehydrated, and digested in 0.5% pepsine (Sigma) in 0.02 M HCl for 20 min at 37°C. Slides were rinsed in H₂O and PBS. PBS was carefully removed, and the reaction mixture containing 3 U terminal deoxynucleotidyl transferase (Fermentas, St. Leon-Rot, Germany), 0.5 μM biotinylated dUTP (Roche Diagnostics, Mannheim, Germany), and 5 \times terminal deoxynucleotidyl transferase buffer (Fermentas) in an end volume of 50 μl was applied, sealed with a coverslip, and incubated at 37°C for 1 h. Slides were rinsed in PBS containing 0.1% Tween 20 (Sigma). The incorporated biotin was visualized with a biotinylated

Table 1 Baseline patient characteristics by patient group

Mature teratoma components occurring in tumor samples from patients of all groups (five in unselected, two in chemosensitive, and three in refractory patients) were analyzed separately from the invasive tumors. NA, not available; NR, not reached.

	Unselected (n = 20)	Chemosensitive (n = 12)	Chemorefractory (n = 24)
Median age (range) (yrs)	32 (18–43)	29 (21–47)	29 (16–56)
Histology			
Seminoma	8	1	1
Nonseminoma	12	11	23
Stage at initial diagnosis (according to UICC) ^a			
I	NA	0	2
II	NA	0	6
III	NA	12	16
Median follow-up (range) (mo)	NA	33 (12–71)	35 (11–180)
Median relapse-free survival (range) (mo)	NA	N.R.	5 (0–150)
Response to initial systemic treatment			
Complete remission	NA	0	4
Partial remission, marker negative	NA	12	9
Partial remission, marker positive	NA	0	2
Progressive disease	NA	0	4
Unknown	NA	0	5
Median number of salvage regimen (range)	NA	0	3 (1–7)

^a UICC, Union Internationale Contre le Cancer.

Table 2 Details on primary antibodies used for immunohistochemical stainings

All incubations with the primary antibodies were done at room temperature for 2 h.

Antigen	Antibody	Species	Source	Antigen retrieval	Dilution	Positive control	Localization of staining
Pgp	JSB1	Mouse	Rik Scheper	AC ^a sodium-citrate, 0.01 M (pH 6)	1:20	Liver	Membranous
MRP1	MRP R1	Rat	Rik Scheper	AC sodium-citrate, 0.01 M (pH 6)	1:20	Lung cancer	Membranous
MRP2	M ₂ III5	Mouse	Rik Scheper	AC sodium-citrate, 0.01 M (pH 6)	1:20	Liver	Membranous
BCRP	BXP 21	Mouse	Rik Scheper	AC EDTA, 0.001 M (pH 8)	1:20	Placenta	Membranous
LRP	Clone LRP06	Mouse	Neomarkers	AC sodium-citrate 0.01 M (pH 6)	1:200	Breast cancer	Cytoplasmic
GST π	Ab1	Rabbit	Neomarkers		1:50	Liver	Cytoplasmic
Met	Clone E9	Mouse	Neomarkers		1:50	Breast cancer	Cytoplasmic
p53	Clone Do7	Mouse	DAKO	AC sodium-citrate 0.01 M (pH 6)	1:50	Colon cancer	Nuclear
p21	Clone EA10	Mouse	Oncogene Science	AC sodium-citrate 0.01 M (pH 6)	1:75	Colon	Nuclear
RB	Clone IF8	Mouse	Neomarkers	AC EDTA 0.001 M (pH 8)	1:100	Colon	Nuclear
BAX	Clone 2D2	Mouse	Neomarkers	AC sodium-citrate 0.01 M (pH 6)	1:100	Tonsil	Cytoplasmic
BCL-2	Clone 124	Mouse	DAKO	AC sodium-citrate 0.01 M (pH 6)	1:50	Tonsil	Cytoplasmic
BCL-X _L	Clone 7D9	Mouse	Neomarkers	AC EDTA 0.001 M (pH 8)	1:100	Melanoma	Cytoplasmic

^a AC, autoclaving at 120°C/1.2 bar; Met, metallothioen.

horseradish peroxidase-streptavidin complex and diaminobenzidine identical to the immunohistochemical stainings. A brown staining of the nucleus/nuclear remnants was considered positive. The apoptotic index was evaluated by counting the fraction of apoptotic cells in five high-power fields.

Statistical Analysis. The results of the responders and nonresponders and of the unselected invasive tumors and the mature teratomas were compared by χ^2 test for immunohistochemical parameters other than p53, p21, and the apoptotic index. A possible correlation between the apoptotic index and the fraction of cells positive for p53 or p21, respectively, was analyzed by Spearman rank correlation. Differences in the fraction of p53- or p21-positive cells and apoptotic index between mature teratomas and invasive nonseminomas and between the different treatment response groups were analyzed by the Kruskal-Wallis test. Differences were considered significant when P was <0.05 .

RESULTS

Markers of Drug Export and Inactivation in Invasive GCTs and Mature Teratomas.

The results of the immunohistochemical analysis are summarized in Table 3. Four ABC transporters (Pgp, MRP1, MRP2, and BCRP) and the major vault protein LRP were investigated. Pgp, MRP2, and LRP (Fig. 1A) were rarely detected in invasive components of any of the groups. BCRP was demonstrated in the syncytiotrophoblastic cells of choriocarcinomas, consistent with its reported expression in normal placenta (25). The majority of invasive tumors stained positive for MRP1. No significant differences were detected between responding and nonresponding patient groups regarding any of the proteins analyzed. However, individual patients of the chemotherapy-refractory group showed a staining for MRP2 or LRP (3 of 24 and 1 of 23, respectively), whereas this was never observed in the group of responding patients

Table 3 Summary of immunohistochemical results

Fraction of tumors scored positive by evaluable tumors (percentage of tumors scored positive). Column A gives the *P* for the difference between unselected tumors and the mature teratomas. Column B gives the *P* for the difference between responding and nonresponding tumors as determined by χ^2 test. Statistically significant values are indicated in **bold**.

Antigen	Unselected tumors	Refractory tumors	Responding tumors	Mature teratomas	A	B
RB	4/20 (20%)	9/22 (41%)	3/12 (25%)	9/10 (90%)	<0.001	0.354
BCL-2	3/20 (15%)	0/24 (0%)	1/12 (8%)	3/10 (30%)	0.333	0.151
BCL-X _L	2/20 (10%)	7/23 (30%)	5/12 (42%)	10/10 (100%)	<0.001	0.632
Pgp	0/20 (0%)	1/22 (5%)	0/12 (0%)	10/10 (100%)	<0.001	0.453
MRP1	19/20 (95%)	15/23 (65%)	10/11 (91%)	2/9 (22%)	<0.001	0.112
MRP2	0/17 (0%)	3/24 (13%)	0/12 (0%)	7/10 (70%)	<0.001	0.201
BCRP	6/20 (30%)	5/22 (23%)	2/12 (17%)	7/10 (70%)	0.037	0.676
LRP	2/20 (10%)	1/23 (4%)	0/12 (0%)	8/10 (80%)	<0.001	0.464
GST π	0/17 (0%)	5/24 (21%)	0/12 (0%)	10/10 (100%)	<0.001	0.125
Met	7/20 (35%)	10/22 (45%)	7/12 (58%)	2/10 (20%)	0.398	0.473

(*P* = 0.20 and *P* = 0.68). Yolk sac components were the only histological subtype showing MRP2 staining and lacking MRP1 staining. Besides the transporters, metallothionein and GST π were included as proteins associated with inactivation of CDDP by detoxification. GST π was demonstrated in 5 of the 24 samples of chemotherapy-refractory patients compared with none in the responding cases. However, this difference did not reach significance (*P* = 0.13). Again, only yolk sac tumors expressed GST π protein. Metallothionein was detected in about one-third of the invasive tumors regardless of treatment outcome or histology.

Mature teratomas, both residual and nonresidual, differed significantly from the other histological components in most of the investigated markers. In contrast to the other histological elements, Pgp, MRP2, BCRP, LRP (Fig. 1B), and GST π showed an intense staining in the majority of mature teratomas. MRP1, which was demonstrated in most invasive tumors, was seen only rarely in mature teratomas. Apart from metallothionein, the observed differences between mature teratomas and the tumors of the unselected group of patients differed significantly using the χ^2 test (*P* = 0.038 for BCRP, and *P* < 0.001 for the remaining parameters). Within the group of chemotherapy-refractory patients, no parameters differentiated patients relapsing after initial remission and those progressing under treatment.

Spontaneous Apoptosis and Effectors and Regulators of Apoptosis. With p21, BAX, and the TUNEL assay to demonstrate apoptotic cells, two competing downstream effects of p53 (apoptosis versus cell cycle arrest) were assessed. Sixteen of the chemotherapy-refractory cases were previously shown to have wild-type p53 by single-strand conformational polymorphism (14); in the remaining cases, the quality of extracted DNA or the amount of available tissue precluded mutation analysis. The fraction of cells positive for p53 or p21 and the apoptotic index did not differ between unselected, responding, and nonresponding patients (*P* = 0.82, *P* = 0.92, and *P* = 0.08). However, the fraction of p53-positive cells was correlated with the apoptotic index ($r_s = 0.66$ and *P* < 0.001 for all invasive tumors together), but not with the percentage of p21-positive cells ($r_s = 0.26$; *P* = 0.16). Seminomas had a lower apoptotic index and a lower number of p53-positive cells than nonseminomas. Within the group of nonseminomas, no differences between the histological subtypes were detected for p53 or

apoptotic index. p21 was hardly detected in invasive tumors with the exception of the syncytiotrophoblastic cells. RB was demonstrated only in part of the yolk sac tumors and in syncytiotrophoblastic cells of choriocarcinoma, irrespective of the treatment outcome. A markedly increased BAX signal was detected in up to one-third of apoptotic cells (Fig. 1C). BCL-2 and BCL-X_L were scarcely seen in vital invasive components.

Analyzing mature teratomas, no significant correlations between the fraction of p53-positive cells and the fraction of p21-positive cells or apoptotic index were observed, possibly due to the limited number of cases studied. Despite a higher percentage of P53-positive cells (mean \pm SD, 14% \pm 9% versus 6 \pm 9.0%; *P* = 0.005) and of P21-positive cells (16% \pm 7% versus 1% \pm 3%; *P* < 0.001), mature teratomas showed a lower apoptotic index (2% \pm 4% versus 4% \pm 3%; *P* = 0.04) than the remaining components. Fig. 2 illustrates the correlation between p53 and p21/apoptotic index for invasive components and mature teratomas.

DISCUSSION

The exquisite chemosensitivity of GCTs has been attributed to their propensity to rapidly undergo apoptosis upon exposure to external stress. A high level of functional p53 has commonly been regarded as the crucial determinant of this disposition (26). However, we (14) have demonstrated previously that this notion is an oversimplification because p53 level and status did not correlate with treatment outcome. The results of the current study offer a different explanation for the chemosensitivity of GCTs. The profile of the tumor cells favors efficacy of chemotherapeutic substances, in particular of CDDP, on multiple levels: neither LRP nor any of the investigated export pumps with affinity for CDDP was found in high levels in responding and unselected invasive tumors. GST π , an enzyme able to inactivate CDDP by conjugation to glutathione, was hardly detected by immunohistochemistry. In accordance with data on GCT-derived cell lines, metallothionein was detectable in some of the samples, regardless of treatment outcome (19). Thus, the presence of metallothionein is not sufficient to confer resistance to GCTs. Further contributing to the chemosensitivity, it has previously been shown that the repair of CDDP-induced DNA damage may be impaired in GCTs (20). The

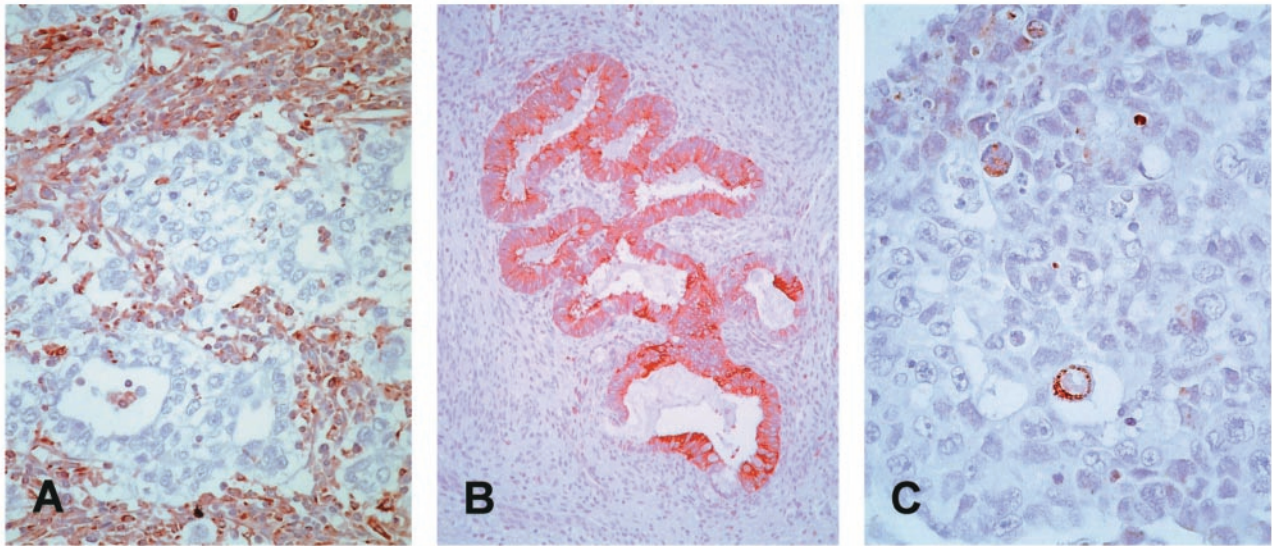


Fig. 1 Representative examples of immunohistochemical stainings. Representative examples of immunohistochemical stainings are shown: **A**, LRP staining in an embryonal carcinoma (note the absence of LRP in tumor cells); **B**, LRP staining in a mature teratoma; and **C**, apoptotic tumor cell showing staining for BAX.

current investigation did not include any parameter in this regard.

Various parameters were analyzed to assess the downstream effects of p53 and regulators of apoptosis of the BCL-2 family in GCTs. The presence of high numbers of spontaneously apoptotic cells, the correlation with p53 positivity, and the presence of BAX in apoptotic cells suggest that the apoptotic cascade of the p53-dependent mitochondrial pathway is intact and activated in spontaneous apoptosis in untreated invasive GCTs. None of the investigated antiapoptotic members of the BCL-2 family was detected at high levels. In line with these findings, a high ratio of BAX:BCL2 has previously been proposed as a possible explanation for the sensitivity of GCT-derived cell lines to etoposide (21). However, it is important to note that it has not been demonstrated thus far which apoptotic pathway is used by the tumor cells in response to treatment with CDDP. The low level of p21 and the lack of correlation between wild-type p53 and p21 suggest that GCT cells do not go into a G₁-S-phase arrest upon induction of p53, at least in the untreated situation (27). The absence of RB in invasive tumors, confirmed in this study, provides an additional argument for this assumption (28, 29). It has previously been proposed that a defect in the G₁-S-phase cell cycle check point represents a crucial step in the progression from preinvasive to invasive stages of GCTs (28). However, the demonstration of both p21 and RB in residual and nonresidual mature teratomas and in the syncytiotrophoblastic cells of choriocarcinomas argues against an acquired defect in cell cycle control on the level of p21 or RB expression. In our opinion, the differential expression of these proteins reflects a differentiation-dependent preference for G₁-S-phase arrest in (terminally differentiated) syncytiotrophoblasts and mature teratoma cells, which is not found in the tumor cells that contain embryonic characteristics. The p53 levels found in the invasive tumors probably represent a response of the tumor cells to

external stress such as hypoxia, malnutrition, or changes in the microenvironment rather than high intrinsic levels of p53. Thus, induction of apoptosis by CDDP is supported by multiple intrinsic features of the tumor cells with embryonic characteristics rather than by a single characteristic, such as a high level of p53.

The next objective of the study was to seek explanations for the rare but clinically important occurrence of chemotherapy resistance of invasive GCTs. No significant differences were observed between the samples of patients with responding or chemotherapy-refractory tumors in any of the parameters analyzed. Adding to our previous findings on p53, the identical behavior regarding the correlation between p53 positivity and apoptosis indicates that alterations of the p53-dependent mitochondrial apoptotic pathway are not a common means to achieve treatment resistance in GCTs. This finding allows two conclusions. (a) The CDDP-induced apoptosis could be executed via a different pathway than spontaneous apoptosis, possibly not depending on functional p53 in the same way. Supporting this hypothesis, we have recently detected a high incidence of microsatellite instability in refractory GCTs (30). Microsatellite instability is a consequence of a defect in the DNA mismatch repair pathway. It has been proposed, that this particular pathway is involved in induction of apoptosis on recognition of DNA damage (31). Interestingly, inactivation of p53 in cell lines with a defective MMR resulted in hypersensitivity toward CDDP in various models (Ref. 32 and the references cited therein). (b) Resistance in GCTs could be related to factors acting upstream of p53. The presence of GST π , LRP, and MRP2 in some chemotherapy-refractory tumors might for example explain the chemotherapy-resistant phenotype in these cases. Due to the small numbers of tumors positive for either LRP or MRP2, it is obvious that overexpression of these proteins is not a common mechanism of resistance. However, it might contribute to the chemotherapy-resistant phenotype in

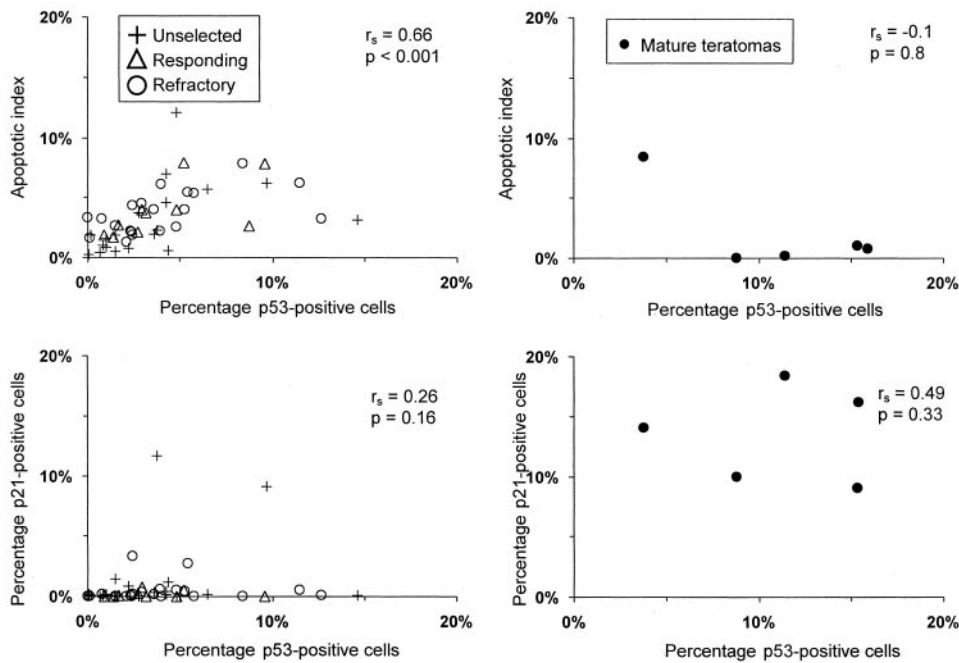


Fig. 2 Correlation between p53 and p21 and between p53 and apoptotic index. Correlation between the percentage of p53-positive cells and the apoptotic index is shown (as determined by immunohistochemistry and TUNEL assay, respectively). Regression coefficients and P s are calculated for invasive tumors of all patient groups together (top and bottom left panels) and for mature teratomas (top and bottom right panels) separately, by Spearman rank correlation. The five remaining teratomas could not be included because TUNEL staining gave unacceptable background ($n = 2$) or because the evaluated areas could not be sufficiently matched for three stainings.

selected cases, as has been postulated for GST π in GCT-derived cell lines (19).

In contrast to the other histological elements of GCTs, the mature teratomas, both residual and nonresidual, are intrinsically resistant to chemotherapy (4). The three ABC transporters, P-glycoprotein, MRP2, and BCRP, are present in this type of tissue, and the latter two are supposed to have a role in CDDP export (17). Whereas P-glycoprotein and MRP1 have been analyzed in GCTs (33), to our knowledge, MRP2 and BCRP have not been investigated in this context thus far. Drug export by the ABC transporters might be further facilitated in mature teratomas by conjugation of CDDP with glutathione because GST π was demonstrated as well. Similarly, the major vault protein LRP, which has been correlated with the response of small cell lung cancer and ovarian cancer to CDDP (34, 35), is regularly detected in mature teratomas. On the level of apoptosis and cell cycle control, additional differences were observed. As indicated above, mature teratomas show immunohistochemically detectable levels of p21 and RB. It is therefore likely that the tumor cells of mature teratoma can arrest at G₁-S, *i.e.*, they have an intact G₁-S checkpoint control. This might allow DNA damage repair to occur, instead of apoptotic cell death. Finally, the antiapoptotic BCL-2 was detected in mature teratomas. Similar to the proposed multifactorial explanation for the general chemosensitivity of invasive GCTs, the resistance of mature teratomas seems to be determined on multiple levels, overall with features opposite to those of the other GCT components. Most likely, the chemotherapy-resistant phenotype is a consequence of loss of embryonic features and gain of complete somatic differentiation. Accordingly, the mature teratoma cells should be as sensitive/resistant as nontumorous, differentiated cells of the body. Therefore, doses of chemotherapy needed to eliminate mature teratoma would be associated with unaccept-

able toxicity. Thus, complete surgical removal will probably remain the appropriate intervention to handle these lesions.

The current study is based on immunohistochemical assessment of potential regulators of chemotherapy sensitivity in GCTs. Multiple caveats have to be kept in mind regarding immunohistochemical studies, such as the effect of pretreatment and tissue preservation. A "negative" finding might be due to a concentration of the investigated target just below the detection threshold of the method applied, rather than the complete absence of the protein. Accordingly, the results have been interpreted primarily comparing different histological elements of GCTs and clinically defined subgroups rather than in absolute terms. Therefore, the presented data provide valuable information and suggest new concepts to understand the differential behavior of subgroups of GCTs to chemotherapy.

In summary, the chemotherapy-refractory phenotype in invasive GCTs is unlikely to be caused by aberrations in the apoptotic pathway downstream of p53. Although it does not explain all chemotherapy-refractory GCTs, overexpression of GST π , MRP, or LRP might confer resistance in individual cases. The unique treatment sensitivity of most GCTs is probably a consequence of a cellular profile supporting optimal efficacy of CDDP in particular on multiple levels. Mature teratomas differ from this profile on each of these levels, most likely reflecting their loss of embryonic features. The intrinsic resistance of mature teratomas to chemotherapy is probably the consequence of presence of a whole spectrum of resistance markers from drug export to cell cycle control.

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