Understanding the cellular basis of chemotherapy response in germ cell tumors

Understanding the cellular basis of chemotherapy response in germ cell tumors

Begrijpen van de cellulaire basis voor chemotherapie gevoeligheid van kiemceltumoren

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Chapter 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

Germ cell tumors are a heterogeneous group of neoplasms derived from the germ cell lineage. Most of these tumors occur within the gonads, both ovary and testis. Rarely, they are also encountered at a variety of extragonadal locations, mostly along the midline of the body[1,2]. This specific distribution has been linked to the migration route of the primordial germ cells from the yolk sac to the genital ridge during embryogenesis[3,4]. Three major groups of germ cell tumors can be distinguished based on differences in age at clinical manifestation, histology and clinical behavior: the germ cell tumors of neonates and infants, the germ cell tumors of adolescent and young adults, and the so-called spermatocytic seminomas of elderly men[3]. The latter type is only found in the testis. This thesis is focussed on the largest group of malignant germ cell tumors, those of adolescent and young males, which will be referred to as GCTs. Reference to the other two groups is only done when a comparison with the GCTs is meaningful.

1.1. Histopathology of GCT

Based on clinical and biological characteristics, GCTs are divided into two major groups, the seminomas and nonseminomas. The seminomas are uniform tumors, composed of tumor cells resembling primordial germ cells/gonocytes[5,6]. In contrast, the nonseminomas are heterogenous and can contain one or more histologic subtypes representing various differentiation lineages and stages of normal intra-uterine development. The embryonal carcinoma cells are the stem cell component, which may differentiate towards extra-embryonal tissues (yolk sac tumor and choriocarinoma) and embryonic tissues with mesenchymal, epithelial and neuronal appearance (immature and mature teratoma)[7].

1.2. Embryonic origin of GCTs

It is generally accepted, that all GCTs of the testis develop from the precursor lesion "carcinoma *in situ*" (CIS)[8], or intratubular germ cell neoplasia[9]. CIS cells are located at the inner side of the basal membrane of the seminiferous tubules, where normally the spermatogonia reside[9]. Various phenotypical characteristics support the model, that the primordial germ cell/gonocyte is the benign counterpart of the CIS cell[10,11]. Primordial germ cells migrate from the allantois along the midline of the body, and reach the gonadal blastema during the 6-7th gestational week. From this stage onwards, the cells are called gonocytes, even though they are probably biologically identical to the primordial germ cells[3,4]. Gonocytes can be detected in the neonatal and infantile testis until they mature further at the end of the first year of life[12]. Accordingly, the initiation of tumorigenesis has to occur during intra-uterine development, if the primordial germ cell/gonocyte is the cell of origin for testicular GCT. It has been suggested, that the CIS cells are formed before the 9th gestational week. Various epidemiological and tumorbiological arguments support the concept of early initiation[13] for review. Despite the early initiation, testicular GCTs are only clinically manifest after puberty. This lag period is most likely explained by hormonal influences on the growth and development of transformed (initiated) primordial germ cells/gonocytes. The exact nature of these factors remains unknown.

So far, it is not resolved whether CIS cells are programmed to form only seminoma, nonseminoma, or a combination of the two, or whether CIS cells are multipotent. A strong argument in favor of a

multipotent nature of CIS cells is the influence of an orchidopexy of an undescended testis on the histology of a GCT occuring later in this testis[14,15]. On the other hand, differences between CIS surrounding nonseminomas and seminomas have been described regarding immunohistochemical markers and chromosomal constitution[16,17] suggesting already a determination of the later tumor histology at the stage of CIS. However, the differences could also be an effect of the adjacent tumor on the CIS cells.

1.3. Genetic changes in germ cell tumors and their precursor

GCTs are characterized by a range of genetic changes. Already the precursor lesion CIS is consistently aneuploid[18]. At this stage, when the invasive tumor has not yet occurred, the number of chromosomes is approximately tetraploid. During the progression to either seminoma or nonseminoma, (parts of) chromosomes are lost resulting in a hypertriploid DNA content for seminomas and a hypotriploid DNA content for nonseminomas. All invasive gonadal GCTs share various genomic imbalances, of which overrepresentation of the short arm of chromosome 12 as the most consistent one is found in all invasive GCTs. In 80% of the cases, this is achieved by formation of an isochromosome 12p. In part of the remaining cases, the gain is caused by a high level amplification of a restricted region of 12p[19,20]. As this feature is absent in the pre-invasive CIS, the overrepresented genes have been correlated with invasiveness/Sertoli cell independent growth[21]. Recently, *DAD-R* has been identified as a promising candidate gene allowing survival of the invasive cells outside the micro-environment of the seminiferous tubule[22].

1.4. Epidemiology

The most common anatomical site of GCTs is the adult testis. Testicular GCTs account for up to 60% of all malignancies diagnosed in men between 20-40 years of age[23]. An annual increase in incidence of 3-6% has been reported for both histological variants during the last decades in white populations throughout the world[24,25] resulting in an incidence of currently 6-11/100.000 in central Europe. In some European countries like Denmark and Switzerland, the lifetime risk to develop a testicular GCT is almost 1%. While most patients with a testicular seminoma present in their 4th decade of life, the incidence of nonseminomas peaks in the 3rd decade[23]. The tumors with both a seminoma and a nonseminoma component, present at an age between that of pure seminomas and nonseminomas[26,27].
In most cases of GCTs, the tumorigenesis cannot be explained by known external influences. However, epidemiologic studies have revealed a number of risk factors. In line with the model of tumor initiation during early development, the established risk factors seem to target intra-uterine development or the first year of life. Patients suffering from cryptorchidism show a 4-5 fold increase in risk of developing a GCT, patients with bilateral cryptorchidism have an even higher risk[28,29]. Another risk factor is a positive family history. Gonadal dysgenesis associated with the presence of at least a part of the Y chromosome, androgen insensitivity, and a low birth weight are further established risk factors[30,31]. A weak increase in risk is assumed for neonatal jaundice and hypospadias[32]. Possible influences have been postulated for various viral infections affecting the testis, high social status, and hydrocele amongst others[33]. The higher incidence in the white compared to the black population and the risk associated with preterm birth have been attributed to hormonal influence,

i.e., a higher level of estrogens might increase the risk, while higher levels of testosteron may reduce the risk[10,11,34].

An association between risk to develop a testicular GCT and disturbances in spermatogenesis has been described. The correlation is apparent from the fact, that 1% of males visiting the Andrology clinic for fertility problems, have a testicular GCT, compared to a prevalence of 1 in 11,000 in the general population. On the other hand, about 25% of patients with a testicular GCT have a disturbed spermatogenesis in the contralateral testis as well. It is unclear, however, whether the GCT is adversely affecting spermatogenesis, whether both are caused independently by the same influences or whether disturbances in spermatogenesis predispose to developing a GCT[35,36].

1.5. Stage specific treatment and prognostic factors

Modern treatment of GCTs is based on all available treatment options in the field of oncology: surgery, radiotherapy and chemotherapy. Whereas in many situations different treatment modalities result in comparable long term results, they differ significantly in their side effects. Even in metastatic stages, cure rates of 80% can be achieved by multiagent, cis-diamino-dichlorid-platin (CDDP)-based chemotherapy. These treatment results are unique among the solid tumors of adults. GCTs are therefore considered a model for a curative maligant disease[37].

1.5.1. Treatment and prognostic factors for stage I and II disease

Patients with seminoma in stage I achieve long term survival of 99%. Standard of care is orchidectomy followed by adjuvant radiation of para-aortic lymph nodes. Alternatively, a careful "wait and watch" strategy can be offered with a relapse rate of 15-20%. These patients can usually be cured by radiation or chemotherapy. In stage IIA and IIB irradiation of the infradiaphragmatic para-aortic and the ipsilateral iliac lymph nodes is standard of care resulting in relapse rates between 0-18%. In stage IIC radiation cures only 60% of the patients. Standard of care is therefore treatment with either 4 cycles of CDDP/etoposide (PE) or 3 cycles of CDDP, etoposide, and bleomycin (PEB). Less toxic regimens are being evaluated in clinical studies[38].

For stage I nonseminomas long term survival of 97% can be achieved by orchidectomy followed either by a "wait and watch" strategy, nerve sparing retroperitoneal lymph node dissection (RPLND), or primary chemotherapy with 2 cycles of PEB. The only established prognostic factor for the risk of relapse is angio-invasive growth. Patients suffering from nonseminomas stage IIA/B can also be offered different strategies: Primary RPNLD followed by two adjuvant cycles of PEB, surveillance after RPLND, or primary chemotherapy followed by secondary RPLND only in case of a residual mass. For patients with nonseminomas stage IIC three cycles of PEB are standard of care[39].

1.5.2. Treatment and prognostic factors for advanced stage seminomas and nonseminomas

Once the tumor has spread beyond the retroperitoneal lymph nodes, local treatment strategies are no longer sufficient. Based on a large data base derived from Institutions of four countries, the International Germ Cell Cancer Collaborative Group (IGCCCG) developed a prognostic model for patients with metastatic GCTs[40]. Prognostic factors are histology (seminoma versus nonseminoma), site of the primary tumor (mediastinal versus gonadal/retroperitoneal), presence of extrapulmonal metastatic sites, levels of serum markers LDH, AFP and ßHCG. The model identifies three prognostic

groups among patients with metastatic spread beyond the retroperitoneal lymph nodes. 60% of these patients present with good prognostic features resulting in a cure rate of 90%. These patients are currently treated with three cycles of PEB or four cycles of PE. Patients with intermediate prognostic features represent about 25% of all patients with advanced disease. Standard treatment here are four cycles of PEB resulting in cure rates of approximately 80%. Nonseminomas from a mediastinal primary or nonseminomas with nonpulmonary visceral metastasis or poor serum markers belong to the smallest group (constituting about 15%) with a poor prognosis. The standard treatment of four cycles of PEB achieves long term disease free survival only in 50% of patients. Treatment intensification using high-dose chemotherapy in first line treatment shows promise in phase I/II trials[41]. Recently it has been become clear, that within the poor prognosis group, a subpopulation of patients with an even worse prognosis can be identified[42]. These include patients with a primary mediastinal GCT with lung metastases (progression free survival 28% and two years overall survival of 49%), while patients with a primary gonadal or a retroperitoneal GCT without visceral metastases showed the highest progression and overall free survival (75 and 84%).

The IGCCCG criteria are helpful but clearly not sufficient as prognostic markers. Using highly toxic treatment regimen to improve results, better prognostic markers are needed to select patients, who benefit from intensified strategies rather than harboring unneccessary toxicity. Furthermore, treatment results are not satisfying and improvements are warranted for all prognostic groups with disease spread beyond the retroperitoneal lymph nodes.

1.5.3. Management of mature teratoma

In contrast to the exquisite chemosensitivity of seminomas and the invasive components of nonseminomas, mature teratomas are not eradicated by systemic chemotherapy. In case of a teratoma component in tumor manifestations, residual mature teratoma will remain viable after systemic treatment. Secondary resection of these masses is an integral part of treatment[43]. Even in case of normalized tumor markers, 15-20% of resected masses contain viable, malignant tumor and 30-40% contain residual mature teratoma, whereas in the remaining 45-50% only necrosis is found. The presence of residual mature teroma is related to the presence of a teratoma element in the primary GCT, which favors a model of selection in stead of induction of differentiation[44]. However, somatic differentiation of totipotent GCT cell lines could be induced by chemotherapy in a xenograft model[45], indicating that induction of differentiation by chemotherapy is possible at least in this experimental setting. Based on histological and clinical features like serum markers, pre- and post-chemotherapy tumor size and shrinkage of the mass during chemotherapy, the presence of pure necrosis in the resected specimen can be predicted[46]. However, occult malignant cells can not be ruled out reliably nor can they be discriminated adequately from mature teratoma. In any case, surgical removal of residual mature teratoma lesions is important because of their chance to progress to growing teratoma or so called secondary non-germ cell malignancies, i.e., malignant tumors like sarcomas or carcinomas[47,48]. These tumors behave like their histologically identical counterparts developing from normally differentiated tissues and cannot be cured by chemotherapy.

1.5.4. Treatment of relapsed and refractory disease

Despite the high sensitivity of GCTs to the available treatment protocols, 10-30% of the patients will not achieve a durable complete remission, either due to progressive disease or relapse. These patients

become candidates for salvage treatment[49]. Phase II data indicate an improved outcome with high-dose chemotherapy followed by autologous stem cell transplantation compared to standard salvage regimen[50]. About 40% of patients in first and 20% in subsequent relapse can be rescued by this treatment. Again, based on clinical parameters (response to initial treatment, level of tumor markers and site of the primary tumor), the response to salvage high-dose chemotherapy can be roughly predicted[51].

Once a patient has failed a salvage regimen, further treatment is palliative in most cases. Various substances have been tested in this situation. Paclitaxel, gemcitabine and oxaliplatin achieve responses in the range of 20% in refractory GCTs[52-55]. However, the responses seen are not durable. Clearly, better strategies for these mostly young patients are warranted. The poor outcome of the treatment in the salvage situation further underlines the need to identify patients at risk for relapse early in the course of the disease to be able to offer optimal treatment.

1.5.5. Receptor tyrosine-kinases as treatment targets in chemotherapy resistant solid tumors

The increasing knowledge of tumor biology has led to the identification of various receptor tyrosine-kinases as interesting treatment targets. The tyrosine-kinases can be targeted by specific antibodies or by small molecules inhibiting the kinase activity. Currently, drugs directed against members of the epidermal growth factors receptor (EGFR) family and against c-KIT and BCR-ABL have been approved for clinical use. The treatment results either in a direct or indirect killing of the cell or blocks the growth stimulatory effects of the tyrosine-kinase signalling. The humanized monoclonal antibody against Her2/neu (c-ERB-B2) has improved treatment outcome in Her2-positive breast cancer[56]. Gastrointestinal stromal tumors (GIST), a rare tumor entity, that used to be resistant to systemic chemotherapy, can be treated successfully with the tyrosine kinase inhibitor STI571[57]. In GIST, STI571 is targeting the receptor-tyrosine kinase, which is consistently overexpressed, frequently in a constitutively active mutant form.

Expression of EGFR and ERB-B2 has been correlated to poor prognosis in breast cancer and resistance to CDDP[58]. More than 30% of all epithelial cancers express any of EGFR-family members[59]. Particularly with the link to CDDP-resistance, it seems worth investigating the expression of these tyrosine-kinase receptors in a variety of refractory tumors, where new treatment options are warranted. However, only a case report on a patient with a Her2/neu-positive refractory GCT described a meaningful response[60]. No larger series assessing the expression of any receptor tyrosine-kinase as potential new treatment target has been described.

1.6. Biologic basis of treatment sensitivity of GCTs

The exquisite chemosensitivity of GCTs has been regarded as a reflection of the characteristics of their cells of origin, the primordial germ cell/gonocyte and the early embryonal cells derived from them. From a teleologic point of view, it is assumed that it is of paramount importance to maintain the genetic information of stem cells intact, so that already cells with minor DNA damage are eliminated by apoptosis rather than trying to repair them in order to avoid faults associated with DNA-repair which could be transmitted to their progeny[61]. The following considerations are focussed primarily on CDDP, as CDDP is considered the most active drug in the systemic treatment of GCTs. However, it is clear from clinical studies, that other substances like etoposide and bleomycin contribute to the

success of the systemic treatment of these tumors[62]. CDDP as most if not all cytotoxic agents is killing the cell by activation of an apoptotic program, in which the P53-protein has been regarded as the most important determinant.

1.6.1. P53 and chemotherapy sensitivity

The *P53*-gene and the encoded protein have become the center of interest in cancer research, when it became clear, that almost 50% of human malignancies harbour inactivating *P53* mutations[63]. The majority of these mutations are missense mutations resulting in a truncated protein with an impaired function and a prolonged halflife. Multiple downstream targets of P53 have been identified and correlated to different functions of the protein[64]. The first pathways identified in which P53 plays an important role were cell cycle control and apoptosis. However, subsequent observations indicate, that the role of P53 extends far beyond. For example, P53 seems to be involved in DNA-repair, the regulation of centrosome numbers and ploidy and regulation of telomerase activity[65,66].

P53 can mediate a G1/S-phase cell cycle arrest via transactivation of the downstream target p21. p21 inhibits the cyclin-dependent kinase 4, whose kinase activity is required for phosphorylation of Retinoblastoma protein (RB) in order to release E2F-1 and 2 from the complex with RB. E2F is necessary for the progression of cell cycle beyond the G1/S-checkpoint. P53-mediated G1/S-cell cycle arrest therefore depends on its ability to transactivate p21[67].

Alternatively, P53 can lead to apoptotic cell death via the mitochondrial pathway by induction of BAX, a pro-apoptotic member of the BCL2 family. Formation of BAX-homodimers results in release of cytochrome c from the mitochondria, activation of APAF-1 and finally of procaspase-9. Caspase-9 activates effector caspases like caspase, which are responsible for degradation of cellular structures and finally apoptotic cell death. Probably, P53 can induce apoptosis by means unrelated to transactivation of BAX, possibly by direct protein-protein interaction. A cross-talk between the P53-mediated pathways of cell cycle arrest and apoptosis exists e.g., in inhibition of caspases by p21.

P53 is upregulated on exposure of a cell to stress like DNA-damage or hypoxia. The halflife of the wild-type protein is short (about 20 min.), but it is stabilized upon activation. Its degradation via ubiquitination is promoted by binding to MDM-2 in a feedback loop, as MDM-2 is transactivated by P53. The P53-status has been correlated to prognosis and treatment outcome in a variety of tumor entities. In contrast to most other human cancers, GCTs hardly ever show P53-mutations. At the same time, most GCTs have been described positive for P53 by immunohistochemistry (references cited in Table 1 and 2, chapter 2). Therefore, the high level of P53 in GCTs is commonly considered the biologic explanation for their chemosensitivity. However, no study has demonstrated a correlation of the P53-level with treatment outcome in these tumors. The experimental evidence supporting the role of P53 is based on studies on cell lines, partly the mouse P19 teratocarcinoma cell line[68,69]. Data on human cell lines partly resulted in conflicting results. For example, the P53-status did not correlate with chemosensitivity in a panel of human GCT-derived cell lines: inactivation of P53 by the E6 protein did not affect chemosensitivity[70,71].

1.6.2. Alternative explantations for chemosensitivity of GCTs

Besides the possible role of P53, other mechanisms can be involved in the specific chemosensitivity of GCTs. In order to exert its effect, CDDP has to enter the cell. Recent data suggest an energy-

dependent uptake of the CDDP-derivative carboplatin in human hepatoma cells[72], although others assume rather a passive diffusion. Having reached the cytoplasm, CDDP binds to various molecules including RNA and proteins. Only a minor fraction reaches the nucleus and binds to DNA. The most common CDDP-DNA adducts are intrastrand crosslinks between two guanidin residues. In addition, other adducts including highly toxic interstrand crosslinks are also formed. The induced DNA-damage is sensed, which leads to activation of an apoptotic program.

GCTs are obviously not capable of repairing the CDDP-induced DNA damage effectively in order to prevent cell death. An impaired DNA-repair by the nuclear-excision repair pathway has been found in cell lines of GCTs due to low levels of XPA[73]. Looking at different members of the BCL-2-family downstream of P53, the sensitivity towards etoposide, the second most commonly used substance in the treatment of GCTs, was ascribed to a high ratio of pro-apoptotic BAX to anti-apoptotic BCL-2[74]. However, the cited investigations are either based on cell lines or lack clear correlations with the clinical course. Therefore, an unequivocal explanation for the curability of GCTs is still pending.

1.7. Possible resistance mechanisms to CDDP

Resistance mechanisms against CDDP can be active at all stages of its action beginning with the uptake of CDDP into the cell down to the execution of apoptosis. For reasons of clarity, the multiple resistance mechanisms are introduced in four separate groups. Members of the first group lower the intracellular concentration of CDDP, so that less damage is inflicted on the DNA. The second group repairs the CDDP-induced DNA-damage before it is recognized by factors intializing an apoptotic pathway. The mechanisms of the third group prevent the initiation of an apoptotic cascade upon damage recognition. Recently, it has become clear that there is a significant overlap between the latter two groups. However, the effective role taken over by specific pathways in handling CDDP-induced DNA damage in GCTs is probably primed in one way or the other justifying a separate discussion[75]. Finally, resistance can be the consequence of failure of execution of apoptosis despite initiation of an apoptotic cascade due to predominance of anti-apoptotic signals or defects in downstream apoptotic effectors. Figure 1 schematically illustrates the intracellular sequence leading to CDDP-induced apoptosis.

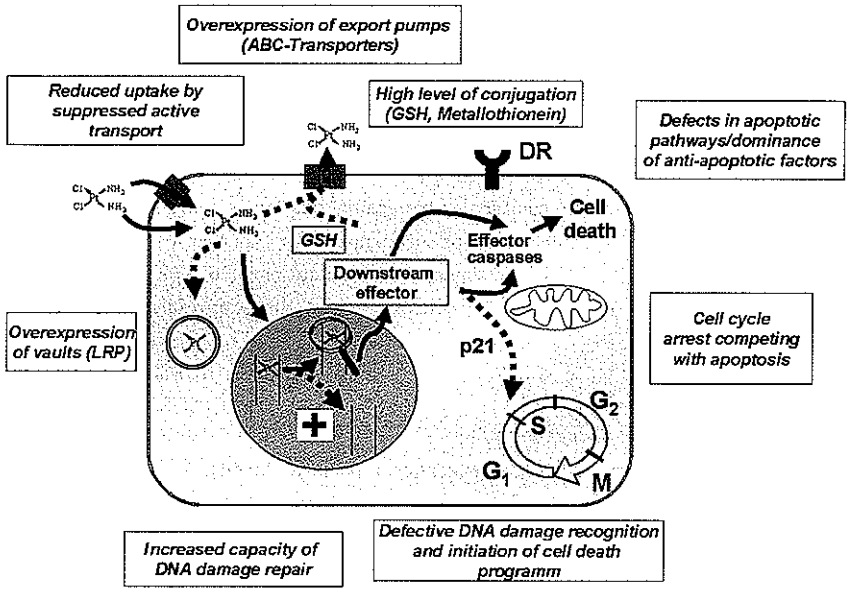

Figure 1: schematic illustration of CDDP-effect and possible resistance mechanisms.

In order to induce apoptotic cell death, CDDP has to reach the nucleus and form DNA-adducts. This DNA-damage has to be recognized to initiate a cascade resulting in cell death. Putative resistance mechanisms - indicated by dashed arrows – can inactivate or remove CDDP before reaching the DNA, the DNA-damage can be repaired before a cell death program is activated, the DNA-damage can be tolerated due to defective damage recognition, or the execution of the cell death program can be disturbed. (DR – death receptor; GSH – glutathione; LRP – lung resistance protein; ABC – ATP binding cassette)

1.7.1. Group 1: Reduction of intracellular concentration of active CDDP

The intracellular concentration of active CDDP can theoretically be lowered by a reduced uptake, increased export or detoxification. It has been suggested, that a reduced ATP-dependent uptake of the CDDP-derivative carboplatin results in resistance of hepatoma cells[72]. Other authors assume a passive diffusion rather than an active transport, in which case a reduced uptake would not be a likely means of developing resistance. Even though CDDP is not a substrate for the P-glycoprotein, the product of the multidrug resistance gene *MDR*, other members of the ATP-binding cassette superfamily of transporters (ABC-transporters) do have an affinity for CDDP[76,77]. Overexpression of these pumps therefore can result in resistance to the drug, as has been demonstrated for cell lines[77]. Probably pursuing a comparable effect by unrelated means, overexpression of the major vault protein or lung resistance protein (LRP), can also lead to elimination of CDDP before it reaches the DNA to exert its action[78]. Data on any of these possible resistance mechanisms regarding GCTs are not available in

relation to treatment response. The ABC-transporters work more efficiently when the substrate is conjugated, for example to glutathion. Furthermore, such a conjugation by itself can interfere with the toxic effect of CDDP. An increased content of metallothionein or glutathion, or overexpression of glutathion-S-transferase (GST) are further possible resistance mechanisms. A correlation of increased GST content with chemotherapy resistance has been described for GCT cell lines[79].

1.7.2. Group 2: increased capacity for DNA-repair

Once CDDP is bound to DNA, the damage can be repaired before it is recognized and an apoptotic program initiated. Responsible for the repair of such damage is the nuclear excision repair pathway (NER)[80]. As mentioned earlier, GCTs are supposed to have an impaired NER due to low levels of XPA. It is unclear, whether upregulation or overexpression of XPA can result in clinically relevant levels of resistance in GCTs.

1.7.3. Group 3: impaired initiation of apoptotic cascades upon DNA-damage recognition

Another DNA repair pathway, the DNA mismatch repair pathway (MMR), does not seem to repair the CDDP-induced damage efficiently, but probaby belongs rather to the third group of resistance mechanisms interfering with damage recognition and consecutive initiation of an apoptotic program. The MMR removes nucleotides, that have been mispaired by DNA-polymerases. Loops resulting from slipping of the polymerases during replication of repetitive sequences are also corrected. Defects of MMR-factors lead to instability of short repetitive sequences called microsatellites. Therefore, analysis of microsatellites provides information about the functionality of the MMR system. Germ line mutations in MMR factors, particularly of *hMLH1* and *hMSH2* lead to familial colon cancer. Loss or defects of MMR factors has been correlated with a better response to topo-isomerase I inhibitors like topotecan or irinotecan[81]. At the same time, the identical alterations of MMR confer resistance to CDDP, alkylating agents, methotrexate, and topo-isomerase II inhibitor doxorubicin[82-85]. Two different explanations link the loss of MMR-factors to resistance to CDDP. Some MMR-factors, in particular MLH1 and MSH2, probably play a role in detection of DNA-damage and subsequent induction of apoptosis. A loss of this function results in reduced apoptotic response to DNA-damage[84,86]. According to the second hypothesis, the defective MMR results in microsatellite instability and the accumulation of secondary mutations providing a selection advantage to the tumor cell in case of treatment[87]. MMR or microsatellite instability has been investigated in a few small series of GCTs indicating that most GCTs are microsatellite stable[88-91]. No correlations with the clinical course have been described so far. Next to the MMR, P53 has been associated with recognition of DNA-damage and induction of apoptosis. In a study on relapsed GCTs, *P53* mutations were detected in four out of 28 tumors, three of them being mature teratomas, the remaining one being a secondary non-germ cell malignancy derived from a mature teratoma. The finding has been regarded as a further proof of the relevance of P53 in determining the chemotherapy response of GCTs[92]. However, all tumors were previously treated and the mutation-containing tumors were all intrinsically chemotherapy resistant. Therefore, the contribution of the P53-status to the clinical behavior is difficult to estimate.

1.7.4. Group 4: Inhibition of activated apoptotic cascade

Once the CDDP-induced DNA-damage is sensed, an apoptotic program is activated. Most cytotoxic substances act via the mitochondrial apoptotic pathway. Defects in components of the apoptotic cascade like loss of caspases or overexpression of anti-apoptotic proteins of the BCL-2-family can result in chemotherapy resistance on the last step of CDDP-action. The level of BCL-2 has been correlated with poor response to chemotherapy in myeloid leukemia, neuroblastoma, and transitional cell carcinoma amongst others[93]. At least *in vitro*, inhibition of the function of caspases results in increased resistance to various apoptotic stimuli.

1.7.5. Aneuploidy as a mechanism to achieve chemotherapy resistance

Whereas the described putative mechanisms of chemotherapy resistance are based on mutations of changes in the expression of specific genes, an alternative means of developing chemotherapy resistance has been proposed. According to this model, aneuploidy as the most consistent genomic alteration in solid tumors allows the tumor cells to achieve a resistant phenotype by chromosome reassortments. Apart from theoretical considerations, the model is based on experimental evidence[94,95]. Various studies on different tumor entities support at least a correlation between aneuploidy and prognosis, even though this correlation does not necessarily depend on chemotherapy resistance. The regular aneuploidy of GCTs casts doubts on the general applicabilty of the hypothesis.

1.8. Aneuploidy and centrosomes

Aneuploidy is the most consistent genetic aberration in solid tumors, including GCTs (see above). Depending on the type of tumor, aneuploidization has been regarded as an early event associated with tumorigenesis[96]. At the same time, others have considered it a late event associated with tumor progression. Recently, it has become clear that amplification (hypertrophy) of centrosomes can be a driving force for aneuploidization[97]. Centrosomes are cell organelles involved in the organization of microtubules including the formation of the bipolar spindle during mitosis and meiosis. In normal cells, each cell contains one centrosome composed of two centrioles. Centrosomal abnormalities can be caused by various mechanisms, including overexpression of the serine-threonine-kinase 15 (STK15), also called Aurora-A, or loss of P53-function amongst others [65,98,99].

In GCTs aneuploidization occurs very early in the course. Already the precursor lesion CIS is consistently aneuploid, and this feature is maintained in the invasive tumors, including the mature teratomatous components as part of nonseminomas[100]. In contrast to the GCTs of adolescents or adults, the mature teratomas of infants, even though histologically identical to some of the tumors found in the adult, are diploid. Aneuploidization is only observed when these tumors progress to invasive yolk sac tumors[101]. The spermatocytic seminomas are polyploid rather than aneuploid; the cells show a total DNA content of 2n, 4n or 8n[102]. No explanation for these differences is available yet.

1.9. Aims of the thesis

The objective of this thesis is to understand the cellular basis of the behavior of different types of GCTs towards chemotherapy. The background of the exquisite chemotherapy sensitivity of invasive GCTs and the intrinsic chemotherapy resistance of mature teratomas are investigated.
Differences are sought in comparison of sensitive tumors with invasive tumors refractory to currently available agents. Potential markers that could predict chemotherapy response based on an analysis of the characteristics of primary tumor are evaluated.

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Chapter 2

ROLE OF P53 AND MDM2 IN TREATMENT RESPONSE OF HUMAN GERM CELL TUMORS

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By Anne-Marie F. Kersemaekers, Frank Mayer, Michel Molier, Pascale C. van Weeren, J. Wolter Oosterhuis, Carsten Bokemeyer, and Leendert H.J. Looijenga

Role of P53 and MDM2 in Treatment Response of Human Germ Cell Tumors

By Anne-Marie F. Kersemaekers, Frank Mayer, Michel Molier, Pascale C. van Weeren, J. Wolter Oosterhuis, Carsten Bokemeyer, and Leendert H.J. Looijenga

Purpose: **Testicular germ cell tumors (TGCTs) of adolescents and adults are very sensitive to systemic treatment. The exquisite chemosensitivity of these cancers has been attributed to a high level of wild-type P53.**

Materials and Methods: **To clarify the role of P53 in treatment sensitivity and resistance of TGCTs, we performed immunohistochemistry and Western blotting analysis on a series of 39 fresh-frozen primary TGCTs before therapy (unselected series). In a series of forma-lin-fixed paraffin-embedded TGCTs of patients with fully documented clinical course, including treatment-sensitive (n = 17) and -resistant (n = 18) tumors, P53 status was assessed by immunohistochemistry and mu-tation analysis. In addition, the involvement of MDM2, a P53 antagonist, was investigated by immunohisto-chemistry, reverse transcriptase polymerase chain re-action, and in situ hybridization.**

Results: **Immunohistochemistry demonstrated ab-sence of staining for P53 in 36%, 41%, and 17% of the unselected, responding, and nonresponding TGCTs, respectively. Of the positive TGCTs, most tumors, ie, 49%, 41%, and 33%, showed 1% to 10% positive nuclei. This overall low level of P53 was confirmed by Western blotting. Mutation analysis revealed only one silent *P53* mutation in one of the responding patients. All embry-onal carcinomas were homogeneously positive for MDM2, encoded by the full length mRNA, while a heterogeneous pattern was found for the other histo-logic components. Amplification of *MDM2* was detected in one out of 12 embryonal carcinomas.**

Conclusion: **Although our results are in line with previous findings of the presence of wild-type P53 in TGCTs, they show that a high level of P53 does not relate directly to treatment sensitivity of these tumors, and inactivation of P53 is not a common event in the development of cisplatin resistance.**

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TESTICULAR GERM CELL tumors (TGCTs) of adolescents and adults, ie, seminomas and nonseminomas, are of particular interest from a clinical point of view because of their exquisite sensitivity to treatment. While seminomas and nonseminomas can be effectively treated with cisplatin-based chemotherapy, seminomas are also highly sensitive to irradiation (reviewed in [1-3]). Approximately 80% of patients with metastatic disease can be cured by systemic treatment.[4] In addition, TGCTs are also biologically interesting, since they mimic embryonal development to a certain extent (reviewed in [5-8]). Seminomas show characteristics of early (primordial) germ cells, whereas nonseminomas can be composed of embryonal carcinoma, being the stem-cell component, which may differentiate to either yolk sac tumor and choriocarcinoma (the extra-embryonic lineages) or teratoma (the somatic lineage).[9] Another intriguing clinical finding is that fully differentiated ("mature") teratoma components are resistant to chemotherapy. [10-12] Surgery is needed to remove these lesions because of their potential for secondary malignant transformation, which can lead to non-germ cell malignancies.[13]

Various investigations have focused on the P53 pathway to explain the chemosensitivity of TGCTs. Most studies reported a high level of wild-type P53 protein based on immunohistochemistry. An overview of the different studies is given in Table 1,[14-31] indicating that although a high percentage of positive tumors are reported, the majority of these cases show less than 30% of positive tumor nuclei. In fact, a significant number of cases are scored as containing between 1% and 10% positive nuclei, and some of the tumors showed no staining at all. In contrast to many other solid cancers, *P53* mutations have hardly been identified in unselected TGCTs (see Table 2 for publications and results [20,21,29,31-40]). Out of the 281 sequence-verified tumors, [19] (6.7%) were demonstrated to have a mutation. Comparison of two independent human TGCT-derived cell lines, one with functional and one with a mutant P53, showed that the former was more

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Table 1. Overview of Selected Immunohistochemical Studies on P53 in Germ Cell Tumors

Frist Author (ref)	Histology	No. Patients	Antibody*	Material	Overall positive Staining (%)	Fraction of Positive Cells (%)		
						0-5 or 0-10	5-30%, 10-30% or 0-25	>30% or >25%
Baltaci [14]	NS, SE	24	Do-7	P	83	—†	—	—
Heidenreich [15]	NS, CT	149	Signet Laboratories	P	95	—	—	—
Heidenreich [16]	NS, CT	15	Signet Laboratories	P	94	—	—	—
Eid [17, 18]	SE, NS	77	Do-7	P	91	—	—	—
Chou [19]	NS	10	Do-1	P	80	0	0	80%
Giulou [20]‡	SE, NS	35	PAb 240 CM1 Pab1801	F	65 100 35	15 6 —	15% 26% —	35% 68% —
Lothe [21]	SE, NS	14	PAb 1801 CM1	P	78 85	64§ 71§	14%‖ 14%‖	0% 0%
Albers [22]	NS	62	PAb 1801	P	99	24	23%	52%
Riou [23]	SE, NS	24	CM1 and Do-7	P	67¶	8	42%	25%
Lewis [24]	SE, NS	113	-	P	93		52#	41%**
Ulbright [25]	NS	69	PAb 1801	P	49	26	20%	3%
Bartkova [27]	NS, SE	107	CM1, PAb1801; PAb 421	P	84	37	47%††	
Heimdal [29]	Familial, bilateral Se, NS	15	CM1	F	0	0	0%	0%
deRiese [28]	NS	84	—	—	40	19	19%	2%
Bokemeyer [31]	SE, NS	16	Pab 1801	P	88	25	13%	62%
Ulbright [26]	NS	50	—	—	50	—	—	—
Przgodzki [30]	Mediastinal, testicular SE	26	Do-7	P	30 77	0 0	30% 77%	0% 0%

Abbreviations: SE, seminoma; NS, nonseminoma; CT, combined tumor; P, formalin-fixed, paraffin-embedded; F, frozen.
* Antibodies used in the studies: PAb 1801, PAb 240, Do-1 (Oncogene Science, Cambridge, MA); CM1 (NovoCastra); Do-7 (Dako, Glostrup, Denmark); PAb 421 (as reported in Bartkova et al [27]).
† Dashes (—) indicate "not specified" in the article.
‡ The authors selected field for quantification of P53-positive cell fraction for areas with high P53 staining.
§ 0% to 10% positive cells.
‖ 10% to 30% positive cells.
¶ > 10% positive cells.
# 0% to 25% positive cells.
** > 25% positive cells.
†† 1% to 50% positive cells.

sensitive to cisplatin than the latter.[33] These studies led to the conclusion that high levels of wild-type P53 account for the exquisite chemosensitivity of TGCTs. However, we found no differences in treatment sensitivity between a well-characterized TGCT-derived cell line with functional P53 (NTera2) and a line without (NCCIT).[41] Inactivation of P53 by the HPV16-E6 protein in the cisplatin-sensitive NTera2 cell line did not result in resistance. In addition, the only resistant TGCT-derived cell line (2102Ep) had functional P53.

Under physiologic conditions, the function of P53 can be inactivated by MDM2. MDM2 binds to the transactivation domain of P53, thereby directly inhibiting P53 function, and in addition, binding of MDM2 to P53 results in degradation of P53 by ubiquitination.[42,43] Overexpression of *MDM2*, mostly due to gene amplification, has been correlated with a poor prognosis and resistance to chemotherapy in various malignancies.[44-48] Two studies dealt with the presence of MDM2 in TGCTs.[20,49] Immunohistochemistry demonstrated that more than 50% of the tumors showed a positive staining. However, only three out of 65 TGCTs—one seminoma, one teratoma, and one choriocarcinoma—contained amplification of *MDM2*.[23,36] None of these studies correlated their findings with clinical outcome.

The objective of the present study was to clarify the role of P53 and MDM2 in sensitivity and resistance of TGCTs to cisplatin-based chemotherapy. To exclude interobserver variability, we applied both Western blotting and immunohistochemistry to assess P53 protein level. By assuming a crucial role of a high-level wild-type P53 in chemosensitivity of TGCTs and an association between *P53* mutations and chemotherapy resistance, high levels of P53 protein andno or only small numbers of mutations were expected

Table 2. Summary of Selected Published Data on Mutation Analysis of *P53*

First Author (ref)	Group	No. Patients	Material	Methods	Analyzed exons	Finding (%)
Kim [32]	Intracranial GCT	19	P	Dideoxyfingerprinting, sequencing	5-8	26%
Houldsworth [33]	Resistant SE, NS, NGCM	23	-	SSCP, direct sequencing	5-9	17%
Kuczyk [34]	CIS	18	F	RNA SSCP direct DNA sequencing	5-8	39%
Giullou [20]	TGCT	35	F	SSCP	5-9	0%
Lothe [21]	SE and NS	23	F	CDGE	Hotspots in 5,7,8	0%
Schenkman [35]	SE and NS	30	F, P	SSCP, direct sequencing	5-8	0% (1/30 silent mut)
Fleischacker [36]	SE and NS	39	F	SSCP, direct sequencing	5-8	0%
Peng [37]	SE and NS	22	F	SSCP, CDGE, direct Sequencing	4-9	0%
Helmdal [29]	SE and NS	15	F	CDGE, denaturing gel electrophoresis	5-8	0%
Serth [38]	SE and NS	31	-	Allelotyping, SSCP	5-7	5/16 SSCP shift 9/29 LOH
Bokemeyer [31]	SE and NS	15	-	RNA-SSCP, direct sequencing	5-8	73%
Wei [39]	SE	17	F	SSCP, direct sequencing	5-8	23.5%
Strohmeyer [40]	TGCT	40	F	SSCP, direct sequencing	4-8	0%

Abbreviations: SE, seminoma; NS, nonseminoma; (T)GCT, (testicular) germ cell tumor; NGCM, non–germ cell malignancy; P, formalin-fixed, paraffin-embedded; F, frozen; SSCP, single-strand confirmation polymorphism; CDGE, constant denaturant gel electrophoresis; LOH, loss of heterozygosity.

in agroup of unselected cases and in chemosensitive cases. In contrast, resistant cancers would show either a low level of wild-type P53 and/or an increased frequency of *P53* mutations or inactivation by overrepresentation of MDM2 encoded by the full-length mRNA.

MATERIALS AND METHODS

Patient Material

Fresh-frozen and formalin-fixed paraffin-embedded tissue blocks from 39 unselected patients (23 nonseminomas and 16 seminomas) were collected between 1991 and 2000 in close collaboration with urologists and pathologists in the southwestern part of the Netherlands. They were retrieved from the archive of the Laboratory for Experimental Patho-Oncology, Department of Pathology, University Hospital Rotterdam/Daniel. For these patients, no data on the clinical course were available.

From 17 patients treated at the University Hospital Rotterdam/Daniel between 1991 and 1994 who remained continuously disease-free after initial treatment, formalin-fixed paraffinembedded material of the primary TGCT was collected. The series consisted of nine seminomas and eight nonseminomas.

Formalin-fixed, paraffin-embedded samples from 18 chemotherapy-refractory patients diagnosed between 1991 and 1998, treated within various trials led by Tu" bingen University, Germany, were investigated. Patients were considered refractory when progression or relapse occurred despite adequate initial and salvage treatment, including high-dose chemotherapy with autologous stem-cell transplantation. The material of nine patients was obtained at initial diagnosis; in eight cases, the material was sampled after exposure to chemotherapy, and in one case, material from both the primary tumor and a metastatic tumor in relapse was available. The group of refractory tumors consisted of 16 nonseminomas, one seminoma, and one secondary non–germ cell malignancy. Table 3 summarizes the characteristics of the responding and refractory patients. All cases were reviewed and diagnosed by J.W.O. according to the World Health Organization classification, and the fully documented clinical course was available for these patients.

Cell Lines

The TGCT-derived cell lines NTera2, NCCIT, and 2102Ep were maintained in principle as previously described.[41] The breast cancer–derived cell lines SKBR-3, T47D, and MCF-7 were grown as monolayers and maintained at 37°C in a humidified cell-culture incubator with 8.5% carbon dioxide in HEPES-buffered RPMI 1640 supplemented with 10% fetal calf serum (Gibco BRL, Paisley, United

Table 3. Patient Characteristics

	Chemosensitive (n = 17)	Refractory (n = 18)
Age, years		
Median	33	31
Range	17-49	17-56
Histology		
Seminoma	9	1
Nonseminoma	8	16
Non germ cell tumor malignancy	0	1
Stage at diagnosis (according to UICC)		
Initial treatment after surgery		
surveillance	0	2
radiation	8	0
chemotherapy	9	16
Follow-up, months (range)		
Median	81.4	45.3
Range	30-118	12.5-180
Relapse-free survival, months		
Median	N.R.	18.0
Range		0-150
Response to initial treatment		
Complete remission	17	4
Partial remission, marker negative	0	8
Partial remission, marker positive	0	2
Progressive disease	0	2
Unknown	0	2
No. of salvage regimens		
Median		3
Range		1-7

Abbreviations: NR, not reached; UICC, International Union Against Cancer.

Kingdom), penicillin 100 IU/mL (Sigma-Aldrich, Zwijndrecht, the Netherlands), streptomycin 100 µg/mL (Sigma-Aldrich), and L-glutamine 2 mmol/L (Gibco).

Immunohistochemical Detection of P53 and MDM2

Paraffin sections of 3-µm thickness were mounted on aminopropylethoxysilane-coated slides, deparaffinized in xylene, and rehydrated. Pressure cooking in citrate buffer 0.01 mol/L, pH 6.0 (1.2 bar), was used for antigen retrieval. All antibodies were diluted in phosphate-buffered saline (PBS) with 1% bovine serum albumin. Primary antibodies (mouse monoclonal anti-P53, Do-7, 1/50 [Dako, Glostrup, Denmark]; mouse monoclonal anti-MDM2, Ab1, clone smp14, 1/50 [Neomarkers, Fremont, CA]) were incubated at room temperature for 1 hour (P53) or overnight (MDM-2). Biotin-labeled rabbit-antimouse immunoglobulins and a biotinylated AP-streptavidin complex (both Dako) were subsequently applied for 30 minutes each. A solution of new fuchsin, naphthol, and levamisole in a Tris HCL buffer 0.2 mol/L (pH 8.0) was used as chromogen; slides were counterstained with Mayer's hematoxylin stain. Only red nuclear staining was considered positive. For a negative control, the primary antibody was omitted on serial slides. Appropriate positive control sections were stained simultaneously (colon cancer for P53, breast cancer for MDM2). Two investigators (A.-M.F.K. and F.M.) independently assessed samples. In case of discordance, slides were re-evaluated. For evaluation of P53, 300 cells were scored in three randomly selected high-power fields. Furthermore, four randomly selected slides were stained and evaluated independently in the pathology department of the University of Tromso (Norway), as described before.[50]

Immunoprecipitation and Western Blotting

Cultured cells and fresh-frozen tissue samples were used for immunoprecipitation and Western blotting. Samples containing only a limited nontumor component were selected. For immunoprecipitation studies, cells were washed twice in ice-cold PBS and harvested in lysis buffer (Tris HCl 50 mmol/L [pH 8.0], EDTA 5 mmol/L, NaCl 150 mmol/L, 0.75% NP40, Pefabloc SC 1 mmol/L [Roche, Mannheim, Germany], sodium fluoride 50 mmol/L, sodium o-vanadate 10 mmol/L, and one tablet of Complete mini [Roche] per 10 mL of lysis buffer). Protein was subjected to immunoprecipitation by incubating 1 µg of monoclonal antibody against MDM2 (IF-2; Oncogene Science, Cambridge, MA). Specific complexes were collected after incubation with Protein A agarose (Roche). After the precipitates were washed in lysis and wash buffer (Tris HCl 10 mmol/L [pH 8.0], EDTA 1 mmol/L, and sodium o-vanadate 1 mmol/L [Sigma-Aldrich]), they were electrophoresed in 8% sodium dodecyl sulfate (SDS)–polyacrylamide gels, transferred to PVDF membrane (Amersham/Pharmacia, Buckinghamshire, United Kingdom), and probed for MDM2 as described below.

For Western blot analyses, cells were washed with ice-cold PBS, scraped into lysis buffer, and cooled on ice. After centrifugation and denaturation of the proteins at 95°C, the protein lysate (50 µg/lane) was electrophoresed on 10% SDS-polyacrylamide gels and transferred to PVDF membranes. The filters were blocked with PBS containing 3% nonfat dried milk and 0.1% Tween-20 and probed with the respective antibodies. MDM2 was detected using IF-2 antibody 5.0 µg/mL (Oncogene Science), and P53 was detected using Do-7 antibody 0.08 µg/mL (Dako). The input for Western blotting was standardized using the nuclear replication protein A, a nuclear protein present at relatively constant levels in human cells.[51,52] After washing and subsequent incubation with horseradish peroxidase–conjugated rabbit-antimouse antibody (Dako), specific complexes were detected using a chemiluminescent technique according to the manufacturer's recommendations (ECL kit; Amersham/Pharmacia).

Amplification Analysis for MDM2 Using Fluorescence In Situ Hybridization

Frozen tissue sections of 5 µm were mounted on aminopropylethoxysilane-coated slides, air dried, submerged in methanol/acetone (1:1) at -20°C for 20 minutes, and air dried again. Sections were digested with 0.0005% pepsin (Sigma-Aldrich) in HCl 0.01 mol/L for 1 minute at 37°C, rinsed in demi-water, and dehydrated. YAC-MDM2 probe 75la4 (chromosome 12 band q14, obtained from CEPH, Paris, France) was used in combination with a chromosome 12 centromeric probe (pα12H8).[53,54] DNA of the YAC-MDM2 was isolated and human sequences were amplified by inter-ALU polymerase chain reaction (PCR), using the primers Alu-1 and Alu-2. [55] The centromeric probe was labeled with digoxigenin-11-deoxyuridine triphosphate and the YAC-MDM2 probe was labeled with biotin-16-deoxyuridine triphosphate (Roche) using a nick-translation kit (Gibco). After denaturation (73°C for 5 minutes in Hybmix), the probes were preannealed with an excess of Cot-1 DNA (Life Technologies/Gibco). The denatured probe mix was added to denatured slides (3 minutes in 70% formamide/two times standard saline citrate (2xSSC) [pH 7.0] and 5 minutes in 70% ethanol at -20°C and then dehydrated) and hybridized for 48 hours. Slides were washed in 50% formamide/2xSSC. The hybrids were visualized with fluorescein isothiocyanate–conjugated sheep-anti-digoxigenin antibody (Roche) and Cy3-conjugated avidin antibody (1/50; Jackson ImmunoResearch, West Grove, PA). The *MDM2* gene was considered amplified when at least twice as many hybridization signals were found for *MDM2* than for the centromeric probe.

DNA Isolation

Of formalin-fixed paraffin-embedded tissue blocks considered suitable for DNA isolation without further processing, four sections of 25 µm were used. In case of significant nontumor components, tumor areas were microdissected from four to eight 10-µm-thick unstained sections. The material was deparaffinized with xylene, washed in 100% ethanol, and air dried. The tissue was incubated in lysis buffer (Tris 50 mmol/L [pH 8], EDTA 100 mmol/L, NaCl 100 mmol/L, and 1% SDS) containing 25 µL of proteinase K (10 mg/mL) overnight at 55°C followed by incubation with RNase at 37°C for 30 minutes. After phenol/chloroform extraction, DNA was precipitated with isopropanol, washed with 70% ethanol, air dried, and resuspended in a suitable volume of TE buffer. Fresh-frozen material was processed in an identical manner, apart from omission of deparaffinization and rehydration.

Single-Strand Conformation Polymorphism and Sequencing of P53 Exons 5 to 8

Isolated DNA was used as a template for radioactive PCR using alpha-32 P as label. PCR settings and primer sequences are given in Table 4. The PCR products were run on a nondenaturing gel (8% acrylamide/bisacrylamide 49:1, 10% glycerol) for 16 hours at 8 watts. Samples showing band shifts or extra bands on single-strand conformation polymorphism (SSCP) were subjected to sequencing of the respective exons. Sequencing was done using a commercial kit (Fermentas, St Leon-Rot, Germany) according to the manufacturer's instructions, except for the addition of 1% dimethylsulfoxide in the reaction mix. Sequencing was done with both forward and reverse primers spanning the whole exon. The cell lines DU145, Caco2, and

Table 4. Primers for SSCP and Sequencing

Exon 5	Primer A	F	5'- GACTTCAACTCTGTCTC -3'
	Primer NB	R	5'- ACTGCTTGTAGATGGCCATG -3'
	Primer NA	F	5'- CAGCTGTGGGTTGATTCCAC -3'
	Primer B	R	5'- ACCCTGGGCAACCAGCCCTGT -3'
Exon 6	Primer A	F	5'- AGGCCTCTGATTCCTCACTG -3'
	Primer NB	R	5'- GCACCACCACACTATGTCGA -3'
	Primer NA	F	5'- CTCCTCAGCATCTTATCCGA -3'
	Primer B	R	5'- CCACTGACAACCACCCTT -3'
Exon 7	Primer A	F	5'- AGGCGCACTGGCCTCATCTT -3'
	Primer NB	R	5'- TCCAGTGTGATCATGGTGAGG -3'
	Primer NA	F	5'- CATGTGTAACAGTTCCTGCATG -3'
	Primer B	R	5'- GCGGCAAGCAGAGGCTGG -3'
Exon 8	Primer A	F	5'- CCTTACTGCCTCTTGCTTCTC -3'
	Primer NB	R	5'- CTTGCGGAGATTCTCTTCCTC -3'
	Primer NA	F	5'- TTGTGCCTGTCCTGGGAGAG -3'
	Primer BB	R	5'- CTCCACCGCTTCTTCTCCT -3'

NOTE. Primers A forward and B/BB reverse were used for sequencing of the different exons. PCR settings: 95°C, 30 minutes; 58°C, 30 minutes; 72°C, 40 minutes; 35 cycles

NCCIT together with two colon cancer samples with known *P53* mutations served as positive controls for SSCP and sequencing.

Reverse Transcription PCR for the MDM2 Splice Variants

RNA was isolated from 14 snap-frozen TGCTs (10 seminomas and four embryonal carcinomas) with Trizol reagent (Life Technologies/Gibco) according to the manufacturer's instructions. For reference, the histologic composition of the tumor under investigation was checked by microscopic analysis of a parallel section stained with hematoxylin and eosin. RNA was isolated in the same way from four cell lines (Tera1, NTera2, 2102EP, and NCCIT). All RNA samples were pretreated with RNase-free DNase I according to the standard method.[56] The RNA pellets were dissolved in DEPC-treated water. First-strand cDNA was synthesized from 1 µg of oligodeoxythymidine and random hexamer–primed DNase I–treated RNA in a total volume of 40 µL according to standard procedures. The cDNA quality was checked by PCR with the primers HPRT 244 and HPRT 246, which amplify a specific 587-base pair fragment from mRNA encoding the housekeeping gene hypoxanthine phosphoribosyltransferase (*HPRT*).[57]

Presence of *MDM2* splice variants was determined by PCR amplification on the generated cDNA, using gene-specific primer combinations spanning the complete coding region. The following primers were used: forward, 5'-GGCCCGGAGAGTGGAATG-3'; reverse, 5'-ATAAATTTCAGGTTGTCTAAATTC- 3' (annealing at 58°C). The expected size of the full-length transcript is 1,685 base pairs.

RESULTS

Immunohistochemistry for P53 and MDM2

Unselected tumor samples. To study the presence of P53 and MDM2 protein in a randomly collected series of TGCTs, immunohistochemical analysis was performed on paraffin-embedded tissue sections of a series of 39 TGCTs (16 seminomas and 23 nonseminomas). In this series, the clinical outcome of which is not known, but frozen tissue is available for Western blotting (see below), 36% of the tumors showed no staining for P53 at all, including seven seminomas and 10 nonseminomas (see Table 5). In 49% of the cases, 1% to 10% of the tumor nuclei were positive. In 15% of the cases, there was 10% to 30% positive staining. None of the tumors showed more than 30% positive tumor nuclei. Positive cells were found in all different histologic elements except choriocarcinoma, which was completely negative. Representative examples are shown in Fig 1A and 1B. In total, 1% or more positive tumor nuclei for P53 were identified in 25 (64%) of the 39 TGCTs. To confirm our results, parallel sections of a selected number of TGCTs (n = 4) were stained and evaluated independently; the results were the same.

Immunohistochemical analysis of the same series of TGCTs as studied for P53 showed that all seminomas were weakly positive for MDM2 in a heterogeneous pattern (Table 5, and see Fig 1C), while no staining was found in choriocarcinoma. A strong positive staining was found in all tumor cells of embryonal carcinoma (see Fig 1D), while only scattered positive cells were observed in the yolk sac tumors and teratomas.

Table 5. Results of the Immunohistochemical Findings for P53 and MDM2 in TGCTs of Unselected, Chemosensitive, and Refractory Patients (% of positive nuclei)

	Unselected patients (n=39)		Responding patients (n=17)		Nonresponding patients (n=18)	
	%	SE/NS	%	SE/NS	%	SE/NS
P53						
0%	36	6/8	41	6/1	17	0/3
1-10%	49	8/11	41	3/4	33	1/5
>10%-30%	15	2/4	12	0/2	33	0/6
>30%	0	0/0	6	0/1	17	0/3
MDM2						
0%	28	10/1	35	5/1	17	0/3
1-10%	26	6/4	35	4/2	11	1/1
>10%-30%	10	0/4	6	0/1	17	0/3
>30%	36	0/14	24	0/4	55	0/10

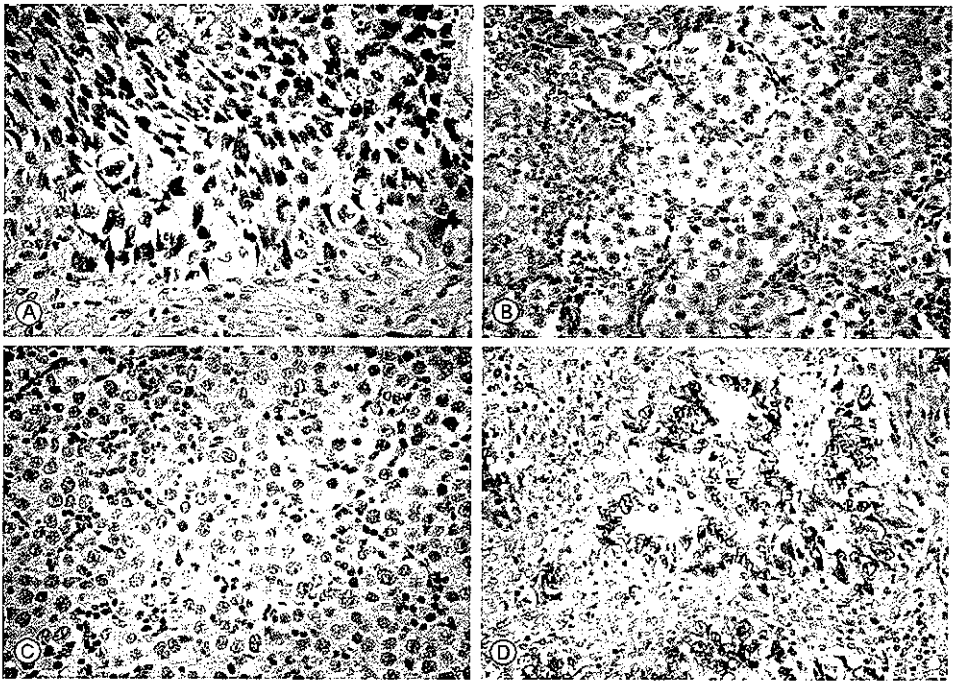

Fig 1. Representative examples of the immunohistochemical stainings for P53 and MDM2. (A) Nonseminoma with strong nuclear P53 staining; (B) seminoma with single P53-positive cells; (C) seminoma without positive MDM2 staining; (D) embryonal carcinoma with strongly positive nuclear MDM2 staining (all x200).

Chemosensitive *and refractory tumor samples.* To shed light on the actual role of P53 and MDM2 in chemosensitivity and resistance, we investigated a series of patients with TGCTs responding (n >17) or not responding (n >18) to chemotherapy by using immunohistochemistry for P53 and MDM2. The clinical data of the patients and histology of the tumors are listed in Table 3. The results of immunohistochemistry are also summarized in Table 5, indicating that the findings for P53 in the responding TGCTs are comparable to the results in the unselected series; 41% of the responding TGCTs (six seminomas and one nonseminoma) showed no staining at all; 41% had 1% to 10% positive nuclei, and 12% of the TGCTs had 10% to 30% positive nuclei. Only one tumor showed more than 30% positive nuclei. In the series of refractory cases, a trend toward a lower number of completely negative cases (17%, three nonseminomas) and a higher number of ositive cases (33% for 10% to 30% positive nuclei, and 17% for >30% positive nuclei) was observed, compared with the unselected and responding series. No differences were identified between cases obtained before (n =11) and after (n =7) exposure to chemotherapy. MDM2 also showed a higher number of positive cases in the nonresponding series (15 of 18) compared with the unselected (28 of 39) and responding (11 of 17) series. This is explained by the higher number of nonseminomas in the first series. Again, no differences between chemotherapy-naive and exposed tumors were observed.

Western Blotting and Immunoprecipitation of P53 and MDM2

As immunohistochemistry can be influenced by differences in fixation, pretreatment protocols, the method of staining and scoring, and particularly by selection of regions in case of tumor heterogeneity, we performed Western blotting and immunoprecipitation for P53 and MDM2.

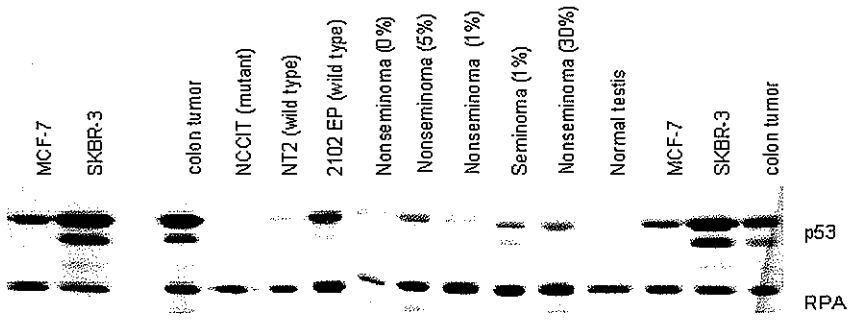

Fig 2. Western blotting for P53. All nonseminomas and seminomas showed low levels of P53 compared with SKBR-3 and colon tumor (high expression) and MCF-7 and normal testis (low expression). Replication protein A (RPA) was used as a loading control. Percentage of positive nuclei by immunohistochemistry is given in parentheses

Fourteen snap-frozen TGCTs (seven seminomas and seven nonseminomas, all with a limited nontumor component), one normal testicular parenchyma sample, and three well-characterized TGCT-derived cell lines (NTera2, NCCIT, and 2102Ep) were investigated. All TGCTs, the normal testis, and the NTera2 and NCCIT cell lines showed a low level of P53. This was comparable to the level found in the breast carcinoma–derived cell line MCF-7, known to have a wild-type and low-level P53. The level was lower than that found in the breast carcinoma cell lines SKBR-3 and T47D and a colon cancer cell line, all of which are known to have mutant P53 and thus a higher protein level (see Fig 2). The only resistant cell line (2102Ep) showed the highest level of P53 in our series, in accordance with our previous data.[31] Immunoprecipitation of MDM2 performed on one seminoma and two nonseminomas demonstrated, in concordance with the immunohistochemical results, a high level in embryonal carcinomas and a low level in the other histologic components. Rehybridization of the blots with the P53-specific antibody showed that most of P53 was bound to MDM2.

MDM2 Amplification Using Fluorescence In Situ Hybridization, Alternative Transcripts, and P53 Mutation Analysis

Due to the specific high level of MDM2 in embryonal carcinoma, and the finding that gene amplification is the most frequent mechanism resulting in *MDM2* overexpression, we performed double-color fluorescence in situ hybridization on tissue sections to screen for amplifications of *MDM2*. Six seminomas and 14 nonseminomas (including 12 tumors containing an embryonal carcinoma component) of the unselected series, for which frozen tissue was available, were analyzed with a centromere 12–specific probe and a *MDM2*-specific probe. Representative results are indicated in Fig 3A and 3B. Only one embryonal carcinoma (8%) showed amplification of *MDM2*. Reverse transcription PCR using primer sets to amplify all described alternative transcripts of *MDM2* demonstrated that indeed a higher level of expression is found in embryonal carcinoma compared with seminomas and that only the full-length transcript (1,685 base pairs) is present (see Fig 3C).

Mutation analysis of exons 5 to 8 of *P53* by SSCP and sequence verification demonstrated a silent mutation (exon 6, codon 213, CGA to CGG)[58] in one of 17 TGCTs of the responding patients and none of the 18 TGCTs of the nonresponding patients. Because of the purification step of tumor cells before DNA isolation in case of a significant stromal component, the lack of mutation detection was not due to a limited amount of tumor DNA in the assay. All positive controls revealed the expected mutations (see Materials and Methods).

DISCUSSION

TGCTs, ie, seminomas and nonseminomas, account for 1% to 3% of all neoplasms in men. They are the most common cancers in white males between 15 and 45 years of age.[59] These tumors are unique in their responsiveness to cisplatin-based chemotherapy and are considered a model for curative disease.[1] This phenomenon has been explained by high levels of wild-type P53, as demonstrated by immunohistochemistry in a number of studies (see Tables 1 and 2). On average, 70% of the tumors were considered positive, although in most cases, only a minority of tumor cells showed a P53 signal and even completely negative

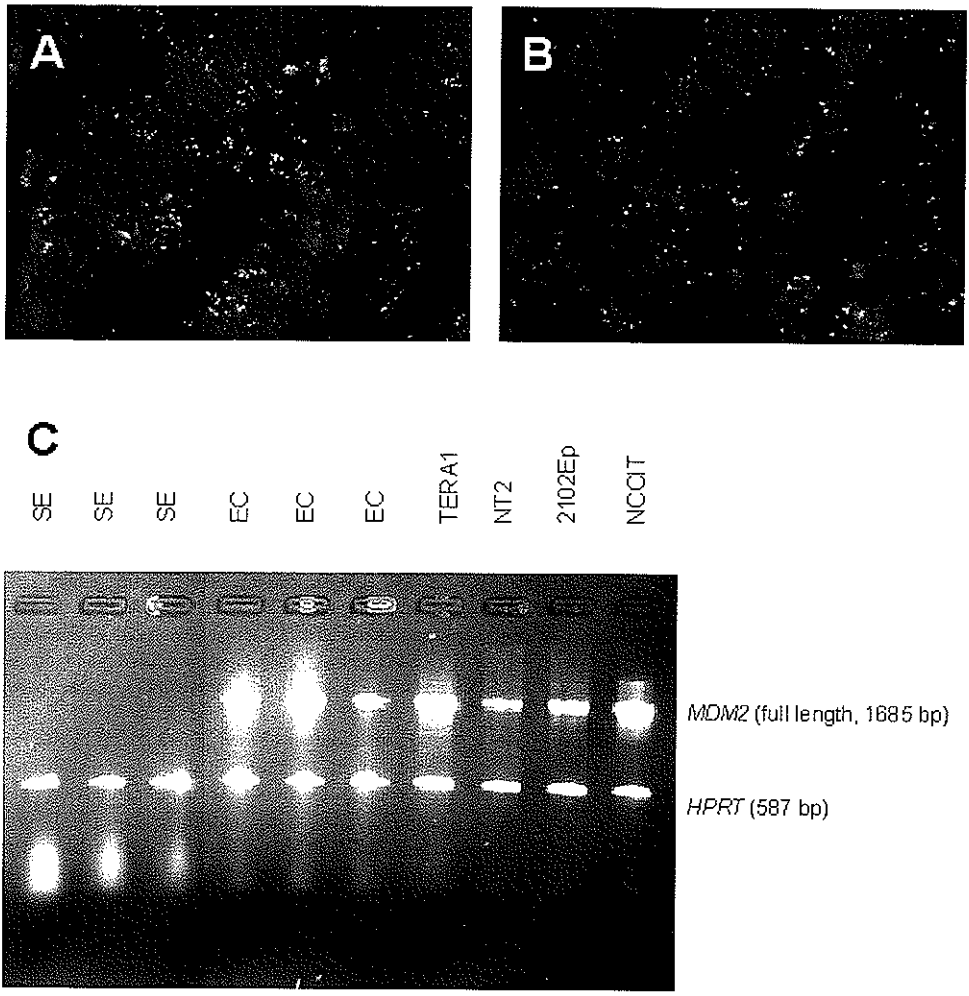

Fig 3. Representative examples of fluorescence in situ hybridization with centromere 12 (red) and *MDM2* (green) probes on frozen sections of (A) an embryonal carcinoma and (B) a seminoma. Note the increased *MDM2* copies compared with the centromere in the embryonal carcinoma. (C) Examples of reverse transcription PCR for expression and alternative transcripts of *MDM2*. Only the full-length mRNA (1,685 base pairs) is present in the embryonal carcinomas (ECs) and nonseminoma-derived cell lines but not in seminoma (SE).

tumors were identified. In analyses of these data by the fraction of positive cells, approximately 32% of the tumors contained no P53-positive cells, approximately 45% showed positivity in more than 5% of tumor cells, and approximately 23% had a P53 signal in 30% or more tumor cells. These numbers are in the same range as our results (see Table 5). Regarding the putative role of P53 in the favorable response of TGCTs to therapeutic interventions

it is important to realize that in both the unselected series and the series of responsive tumors, more than one third of the cases showed no P53 staining.

To assess the actual level of P53 protein in TGCTs, we performed Western blotting on seminomas, nonseminomas, and well-characterized nonseminoma-derived cell lines. These experiments demonstrated convincingly that the protein level of P53 is in the range of control samples with a low level of P53, ie, normal testis and the breast carcinoma cell line MCF-7. [60] The protein level was far lower than that found in a colon cancer–derived and two breast carcinoma– derived cell lines, all known to have a high level of P53. The discrepancy between our results and the finding of a high level of P53 in mouse teratocarcinomas [61] may indicate that mouse teratocarcinomas are not a proper model for human TGCTs. This assumption is supported by further discrepancies such as the absence of seminoma components, a prepubertal clinical presentation, and a diploid chromosomal constitution of mouse tumors (reviewed in [62-64]).

To study the role of P53 in chemotherapy resistance in vivo, we investigated the presence of P53 by immunohistochemistry on a clinically well-defined group of patients with nonresponding/ refractory TGCTs. All patients had failed adequate first-line treatment. In the course of the disease, most of them had been considered suitable candidates for salvage high-dose chemotherapy but had progressed or relapsed afterward. The treatment-resistant tumors did not show reduced levels of P53 compared with the responsive and the unselected groups. In contrast, the resistant group contained fewer cases without P53 signal in any fraction of cells (17%) and more cases with a high percentage of positive tumor nuclei (both between 10% and 30% and > 30%). This finding cannot be explained by a prior exposure of tumor cells to chemotherapy in the resistant group, as no difference was seen between tumors sampled before and after chemotherapy. Furthermore, mature teratomas, known to be intrinsically resistant to cisplatin-based chemotherapy,[10-12] also showed positive staining for P53 in all three investigated groups. Therefore, a loss or lack of high levels of P53 protein does not account for a resistant phenotype in our series.

None of the tumors of chemosensitive and of the nonresponding patients contained a mutation within exons 5 to 8 of the *P53* gene, supporting the previously described low frequency of *P53* mutations in TGCTs. At the same time, the findings indicate that *P53* mutations are not a common means by which these tumors develop chemotherapy resistance. This is in line with data from TGCT-derived cell lines, where inactivation of P53 in a cisplatin-sensitive cell line did not alter the response to cisplatin. Furthermore, a cell line with inactive P53 was still highly sensitive to chemotherapy, and another cell line with a high level of functional P53 has been demonstrated to be resistant.[41,65]

In contrast, one study described *P53* mutations in specimens from four out of 23 patients sampled at relapse. All of these patients died of their disease. Histologically, three patients were diagnosed as having pure mature teratoma and one patient had both a mature teratoma and a rhabdomyosarcoma component, the latter probably representing a secondary non– germ cell malignancy.[33] The authors connected the resistant phenotype to the presence of a *P53* mutation. However, since mature teratomas are intrinsically resistant to chemotherapy, it remains difficult to judge the impact of the *P53* mutation in the development of cisplatin resistance. Moreover, *P53* mutations are more frequently detected in rhabdomyosarcoma than in TGCTs,[66] which might explain the presence of the *P53* mutation in the mixed tumor. An additional explanation for the different results between the studies may be a lower number of mature teratomas and a lower number of specimens sampled at relapse in our series. In any case, the majority of resistant cases both in the former and in the present study cannot be explained by inactivating *P53* mutations. This is in agreement with our conclusion that the sensitivity to treatment in a significant number of TGCTs cannot be explained by a high level of wild-type P53.

In the absence of mutations, P53 can be inactivated by alternative mechanisms, including overrepresentation of MDM2. A high level of MDM2 has been described in TGCTs in two investigations, with no correlation of the findings to treatment response.[20,49] Both studies showed more positive staining in nonseminomas compared with seminomas. We found high levels of MDM2, specifically in embryonal carcinoma components regardless of the treatment outcome, associated with amplification of the *MDM2* gene in only one case. The MDM2 protein was encoded by the full-length mRNA, indicating the absence of the formerly reported alternative splice products.[67,68]

In summary, our data show that not all TGCT are characterized by a high level of P53. Therefore, the level of wild-type P53 in TGCTs does not explain their overall sensitivity to cisplatin-based chemotherapy. This does not exclude the possibility that functional P53 is necessary for a favorable response of TGCTs to chemotherapy. Loss of high levels of P53 protein or inactivation of P53 by mutation might contribute to the resistant phenotype in a minority of cases at most. It is the strength of the current investigation that immunohistochemical and molecular studies were performed not only on cell lines and unselected samples but specifically on clinically clearly defined samples from patient cohorts with documented treatment-sensitive and cisplatin-refractory disease. The following hypothesis might explain the P53 findings so far.

First, positive staining for P53 in TGCT cells might reflect a physiologic reaction of these cells to apoptotic stimuli rather than being an indication of an overall high level of wild-type P53 in these tumor cells. This is supported by the spatial relation between positive staining for P53 and the presence of apoptotic bodies in most of the tumors studied (see Fig 1A). Second, exposure to DNA-damaging agents, particularly cisplatin-based chemotherapy, induces apoptosis in the TGCTs by a P53-independent mechanism with a lower threshold than the P53-dependent mechanism. In refractory tumors, inactivation of P53 might become secondarily relevant, resulting in positive selection of tumor cells with inactive P53. Again, inactivation of P53 by MDM2 does not seem to interfere with chemoresponsiveness in these tumors. Thus, future experiments need to seek alternative explanations for the unique chemosensitivity of TGCTs.

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Chapter 3

MOLECULAR DETERMINANTS OF TREATMENT RESPONSE IN HUMAN GERM CELL TUMORS

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Molecular Determinants of Treatment Response in Human Germ Cell Tumors [1]

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ABSTRACT

Purpose: Germ cell tumors (GCTs) are highly sensitive to cisplatin-based chemotherapy. This feature is unex-plained, as is the intrinsic chemotherapy resistance of ma-ture 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-refrac-tory 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 correla-tion 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 Ps were >0.08), even though only individual samples from chemotherapy-resistant cases showed a strong staining for MRP2 and GSTπ. In contrast to other components, mature teratomas showed an intense p21 and RB staining and were mostly positive for MRP2, lung resistance protein, and GSTπ.

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 explana-tion 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 fre-quent 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 unresponsive to CDDP-based chemotherapy or will relapse and

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[4] The abbreviations used are: GCT, germ cell tumor; CDDP, cisdia-minodichloroplatinum; 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 deoxynucle-otidyl transferase-mediated nick end labeling.

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 upregulation of GSTπ (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 pathologists 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 high temperature/high pressure in different buffers [120°C, 1.2 bar in 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, GSTπ, 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 H2 O and PBS. PBS was carefully removed, and the reaction mixture containing 3 IE terminal deoxynucleotidyl transferase (Fermentas, St. Leon-Rot, Germany), 0.5 μM biotinylated dUTP (Roche Diagnostics, Mannheim, Germany), and 5' 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 in months (range)	NA	33 (12-71)	35 (11-180)
Median relapse-free survival in months (range)	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] Na-Citrate 0.01M, pH 6	1:20	Liver	Membranous
MRP1	MRP R1	Rat	Rik Scheper	AC Na-Citrate 0.01M, pH 6	1:20	Lung cancer	Membranous
MRP2	M_2III5	Mouse	Rik Scheper	AC Na-Citrate 0.01M, pH 6	1:20	Liver	Membranous
BCRP	BXP 21	Mouse	Rik Scheper	AC EDTA 0.001m, pH 8	1:20	Placenta	Membranous
LRP	Clone LRP06	Mouse	Neomarkers	AC Na-Citrate 0.01M, pH 6	1:200	Breast cancer	Cytoplasmic
GST_{pi}	Ab1	Rabbit	Neomarkers	-	1:50	Liver	Cytoplasmic
MET	Clone E9	Mouse	Neomarkers	-	1:50	Breast cancer	Cytoplasmic
p53	Clone Do7	Mouse	DAKO	AC Na-Citrate 0.01M, pH6	1:50	Colon cancer	Nuclear
p21	Clone EA10	Mouse	Oncogene Science	AC Na-Citrate 0.01M, pH 6	1:75	Colon	Nuclear
RB	Clone IF8	Mouse	Neomarkers	AC EDTA 0.001M, pH 8	1:100	Colon	Nuclear
BAX	Clone 2D2	Mouse	Neomarkers	AC Na-Citrate 0.01M, pH 6	1:100	Tonsil	Cytoplasmic
BCL-2	Clone 124	Mouse	DAKO	AC Na-Citrate 0.01M, pH 6	1:50	Tonsil	Cytoplasmic
$BCL\text{-}X_L$	Clone 7D9	Mouse	Neomarkers	AC EDTA 0.001m, pH 8	1:100	Melanoma	Cytoplasmic

[a] AC, autoclaving at 120°C/1.2 bar; Met, metallothionein.

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-XL	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
GSTπ	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 GSTπ were included as proteins associated with inactivation of CDDP by detoxification. GSTπ 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 GSTπ protein. Metallothionein was detected in about one-third of the invasive tumors regardless of treatment out-come or histology.

Mature teratomas, both residual and nonresidual, differed significanty from the other histological components in most of the investigated markers. In contrast to the other histological elements, Pgp, MRP2, BCRP, LRP (Fig. *1*B), and GSTπ 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 remaing 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. *1*C). 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 ± SD, 14% ± 9% *versus* 6 ± 9.0%; *P* = 0.005) and of p21-positive cells (16% ± 7% *versus* 1% ± 3%; *P* = 0.001), mature teratomas showed a lower apoptotic index (2% ± 4% *versus* 4% ± 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 chemo-sensitivity 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. GSTπ, 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.

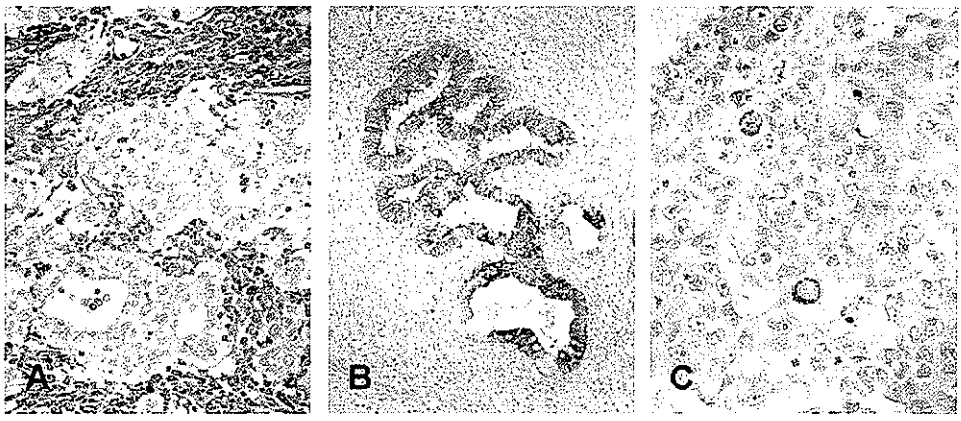

Fig. 1 Representative examples of immunohistochemical stainings. Representative examples of immunohistochemical stainings are shown: *A*, LRP staining in an embryonal carcinoma (note the absence of LRP in tumor cells); *B*, LRP staining in a mature teratoma; and *C*, apoptotic tumor cell showing staining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

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 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 pro-posed 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 G_1-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 G_1-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 G_1-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 GSTπ, 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

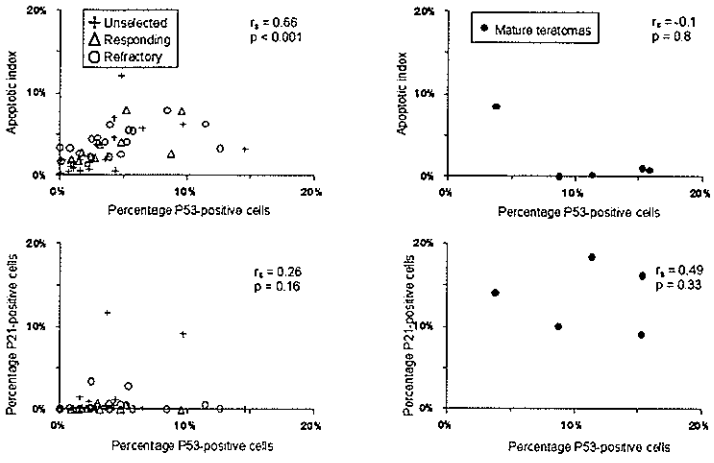

Fig. 2 Correlation between p53 and p21 and between p53 and apoptotic index. Correlation between the percentage of p53-positive cells and p21- positive cells and the apoptotic index is shown (as determined by immunohistochemistry and TUNEL assay, respectively). Regression coefficients and Ps are calculated 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.

chemotherapy-resistant phenotype in selected cases, as has been postulated for GSTπ 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 GSTπ 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 G_1-S, *i.e.,* they have an intact G_1-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 unacceptable 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 GSTπ, 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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Chapter 4

XERODERMA PIGMENTOSUM PROTEIN GROUP A PROTEIN AND CHEMOTHERAPY-RESISTANCE IN HUMAN GERM CELL TUMORS

Manuscript in preparation

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Xeroderma Pigmentosum Protein Group A Protein and Chemotherapy-Resistance in Human Germ Cell Tumors

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Summary

The exceptional sensitivity of germ cell tumors of adolescents and adults (GCTs) to chemotherapy, in particular to cisplatin, has been attributed to low levels of Xeroderma Pigmentosum Protein Group A (XPA), a crucial component of the nucleotide excision repair (NER) DNA repair pathway. In different types of solid tumors, resistance to cisplatin has been associated with enhanced expression of XPA. To assess the role of XPA levels in clinical sensitivity and resistance of GCTs to chemotherapy, immunohistochemistry was performed on tumor samples of both unselected patients before therapy and patients with fully documented clinical course before and after therapy. In case of high XPA levels, fluorescent *in situ* hybridization was applied to assess the possibility of gene amplification. XPA protein levels were investigated by Western blot analysis after repeated exposure to cisplatin in different GCT-derived cell lines. Finally, XPA-levels of both sensitive and cisplatin-resistant GCT-cell lines were compared to cell lines derived from other neoplasms.

Overall, the differentiated nonseminomatous GCT subtypes (yolk sac tumor and teratoma) contained more XPA than embryonal carcinoma as well as seminoma, irrespective of clinical outcome. No XPA gene amplification was found. Interestingly, all tumors resected in relapse after chemotherapy in the refractory group stained positive for XPA. However, XPA was not induced by repeated courses of sublethal doses of cisplatin in GCT-derived cell lines *in vitro*. Moreover, no correlation between cisplatin-sensitivity and intrinsic XPA levels was observed. Therefore, our data indicate that the level of XPA in GCTs is predominantly determined by histology but does not play an important role in determining treatment outcome.

Introduction

GCTs of the testis are the most frequent solid tumor in Caucasian males between 20 and 45 years of age (Adami et al, 1994). Histologically, they display patterns that resemble stages of embryonal development (Looijenga et al, 1999; Looijenga et al, 2002)[for review]. Seminomas show characteristics of early germ cells, (primordial germ cells or gonocytes), whereas nonseminomas can contain different, both embryonic and extra-embryonic, elements. The embryonal carcinoma is the stem cell component of nonseminomas, which may differentiate into yolk sac tumor, choriocarcinoma, and teratoma.

Seminomas and nonseminomas are highly sensitive to chemotherapy, notably to cisplatin (Einhorn 2002, for review). Presently, more than 80% of patients with metastatic disease can be cured by cisplatin-based combination chemotherapy. However, mature teratomas are intrinsically resistant to chemotherapy. This histology is found in about 40% of residual lesions after chemotherapy and should be resected to prevent malignant transformation and development of secondary non-germ cell malignancies (Oosterhuis et al, 1983; Fizazi et al, 2001) The biological basis of the overall high sensitivity to chemotherapy and the infrequent but mostly lethal occurence of resistant phenotypes remains unclear.

Different mechanisms of cellular resistance to cisplatin have been described, including inhibition of drug uptake and increased efflux; inactivation by sulfur-containing proteins; enhanced replicative bypass of platinum-DNA adducts; changes in concentrations of regulatory proteins; and enhanced repair of DNA crosslink lesions caused by cisplatin (for reviews see (Reed, 1998; Kartalou et al, 2001; Mayer et al, 2003)). Nucleotide excision repair (NER) is believed to be the main mechanism by which damage caused by cisplatin through formation of bulky DNA-adducts is repaired in mammalian cells (Zamble et al, 1995; Chaney et al, 1996). XPA, involved in the NER system, is a zinc finger protein that is absent or aberrant in cells of patients with xeroderma pigmentosum complementation group A (Bootsma et al, 2001). Binding of XPA to the replication protein A is the initiating and rate-limiting step of NER (Matsuda et al, 1995). It subsequently recruits other factors to damaged substrates (Buschta-Hedayat et al, 1999). GCT-derived cell lines were found to have low XPA levels and a low NER capacity. This finding was proposed as the major reason for the high sensitivity of GCTs to chemotherapy (Köberle et al, 1999). The XPA gene maps to chromosome 9q22, one of the regions found to be specifically amplified in GCTs showing chemotherapy resistance (Rao et al, 1998).

The objective of the present study was to investigate the presence of XPA in GCTs and clarify its role in sensitivity and resistance to cisplatin-based chemotherapy. XPA status was assessed by immunohistochemistry in tumor samples from patients with histologically defined GCTs without information on clinical outcome. To correlate immunohistochemical findings with clinical outcome, tumors from patients with fully documented clinical course, including chemotherapy-sensitive and refractory cases, were investigated. In addition, the correlation between XPA protein levels and cisplatin sensitivity was investigated by immunoblotting in different cell lines, including GCT cell lines with defined sensitivity and resistance to cisplatin.

Results

The presence of XPA protein was investigated in different histological elements of GCTs, including carcinoma *in situ* (CIS), their obligatory precursor lesion. Immunohistochemistry was performed on paraffin-embedded tissue sections of 85 unselected tumors of all histological variants (see Table 1). XPA showed a nuclear localization in the tumor cells with differing frequency and intensity in the different subtypes and histologic elements (see Table 1 and Fig 1 for representative examples). Embryonal carcinomas showed staining in 26% of cases (4/15), followed by seminomas (16/33; 48%), choriocarcinoma (2 out of 4; 50%), and yolk sac tumors (11/18; 61%). Interestingly, all teratomas (19 primary cases and four residual mature teratomas) were strongly positive. The overall percentage of positive tumor cells varied between different subtypes and histologies. Seminomas and embryonal carcinomas displayed a heterogenous staining pattern with only a few tumors (n=8/33 and 2/15 , respectively) showing more than 50% positivity. Yolk sac tumors, choriocarcinomas and teratomas revealed a much more homogenous staining with up to 100% of nuclei being positive. About 25% of the CIS cells, as present in adjacent testicular parenchyma of invasive seminomas or nonseminomas (n=5), showed a positive staining for XPA. This positivity was confirmed using a double-staining for c-KIT and XPA (see Figure 1).

To assess a possible correlation between the presence of XPA and clinical outcome, we investigated samples of GCTs from patients with chemotherapy-sensitive (n=12) and refractory (n=23) tumors. Clinical information is given in Table 2. The immunohistochemical results demonstrated no difference between the two groups: 9 of 12 (75%) tumors in the chemosensitive and 16 of 23 (70%) tumors in the refractory group stained positive for XPA (see Table 2). However, all tumors sampled after chemotherapy (n=9) in the refractory group were positive for XPA. The histologies found in these cases were yolk sac tumor (n=7); teratoma (n=2) and choriocarcinoma (n=2; some of the tumors exhibited more than one histological subtype). No gene amplification of XPA was found using double-colour fluorescent *in situ* hybridization

Table 1. Immunohistochemical Detection of XPA in CIS and in GCTs of different histologies (unselected tumor samples). The majority of cases showed more than one histological subtype.

Histology	% of cases showing any positivity	% of cases with >50% positive nuclei
EC (n=15)	26%	13%
SE (n=33)	48%	24%
CC (n=4)	50%	50%
YS (n=18)	61%	44%
TE (n=23; including 4 RMT)	100%	95%
CIS containing samples (n=5)	25% positivity of all CIS cells	

Abbreviations: CIS, *carcinoma in situ*; GCTs, germ cell tumors; EC, embryonal carcinoma; SE, seminoma; CC, choriocarcinoma; YS, yolk sac tumor; TE, teratoma; RMT, residual mature teratoma

(FISH) on tumors showing strong XPA expression in the refractory group.

To investigate possible mechanisms related to the consistent presence of XPA in the refractory GCTs after cisplatin exposure, we investigated the total amount of XPA protein in three well-characterized GCT-derived cell lines (NT2, 2102Ep, and NCCIT) by Western blotting. Whereas NCCIT showed a low expression level, NT2 and 2102 EP contained XPA at a level comparable to that of various other tumor cell lines (see Figure 2A). Furthermore, in cytotoxicity assays no correlation between XPA level and cisplatin sensitivity was observed (see Figure 2C). Subsequently, NT2 and 2102Ep cells were repeatedly treated with sublethal doses of cisplatin, resulting (after nine cycles of treatment) in cell lines relatively resistent to cisplatin. The NT2 subline (NT2/CDDP) showed a 2.5-fold and the 2101EP subline (2102EP/CDDP) a 2-fold resistance to cisplatin at the IC50. However, this was not accompanied by an increase in the level of XPA in these sublines (see Figure 2B).

Table 2. Patient Characteristics

	chemosensitive (n=12)	Refractory (n=22)
Age, years		
Median	28	29
Range	20-47	17-56
Histology		
Seminoma	1	1
Non-Seminoma	11	21
Stage at diagnosis (acc. to UICC)		
I	0	3
II	0	7
III	12	12
Initial treatment after surgery		
Surveillance	0	2
Chemotherapy	12	20
Follow-up, months		
Median	49	39
Range	12-67	11-180
Relapse-free survival, months		
Median	NR	7.1
Range		0-150
Response to initial treatment		
Complete remission	8	5
Partial remission, marker negative	4	8
Partial remission, marker positive	0	3
Progressive disease	0	3
Unknown	0	3
No. of regimens in relapse		
Median	0	3
Range	0	1-9
% cases showing XPA positivity	75% (9/12)	70% (16/23)

Abbreviations: UICC, International Union Against Cancer; NR, not reached

Figure 1.

Representative examples of the immunohistochemical stainings for XPA in GCTs of different histologic subtypes. (A) double staining for c-KIT (red) and XPA (blue) in a seminiferous tubule containing CIS cells. While all these cells were positive for c-KIT, they can be either positive (arrow) or negative (arrowhead) for XPA; (B) tumor containing teratoma (arrow) positive for XPA and embryonal carcinoma (arrowhead) negative for XPA; (C) teratoma showing epithelial differentiation, positive for XPA; (D) yolk sac tumor positive for XPA. Note that stromal cells and infiltrating lymphocytes can be positive for XPA.

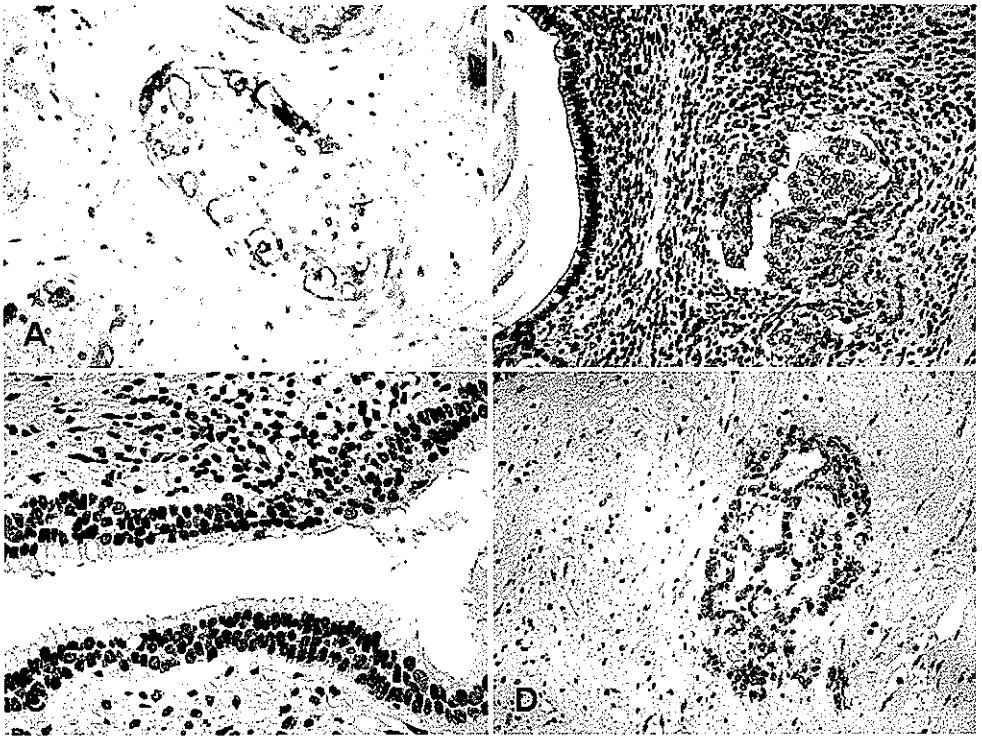
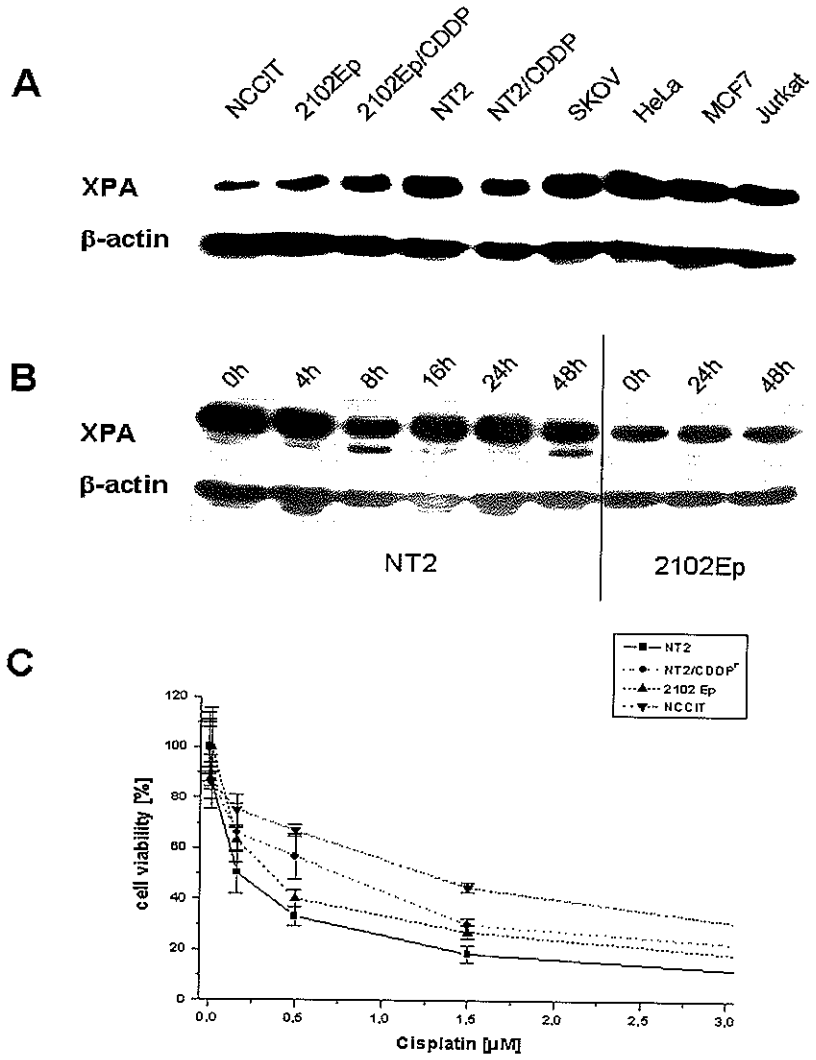


Figure 2.

(A) Immunoblot analysis for XPA and β-actin on different cell lines derived from germ cell tumors including NT2/CDDP and 2102Ep/CDDP - cell lines with acquired resistance to CDDP following repeated sublethal exposure. SKOV, HeLa, MCF7, and Jurkat are cell lines derived from ovarian cancer, cervical cancer, breast cancer and leukemia cells. (B) Immunoblot for XPA and β-actin on NT2 and 2102Ep cells during treatment with CDDP. (C) Cytotoxicity assay for selected GCT-cell lines. Note the lack of correlation between CDDP-sensitivity and XPA-level.



Discussion

The unique chemosensitivity of GCTs is poorly understood on a molecular level so far (Mayer et al, 2003). Based on studies on the mouse teratocarcinoma cell line P19, it has been assumed that a high level of wild type p53 results in a low threshold for induction of apoptosis (Lutzker et al, 1996)(Lutzker et al, 2001). However, this model differs from human GCTs in various parameters, and we recently demonstrated that treatment outcome in patients with GCTs does not correlate with p53 status (Kersemaekers et al, 2002). It has also been suggested that the unique treatment sensitivity of GCTs could be explained by a defective repair of cisplatin-induced DNA damage related to low XPA (Koberle et al, 1999). Several observations seem to support this model. For example, mammalian cells deficient in NER are more sensitive to cisplatin than corresponding wild type cells (Poll et al, 1984; Dijt et al, 1988). Expression of a truncated XPA protein exerting a dominant-negative effect sensitizes human tumor cells to UV light and cisplatin (Rosenberg et al, 2001). Moreover, an enhanced XPA expression is associated with resistance to cisplatin treatment in human ovarian cancer (Dabholkar et al, 1994). However, studies correlating NER with clinical data result in conflicting data for different tumor entities. For example, expression of several NER factors in patients with chronic lymphocytic leukemia did not correlate with resistance to nitrogen mustard (Bramson et al, 1995), while an increased NER capacity in patients with non-small-cell lung cancer may be associated with poorer survival (Bosken et al, 2002). It is of specific interest that a link between NER and differentiation has been observed in normal tissues: somatic stem cells are highly sensitive to the effects of physical and chemical mutagens (Potten, 1977; Ijiri et al, 1987) and avoid certain forms of potentially error-prone DNA damage repair (Roth et al, 2002). It has been suggested that the choice of death rather than defective DNA repair in case of damage serves to avoid accumulation of replication errors with fatal consequences for the progeny (Cairns, 2002). So far, however, no data are available regarding NER and clinical outcome in GCTs.

The aim of this study was to assess the role of XPA in clinical resistance of GCTs. Immunohistochemistry in the unselected group of patients revealed that the staining pattern of XPA correlated with histology. In seminomas and in embryonal carcinoma, only a minority of cells contained detectable levels of XPA. In contrast, the majority of cells from choriocarcinomas, yolk sac tumors, or teratomas were positive for XPA. Three teratomas and yolk sac tumors of infants and neonates that were included in this series showed a similar staining pattern as the tumors in histological counterparts found in testis of the adults. No differences in the presence of XPA was evident comparing tumors of patients with cisplatin-sensitive and refractory disease. The overall picture was heterogenous, as high expression of XPA was observed in several tumor samples of chemosensitive cases on the one hand, and lack of expression of XPA was seen in some refractory cases on the other hand. Results were in the same range as in the unselected group. Therefore, we conclude that XPA detection by immunohistochemistry has no prognostic or predictive value for patients with newly diagnosed GCTs.

None of the resistant tumors with detectable levels of XPA showed amplification of the respective locus using FISH analysis. This excludes amplification of the XPA-gene as a common mechanism of

chemotherapy resistance in GCTs. However, it is noteworthy that all tumors sampled at relapse in the refractory group, i.e., after exposure to chemotherapy, were positive for XPA. This could simply be due to the particular histology (yolk sac tumors, choriocarcinoma and teratoma) found in this limited series. Alternatively, induction of XPA or selection for XPA positive cells could have occurred during treatment. To test this hypothesis, an *in vitro* model was used, which showed no correlation between XPA levels and sensitivity to cisplatin in the three GCT-derived cell lines investigated. We found a low level of XPA only in NCCIT, whereas the other two GCT cell lines showed levels only slightly lower than that found in cell lines derived from other neoplasms. In addition, XPA expression was not induced in GCT-cells which acquired cisplatin resistance by repeated exposure to the drug, differing from findings reported from ovarian cancer derived-cell lines (Hector et al, 2001). In this context it is of interest that it has been shown that even a low level of XPA expression in deficient cells is sufficient for total complementation of cellular sensitivity and DNA repair activity (Muotri et al, 2002). In accordance with this, it has been reported that sensitivity to 170 different compounds, including cisplatin, tested in a cytotoxicity assay did not correlate with XPA levels in 60 human tumor cell lines (Xu et al, 2002).

In conclusion, we demonstrated that the presence of XPA protein as assessed by immunohistochemistry differs between the various histologies of GCTs. XPA is found more frequently and with a more homogenous staining pattern in the histological subtypes with a more differentiated phenotype. Overall, no differences in the presence of XPA were observed between samples of tumors refractory or sensitive to chemotherapy. Furthermore, we did not find a correlation between XPA protein levels and sensitivity to cisplatin in three GCT-derived cell lines. We therefore conclude that XPA does not play a critical role in treatment resistance of GCTs. Further research is needed to elucidate the mechanisms of chemotherapy sensitivity and resistance in these tumors.

Materials and Methods

Patient material

Unselected group: formalin-fixed paraffin-embedded tissue blocks were retrieved from the archive of the Laboratory for Experimental Patho-Oncology, Department of Pathology, Erasmus MC, Rotterdam. The material was collected between 1991 and 2001 in close collaboration with urologists and pathologists in the South West of the Netherlands. 81 tumors were collected before therapy, in 4 cases residual mature teratomas were removed after chemotherapy. No data on the clinical course of the patients was available.

Chemosensitive group: Formalin-fixed, paraffin-embedded samples collected before therapy from 12 patients diagnosed between 1995 and 1998 were investigated. Only patients with a complete remission or a marker negative partial remission after high-dose chemotherapy and a relapse-free follow–up of more than one year were included. The series consisted of 11 non-seminomas and one seminoma. Refractory group: 23 formalin-fixed, paraffin-embedded samples from 22 patients with chemotherapy-refractory disease diagnosed between 1991 and 1998 were investigated. Patients were considered refractory when progression or relapse occured despite adequate initial and/or salvage treatment, including high-dose chemotherapy in some cases. The material of 14 patients was obtained at initial diagnosis; in nine cases, the material was sampled after exposure to chemotherapy from metastatic lesions in relapse. In one case, material from both the primary tumor and a metastatic tumor at relapse was available. The series consisted of 22 nonseminomas and one seminoma. Table 2 summarizes the characteristics of the patients with refractory and chemosensitive tumors. All cases were reviewed and diagnosed by J.W.O. according to the WHO classification and the fully documented clinical course was available for these patients.

Cell lines and Culture Conditions

The CT-derived cell lines NT2 and NCCIT were purchased from the American Type Tissue Culture (Manassas, VA, USA), 2102Ep, was a kind gift from M. Pera. MCF-7 (breast cancer), OVCAR-3 (ovarian cancer), HeLa (cervical cancer), were gifts from P. Brossart and K562 (chronic myeloid leukemia) was a gift from T. Brümmendorf (University of Tuebingen Medical Center). Cells were grown as monolayer and maintained at 37°C in a humidified cell culture incubator with 5% carbon dioxide. NT2 were cultured in DMEM/glutamax supplemented with 10% fetal calf serum (Gibco BRL, Paisly, UK), penicillin (Biochrom) and streptomycin (Biochrom). NCCIT and 2102Ep were cultured in DMEM/nut mix F12 supplemented with 10% FCS, pencillin, streptomycin and glutamin. The remaining cells were kept in HEPES-buffered RPMI (Gibco).

Immunohistochemistry

Paraffin sections of 3 micron thickness were mounted on Starfrost slides (Knittel Gläser, Germany), dried at 50°C over night, deparaffinized and rehydrated. For antigen retrieval, pressure cooking at 1.0 bar in citrate buffer 0.01 mol/L, pH 6.0, was used. All antibodies were diluted in phosphate-buffered saline (PBS)

with 1% bovine serum albumin (Sigma, Zwijndrecht, The Netherlands). The primary antibody anti-XPA (mouse monoclonal antibody, Ab-1 Clone 12F5; NeoMarkers, Freemont, CA) was used in a dilution 1:100 and incubation time was 60 minutes at room temperature. Biotin-labeled rabbit-antimouse immunoglobulins and an avidin-biotin-HRP complex (both DAKO A/S, Glostrup, Denmark) were subsequently applied for 30 minutes each. DAB (Fluka, Switzerland) was used as a chromogen and slides were counterstained with Mayer's hematoxylin stain. For a negative control, the primary antibody was omitted; lymphocytes and normal spermatogenesis (pachytene spermatocytes) as present in the histological sections under investigation were used as positive control. Two investigators (F.H., F.M. or H.S.) assessed samples independently. For evaluation of percentage of positive cells, 200 cells were scored in three randomly selected high-power fields. Only clearly nuclear staining of intact cells was considered positive.

Western Blotting/ Amplification Analysis for XPA Using Fluorescence In Situ Hybridization

Protein harvest, Western blotting procedure, and Fluorescence *in situ* Hybridization (FISH) were performed as previously described (Kersemaekers et al, 2002). The XPA-probe for FISH was derived from BAC clone RP11-414C23.

Cisplatin Treatment and Toxicity/Resistance Assay/Cytotoxicity Assay

In vitro-cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide-(MTT-)assay, which was performed according to standard protocols. 1000 cells/well were seeded in 96-well plates 8-fold, left to attach overnight, exposed to CDDP in increasing concentration continuously for further 72 hours. The CDDP-containing medium was carefully removed, replaced by medium containing MTT (Sigma), incubated for 4 hours at 37°C. The tetrazolium-salt was dissolved in DMSO, the extinction was measured at 570nm on a biorad ELISA-reader. The results are expressed relative to the extinction of the cells grown in the absence of CDDP.

Induction of drug resistance

Cells were treated in increasing sublethal doses of CDDP (ranging from 0,6 μM to 2,0 μM) for two hours, transferred to normal medium, and grown for two weeks before re-exposure to CDDP. Exposure at each dose level was repeated three times.

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Chapter 5

MICROSATELLITE INSTABILITY OF GERM CELL TUMORS IS ASSOCIATED WITH RESISTANCE TO SYSTEMIC TREATMENT

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Microsatellite Instability of Germ Cell Tumors Is Associated with Resistance to Systemic Treatment [1]

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Abstract

Systemic cisplatin-based chemotherapy cures >90% of patients with metastatic germ cell tumors (GCTs). The biological basis of this exquisite chemo-sensitivity and the resistant phenotype encountered in 10–15% of patients with GCT is yet unclear. A defective mismatch repair pathway leading to microsatellite instability (MSI) has been related to resistance to cytotoxic drugs. We investigated 100 unselected GCTs and 11 clinically defined chemotherapy-resistant GCTs for MSI using 8 mono- or dinucleo-tide markers and the presence of the mismatch repair factors MLH1, MSH2, and MSH6 by immunohistochemistry. The resistant tumors, both chemo-naïve (*n* = 8) and pretreated (*n* = 3), showed a significantly higher incidence of MSI compared with the unselected series (45 *versus* 6% in at least one locus and 36 *versus* 0% in >2 of 8 loci, both *P* <0.001). In 5 of all 11 unstable tumors, MSI correlated with immunohistochemical find-ings. This study demonstrates for the first time a positive correlation between MSI and treatment resistance in GCT.

Introduction

GCTs [3] of adults are the most frequent solid tumor of Caucasian males between 20 and 45 years of age (1). On the basis of histological, biological, and clinical differences, GCTs are divided in seminomas and nonseminomas (2). Even in metastatic stages, most patients with GCTs can be cured with multiagent, CDDP-based chemotherapy (Ref. 3, for review). Despite this success, 10–15% of the patients do not achieve a long-lasting remission with the available treatment strate-gies and eventually die of their disease. Up to now, the biological basis for the exquisite treatment sensitivity of most GCT has not been elucidated. This also accounts for the mechanism of chemotherapy resistance (Ref. 4, for review). Response to CDDP-based chemother-apy of ovarian carcinoma has been related to MSI, *i.e.*, alterations in length of short repetitive sequences of the genome by small deletions or insertions. MSI is caused by genetic or epigenetic changes in genes of the DNA MMR pathway. Several proteins of this pathway have been identified, including MLH1, MSH2, and MSH6 (Ref. 5, for review), whose inactivation might result in MSI. A number of reports deal with MSI in GCT. Although most of them showed a negative result (6 –8), one study indicated locus-specific instability (9). Thus far, MSI has not been correlated with treatment resistance of GCT. Here we report the results of an analysis of the presence of MSI and the related factors MLH1, MSH2, and MSH6 in a large series of unselected GCT and a unique series of treatment-resistant GCT.

Materials and Methods

Patients and Samples. Fresh frozen and formalin-fixed, paraffin-embed-ded tissue from 100 unselected cases (50 seminomas and 50 nonseminomas), in most cases together with peripheral blood, was collected between 1991 and 2001 in collaboration with urologists and pathologists in the Southwestern part of the Netherlands. These cases were retrieved from the archive of the Laboratory for Experimental Patho-Oncology (Department of Pathology). Complete data on the clinical course were not available of these patients. The resistant series consisted of 11 patients diagnosed between 1991 and 1998, treated within various trials led by Tübingen University, Germany. Patients were considered refractory, when progression or relapses of the disease occurred despite adequate initial and salvage treatment. The tumors were obtained either at initial diagnosis *(i.e.,* before chemotherapy, *n* = 8) or by resection of a metastatic lesion in relapse *(n* = 3). Table 1 summarizes the characteristics of the unselected and refractory patients. All cases were reviewed and diagnosed by an experienced pathologist (J. W. O.) according to the WHO classification (2).

DNA Isolation and Microsatellite Analysis. Normal DNA was isolated either from peripheral blood or, if not available, from nontumor tissue specif-ically dissected from the tissue blocks as described before (10). DNA of fresh-frozen tissue or paraffin-embedded tissue was isolated according to standard procedures as described previously (4). Microsatellite analysis of pairs of normal and tumor DNA was performed as described previously using eight mono- or dinucleotide markers (D2S123, BAT25, BAT26, D5S346, D17S250, BATRII, MSH6, and BAT40) with an input of 50 ng of DNA/PCR reaction (11).

Immunohistochemistry. Paraffin sections of 3- μm thickness were mounted on APES-coated slides, deparaffinized, and rehydrated. Antigens were unmasked by high temperature/high pressure [120°C, 1.2 bar in Na-citrate 0.01 M (pH 6)]. MLH1, MSH2, and MSH6 were demonstrated using mouse monoclonal antibodies (MHL1: clone G168–15, diluted 1:500; MSH2: clone G219–1129, diluted 1:400; MSH6: clone 44, diluted 1:1000; all BD Biosciences, Alphen aan den Rijn, the Netherlands). The sections were incu-bated with the primary antibodies overnight at 4°C (MLH1 and MSH2) or room temperature (MSH6). Biotin-labeled rabbit-antimouse immunoglobulins and a biotinylated horseradish peroxidase-Streptavidin complex (both DAKO, Glostrup, Denmark) were applied subsequently for 30 min at room temperature each. Diamino-benzidin was used as chromogen. Normal tonsil and colon tissue served as positive controls. Nuclear staining was scored as "strong," "weak," or "absent" compared with lymphocytes as internal positive controls, known to be positive for all markers (12).

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[3] The abbreviations used are: GCT, germ cell tumor; CDDP, *cis*-diamino-dichlorid-platin; MSI, microsatellite instability; MMR, mismatch repair.

Table 1: *Baseline patient characteristics*[a].

	unselected (n=100)	Refractory (n=11)
Median age (years)	32 (14-66)	29 (18-55)
Histology		
• Seminoma	50	1
Nonseminoma	50	10
mixed nonseminoma	29	7
embryonal carcinoma	7	0
yolk sac tumor	5	3
mature teratoma	9	0
Time of tissue sampling		
• at initial diagnosis	99	8
• after chemotherapy	1	3
Systemic treatment		
• standard PE/PEB/PVB/PEI	NA	11
• salvage high-dose chemotherapy	NA	7
Median time to progression (months)	NA	10 (2-149)
Median overall survival (months)	NA	40 (11-180)

[a]P – cisplatin, E – etoposide, B – bleomycin, V – vinblastine, I – ifosfamide, NA – not available

Table 2: *Summary of MSI and immunohistochemical analysis on control and refractory GCT.*

	Unselected (n=100)	Refractory (n=11)	p-value[a]
Microsatellite instability	6 (6%)	5 (45%)	0.001[a]
MSI in 1out of 8 loci	6 (6%)	1 (9%)	0.558[a]
MSI in ≥2 out of 8 loci	0	4 (36%)	<0.001[a]
IHC negative/weak any marker	3 (3%)	4 (36%)	
hMMLH1	0	4 (36%)	
hMMSH2	2 (2%)	1 (9%)	
hMMSH6	2 (2%)	1 (9%)	
MSI correctly predicted by IHC	2	3	
MSI not predicted by IHC	4	2	
MSI falsely predicted by IHC	1	1	

[a] p-values are given for differences between all tumors of the two study groups as determined by a two-sided Fisher's exact test.

[b] Analyzing only the nonseminomas of both groups, the p-values are 0.002, 0.508, and <0.001, respectively.

[c]Abbreviations: IHC – immunohistochemistry

Statistical Analysis. The two groups of unselected and refractory GCT were compared for differences in the incidence of MSI overall and by number of unstable loci using a two-sided Fisher's exact test. Differences were con-sidered significant, when the P was < 0.05. Differences in time to progression and overall survival between refractory cases with and without MSI were analyzed by a Log-rank test.

Results

In total, a series of 111 GCT and matched normal DNA was studied using eight microsatellite markers. Instability was found in 6 of the 100 control GCTs (6%, three seminomas and three nonseminomas), all of them affecting only one locus (four in BAT40, one in MSH6, and one in D17S250). In contrast, the series of refractory GCTs showed MSI in 5 of 11 cases (45%, all nonseminomas), 4 of which were sampled before chemotherapy exposure. This difference was statistically significant ($P < 0.001$; analyzing only nonseminomas: $P = 0.002$). Four of these refractory tumors (three obtained before chemotherapy and one after) showed instability in two or more loci, which was never observed in the unselected series ($P < 0.001$, also for analysis of nonseminomas only). The results are summarized in Table 2, and representative examples of the microsatellite analysis in the refractory GCTs are shown in Fig. *1*A. The refractory cases showing MSI and those without were analyzed regarding their progression-free survival and overall survival by the Kaplan-Meier method and compared using the Log-rank test. The microsatellite stable cases had a median progression-free survival time of 6 months compared with 26 months in the group of tumors with MSI ($P = 0.05$; see Fig. *1*B); the data for the median overall survival were 21 and 41 months, respectively ($P = 0.43$; data not shown). All tumors were investigated by immunohistochemistry for the presence of the MLH1, MSH2, and MSH6 proteins. Most cases showed an intense homogeneous nuclear staining of nearly all tumor cells, irrespective of their histology. Three of the 100 (3%) control tumors and 4 of the 11 refractory tumors (36%) showed a markedly reduced signal in any of the three stainings. Examples of tumors with a staining for MSH6 rated as "absent" and "strong" are shown in Fig. *1C*, and the results are summarized in Table 2. Of note, one GCT (of the control series) showed instability within the *MSH6* locus with concomitant loss of MSH6 protein. The immunohistochemical

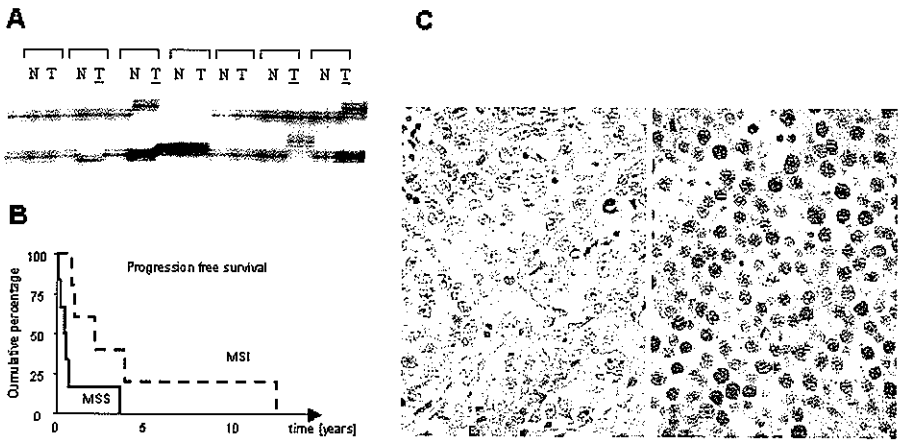

Fig. 1. *A*, microsatellite analysis on pairs of normal DNA/tumor DNA of seven refractory cases using D2S123 as a marker (the unstable tumors are *underlined*); *B*, Kaplan-Meier curve for progression-free survival of patients with refractory tumors with MSI and those with microsatellite stability (MSS). The difference was borderline significant by Log-rank test ($P = 0.05$); *C*, representative examples of immunohistochemical detection of the MSH6 protein. *Left panel*, tumor scored "absent" (same case showed MSI in MSH6 locus); *right panel*, tumor scored "strong."

findings in this study did only successfully predict the presence of MSI in two of the six unstable control GCTs and in three of the five unstable refractory GCTs. In addition, a "weak/absent" immunohistochemical finding was found in two GCTs (one control and one refractory) in the absence of MSI.

Discussion

The unique chemo-sensitivity of GCT is poorly understood at the molecular level thus far. We demonstrated recently that the model assuming a high level of wild-type P53 resulting in a low threshold for induction of apoptosis is not correct (4). This particular study also showed that genetic inactivation of P53 is not a major way to induce treatment resistance in GCT as proposed previously (13).

In vitro analyses of cell lines of various origin have suggested a correlation between MSI, MMR, and sensitivity toward CDDP, the key substance of all combination regimens in the systemic treatment of GCT (14, 15). A number of groups has analyzed MSI and the MMR pathway in GCT and found a low rate of instability without correlating the results to clinical outcome (6–9). Our findings confirm these results in a large series, including all different histological subtypes of GCT. However, in a clinically defined group of treatment-resistant GCT, 45% of the tumors showed MSI, most of them in two of the eight investigated markers, a feature never encountered in any of the control tumors. A correlation between MSI and treatment resistance can be explained by two different mechanisms (Ref. 16, for review): (a) the MSI renders the genome of the cancer cell prone to harbor secondary mutations, which could be responsible for the resistant phenotype; or (b) MMR factors could directly be involved in induction of apoptosis (17). This could result in resistance to apoptosis independent from the presence of actual MSI. It remains to be determined to what extent these two mechanisms contribute to the resistance of GCT. It is important to note that the majority of the unstable refractory GCTs investigated in this study was sampled before exposure to chemotherapy. Our data differ in this feature from *in vitro* data and a study on ovarian cancer, where MSI was induced by CDDP-based treatment (14, 18). To study the biological relevance of MSI, we compared the progression-free and overall survival between the refractory patients with and without MSI. The shorter progression-free survival in the subgroup of refractory tumors without MSI suggests a different clinical behavior of refractory tumors depending on the underlying resistance mechanism. A similar observation was made in colon cancer (19). The difference in behavior may be explained by a higher level of resistance mediated by mechanisms other than MSI in these tumors. The lack of a difference in overall survival might be caused by accumulation of secondary mutations in tumors with MSI leading to a more aggressive phenotype in the later course of the disease. However, with the limited number of patients in the refractory group, these considerations remain speculative at this point.

Immunohistochemical demonstration of MMR factors has been applied successfully to predict MSI in colon cancer (20). In contrast, low protein levels of MMR factors did not correlate with MSI in cell lines derived from gastric carcinoma (21). Our findings indicate that assessing MLH1, MSH2, and MSH6 by immunohistochemistry is not sensitive and specific enough to predict MSI in GCT. This difference to the situation in colon cancer could be related to a more prominent role of other MMR factors, like PMS1 and PMS2, in the development of MSI in GCT, as has been suggested for prostate cancer (22). However, the data suggest a reduced protein level of specific MMR factors as an explanation of MSI at least in some GCT. In summary, we demonstrate a correlation between chemotherapy resistance and MSI in GCT. The results offer the first plausible explanation for the clinical behavior of refractory GCT. Furthermore, analyzing MSI shows promise to predict treatment response based on characteristics of the primary tumor in a significant number of cases. In this regard, the findings have to be validated prospectively.

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Chapter 6

ABSENCE OF C-KIT AND MEMBERS OF THE EPIDERMAL GROWTH FACTOR RECEPTOR FAMILY IN REFRACTORY GERM CELL CANCER

Cancer 2002;95:301–308

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Absence of c-KIT and Members of the Epidermal Growth Factor Receptor Family in Refractory Germ Cell Cancer

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BACKGROUND. Germ cell tumors (GCTs) in adolescent and young males are very sensitive to cisplatin-based chemotherapy. However, 10–20% of the patients cannot be cured by currently available therapeutic options. Once a tumor does not respond to cisplatin, current therapeutic modalities offer only a chance for short palliation. Recently, new treatment options that interfere with various receptor tyrosine kinases, including c-KIT and members of the epidermal growth factor receptor (EGFR) family, have been used successfully in chemotherapy-resistant tumors overexpressing c-KIT, ERB-B2, or EGFR.

METHODS. We studied the presence of c-KIT and the four members of the EGFR family by immunohistochemistry, as well as by *ERB-B2* gene amplification using fluorescent in situ hybridization, in a series of 22 patients with cisplatin-resistant GCTs in search of new treatment targets. The results in these refractory tumors were compared with those of 12 patients with chemosensitive GCTs diagnosed in an advanced metastatic stage.

RESULTS. The data obtained in both groups did not differ in any of the investigated biologic markers. c-KIT was detected in the one case of pure seminoma studied and in the seminomatous components of combined tumors. The presence of EGFR was restricted to trophoblastic giant cells and the syncytiotrophoblastic elements of four nonseminomas including one pure choriocarcinoma and to a secondary non–germ cell malignancy, which had developed most likely from a mature tera-toma. ERB-B2 was moderately positive in the secondary non–germ cell malig-nancy, in one mature teratoma component of a mixed nonseminoma, and together with EGFR in the syncytiotrophoblastic cells of a pure choriocarcinoma. Of all samples investigated, this latter case was the only one showing an amplification of the *ERB-B2* gene in the syncytiotrophoblasts. ERB-B3 and ERB-B4 were detected rarely.

CONCLUSION. The majority of refractory GCTs do not qualify for treatment with new biologic agents targeting the receptor tyrosine kinases EGFR, ERB-B2, or c-KIT. The lack of differences between the tumors of refractory and the responsive patients indicates that overexpression of any of these receptor tyrosine kinases does not contribute to a resistant phenotype in GCTs. *Cancer* 2002;95:301–8.

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KEYWORDS: germ cell cancer, EGFR, Her2/neu, c-kit, cisplatin-refractory.

Germ cell tumors (GCTs) are the most common malignancies in males between 15 and 45 years of age. Most GCTs develop in the testis and 5–10% occur at extragonadal locations, including the retroperitoneum and the mediastinum.[1,2] GCTs originate from trans-formed embryonic germ cells. In the testis, the earliest precursor

lesion identified is the carcinoma in situ (CIS) consisting of premalignant cells confined to the seminiferous tubules.[3] Histologically and clinically, GCTs are separated into seminomas and nonseminomas. The latter are a heterogenous group containing one or more of the following components: embryonal carcinoma, yolk sac tumor, choriocarcinoma, and teratoma.[4] GCTs are highly sensitive to chemotherapy. Even in metastatic stages, 70–80% of the patients achieve long-term survival with multiagent, cisplatin-based chemotherapy.[1] However, approximately 10–20% of all patients with GCTs will suffer from recurrence or progression of their disease after systemic treatment.[1,2] Once the tumor no longer responds to cisplatin, the treatment of these patients becomes palliative.[5] Although paclitaxel, gemcitabine, and oxaliplatin have been identified as new active agents, with each drug achieving partial remissions of short duration in about 20% of the patients, therapeutic options are still very limited in this situation.[6] Identifying new treatment strategies for patients with cisplatin- refractory disease remains a priority.

The recent development of tyrosine kinase interactive agents addressing the epidermal growth factor receptor (EGFR), Her2-neu, and the stem cell factor receptor, c-KIT, has offered new therapeutic options for patients with tumors overexpressing any of these receptor tyrosine kinases. Several strategies to attack these receptors have been developed. Receptor activation can be blocked by monoclonal antibodies such as cetuximab and trastuzumab, which bind to the extracellular domains of EGFR and ERB-B2, respectively.[7,8] Tyrosine kinase activity can also be inhibited by small molecules such as ST1571, ZD 1839 (iressa), or OSI-774, which selectively and competitively inhibit the enzyme by blocking the adenosine triphosphate binding site of the tyrosine kinase domain.[8–10] A number of mechanisms may contribute to the antitumor efficacy of the tyrosine kinase blockade, including the inhibition of cell cycle progression, induction of apoptosis, inhibition of angiogenesis, and potentiation of drug-induced cytotoxic damage.[8]

The presence of c-KIT is a diagnostic marker for both CIS and seminoma.[11] In addition, scattered c-KIT–positive cells have been reported in 9 of 29 (32%) nonseminomas.[12] It is noteworthy that an activating mutation in this receptor has been found in 2 of 33 human GCTs, one testicular seminoma and an ovarian dysgerminoma/yolk sac tumor.[13] However, these results were not correlated to the clinical course of the patients.

Four members of the EGFR tyrosine kinase family have been described so far: EGFR, ERB-B2, ERB-B3, ERB-B4. They are expressed at high levels in approximately one third of epithelial cancers.[14] Overexpression of ERB-B2 has been associated with a poor response to different chemotherapeutic agents (e.g., cyclophosphamide).[15] We have reported a patient with cisplatin-refractory GCT overexpressing ERB-B2.[16] This patient achieved a partial response after treatment with trastuzumab, suggesting a potential role of targeting ERB-B2 in this difficult treatment situation. To explore potential new therapeutic targets, the present study has systematically investigated the presence of all members of the EGFR family and of the receptor tyrosine kinase, c-KIT, in tumor tissue from a homogenous group of patients with cisplatin-refractory GCT. The expression pattern of tumors from patients with refractory disease was compared with that of tumors from patients with advanced metastatic disease ("poor prognosis" according to the International Germ Cell Cancer Cooperative Group [IGCCCG] criteria) at initial diagnosis, who achieved remission of disease following chemotherapy and who remained long-term disease free after first-line chemotherapy.[17]

MATERIALS AND METHODS

Patients and Tissue Samples

Formalin-fixed paraffin-embedded tissue samples were collected retrospectively from 22 patients with cisplatin-resistant disease (resistant tumors) and from 12 patients with advanced metastatic disease (poor prognostic characteristics according to the IGCCCG classification), who had achieved long-term survival after initial treatment (responsive tumors). Cisplatin resistance was defined clinically as progressive disease or recurrence after adequate cisplatin-based primary and salvage therapy.[18]

Patients with responsive tumors had been treated within the multicenter first-line high-dose VIP (cisplatin/etoposide/ifosfamide) chemotherapy trial of the German Testicular Cancer Study Group (GTCSG). The exact regimens and the results of this trial have been published elsewhere.[19]

Twenty-two patients with resistant disease had been treated initially with standard platinum-based regimens (*n*=18; PE [cisplatin/etoposide], VIP, PVB [cisplatin/vinblastin/ bleomycin], or BEP [cisplatin/ etoposide/bleomycin]) or with first-line high-dose VIP followed by autologous stem cell transplantation (*n*=4) as previously described.19 Salvage high-dose che-motherapy was performed in 15 patients in a multi-center trial of the GTCSG investigating the role of high-dose chemotherapy for patients with recurrent or refractory GCTs.[20] The remaining seven patients, as well as patients with recurrence of disease after salvage high-dose chemotherapy, received further palliative treatment for refractory disease with

various agents such as orally administered etoposide, oxaliplatin, bendamustine, or gemcitabine within trials of the GTCSG.[21–24]

The tissue samples from patients with resistant disease were derived from tumors resected at initial diagnosis (n = 14), from secondary resection of residual masses containing viable tumor cells (n = 4), or from metastatic tumors (n = 4). The specimens of the responsive cases were derived from orchiectomy (n = 10) or from retroperitoneal biopsies (n = 2). All samples were reviewed by two independent pathologists (J.W.O, H.P.) and classified according to World Health Organization (WHO) classification.[25]

Immunohistochemistry

Three-micron thick sections of formalin-fixed paraffin- embedded tissue blocks were mounted on APES (3-aminopropyl-triethoxysilane)-coated slides, baked overnight at 50 °C, deparaffinized, and rehydrated. ERB-B2 was demonstrated by the Herceptest kit (Dako, Glostrup, Denmark) according to the manufacturer's instructions. The other antibodies were diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (PBS/BSA). For detection of EGFR, antigen retrieval by digestion with 0.1% protease (Sigma, Zwijndrecht, The Netherlands) in HCl 0.01 M for 15 minutes at 37 °C was performed before the incubation with the primary antibody (EGFR, Ab-10, Clone 111.6, Neomarkers, Union City, CA). The antibody was diluted 1:50 and incubated overnight at 4°C. The antibodies against ERB-B3 (Ab-10) and ERB-B4 (Ab-4, Clone HFR-1; Neomarkers) were both diluted 1:50 and incubated at room temperature (RT) for 2 hours. The c-KIT antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:500 and incubated for 2 hours at RT. Primary antibodies were detected with biotinylated rabbit-antimouse or swine-antirabbit F(ab')2-fragments (Dako), respectively, both diluted 1:200 for 30 minutes at RT, followed by incubation with avidin-biotin-alkaline phosphatase (Vector, Burlingame, CA) diluted 1:100 for 30 minutes at RT. Visualization was achieved with New Fuchsin (Sigma; Zwijndrecht, the Netherlands) as the chromogen. Sections were counterstained with Mayer's hematoxylin. Negative controls replacing the primary antibody by PBS/BSA and adequate positive controls (epidermis for EGFR, breast carcinoma for ERB-B2, skeletal muscle for ERB-B3 and ERB-B4, and CIS for c-KIT) were stained together with each batch.

The immunostaining for ERB-B2 was scored according to the criteria specified by Dako for the interpre-tation of the Herceptest, which have been used in the published Herceptin trials.[26,27] The stainings for EGFR, ERB-B3, and ERB-B4 were evaluated according to the scoring system adopted from Adams et al.28 In line with recommendations of the European Organization for Research and Treatment of Cancer and a recent study on EGFR family receptors in transitional cell carcinoma, cytoplasmic staining was not regarded as relevant.28–30 Staining was scored from 0 (negative) to 3+ (strongly positive) with both systems (Table 1). Tumors rated as 2+ or 3+ were considered positive.

TABLE 1
Criteria for the Evaluation of Immunohistochemical Staining for Epidermal Growth Factor Receptor, ERB-B3, and ERB-B4

Score	Criteria
3+	Dark membrane staining easily visible with low-power objective involving _ 50% of cells
2+	Focal darkly staining areas (_ 50% of cells) or moderate membrane staining _ 50% of cells
1+	Focal moderate membrane staining (_ 50% of cells) or pale membrane staining in any portion of cells not easily seen with low-power objective
0	Membrane staining in widely scattered cells or none of the above

Adapted from Adams et al.[28] Only tumors scored as 2+ or 3+ were considered positive

Fluorescent in situ hybridization (FISH) for the detection of ERB-B2 gene amplification

Copy numbers of the *ERB-B2* gene were determined using the PathVysion kit (Vysis, Downers Grove, IL). Three-micron thick sections were mounted on APES-coated slides and baked overnight at 50 °C. Slides were pretreated, hybridized, and scored according to the manufacturer's instruction. Signals of the *ERB-B2* gene and of the control probe (centromeric region of chromosome 17) were evaluated in 60 randomly selected nuclei per case. A ductal carcinoma of the breast with known amplification of the *ERB-B2* gene served as the positive control.

RESULTS

Tumor Samples

Specimens of 22 therapy refractory patients obtained either from the primary tumor before treatment (n = 14), from secondary resections of residual tumor masses containing viable cells (n = 4), or from resections of metastases at recurrence (n = 4), as well as pathologic specimens of the primary tumor from 12 patients with chemosensitive widespread metastatic disease, were included in the analysis. Patients characteristics are listed in Table 2. The refractory patients had received a median of three treatment regimens (range: 1–7) and of eight cisplatin-containing chemotherapy cycles (range: 2–20). All patients with therapy-refractory GCT have died, whereas all patients with

TABLE 2
Patient Characteristics

	Therapy-refractory patients (n = 22) (%)	Therapy-responsive patients (n = 12) (%)
Median age	29 yrs (range, 17–56)	35 yrs (20–51)
Primary tumor localization		
Gonadal	17 (77)	10 (83)
Retroperitoneal	4 (18)	2 (17)
Mediastinal	1 (5)	0
Tumor stage at initial diagnosis according to the IGCCCG		
Good	13 (59)	0
Intermediat e	2 (9)	0
Poor	7 (32)	12 (100)
First-line treatment		
PEB/VIP/PVB/PE	18 (82)	0
High-dose chemotherapy	4 (18)	12 (100)
Median no. of salvage regimens (range)	3 (1–7)	—
No. of patients receiving high-dose chemotherapy during salvage treatment	15 (65)	—
No. of patients receiving salvage chemotherapy		
Gemcitabine	15 (68)	—
Bendamustine	5 (23)	
Oxaliplatin	4 (18)	
Orally administered etoposide	7 (32)	
Median no. of platin-containing cycles (range)	8 (2–20)	4 (4–5)
Current status		
DOD	22 (100)	0
Alive	—	12 (100)
Histologic diagnosis		
Seminoma	1	0
Nonseminoma	21	12
Tumor components evalutated		
Yolk sac	14 (63)	9 (75)
Mature teratoma	7 (32)	2 (17)
Seminoma	2 (9)	2 (17)
Embryonal carcinoma	4 (18)	6 (50)
Choriocarcinoma	3 (14)	3 (25)
Non-germ cell cancer malignancy	1 (5)	—

IGCCCG: International Germ Cell Cancer Cooperative Group; PE: cisplatin/etoposide; PEB: cisplatin/etoposide/bleomycin; VIP: cisplatin/etoposide/vinblastine; PVB: cisplatin, vinblastin, bleomycin; DOD: dead of disease.

chemosensitive disease are alive and continuously free of disease following first-line treatment for a minimum follow-up period of 18 months.

In the group of refractory patients, one tumor was a pure seminoma and 21 tumors were nonseminomas containing the following histologic elements: yolk sac tumor (present in 63% of the tumors), mature teratoma (32%), embryonal carcinoma (18%), choriocarcinoma (14%), and immature teratoma and seminoma (9% each). One tumor (5%) was diagnosed as a secondary non–germ cell malignancy, which most likely developed from a mature teratoma. In the group of patients with responsive tumors, all 12 had been diagnosed as nonseminomas, containing components of yolk sac tumor (75%), embryonal carcinoma (50%), mature teratoma (17%), choriocarcinoma (25%), and seminoma (17%).

Immunohistochemistry for the EGFR Family and FISH for the ERB-B2 Gene

In general, immunohistochemical detection of the protein of any of the four members of the EGFR family was rare. Within the group of 22 refractory patients, only the secondary non–germ cell malignancy was scored moderately positive for EGFR (+2), even though the tumor also showed completely negative areas. All three tumors containing choriocarcinoma components (one pure choriocarcinoma and two mixed) showed strong membrane staining of the syncytiotrophoblastic cells (Fig. 1A), as did trophoblastic giant cells present in one tumor. ERB-B2 was found at a moderate level (+2) in the syncytiotrophoblast of one mixed nonseminoma, the secondary non–germ cell malignancy, and a mature teratoma component of a mixed nonseminoma. No *ERB-B2* gene amplification was found by

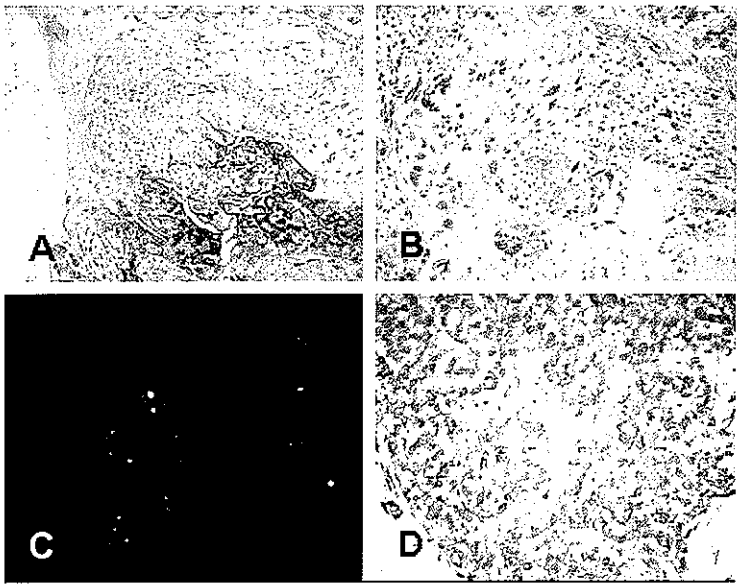

FIGURE 1. (A) Immunohisto-chemical demonstration of epidermal growth fac-tor receptor (EGFR) in a pure choriocar-cinoma of the responsive group. The strong membrane staining is restricted to the syncytiotrophoblast. (B) ERB-B2 immunostaining of the same tumor showing the identical distribution pat-tern. (C) Fluorescent in situ hybridization for the ERB-B2 gene (red) and the cen-tromer of chromosome 17 (green). (D) Moderate EGFR immunostaining in a secondary non- germ cell malignancy, which most likely developed from a ma-ture teratoma

FISH in any of these cases. ERB-B3 was detected in one residual mature teratoma together with ERB-B4 (+2). ERB-B4 (+2) was observed in the non–germ cell malignancy and in two additional nonseminomas (one pure yolk sac, one mixed nonseminoma).

Among the tumor specimens of the 12 chemosensitive patients, none stained positive for EGFR apart from the strongly positive syncytiotrophoblastic cells of the choriocarcinoma components in the two mixed nonseminomas and the one pure choriocarcinoma. ERB-B2 expression Grade +2 and +3 was observed only in the syncytiotrophoblasts of the pure choriocarcinoma. These cells were the only ones showing amplification of ERB-B2 resulting in 8–10 copies per nucleus, whereas the remaining tumor cells had equal signals for ERB-B2 and the centromer of chromosome 17. One mature teratoma component showed moder-ate positive staining for ERB-B3 and ERB-B4. ERB-B4 alone was seen in one pure yolk sac tumor. In both groups, a positive cytoplasmic staining was mainly seen for ERB-B3 and ERB-B4, which did not qualify as a positive tumor. In contrast, this was not observed for EGFR.

Immunohistochemical Demonstration of c-KIT

In the series of both the refractory and the responding GCTs, staining for c-KIT was restricted to seminoma components of mixed nonseminomas (combined tumors according to the British classification) and the refractory pure seminoma. In these cases, clear membrane staining was evident. Nonseminomas showed neither membranous nor scattered intracellular positive staining.

DISCUSSION

With the development of molecular targeted therapy such as receptor tyrosine kinase-interacting agents addressing the EGFR family as well as c-KIT, new options have become available for cancer patients with treatment-resistant tumors like gastrointestinal stromal tumors and refractory breast or colon carcinoma having failed irinotecan.[31–34] These receptor tyrosine kinases, particularly ERB-B2 in breast carcinoma patients, do not only serve as therapeutic targets but may also offer prognostic information for the clinical course of the patient and may be involved in the development of chemotherapy resistance. For example, relations between overexpression of EGFR and ERB-B2 and resistance to cisplatin and paclitaxel have been suggested based on in vitro experiments.[35-39] Clinically, amplification and overexpression of ERB-B2 or EGFR have been correlated with an unfavorable outcome in patients with metastatic breast, ovarian, and esophageal carcinoma.[40–45] Conversely, inhibitors of EGFR and ERB-B2 enhance the

antitumor activity of cisplatin in patients expressing these receptor tyrosine kinases, rendering cisplatin- refractory GCTs a target for a comprehensive analysis of these receptors.[46-48]

The members of the EGFR family dimerize upon ligand stimulation and transduce their signals by activation of the intrinsic protein tyrosine kinase activity and subsequent tyrosine autophosphorylation. This activates different downstream signaling cascades.[36,49,50] Ligand-stimulated heterodimerization results in considerably higher tyrosine kinase activity than homodimerization. This is particularly true for ERB-B2, which depends on colocalization of another EGFR family member to form heterodimers with high tyrosine kinase activity.[51]

These aspects, together with a single case of refractory GCT responding to Herceptin treatment, have formed the rationale to investigate the expression of all members of the EGFR family and of c-KIT in tumor specimens from a homogenous group of 22 patients with clinically established absolutely cisplatin-refractory disease. Furthermore, tumor specimens from a group of 12 patients with widespread metastatic disease at initial diagnosis, all responding favorably to first-line dose-intensive chemotherapy, served as a control cohort to detect a potential prognostic value of the EGFR family.

In the current study, positive staining for the EGFR family members, particularly of EGFR and ERB-B2, was rarely observed. Staining was restricted primarily to one non–germ cell malignancy and to the syncytiotrophoblasts of choriocarcinoma components. In one of the latter cases, an amplification of the *ERB-B2* gene was observed. This pattern of positive staining for EGFR in the syncytiotrophoblasts of choriocarcinoma and trophoblastic giant cells in our series probably reflects the physiologic differentiation of these cells, as it mimics the situation in normal placenta.[52] No differences in staining pattern of any of the receptors studied were found between samples from therapy-refractory and chemotherapy-sensitive GCT patients. This indicates that the EGFR family may neither be of important prognostic value regarding the development of chemotherapy resistance in patients with this disease nor serve as a target for new treatment strategies in GCT in general.

In contrast to our study, Moroni et al.[53] reported that 16 of 18 nonseminomas were positive for EGFR by immunohistochemistry. However, all positive tumors contained a choriocarcinoma component and neither criteria for immunohistochemical evaluation nor description of the distribution of EGFR signal in the positive cases were given. Based on their findings, Moroni et al. encourage clinical trials with tyrosine kinase-interactive agents, particularly because they consider GCTs containing choriocarcinoma components more likely to fail conventional treatment. This seems to be an oversimplification. This is because in our series of patients with documented treatment failure, choriocarcinoma was only present in 15% of the cases. This strongly limits the value for therapeutic modalities aiming at this particular histologic subtype. Even in cases of choriocarcinoma, we convincingly demonstrated that EGFR was restricted to the syncytiotrophoblastic cells. Due to this histologic heterogeneity of GCTs, application of a treatment targeting only one specific cell type within a tumor is not justified. This approach would not affect the majority of the malignant cells, including even the cytotrophoblastic cells of choriocarcinoma.

A study by Soulie et al.[54] reported the ERB-B2 status of gonadal and mediastinal GCTs, tissue from retroperitoneal lymph node dissections and from late recurrences, using immunohistochemistry and FISH. Whereas 22 of 97 cases were scored positive by immunohistochemistry, amplification of the respective gene was only found in 4 cases, 3 of these being late recurrences potentially containing non–germ cell tumor elements. Furthermore, the results from FISH and immunohistochemistry were not correlated. These data are in line with our conclusion that a resistant phenotype in GCTs is not related to alterations in the ERB-B2 signalling pathway. STI571, a newly developed tyrosine kinase inhibitor, which was investigated initially in patients with chronic myelogenous leukaemia targeting the BCR-ABL tyrosine kinase, also inhibits the tyrosine kinase c-KIT.[55] Thus, STI571 has now been investigated in a variety of c-KIT–overexpressing tumors. Durable responses in a high percentage of patients have been reported in gastrointestinal stromal tumors, a tumor entity known to be highly chemotherapy and radiation refractory.[31,32] The most favorable responses were achieved in patients whose tumors showed activating mutations of c-KIT. No expression of c-KIT was found in our patients with refractory nonseminomatous GCTs, suggesting that STI571 will not provide a therapeutic option for these patients. In line with previous reports, c-KIT was only detected in the pure seminoma specimen and in the seminomatous component of a combined tumor.[56,57] In general, seminomatous histology is a favorable prognostic marker in metastatic GCT and the development of chemotherapy-resistant seminoma is unusual. Activating c-KIT mutations are hardly detectable even in seminomas.[13] However, in the rare case of a patient with a refractory seminoma, it might be of interest to consider treatment of the patient with STI571.

In summary, although the new receptor tyrosine kinase inhibitors may offer therapeutic options in a number of malignant diseases, refractory GCTs only show very rarely positive staining for the receptor tyrosine kinases of the EGFR family and of c-KIT. Therefore, EGFR, ERB-B2, ERB-B3, ERB-B4, and c-KIT are not likely targets for treatment.

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Chapter 7

ANEUPLOIDY OF HUMAN TESTICULAR GERM CELL TUMORS IS ASSOCIATED WITH AMPLIFICATION OF CENTROSOMES

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Aneuploidy of human testicular germ cell tumors is associated with amplification of centrosomes

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Running title: Centrosome abnormalities in germ cell tumors

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Abstract

Testicular germ cell tumors occur in three age groups. Seminomas and nonseminomas of adults, including mature teratomas, and the precursor carcinoma *in situ* (CIS) are aneuploid. This holds also true for yolk sac tumors of newborn and infants while the mature teratomas of this age are diploid. In contrast, spermatocytic seminomas occuring in the elderly contain both diploid and polyploid cells. Aneuploidy has been associated with centrosome aberrations, sometimes related to overexpression of *STK15*. Aneuploidy of non-neoplastic germ cells has been demonstrated in the context of male infertility, a risk factor for the development of seminoma/nonseminoma. We investigated aneuploidy, centrosome aberrations and the role of STK15 in different types of testicular germ cell tumors as well as in normal and disturbed spermatogenesis. The aneuploid seminomas and nonseminomas tumors (including CIS) showed increased numbers of centrosomes, without *STK15* amplification or overexpression. Four out of six infantile teratomas had normal centrosomes, the remaining two and an infantile yolk sac tumor showed a heterogeneous pattern of cells with normal or amplified centrosomes. Spermatocytic seminomas had two, four or eight centrosomes. Germ cells in seminiferous tubules with disturbed spermatogenesis shared both aneuploidy and centrosome abnormalities with seminomas/nonseminomas and showed a more intense STK15 staining than those with normal spermatogenesis and CIS. Therefore, aneuploidy of testicular germ cell tumors is associated with amplified centrosomes probably unrelated to STK15.

Introduction

Aneuploidy is the most frequent genomic aberration found in solid tumors and has been proposed as a crucial event in the development of many malignancies (Mitelman, 2000, for review). Recent studies have suggested a link between centrosome anomalies, chromosomal constitution and phenotype. Centrosomes are cellular structures composed of a pair of centrioles associated with so called pericentriolar material, of which pericentrin is an important component (Doxsey et al., 1994). Centrosomes are essential for proper cellular polarity and a balanced distribution of chromosomes during mitosis and meiosis (Hinchcliffe & Sluder, 2001, for review). Centrioles start to duplicate at the late G1/early S phase of the cell cycle, and two functional centrosomes are formed during G2. Centrin as one constituent of the centrioles appears to be important in the proper segregation of centrioles during cell cycle, probably regulated by phosphorylation of a serine residue (Lutz et al., 2002). Aberrant centrin phosphorylation has been observed in breast cancer with supernumerous centrosomes (Lingle et al., 1998).

In vitro experiments proved the link between centrosome amplification induced by inactivation of *P53* (Fukasawa et al., 1996; Meek, 2000), and overexpression of the centrosome-associated serine/threonine kinase *STK 15* (BTAK/Aurora2/AuroraA) (Zhou et al., 1998) and aneuploidy. The *STK15* gene maps to the long arm of chromosome 20, band q13, and it is found to be amplified in primary tumors of the breast, ovary, colon, prostate, cervix and neuroblastoma, and various cell lines. Overexpression of *STK15* has also been reported without gene amplification. Lack of centrosome duplication before mitosis results in polyploidy, while aneuploidy might result from the presence of multiple centrosome duplications during one cell cycle. The presence of abnormal centrosomes, i.e., amplification/hypertrophy and a disturbed cellular distribution, has been observed in various aneuploid cancers of different histogenesis (Ghadimi et al., 2000; Gustafson et al., 2000; Kuo et al., 2000; Lingle et al., 1998; Pihan et al., 2001; Weber et al., 1998).

Aneuploidy is characteristic for all testicular germ cell tumors of adolescents and adults (TGCTs), i.e., seminomas and nonseminomas (El-Naggar et al., 1992; Korn et al., 1996; Looijenga et al., 1991; Looijenga et al., 2000; Mostert et al., 1996; Oosterhuis et al., 1989; Ottesen et al., 1997; Rosenberg et al., 1999; Summersgill et al., 1998; Summersgill et al., 2001). This is also the case for their common precursor carcinoma *in situ* (CIS) (De Graaff et al., 1992; Faulkner et al., 2000; Gillis et al., 1994; Looijenga et al., 1993; Looijenga et al., 2000; Summersgill et al., 2001; Van Echten-Arends et al., 1995). While seminomas are composed of cells highly similar to CIS cells, the nonseminomas can be composed of embryonal carcinoma, yolk sac tumor, choriocarcinoma, and teratoma (Mostofi & Sesterhenn, 1998). Interestingly, gain of the long arm of chromosome 20 has been found in some TGCTs, including CIS (Looijenga et al., 2000; Rosenberg et al., 1999; Summersgill et al., 2001). Due to the consistent aneuploidy of TGCTs, polyploidization has been proposed as one of the initiating events in the formation of CIS, although the mechanisms involved are unknown. Based on this knowledge, attempts have been undertaken to detect CIS cells in semen of patients suspected for the presence of a TGCT (Clausen et al., 1991; Giwercman, 1992; Giwercman et al., 1993). This has not been successful so far, due to the small amounts of CIS cells present, as well as the presence of aneuploid non-malignant germ cells in testicular samples showing atrophy, even

unrelated to the presence of a TGCT. The mechanisms behind this phenomenon have also not been explored so far.

In contrast to the histological diversity of TGCTs, the germ cell tumors of newborn and infants are either teratoma or yolk sac tumor (Scully, 1978). Whereas the yolk sac tumors of newborn and infants are aneuploid, the infantile teratomas are diploid (Looijenga et al., 2000; Mostert et al., 2000; Perlman et al., 1994; Perlman et al., 2000). Within the adult testis, a third entity of germ cell tumors is identified, the so called spermatocytic seminoma (Burke & Mostofi, 1993; Cummings et al., 1994; Dekker et al., 1992; Eble, 1994; Müller et al., 1987; Rosai et al., 1969; Scully, 1961; Talerman, 1974). These tumors have a separate pathogenesis from that of TGCTs, and most likely originate from a germ cell which is able to undergo partial meiosis (Stoop et al., 2001, for review). This is in agreement with the presence of small, intermediate and large cells, representing nuclei with a diploid, a tetraploid and a hypertetraploid DNA content (Looijenga et al., 1994; Rosenberg et al., 1998).

We investigated the presence of centrosome abnormalites and a possible role of STK15, in the pathogenesis of both diploid and aneuploid germ cell tumors of different etiology and histology. In addition, these parameters were studied in the context of the presence of aneuploid germ cells in testicular parenchyma with atrophic features, related and unrelated to TGCTs.

Material and Methods

Tissue samples and patients characteristics

Tumor specimens were collected between 1997 and 2000 in close collaboration with urologists and pathologists in the South-Western part of the Netherlands. Tissue samples were snap frozen directly after surgery. Whenever feasible, samples from tumorous and non-tumorous parts of the specimen were taken. Patients underwent orchidectomy/resection for TGCTs (n = 26), spermatocytic seminoma (n = 3), Leydig cell tumors (n = 4), B-cell non-Hodgkin's lymphoma of the testis (n = 3), non-neoplastic reasons (n = 2), or infantile germ cell tumors (n = 7). Patients characteristics are given in Table 1.

Table 1: Baseline patient characteristics of tumor patients .

	Infantile cases	TGCTs	Spermatocytic seminoma	Disease unrelated to TGCT
Number	7	26	3	9
Median age (years)	1	34 (14-62)		
Diagnosis				
• Seminoma		11		
• Nonseminoma (all cases)	7	15		
mixed nonseminoma		12		
pure embryonal carcinoma		2		
pure yolk sac tumor	1			
pure teratoma	6	1		
• Spermatocytic seminoma			3	
• Disease unrelated to GCT				
B-cell lymphoma				4
Leydig cell tumor				3
non-neoplastic				2

Enzyme- and immunohistochemistry

Sections of 5 µm were air-dried, fixed in acetone for 5 min, air dried, rinsed in phosphate buffered saline (PBS), blocked with PBS containing 1% bovine serum albumin (BSA, Sigma Chemicals, Zwijndrecht, Netherlands). Antibodies were diluted in 1% BSA in PBS, all incubations (primary and secondary antibodies) were done at room-temperature for 1 h. Sections were incubated with: anti-STK15 antibody (rabbit polyclonal, provided by S. Sen, diluted 1:50), and/or a mouse monoclonal anti-centrin (clone 20H5, diluted 1:600, kindly provided by Dr. J.L. Salisbury, Department of Biochemistry and Molecular Biology, Myo Clinic, Rochesterm Minnesota, USA) , or anti-pericentrin (rabbit polyclonal, Babco, Richmond, CA,

diluted 1:200). Slides were rinsed in PBS and incubated with the respective secondary antibody (FITC conjugated swine anti-rabbit immunoglobulin, DAKO, Glostrup, Denmark, diluted 1:50) and/or Cy3-conjugated goat anti-mouse immunoglobulin G (Jackson Immuno Research Laboratories, Westgrove, PA, diluted 1:100), rinsed, post-fixed in 4% formaline and mounted in Vectashield (Vecta Laboratories, Burlingame, CA) containing 0.1% DAPI. Double stainings for centrin and pericentrin were performed on selected cases, and showed identical results. For demonstration of endogenous alkaline phosphatase, which specifically demonstrates the presence of CIS cells (Roelofs et al., 1999), slides were incubated in 60 ml Tris-buffer (0.2 M, pH 8) containing 15 mg Fast Red and 15 mg Naphtol-As-MX-Phosphate (Sigma Chemicals, Zwijndrecht, Netherlands) for 5 min. before fixation in formaline.

Fluorescent in situ hybridization (FISH)

A STK15-specific probe (BAC 3B23, kindly given to use by Dr. S. Sen, MD Anderson Cancer Center, Houston, Texas, USA) was used to check for amplifications of the gene (Zhou et al., 1998). For ploidy analysis, centromere specific probes for chromosome 12 (pα12H8; (Looijenga et al., 1990) and 15 (D15Z1; (Higgens et al., 1985)) were used. FISH was performed as previously described (Kersemaekers et al., 2002).

Evaluation of immunohistochemical stainings, scoring of spermatogenesis in seminiferous tubules and correlation with ploidy, centrosome aberrations and STK15 level

The immunohistologically stained sections and those used for FISH, containing the seminiferous tubules, were evaluated with the DAPI-filter for presence of different stages of spermatogenesis from spermatogonia to spermatozoa. These were analyzed for alkaline phosphatase enzymatic reactivity, centrin, STK15, and the centromeres of chromosomes 12 and 15, respectively. The following "centrin-score" was ascribed to tumors as a whole and to individual tubules: "1": the present germ cells had normal centrosomes composed of 2 centrioles; "2": four to eight centrioles; "3": more than eight centrioles. STK15 was scored subjectively as absent/weak ("1"), moderate ("2") or strong ("3"). A Johnson-Score (assessment of the quality of spermatogenesis based on number and furthest maturation stage present with a score of "10" being optimal with normal numbers of spermatozoae present, and a score of "1" representing total absence of germ cells and Sertoli cells) was attributed to individual tubules. Germ cells within seminiferous tubules were considered aneuploid when the number of signals for centromeres 12 and 15 were not 1/1 or 2/2. Ten randomly selected cells per tubule were assessed in 10 tubules per case. The number of aneuploid cells was correlated with centrosome abnormalities, or STK15 protein level in 10 randomly selected tubules per section.

Statistical analysis

A possible correlation between the number of aneuploid cells and the Johnson score was tested in parenchyma samples of four cases within a testicular germ cell tumor or CIS by ANOVA. A correlation between mean Johnson-score and mean centrin score and between mean Johnson score an mean BTAK score was tested in the parenchyma in patients with (n=13) and without (n=9) in invasive seminoma/nonseminoma/CIS.

Results

We investigated whether aneuploidy of human testicular germ cell tumors correlates with the presence of centrosome anomalies. Therefore, a series of seven germ cell tumors of newborn and infants (six teratomas and one yolk sac tumor), 26 invasive TGCTs (11 seminomas and 15 nonseminomas), 13 testis samples containing CIS, and three spermatocytic seminomas were investigated by immunohistochemistry and FISH. The ploidy of these tumors has been reported before, showing that all TGCTs, including CIS, as well as the only yolk sac tumor of infants are aneuploid, and that the teratomas of newborn and infants are diploid (Looijenga et al., 2000; Rosenberg et al., 1999; Rosenberg et al., 1998). In addition, the spermatocytic seminomas contain diploid, tetraploid and hypertetraploid cells (Looijenga et al., 1994; Rosenberg et al., 1998). The number and conformation of the centrosomes in these tumors was determined using two different antibodies (directed against centrin and pericentrin), which revealed identical results.

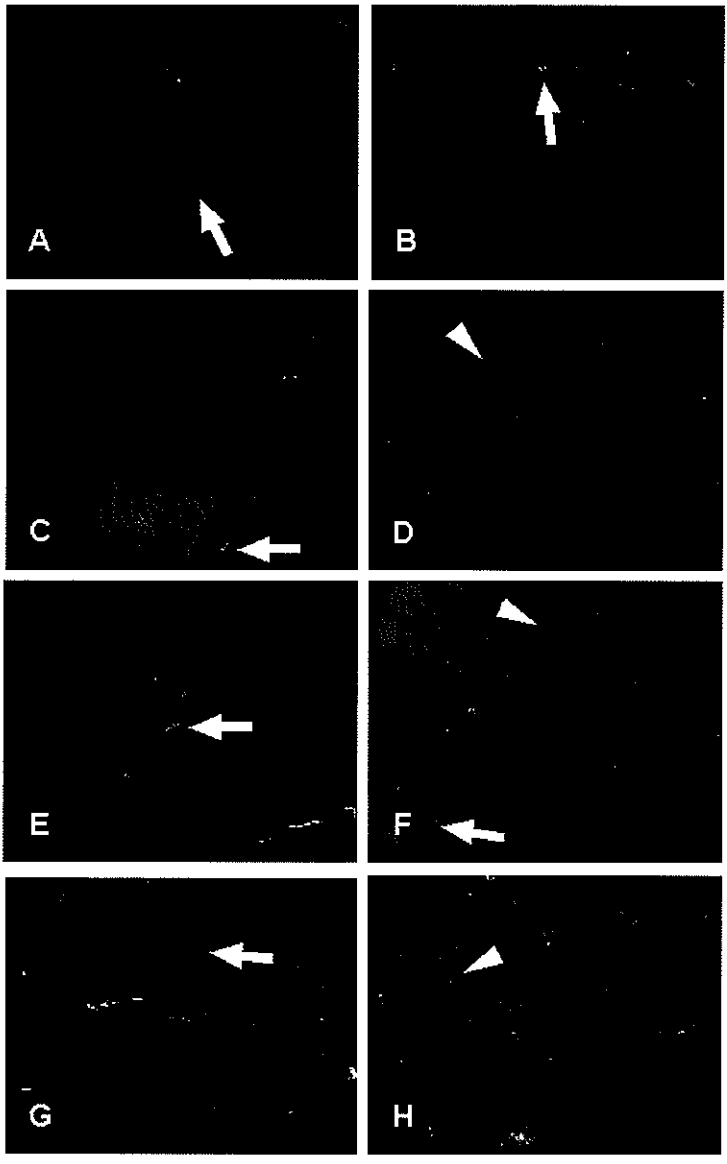
Centrosome organization and STK15 protein in CIS, seminomas and nonseminomas

All seminomas and nonseminomas showed an increased number of centrosomes per cell compared to normal cells. Their numbers per tumor cell varied between 4 and approximately 20, and they were arranged as aggregates at one pole of the nucleus (Figure 1A). Within a single tumor, the pattern was homogeneously distributed, even in case of nonseminomas with different histological elements, including the fully differentiated, but aneuploid teratoma (Figure 1B) (Looijenga et al., 1991). No differences in ploidy status, as demonstrated by the copy numbers of two reference chromosomes (Rosenberg et al., 1997; Rosenberg et al., 1999), were found in three seminomas with the lowest level of centrosome-amplification (about four centrioles per cell) compared to three seminomas with the highest level of amplification (up to 20 centrioles per cell) by ANOVA (mean number of chromosome 12 signals per cell 3.2 versus 2.5, p=0.24; mean number of chromosome 15 signals 2.3 versus 2,5, p=6.4). Immunohistochemistry showed that the STK15 protein was either absent or present at a very low level in TGCTs (see Figure 1A/B). In addition, none of the tumors showed amplification of the *STK15* gene as studied by FISH (data not shown).

CIS cells were identified by the direct alkaline phosphatase staining in the testicular parenchyma adjacent to three seminomas and 10 nonseminomas. Double immunohistochemistry for alkaline phosphatase and centrin demonstrated the presence of amplified centrosomes in all CIS cells (see Figure 1C). This is in agreement with their known aneuploid DNA content (De Graaff et al., 1992; Faulkner et al., 2000; Gillis et al., 1994; Looijenga et al., 1993; Looijenga et al., 2000; Summersgill et al., 2001; Van Echten-Arends et al., 1995), confirmed by FISH in the specific cases studied here (data not shown). The pattern of centrosome amplification in the CIS cells was more heterogeneous than observed in the matched invasive tumor. Similarly as in the invasive TGCTs, no or a very weak staining for STK15 was found. These results indicate that overall the pattern of centrosome organization and presence of STK15 in CIS cells is similar to the invasive components.

Figure 1: Immunohistochemical stainings

A,B,D-H: double-staining for centrin (red) and STK15 (green); C: enzymehistochemical demonstration of alkaline phosphatase activity (red) with immunohistochemical staining for centrin (green). Arrows point to amplified centrosomes, arrowheads to normal centrosomes. A: seminoma. B: mature teratoma of a mixed nonseminoma. C: Seminiferous tubule containing carcinoma *in situ* (positive for alkaline phosphatase). D: infantile mature teratoma. E: infantile yolk sac tumor. F: spermatocytic seminoma. G: seminiferous tubule with disturbed spermatogenesis (no CIS cells). H: seminiferous tubule with intact spermatogenesis.



Infantile germ cell tumors and spermatocytic seminoma of the elderly

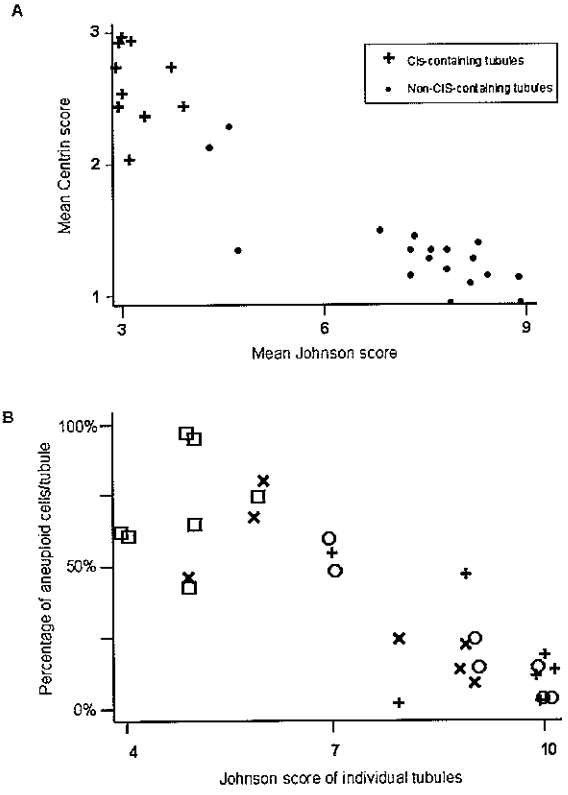
The only infantile yolk sac tumor studied (aneuploid), as well as two out of the six diploid teratomas of this age group showed amplification of the centrosome in about 20% of the tumor cells (see Figure 1E). These cells were spread throughout the whole histological section analyzed. The remaining four teratomas showed normal centrosomes, both in number and organization, in agreement with their diploid DNA content (Figure 1D). Three of these teratomas and the one yolk sac tumors showed no or a weak staining for STK15 protein, while the remaining teratomas showed a strong staining in the tumor cells. Only one of these positive teratomas contained centrosome amplification (see above). All spermatocytic seminomas showed a percentage of the tumor cells with normal centrosome organization (about 40%), whereas the remaining cells had mostly four or eight centrosomes (see Figure 1F). As controls, a number of diploid tumors of different origin (Leydig cell tumors and lymphomas) were included, which all showed a normal pattern of centrosome organization (data not shown).

Ploidy, centrosomes and STK15 status in relation to quality of spermatogenesis

Testicular parenchyma samples of 13 patients with TGCTs (also containing CIS), and of nine patients with unrelated disorders were included in the analysis of the relationship between centrosome organization and atrophy of the seminiferous tubules. Spermatogenesis was rated by means of the Johnson score for 10 individual tubules per case, on which ploidy, centrosome aberrations and the presence of STK15 protein were assessed. The quality of spermatogenesis was in general reduced in samples of patients with a TGCT compared to the other samples (median Johnson score of 6 compared to a median score of 8, with a range of 3 to 9 and 6 to 9, respectively). In the individual patients, the Johnson score of different tubules varied considerably, with occasional tubules showing marked deterioration also in case of an overall well preserved spermatogenesis. In the individual seminiferous tubules a clear correlation between impaired spermatogenesis, and centrosome amplification was evident, regardless of the underlying disease and overall quality of spermatogenesis (Figure 1G/H). This correlation was statistically siginificant ($p<0.0001$) (see Figure 2A). In four samples derived from patients with non-TGCT-related disorders, we analyzed the correlation between Johnson score and the number of aneuploid cells (Figure 2B). Whereas in case of complete maturation to spermatozoa the germ cells were di- or haploid, tubules with disrupted maturation arrested at earlier stages contained significantly more aneuploid cells. The latter showed a more intense staining for STK15. This was also found in the normal germ cells of seminiferous tubules containing CIS.

Figure 2:

A: Correlation between Johnson score and mean centrin score for all tubules containing CIS and all non-CIS containing tubules given as mean for each patient. Score "1": normal centrosomes, "2": 4-8 centrioles, "3": >8 centrioles; B: Percentage of aneuploid cells by Johnson-score given for individual tubules of 4 different patients (data from individual patients are depicted by different symbols)



Discussion

Our data show a correlation between the presence of amplified centrosomes and aneuploid DNA-content for human testicular germ cell tumors. Even though aneuploidy is the most frequent gross genomic aberration in solid tumors (Mitelman 2000), a similar correlation has only been described for a limited number of entities (Ghadimi et al., 2000; Gustafson et al., 2000; Kuo et al., 2000; Lingle et al., 2002; Lingle et al., 1998; Pihan et al., 2001; Weber et al., 1998). Since we observed no difference in ploidy status of tumors with on average 4 or 20 centrosomes, the presence of amplified centrosomes per se and not their actual number seems to be of major importance for the aneuploidy of seminomas and nonseminomas. However, the number of cases investigated in this regard is too small to exclude small differences in ploidy depending on the number of centrioles present. It is of interest to note that these aberrations of centrosomes are already present in the pre-invasive stage of TGCTs, in accordance with its aneuploidy (De Graaff et al., 1992). This indicates that centrosomal changes do not represent a late stage of progression in TGCTs. In this regard TGCTs differ from other solid tumors like prostate cancer, in which the level of centrosome anomalies correlate with Gleason score (Pihan et al., 2001). The more heterogeneous pattern of centrosome amplification in CIS compared to the adjacent invasive tumor could be explained by a selective outgrowth of a certain clone of CIS cells during progression to an invasive tumor. Our data also show that centrosomal aberrations in TGCTs are not related to amplification and/or overexpression of the *STK15* gene, as found in some types of aneuploid tumors (Miyoshi et al., 2001; Zhou et al., 1998). It may, therefore, be that additional genes regulating chromosome segregation contribute towards induction of aneuploidy in TGCT. Two recently identified mammalian members of the STK15 kinase family, AuroraB (also referred to as Aurora1/Aik2) and AuroraC (also referred to as Aik3), have been reported to overexpressed in several human cancers (Kimura et.al. 1999, Tatsuka et al., 1998, Katayama et.al., 1999). However, it cannot be ruled out that overexpression of STK15 is involved in the initial obtainment of supernumerous centrosomes in TGCTs. In addition, it remains to be investigated whether dysregulation of the TGF beta pathway (Glick et al., 1999) is involved in the initial aneuploidization of TGCTs. A role of inactivation of P53 as indicated recently for breast (Meraldi et al., 2002) and prostate cancer (Ouyang et al., 2001), seems unlikely in TGCTs due to the presence of the wild type protein in almost all tumors (Kersemaekers et al., 2002, and references cited therein).

The correlation between ploidy and centrosome status in human TGCTs is not restricted to the seminomas and nonseminomas as demonstrated by the findings in the yolk sac tumor and teratomas of the infantile testis, which have a separate pathogenesis compared to TGCTs (Looijenga, 1999, for review). The aneuploid yolk sac tumor has amplified centrosomes in some of the tumor cells. The only tumors showing a normal centrosome organization are the diploid teratomas of the newborn and infants and to a certain extent the spermatocytic seminomas. The finding of infantile teratomas containing a small cell fraction with amplified centrosomes remains unclear. It is conceivable that aneuploidy of the respective cells has been overlooked due to the techniques used for assessing their ploidy status. Besides the presence of diploid tumor cells, spermatocytic seminomas also contain tumor cells with a polyploid DNA content. Therefore, the subset

of tumor cells containing 4 or 8 centrosomes could be considered as "normal" assuming a polyploid chromosome content. The infantile teratomas and the spermatocytic seminomas are the least malignant subtypes of germ cell tumors, although the teratomas may progress to the (aneuploid) malignant yolk sac tumor and the spermatocytic seminomas to sarcoma (Burke & Mostofi, 1993; Floyd et al., 1988; Matoska & Talerman, 1990; True et al., 1988). Therefore, the data presented are in accordance with the model that aneuploidy and centrosome amplification are related to, and may be a prerequisite for a more malignant phenotype. The presence of STK15 despite normal centrosomes in some of these infantile teratomas, remains unexplained so far. Different localizations of STK15 have been described depending on the model investigated. In contrast to the centrosomal localization in HeLa-cells *in vitro*, a cytoplasmic staining was seen in paraffin-embedded tissue samples from patients suffering from bladder cancer after antigen retrieval using the identical antibody (Zhou et al., 1998; Sen. Et al., 2002). In the fresh-frozen tissue sections of testis tissue and GCTs, the antibody resulted in a circumscript nuclear/perinuclear staining in cells in interphase. Whether these differences have a functional meaning or might just be caused by different experimental conditions remains to be elucidated.

Aneuploidy of germ cells in not confined to the situation after malignant transformation,. It has also been found in semen of patients with a disturbed fertility (Acar et al., 2000; Lahdetie et al., 1997; Shi & Martin, 2000; Van Dyk et al., 2000), both after acute external hazards, like chemotherapy, and primary infertility, the latter condition being a known risk factor for development of a TGCT (Jacobsen et al., 2000a; Jacobsen et al., 2000b; Moller & Skakkebæk, 1999). To investigate whether amplification of centrosomes also plays a role in the generation of aneuploid germ cells under non-malignant conditions, we assessed spermatogenesis quality (by the Johnson score) in relation to ploidy, centrosome organization, and presence of STK15 protein. All samples showed a correlation between the presence of aneuploidy, centrosome number and a poor Johnson score. The data also indicate that detection of supernumerous centrosomes and aneuploidy can not be used as markers for CIS in semen. Of interest is that STK15 protein staining was stronger in germ cells of seminiferous tubules with a low Johnson score compared to seminiferous tubules with a high Johnson score as well as the TGCTs and CIS. This might be related to the model that spermatogonia respond to external stress factors by induction of STK15, which results in surplus numbers of centrosomes and subsequently aneuploidy of germ cells. Subsequently, this could lead to a differentiation block of the germ cells and atrophy. This idea is supported by the uniform picture in the testis of patients suffering from primary TGCTs and unrelated disorders including acute trauma. Another supporting argument is the finding of aneuploidy in oligospermic semen after chemotherapy for various malignancies (Robbins et al., 1997). A similar course of events when occurring in primordial germ cells or gonocytes in the embryonic testis may initiate TGCTs. Role of STK15 in the initiation of centrosome amplification and induction of aneuploidy, as suggested, seems plausible in view of the recent reports that abnormal elevated expression of this protein is detected in a minor fraction of aneuploid bladder epithelial cells of near diploid human bladder tumors (Sen et.al., 2002) and also as an early event in rat mammary carcinogenesis (Goepfert et.al., 2002).

In conclusion, human TGCTs, i.e., seminomas and nonseminomas of the adult testis, as well as teratomas and yolk sac tumors of newborn and infants, and spermatocytic seminoma of the elderly demonstrate a close relationship between aneuploidy and amplification of centrosomes. As this feature is already present in CIS of the testis, it is an early event in development of TGCTs, and not related to invasiveness. Although generation of centrosome aberrations in these tumors seems to be unrelated to STK15, this protein might be involved in the formation of aneuploid germ cells in case of a disturbed spermatogenesis.

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Chapter 8

GENERAL DISCUSSION

8 GENERAL DISCUSSION

Due to their histological and biological diversity, GCTs offer a unique model to investigate the molecular basis for chemotherapy sensitivity and resistance in solid tumors. The spectrum of clinical response ranges from durable complete remissions of advanced metastatic disease after three to four cycles of chemotherapy to absolute chemotherapy resistance of refractory invasive nonseminomas or mature teratomas. The comparison of the various histologic differentation stages present in GCTs allows insight into the effect of differentiation on putative resistance mechanisms, which can influence the efficacy of cytotoxic agents on different levels from drug uptake to execution of apoptosis. In the present work, various of these determinants were analyzed in clinically well defined groups of tumors covering the histologic diversity of GCTs and the spectrum of response to chemotherapy. The results of the different studies are discussed in the context of the overall chemosensitivity of GCTs, the intrinsic chemotherapy resistance of mature teratomas, and the rare occurance of chemotherapy resistance in invasive tumors. These considerations are followed by chapters on receptor tyrosine-kinases as potential treatment targets in refractory disease and on the development of aneuploidy in GCTs.

8.1 Treatment sensitivity of invasive GCTs

The unique chemosensitivity of GCTs amongst solid tumors has been linked to a high level of wild type P53[1]. In vitro experiments on testicular GCT-derived cell lines questioned this notion: a cell line containing no functional P53 at all was found to be sensitive, inactivation of P53 by E6-overexpression in a cell line with functional P53 did not affect CDDP-sensitivity[2,3]. However, the findings were disregarded as representative because the cell lines used did not show a high level of wild type P53[4]. Our study investigating the P53 status (wild type/mutant and level) in clinically well defined patient populations (Chapter 2) clearly showed, that the postulation of a constitutionally high level of wild type P53 in all GCTs is not correct, as a considerable fraction of GCTs had rather low levels of P53 – and still responded to chemotherapy. Consequently, the P53-level itself does not explain the sensitivity of GCTs. On the other hand, in line with previous investigations, most GCTs showed wild type P53. Therefore, it is still conceivable, that a functional P53-pathway rather than a high level of P53 in the untreated situation might be important in the favorable response of GCTs towards chemotherapy[5].

The analysis of multiple markers potentially influencing treatment response adds a number of relevant aspects, which taken together offer an alternative explanation for the chemotherapy sensitivity of GCTs (Chapters 3): Characteristics of the tumor cells favors efficacy of CDDP (and presumably also that of other drugs to a certain extent) on multiple levels: Without interference of export mechanisms with affinity for CDDP like MRP2, BCRP, and LRP, CDDP can accumulate in the cell and bind to the DNA. The observed presence of MRP1 in invasive tumors as opposed to mature teratomas does not contradict the notion of the

lack of CDDP-export systems, as MRP1 has probably no affinity to CDDP[6]. MRP1 expression has even been shown to be inversely correlated with CDDP-sensitivity[7]. GSTπ was also hardly detected in invasive GCTs. The relatively high level of metallothionein detected by immunohistochemistry has been described in GCT-derived cell lines[8]. Obviously, metallothionein is not sufficient to inhibit CDDP in its action in these cells.

Based on data from cell lines, it has been suggested that once CDDP has interacted with the DNA, GCTs were not able to repair the damage efficiently by the NER pathway due to low levels of the xeroderma pigmentosum complementation protein group A (XPA)[9]. Analyzing a series of GCTs of different histologies, XPA could be demonstrated immunohistochemically in the majority of tumors. Seminomas and embryonal carcinoma components showed the lowest rate of XPA-positivity with 48% and 26%, respectively. Yolk sac tumors and choriocarcinomas were positive in 70% and 75%, all mature teratomas showed a strong nuclear staining for XPA. No differences were detected between responding and non-responding cases. Even though GCT-derived cell lines showed a slightly weaker XPA-signal than those of other solid tumors, the differences did by far not reach the extent that has been reported for different GCT cell lines. The results cast significant doubt on the model, that a defect in NER caused by a low XPA-level is responsible for the exquisite chemosensitivity of GCTs. However, our results are purely descriptive and cannot rule out functional defects in NER in GCTs (Chapter 4).

The next group of potential determinants of treatment response affects the recognition of CDDP-induced DNA-damage and subsequent initiation of an apoptotic cascade. At least two potential candidates seem to be functional in GCTs: DNA-MMR and - as previously discussed - P53. The investigated MMR-factors MLH1, MSH2, and MSH6 were detected in high levels in invasive GCTs. The majority of GCTs are microsatellite stable, indicating an intact DNA mismatch repair system (Chapter 5). In addition to the presence of wild type P53, the correlation of the fraction of P53 positive cells with the apoptotic index suggests that P53 is functional, at least as far as the induction of spontaneous apoptosis in GCTs is concerned (Chapter 3). It remains to be investigated, whether further means of damage recognition are involved in the apoptosis of GCT cells exposed to chemotherapy. For example, recent data suggest a role for P73 in CDDP-induced cell death[10]. The execution of apoptosis is the last level determining the response of GCTs to chemotherapy. The lack of knowledge about the exact apoptotic pathway in GCT cells following CDDP-treatment complicates the interpretation of the findings. The anti-apoptotic members of the BCL-2 family BCL-2 and BCL-X_L are present only in low levels in invasive GCTs. The correlation between P53 and the apoptotic index and the demonstration of a strong BAX-staining in apoptotic bodies suggest, that spontaneous apoptosis in untreated GCTs is executed via a P53-dependent, mitochondrial apoptotic pathway. This in turn would indicate, that this particular pathway is intact and that anti-apoptotic influences downstream of P53 are not sufficient to prevent apoptosis at least in the untreated situation. However, these considerations are only relevant in relation to chemotherapy resistance, if CDDP exposure is resulting in cell-kill via the mitochondrial pathway, which remains to be proven (Chapter 3).

In summary, CDDP induced cell death is augmented by the cellular composition of GCT cells on various levels. The sensitivity of these tumors to CDDP-based chemotherapy is determined by the total expression pattern of determinants of treatment response and cannot be attributed to a single factor.

8.2 Intrinsic chemotherapy resistance of mature teratomas

Mature teratomas are the only general exception to the high chemo-sensitivity of GCTs. Their intrinsic chemotherapy-resistance is not limited to CDDP, but extends to all other drugs used in the systemic treatment of GCTs[11,12]. Secondary non-germ cell malignancies derived from mature teratomas share their chemotherapy resistance and have a dismal prognosis[13].

Similar to the supposed multi-factorial explanation for the exquisite chemo-sensitivity of invasive GCTs, the presented investigations suggest a multi-factorial origin for the resistant phenotype of mature teratomas. In contrast to the invasive components, drug export is enabled by the regular presence of ABC-transporters (MDR, MRP2, BCRP) and LRP. The detection of GSTπ suggests, that mature teratomas are able to detoxify CDDP by conjugation to glutathion (Chapter 3). On the level of damage repair, mature teratomas have been shown to have higher levels of XPA compared to unselected invasive tumors (Chapter 4). Therefore, mature teratomas might have a competent NER and be able to repair CDDP-induced DNA-damage[9].

The available data do not indicate a defect in any of the two pathways of damage recognition in mature teratomas. No higher incidence of P53-mutations has been detected in untreated teratomas compared to other histologies (Chapter 2). Only in a series of 28 samples from relapsed or refractory GCT patients, P53 mutations have been described in three teratomas and one secondary non-germ cell malignancy, all of which have been previously exposed to chemotherapy. The fact, that all cases with P53-mutatations were previously treated, indicates, that the P53-mutations were acquired during treatment[14]. However, the resistant phenotype in teratomas is intrinsic and not acquired or induced. The finding therefore does not argue in favor of a contribution of P53-mutations to the resistant phenotype of teratomas.

The MMR-pathway has been considered an alternative means to detect DNA-damage and activate a cell death program (Chapter 5). Immunohistochemistry for hMLH1, hMSH2, and hMSH6 showed a less intense signal in mature teratomas compared to the invasive GCTs (data not shown in the respective article), possibly due to slower growth, which is associated with lower expression of MMR factors[15]. MSI was not more frequently detected in mature teratomas than in the remaining specimen. Therefore, there are no conclusive arguments to assume a defective MMR-pathway in mature teratomas as explanation for their specific chemotherapy resistance.

Regarding the downstream targets of P53, mature teratomas show a different behavior compared to invasive GCTs. Even though P53 can be detected in mature teratomas to a similar extent as in the remaining GCT, its biological effect seems to be altered. Whereas p21 as one of the classic downstream targets of P53 is hardly induced in invasive GCT even in the case of high levels of the wild-type P53 protein, p21 can regularly be detected in mature teratomas. On the other hand, BAX-positive apoptotic bodies – frequently seen in

invasive GCTs – do not occur in teratomas. RB can usually be detected in teratomas in contrast to the remaining components[16]. These data suggest that P53 causes p21-induction and G1/S-arrest in teratomas in contrast to invasive GCTs, where the data favor BAX-induction and apoptotic cell death upon upregulation of P53. Also with regard to the BCL-2 family members, mature teratomas are equipped to resist apoptotic stimuli with higher levels of BCL-X_L and to a lesser extent of BCL-2.

In summary, the resistant phenotype of mature teratomas results from various factors interfering with the efficacy of chemotherapy on multiple levels. The detected resistance mechanisms are not restricted to the action of CDDP, but extent to multiple agents in case of MDR or even to anti-apoptotic mechanisms theoretically effective against all drugs killing via the mitochondrial apoptotic pathway. The occurence of the different resistance markers in mature teratomas is probably the consequence of terminal differentiation. In this way, it is conceivable, that a cell with a somatic differentation sharing biological characteristics with normal somatic cells has a comparable sensitivity and can thus not be killed by systemic treatment without doses associated with unacceptable side effects.

8.3 Predicting and understanding chemotherapy resistance in GCTs

Despite the knowledge of multiple possibilities to achieve chemotherapy resistance *in vitro*, little is known about the development of clinical CDDP-resistance in GCTs.

The investigations on putative mediators of chemotherapy resistance acting before CDDP reaches the DNA did not show upregulation in any of the parameters studied by immunohistochemistry in refractory compared to sensitive cases to a significant extent. The ABC-transporter MRP2, presumably able to transport CDDP when conjugated to glutathion[17], was found in three out of 24 refractory cases, but not in any of the responding or of the unselected cases. GSTπ was only detected in five out of 24 refractory tumors. A high increase in glutathion levels has been described in resistant GCT cell lines compared to sensitive ones[8]. A prognostic value has been described for GSTπ in carcinomas of the head and neck region[18]. Therefore, MRP2 and GSTπ might well have an impact in selected refractory cases and should be evaluated in a larger series.

The option of repair of CDDP-induced DNA-damage was only investigated with regard to one compound of the NER, the XPA. In contrast to data on a limited number of GCT-derived cell lines, XPA could readily be detected in a significant number of GCTs, both in responding and non-responding tumors (Chapter 4). Accordingly, GCTs can respond to chemotherapy despite a level of XPA above the immunohistochemical detection threshold. Nevertheless, two findings do indicate a possible relation of XPA to treatment response: as previously discussed, all mature teratomas were strongly positive for XPA. All seven investigated nonseminomas, that were resected in relapse after previous treatment, showed a clear XPA-signal, suggesting an induction of XPA by CDDP-exposure or a selection of XPA-positive subclone of tumor cells. In this context, it is noteworthy, that XPA was not induced in GCT-derived cell lines treated repeatedly with CDDP. However, given the distribution of histologic subtypes in the group pretreated tumors, the difference

between the seven previously treated refractory samples compared to untreated tumors of the same histology is statistically not significant. In none of the investigated cases positive for XPA by immunohistochemistry, an amplification of the gene could be detected, which might be expected based on chromosomal analyses [19]. Taken together, the data on XPA demonstrate, that in clinical reality, modifications in XPA-level detectable by immunohistochemistry have a limited impact on treatment outcome at best.

No P53 mutations were found in the group of the refractory cases, the P53 level as determined by immunohistochemistry was comparable to that of sensitive and unselected cases, as was the correlation between P53 and apoptosis. In consequence, the P53 protein cannot be a common target in the development of chemotherapy resistance in GCTs. As previously discussed, the finding of P53 mutations in previously treated chemotherapy resistant tumors by Houldsworth and collegues[14] does not contradict this interpretation, as they refer only to mature teratomas. Also for the MMR pathway, a role as damage sensor has been assumed, as defects in this pathway confer resistance to selected drugs[20]. Whereas in a large series of unselected GCT, only 6% of the cases showed MSI in at most one out of eight investigated microsatellite markers, five out of 11 (45%) of resistant cases were microsatellite instable, four of them in at least two loci (Chapter 5). The difference was highly statistically significant ($p<0.001$). Within the group of resistant GCTs, the progression free survival of the patients with MSI in the tumors differed from that of the remaining patients. Even though the longer progression free survival of the patients with MSI cannot be explained at this point, the presence of a difference supports the biological relevance of the finding regarding the clinical behavior. It does not argue against the putative role of a defective MMR pathway in chemotherapy resistance. It is important to realize, that the group of refractory tumors with intact an intact MMR obviously has different, yet unidentified features confering their resistant phenotype. These features obviously result in a more aggressive tumor growth or a higher resistance level than defects in MMR. In addition, the microsatellite instable tumors – even though resistant to CDDP – might respond better to other agents like topoisomerase inhibitors, as has been found in vitro[21].

Looking at the regulation of apoptosis, differences between responding and refractory invasive GCTs could not be detected regarding the apoptotic index, the BCL-2-family members BAX, BCL-2, and BCL-XL, p21 or RB. Therefore, general anti-apoptotic mechanisms like overexpression of anti-apoptotic proteins or defects in effectors like caspases are not suggested by the data.

To summarize, the presented investigations point towards defects in the MMR pathway as a frequent mechanism for clinically relevant chemotherapy resistance in GCTs. The findings will have to be validated in larger patient cohorts and in interventional experimental models. The phenotype of some refractory cases might be explained by alterations in drug export by ABC-transporters or drug inactivation by GSTπ. Mechanisms affecting the execution of P53-dependent apoptosis are not suggested by the available data.

8.4 Aneuploidy of GCTs

Even though the main focus of the study on ploidy, centrosome abnormalities and STK15 (Chapter 7) was to add to the understanding of the pathogenesis of different types of germ cell tumors, the data allow for some

conclusions relating to chemotherapy resistance. Both aneuploidy and centrosome aberrations were evident in CIS, in all seminomas and all nonseminomas including mature teratoma of the adult. Even though no clinical information about the investigated tumors is available, the vast majority will respond favorably to chemotherapy. In contrast, the mature teratomas of the infant – known to be diploid – showed normal centrosomes. On progression to invasive yolk sac tumors, centrosome abnormalities occur together with aneuploidy. Whereas, the former share the chemotherapy resistance of adult mature teratomas, the latter respond to chemotherapy to a similar extent like seminomas and nonseminomas of adults. Thus, a clearcut correlation between aneuploidy and chemotherapy response as postulated by Duesberg and colleagues[22] can be ruled out as far as GCTs are concerned. Assuming the correlation between aneuploidy and chemotherapy resistance in other tumor entities, why do GCTs not stick to the rule? At this point, one can only speculate about the biological background for this difference.

Investigating normal and disturbed spermatogenesis both in the presence and the absence of a testicular GCT, we found centrosome abnormalities and aneuploidy of non-neoplastic germ cells. Given the development of GCTs from primordial germ cells/gonocytes, it can be ruled out, that the aneuploid germ cells are prone to progress to overt maligancy. It can rather be assumed, that centrosome abnormalities and aneuploidization are a regular reaction of germ cells to external stresses. In this way, centrosome abnormalities and aneuploidy found in disturbed spermatogenesis would be a reactive change, and would thus differ from that found in malignancies including GCTs. This might explain the presence of aneuploid germ cells in testes and semen of patients with disturbed spermatogenesis. Again, the considerations have to remain purely speculative at this point.

8.5 Receptor tyrosine-kinases as possible resistance mechanisms and treatment targets in refractory GCTs

Receptor tyrosine kinases have received a lot of attention in recent years, as they have been shown to be promising new targets in a variety of malignancies[23], e.g. the BCR-ABL-tyrosine kinase in CML, c-KIT in gastrointestinal stroma tumors, or ERB-B2 in metastastic breast cancer[24-26].

New therapeutic targets are warranted for patients with refractory disease in particular. A meaningful response has been described in a patient with an ERB-B-positive GCT following treatment with trastuzamab, a monoclonal antibody of ERB-B2[27]. Based on this experience and on considerations of EGFR family members in germ cells during embryogenesis, all four members of the EGFR family –EGFR, ERB-B2 (Her2/neu), ERB-B3, and ERB-B4 – were analyzed in refractory GCTs.

Disappointingly, the results of the investigation revealed staining for EGFR and to a lesser extent ERB-B2 only in the syncytiotrophoblastic cells of choriocarcinoma and in some mature teratomas (Chapter 6). The only investigated secondary non-germ cell malignancy was positive for EGFR. With these results, treatment strategies addressing the members of the EGFR family do not appear to be promising in refractory GCTs and should only be offered in individual cases, when the presence of the respective receptor has been

demonstrated. The presence of EGFR in the syncytiotrophoblastic cells of choriocarcinoma could expected due to the expression in normal placenta[28].

Similar to the EGFR-familiy, c-KIT does not seem to be a suitable treatment target in refractory GCTs, because it is only detected in seminoma and CIS cells. Seminomas have overall a significantly better prognosis than nonseminomas; refractory seminomas are therefore extremely rare [29]. Nevertheless, whenever such tumors are encountered, treatent with STI571 could well be considered

In summary, the data do not support the development of protocols for the regular use of tyrosine-kinase interactive substances in the context of refractory GCTs. None of the investigated receptors seems to play a role in the development of chemotherapy resistance.

8.6 Concluding remarks

The presented investigations add to the understanding of the diverse treatment response of GCTs. Nevertheless, many questions remain to be answered. Rather than seeking explanations in the analysis of single factors, future research should focus on whole pathways or interactions between different cellular systems, in order to elucidate the phenomenon of GCT as a curative disease. Further investigations would be greatly augmented once it is clear, by which pathway or cell death program the GCT cells are killed by CDDP.

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9 SUMMARY/SAMENVATTING

In contrast to most other solid tumors of the adult, germ cell tumors (GCTs) of adolescent and young males can be cured in up to 80% of patients with advanced metastatic disease by chemotherapy followed by resection of residual masses. Despite this overall success, 10-15% of all patients newly diagnosed with a metastastic GCT will not sufficiently respond to available treatment strategies and finally die of their disease. A thorough understanding of the cellular basis of the overall chemotherapy sensitivity of GCTs and of the phenotype of refractory tumors is necessary in order to indicate ways to overcome or circumvene chemotherapy resistance and possibly identify new therapeutic targets. The aim of this thesis was to seek explanations for the exquisite chemosensitivity of GCTs and clinically relevant mechanisms for chemotherapy resistance. The approach concentrated on tumor samples from well-characterized patients. Targets were selected along the intracellular course of cisplatin from uptake into the cell down to eventual execution of apoptosis, i.e. cell death. In addition, receptor tyrosine-kinases were investigated as potential new treatment targets in patients suffering from resistant disease.

A high level of wild-type P53-protein has commonly been regarded as the most likely explanation for the high curability of GCTs of adolescent and young males. Assessing the P53-status by mutation analysis and immunohistochemistry in tumor samples from clinically well defined patients, no correlation between the level and mutation status of P53 and treatment response could be detected. Furthermore, in some of the tumors, P53 could hardly be detected – and still these tumors responded to chemotherapy. In contrast, P53 was commonly demonstrated in mature teratomas despite their intrinsic chemotherapy resistance. The results underlined the need to seek further explanations for the behavior of GCTs following systemic treatment.

Analysis of the occurrence of spontaneous apoptosis and downstream targets of P53 in untreated tumor samples suggested, that the P53 and Bax-dependent apoptotic cascade remains intact, even in the refractory tumors. No differences in apoptotic index and correlation with P53-positivity were detected between sensitive and refractory tumor samples. Therefore, defects of anti-apoptotic stimuli interfering with this cascade are unlikely to be clinically relevant mechanisms mediating chemotherapy resistance in invasive GCTs of adolescents and young adults. However, in mature teratomas, the presence of P53 seems to result in a p21/retinoblastoma protein-mediated cell cycle arrest rather than in induction of Bax and consecutive apoptosis. All together, the data on P53 and on apoptosis suggested, that relevant resistance mechanisms act either upstream of P53 or in an alternative apoptotic pathway different from the P53-dependent mitochondrial cascade.

Various export pumps with proven or at least suspected affinity for cisplatin have been analysed. Potentially contributing to the overall sensitivity of GCTs to cisplatin, none of these pumps were detected in invasive chemotherapy sensitive GCTs. No single pump was expressed commonly in refractory cases, even though the lung resistant protein lung resistance protein (LRP) and the ABC-transporter MRP2 could be detected in individual resistant tumors, where they might contribute to the resistant phenotype. In mature teratomas,

LRP, MRP2 and the breast cancer resistance protein (BRP) was consistently detected. Similarly, glutathion-S-transferase π, an enzyme detoxifying cisplatin by transfering glutathion, was demonstrated in single refractory cases and consistently in mature teratomas.

The last line of defence against the effect of cisplatin before the initiation of an apoptotic cascade is repair of the induced DNA-damage. Previous studies have suggested a low capacity for DNA-damage repair mechanisms in GCTs. This feature has been attributed to a low level of the xeroderma pigmentosum complementation group A protein (XPA). Even though the analysis of DNA-repair mechanisms was restricted to the detection of XPA by immunohistochemistry and the assessment of its gene copy numbers by fluorescence *in situ* hybridisation, our data argue against the idea of a low level of XPA as explanation for the chemosensitivity of GCTs. XPA could be detected in more than half of the tumor samples irrespective of their treatment outcome. Therefore, the mere level of XPA cannot explain the overall chemosensitivity of GCTs. Furthermore, upregulation of this protein is not a common resistance mechanism.

Next to efficient repair of drug-induced DNA-damage, different factors, including DNA-repair mechanisms, can initiate an apoptotic cascade once they have detected DNA-damage. The DNA-mismatch-repair (MMR) pathway seems to be able to induce programmed cell death after exposure to cisplatin. Accordingly, defects in the MMR result in resistance to the drug. The analysis of a large series of unselected GCTs showed an intact MMR in 94% of these tumors together with strong signals of three crucial MMR factors – MLH1, MSH2, MSH6 – as assessed by immunohistochemistry. In contrast, refractory cases showed microsatellite instability – an indication of a defective MMR – in 45% of the tumors.

Extending the focus of interest to the detection of new treatment targets in refractory GCTs, different receptor tyrosine kinases were investigated. Overexpression of different members of the epidermal growth factor receptor (EGFR) family has been associated with a resistant phenotype. When overexpressed, various receptor tyrosine kinases allow for treatment with new agents like specific kinase inhibitors or monoclonal antibodies. Analyzing all four members of the EGFR family, no differences were observed between samples derived from responding and refractory patients. A strong signal for EGFR and – to a lesser extent – of Erb-B2 was detected in all syncytiotrophoblast of choriocarcinoma components. Furthermore, a staining of various members of the EGFR-family was evident in a minority of mature teratomas, mostly restricted to parts of the teratoma components. Accordingly, EGFR-family members do not represent promising treatment targets in patients with chemotherapy-resistant GCTs.

Aneuploidy has been claimed to be the single explanation for the ability of tumor cells to acquire chemotherapy resistance. GCTs, in particular when including the infantile tumors and the spermatocytic seminoma of the elderly, do not fit to this hypothesis, as by no means, a correlation between the ploidy of certain tumor types and their chemotherapy responsiveness can be observed. The last part of the thesis concentrated on the role of centrosomes in the development of aneuploidy in GCTs rather than on the implication of aneuploidy for chemotherapy sensitivity. To widen the spectrum of ploidy, diploid infantile mature teratomas, aneuploid infantile yolk sac tumors and polyploid spermatocytic seminomas were included in this investigation. A clear correlation between aneuploidy and centrosome amplifications was observed

throughout all included tumors. Furthermore, aneuploidy observed in germ cells in the context of disturbed spermatogenesis unrelated to the presence of a GCT shared the correlation with amplified centrosomes. Whereas in the latter case, the serine-threonine-kinase STK15 could be involved in the induction of centrosome abnormalities, neither overexpression of the protein nor amplification of the gene could be observed in any of the aneuploid GCTs.

In summary, the presented investigations indicate, that the exquisite chemosensitivity of GCTs is caused by cellular features promoting induction of apoptosis after treatment with cisplatin on various levels. Mature teratomas differ from the other invasive components in the presence of many of these factors resulting in a resistant phenotype induced by expression of drug export pumps, glutathion-S-transferase pi, anti-apoptotic members of the Bcl-2 family, ability for a p21-dependent cell cycle arrest and possibly a competent nuclear excision repair. The rarely encountered chemotherapy resistant invasive GCTs might partly be explained by defects in the MMR-pathway as demonstrated by the frequent microsatellite instability in these tumors. The precise molecular mechanism connecting the MMR with a failure to induce apoptosis following CDDP-exposure remains to be established in future studies.

Samenvatting

In tegenstelling tot de meeste volwassenen met een kwaadaardige solide tumor, wordt meer dan 80% van de mannen met een uitgezaaide kiemceltumor (KCT) genezen door chemotherapie, eventueel gevolgd door chirurgische verwijdering van restlesies. Dit percentage is ongekend hoog, maar nog steeds reageert 10-15% van de nieuw gediagnosticeerde patiënten met gemetastaseerde ziekte niet of onvoldoende op de behandeling, en zal uiteindelijk overlijden aan de ziekte. Het begrijpen van de moleculaire basis van chemotherapie-gevoeligheid en -resistentie van KCT is een vereiste voor het omzeilen van chemotherapie-resistentie, en voor het vinden van nieuwe aangrijpingspunten voor therapie. Het doel van dit proefschrift is het vinden van de verklaring voor de ongekende chemotherapie-gevoeligheid van KCT en de mechanismen van chemotherapie-resistentie. Het onderzoek is vooral gericht op de analyse van tumoren van goed gedefinieerde patiëntenpopulaties. De te onderzoeken parameters werden geselecteerd op grond van het bekende werkingsmechanisme van cisplatinun, dat via opname in de cel uiteindelijke leidt tot inductie van apoptose (geprogrammeerde cel dood). Tevens werd een aantal receptor-tyrosine-kinases bestudeerd als mogelijk aangrijpingspunt voor de behandeling van patiënten met resistente ziekte.

Aaangenomen werd dat ophoping van wild-type P53 eiwit in de tumorcellen het hoge genezingspercentage van KCT kon verkaren. Gen-mutaties in *P53* en immunohistochemisch aangetoonde aanwezigheid P53-eiwit blijkt niet te correleren met behandelingsrespons. Een aantal gevoelige KCT blijkt zelfs bijna geen P53-eiwit te bevatten. Omgekeerd was P53 vaak aanwezig in mature teratomen die intrinsiek ongevoeligheid zijn voor chemotherapie. Deze resultaten bevestigen dat andere verklaringen gezocht moeten worden voor de reactie van KCT op systemische behandeling.

Onderzoek naar spontane apoptose en de "downstream targets" van P53 in onbehandelende tumoren suggereert dat de P53-BAX apoptose-route intact is, zelfs in resistente tumoren. Het is daarom onwaarschijnlijk dat afwijkingen in anti-apoptotische regulatiemechanismen die van invloed zijn op deze route een rol spelen in chemotherapie-resistentie van invasieve KCT. In mature teratomen daarentegen lijkt de aanwezigheid van P53 te resulteren in een door P21/retinoblastoom-eiwit gereguleerde onderdrukking van celdeling in plaats van inductie van BAX en apoptose. Geconcludeerd kan worden dat de relevante resistentie-mechanismen of "upstream" van P53 functioneren, of onafhankelijk zijn van de P53-mitochondriële cascade.

Verschillende cellulaire pompen zijn in staat cisplatinum te verplaatsen. Ze blijken niet essentieel te zijn voor cisplatinum-resistentie van KCT tumoren. "Lung resistance protein (LRP)" en de ABC transporter MRP2 zijn gevonden in enkele resistente tumoren, waar ze mogelijk van invloed zijn op cisplatinum-gevoeligheid. In mature teratomen daarentegen kunnen LRP, MRP2, en het "breast cancer resistance protein (BRP)" altijd worden aangetoond. Op dezelfde manier is glutathion-S-transferase πi, een enzym dat door middel van glutathion detoxificatie van cisplatinum tot stand kan brengen, slechts in enkele resistente tumoren aanwezig, terwijl het altijd kan worden aangetoond in mature teratomen.

De laatste mogelijkheid voor verweer tegen het effect van cisplatinum is de stap voor de initiatie van het proces van apoptose, en wel het repareren van de geïnduceerde DNA schade. In de literatuur is gesuggereerd dat KCT een lage capaciteit voor DNA herstel hebben omdat het Xeroderma Pigmentosum complementatie groep A eitwit (XPA) laag tot expressie komt. Onze gegevens, verkregen op basis van immunohistochemie voor XPA eiwit en fluorescente *in situ* hybridisatie voor het aantal *XPA* genen geen steun geven aan de hypothese dat het niveau van XPA een rol speelt in chemotherapie-gevoeligheid van KCT. Het eiwit is aanwezig in meer dan 50% van de onderzochte tumoren, onafhankelijk van de behandelingsuitkomst. Met andere woorden, de chemotherapie-gevoeligheid van KCT is niet te verklaren op basis van niveau van XPA-expressie in de tumorcellen. Het blijkt dat overexpressie van XPA niet een veelvoorkomend mechanisme van resistentie kan zijn.

Naast DNA herstelmechanismen, kan ook herkenning van DNA-schade de apoptotische cascade initiëren. Het zogenaamde DNA-mismatch-repair (MMR) mechanisme is in staat celdood te initiëren na blootstelling van de cel aan cisplatinum. Hieraan gerelateerd, blijkt een verstoorde MMR te kunnen resulteren in resistentie tegen dit geneesmiddel. Analyse van een grote serie van willekeurige KCT laat een intact MMR mechanisme in 94% van de tumoren zien, geassocieerd met duidelijke immunohistochemische aanwezigheid van drie cruciale MMR factoren (MLH1, MSH2 en MSH6). De resistente tumoren daarentegen blijken in 45% een verstoorde MMR te vertonen, geïllustreerd door de aanwezigheid van microsatelliet instabiliteit.

Om nieuwe aangrijpingspunten te vinden voor de behandeling van resistente KCT zijn verschillende typen tyrosine-kinase-receptoren bestudeerd. Overexpressie van leden van de "epidermal growth factor receptor (EGFR)" familie is geassocieerd met resistentie. Als deze receptoren tot overexpressie komen in tumoren blijken ze een aangrijpingspunt te zijn voor behandeling met nieuwe geneesmiddelen, zoals specifieke kinase-remmers of monoklonale antilichamen. Er zijn geen verschillen m.b.t. de vier verschillende leden van de EGFR familie tussen gevoelige en ongevoelige tumoren. Een sterk signaal is aanwezig voor EGFR en, maar in mindere mate, voor Erb-B2 in alle syncytiotrophoblast- en choriocarcinoom elementen. Ook blijken verschillende leden van de EGFR-familie aanwezig te zijn in een minderheid van de mature teratomen, en wel in bepaalde componenten van de tumor. Deze resultaten geven aan dat leden van de EGFR-familie geen aangrijpingspunt bieden in de behandeling van patiënten met chemoresistente KCT.

Aneuploïdie (de aanwezigheid van afwijkende aantallen chromosomen) is gesuggereerd als basis voor het vermogen van tumorcellen om chemotherapie-resistentie te ontwikkelen. Het gedrag van de KCT van de neonaten en pasgeborenen en van de spermatocytaire seminomen van de oudere man, is niet in overeenstemming met deze hypothese. Er is in zijn algemeenheid geen correlatie tussen de ploïdie van de verschillende histologische subtypes van KCT en hun chemotherapie-gevoeligheid. Het laatste gedeelte van dit proefschrift betreft de bestudering van de rol van centrosomen in de ontwikkeling van aneuploïdie in KCT, en niet de implicatie van aneuploïdie voor chemotherapiegevoeligheid. Om het geheel in een bredere context te plaatsen zijn ook de diploïde teratomen, de aneuploïde dooierzaktumoren van het jonge kind, en de polyploïde spermatocytaire seminomen in de studie betrokken. Er bestaat een duidelijke correlatie tussen

ploïdie van de tumoren en de aanwezigheid van centrosoom-amplificatie. De diploïde tumoren hebben normale centrosomen, terwijl in alle aneuploïde tumoren amplificatie wordt gevonden. Tevens is een correlatie gevonden tussen centrosoom-amplificatie en aneuploïdie van kiemcellen bij gestoorde spermatogenese, niet gerelateerd aan de aanwezigheid van een KCT. Serine-theonine-kinase STK15 blijkt geen rol te spelen in het ontstaan van centrosoom-amplificatie in KCT. Dit is waarschijnlijk wel het geval bij centrosoom-amplificatie in de spermatogenese.

Samengevat: uit de gepresenteerde studies is gebleken dat de uitzonderlijke chemotherapie-gevoeligheid van KCT het gevolg is van verschillende cellulaire processen, die na behandeling met cisplatinum op verschillende niveaus apoptose induceren. De mature teratomen verschillen van de andere invasieve componenten door hoge expressie-niveaus van factoren die resulteren in een resistente cel: drug- pompen, glutathion-S-transferase π, anti-apoptotische leden van de Bcl2 familie, p21. Mogelijk speelt een functioneel DNA herstel mechanisme door middel van "nucleotide excisie repair" een rol. De soms waargenomen chemotherapie-ongevoeligheid van KCT zou ten dele verklaard kunnen worden door een verstoorde MMR-route, blijkend uit frequente microsatelliet-instabiliteit. De moleculaire mechanismen die MMR koppelen aan het onvermogen tot inductie van apoptose na blootstelling aan cisplatinum moeten verder worden onderzocht.

10 ABBREVIATIONS

BCRP	breast cancer resistance protein
CDDP	cis-dichlor-diamin-platin
CIS	carcinoma *in situ*
DNA	deoxyribonucleic acid
EGFR	epidermal growth factor receptor
GCT	germ cell tumor
GIST	gastrointestinal stroma tumor
IGCCCG	international germ cell cancer collaborative group
LDH	lactate dehydrogenase
MDR	multidrug resistance
MMR	DNA mismatch repair
MRP1/2	multidrug resistance related protein
MSI	microsatellite instability
NER	nucleotide excision repair
PE	cisplatin/etoposid
PEB	cisplatin/etoposid/bleomycin
RB	retinoblastoma gene protein
RNA	ribonucleic acid
RPLND	retroperitoneal lymph node dissection
ßHCG	beta human choriogonadotropin
STK15	serine-threonin-kinase 15
XPA	xeroderma pigmentosum complementation group A protein

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April 2000 – March 2002	Research Fellow at the Department for Pathology Josephine-Nefkens-Institute Pathology/Laboratory for Experimental Patho-Oncology under supervision of Dr. Leendert Looijenga Erasmus MC/Daniel den Hoed Cancer Center Head of the Institute: Prof. Dr. J. W. Oosterhuis

Grants:

1995	German Academic Exchange Service (DAAD) "Grant for scientific education abroad" during the final year in medical school
April 2000 – March 2002	European Society for Medical Oncology Fellowship Program

13 LIST OF PUBLICATIONS

Mayer, F.; Kollmannsberger, C.; Looijenga L.H.J.; Bokemeyer, C.
Autor reply: Absence of c-kit and EGFR-family in refractory germ cell tumors (Letter to the editor). *Cancer* 2003 (in press)

Mayer, F.; Stoop, H.; Sen, S.;Bokemeyer,C.; Oosterhuis, J.W.; Looijenga,L.H.J.
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Chapter 2 :

Figure 1 (Page 25)

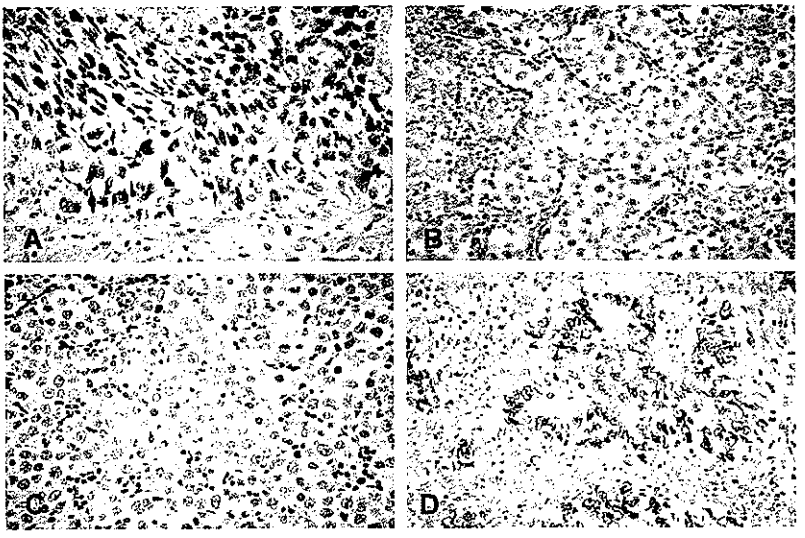
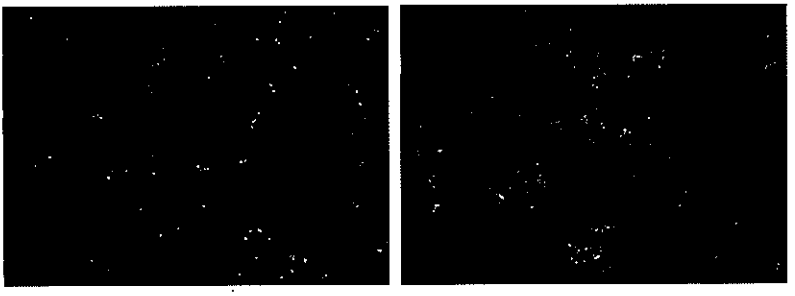
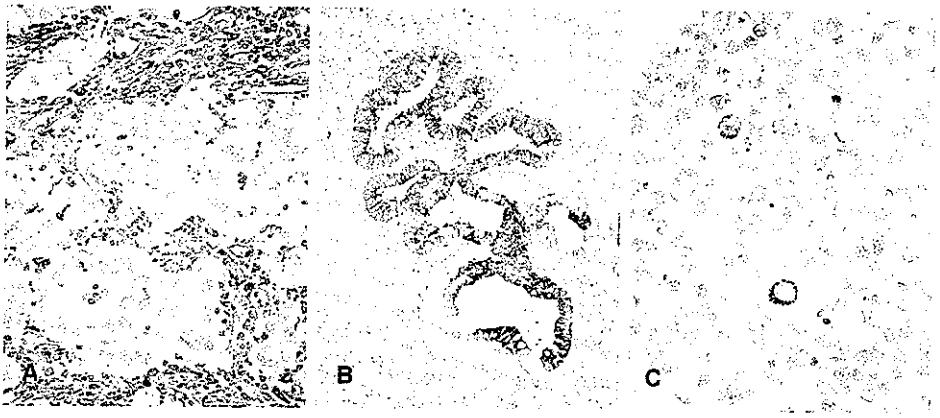


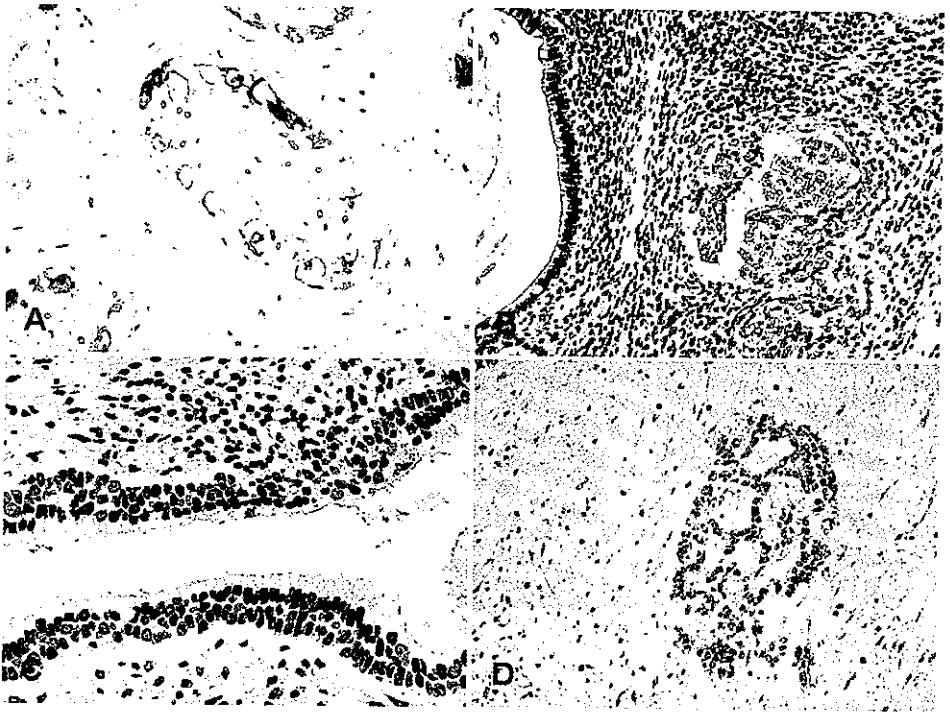
Figure 2 (Page27)



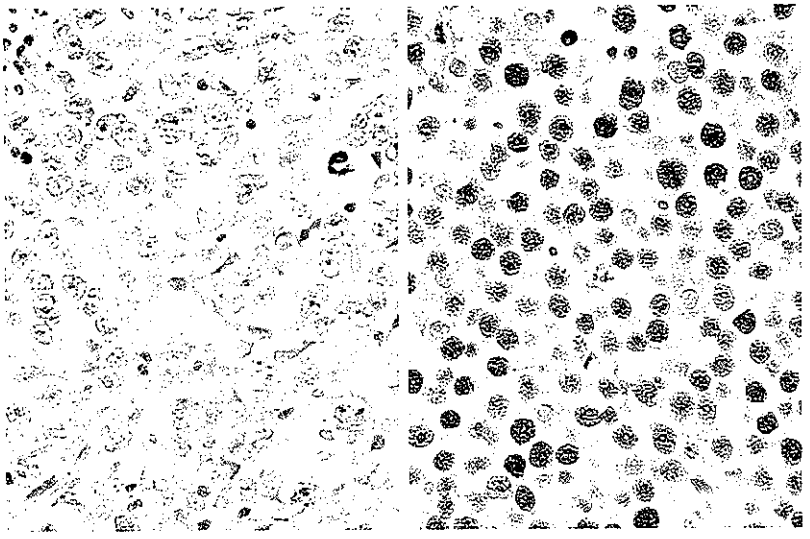
Chapter 3 : Figure 1 (Page 36)



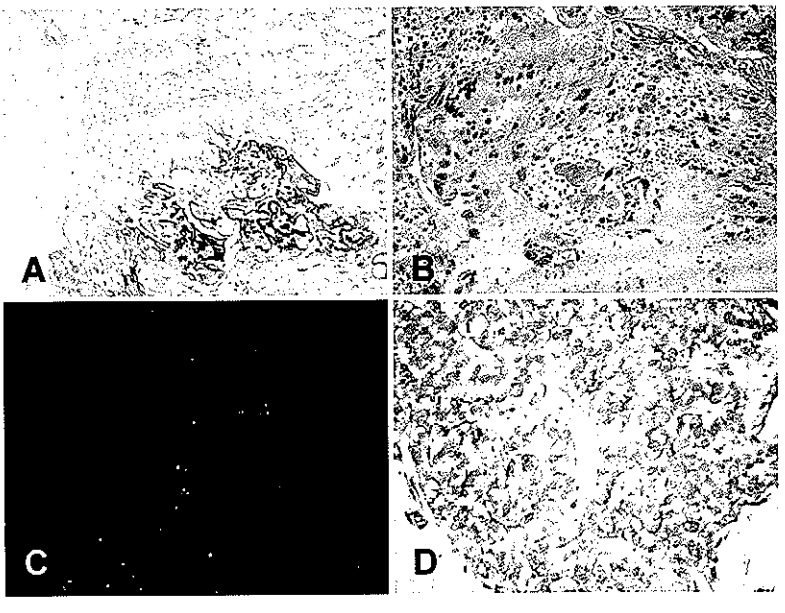
Chapter 4: Figure 1 (Page 45)



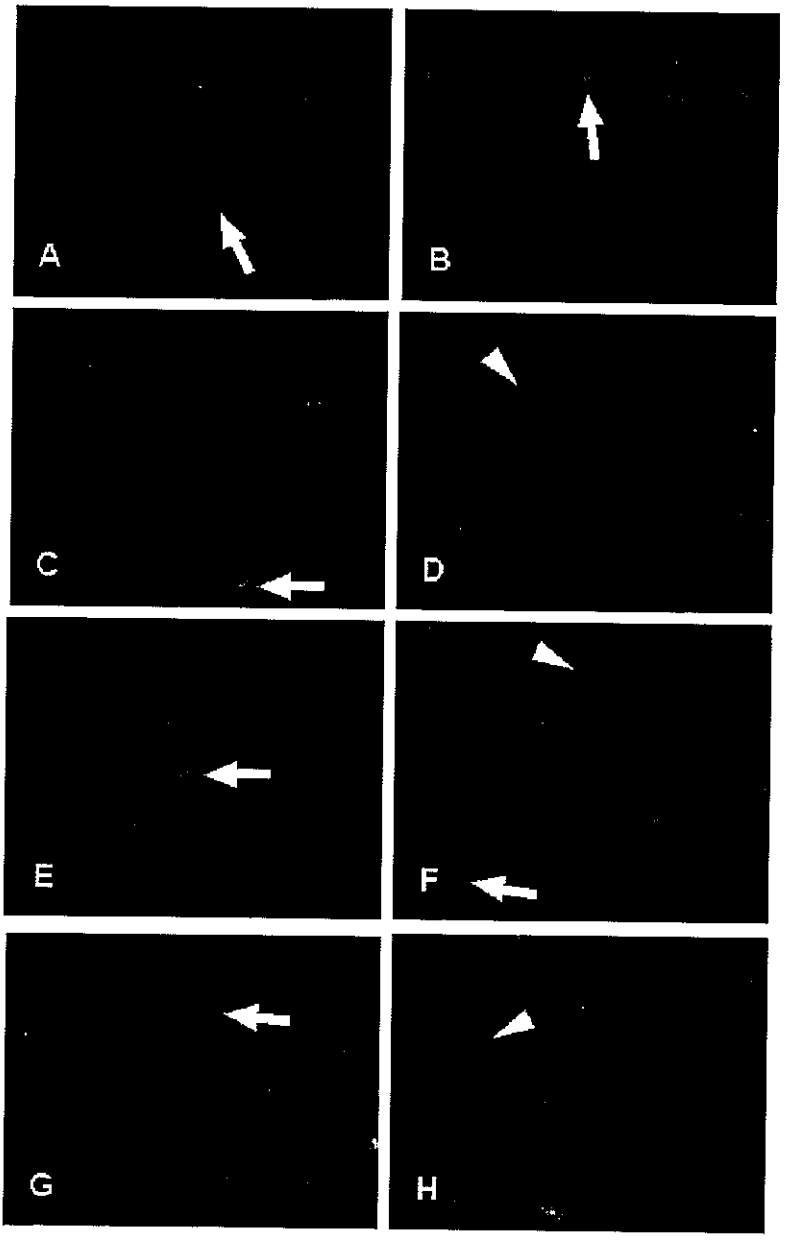
Chapter 5: Figure 1C (Page 55)



Chapter 6: Figure (Page 62)



Chapter 7 : Figure 1 (Page 72)



1. Detectable levels of P53 in part of germ cell tumor samples represent a physiological response of the tumor cells to cellular stress rather than intrinsically high levels of the P53-protein. (this thesis)
2. The increased incidence of microsatellite instability in tumors from chemotherapy refractory germ cell tumor patients compared to unselected patients indicates, that the mismatch-repair system is involved in cisplatin-induced cell death in germ cell tumors. (this thesis)
3. Resembling the behavior of normal adult somatic tissue, the intrinsic chemotherapy resistance of mature teratomas is generated at multiple cellular levels including drug export and cell cycle control and cannot be attributed to a single resistance mechanism. (this thesis)
4. Receptor tyrosin kinases of the epidermal growth factor receptor family do not offer a promising therapeutic target in clinically refractory germ cell tumors in general. (this thesis)
5. Invasive germ cell tumors do not undergo G1/S-phase cell cycle arrest in response to P53 and are thus deprived of time needed for DNA-repair after cisplatin exposure. (this thesis)
6. β-catenin cooperates with K-Ras in upregulating the level of cyclooxygenase 2 in colorectal cancer (Araki et al., Cancer Research 2003; 63: 728-734)
7. The mismatch-repair factor PMS2 can initiate a cell death program in response to cisplatin via a P73-related pathway. (Shimodaira et al., Proceedings of the National Academy of Science USA 2003; 100: 2420-2425)
8. Specific cytotoxic T lymphocyte responses against multiple myeloma cell lines can be induced in vitro using myeloma RNA transduced dendritic cells. (Milazzo et al., Blood 2003; 101: 977-982)
9. Patients suffering from locally advanced colorectal cancer without lymph node involvement do not benefit from adjuvant chemotherapy following adequate local treatment. (IMPACT B2 Investigators, Journal of Clinical Oncology 1999; 17:1356-1363)
10. Optimal pain relief in patients suffering from endstage cancer is an effective treatment against the underlying disease, as it improves the patient's quality of life and prolongs survival. (Smith et al., Journal of Clinical Oncology 2002; 20: 4040-4049)
11. Medical doctors – when left alone in the lab – are unable to design experiments adequate to give answers to the questions, which biologists – when left alone - do not even ask.