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Role of apoptotic pathways in chemosensitivity of testicular germ cell tumour

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2009

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Di Pietro, A. (2009). *Role of apoptotic pathways in chemosensitivity of testicular germ cell tumour*. [S.n.].

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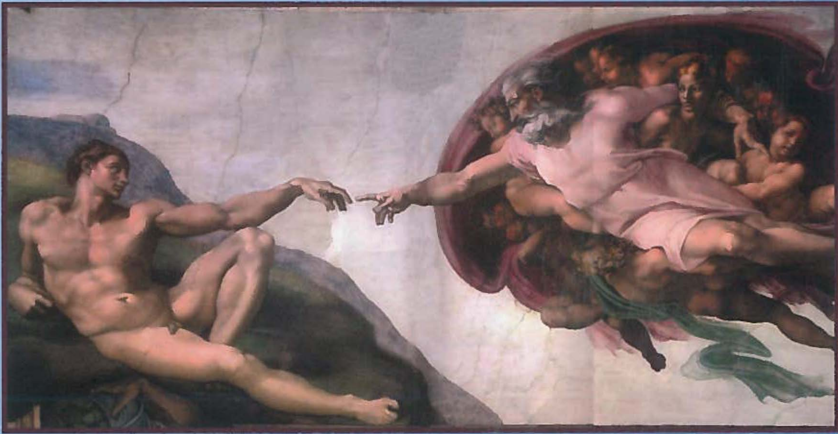
Role of Apoptotic Pathways in Chemosensitivity of Testicular Germ Cell Tumours



ALESSANDRA DI PIETRO

cmo.

RIJKSUNIVERSITEIT GRONINGEN

Role of Apoptotic Pathways in Chemosensitivity of Testicular Germ Cell Tumours

Proefschrift

ter verkrijging van het doctoraat
in de Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de Rector Magnificus, dr. F. Zwarts,
in het openbaar te verdedigen op
Maandag 16 November 2009 16,15 uur

door

ALESSANDRA DI PIETRO

geboren op 1 April 1978
te Rome, Italië

Centrale Medische Bibliotheek Groningen
UMCG

Stellingen aan de proefschrift

1. The association between high level of wild-type p53 expression and loss of $p21^{Waf1/Cip1}$ expression suggests a difference in functionality of p53 in testicular germ cell tumours compared to other tumour types.

(Dit proefschrift)

2. p53 is functional in testicular germ cell tumor cell lines, but its involvement in the response to cisplatin, i.e. its pro-apoptotic or anti-apoptotic effect, is cell context dependent.

(Dit proefschrift)

3. Cytoplasmic but not nuclear localized p21 protects testicular germ cell tumor cells against cisplatin- and irradiation- induced apoptosis.

(Dit proefschrift)

4. Phosphorylation of p21 by p-Akt is essential for p21 localisation in the cytoplasm.

(Dit proefschrift)

5. The expression of positive staining of p16 but not of $p21^{Waf1/Cip1}$ in embryonal carcinoma and yolk sac components of testicular germ cell tumors suggests mutually exclusive expression, which may be an important factor in the responsiveness of embryonal carcinoma to chemotherapy.

(Dit proefschrift)

6. Suppression of p16 by siRNA had no functional consequence on DNA damage-induced apoptosis in an embryonal carcinoma cell model.

(Dit proefschrift)

5. Preclinical data indicate that recombinant human TRAIL deserves exploration as anticancer agent in patients with metastatic triple-negative breast cancer.

(Rahman et al, Breast Cancer Res Treat 2009)

6. The VEGF-VEGFR pathway is of interest to be targeted in triple negative breast cancer, which harbours the amplification of the VEGF coding region on 6p21-p25 as well as high VEGF protein expression.

(Linderholm et al, Ann Onc 2009)

7. Nilotinib, an oral tyrosin kinase inhibitor, should be investigated in a clinical trial for patients with advanced melanoma harbouring a KIT mutation or amplification.

(Woodman SE et al. Mol Cancer Ther 2009)

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8. Tissue Inhibitors of Metallo Proteinase 2 Expression levels and blockage of NF-κB pathway activity should be explored in melanoma since over-expression of Tissue Inhibitors of Metallo-Proteinase 2 protects melanoma cell from apoptosis, by decreasing basal levels of IκBα, increasing phosphorylation of IκBα and NF-κB and increasing NFκB transcriptional activity.

(Sun & Stetler-Stevenson, J Molec Signal 2009)

9. *Colour is a mean to directly influence the soul.*

(Vasily Kandinsky)

10. *Noli foras ire, in te ipsum redi: in interiore homine habitat veritas.* Werkelijkheid en waarheid zijn in ons zelf te vinden, als wij goed kunnen leren kijken.

(St. Augustin, De vera religione, XXXIX, 72)

11. *Est autem fides sperandarum substantia rerum, argumentum non apparentium. Geloven is niet te beschrijven of logisch uit te leggen: het is maar de binnenkant van wat wij hopen en de buitenkant van wat wij niet kunnen zien.*

(St. Tommaso d'Aquino, Summa Theologiae, II-II)

12. *The old form of Argentine Tango called Orillera was based on a very low set on the legs of both partners as they could not be bothered by stones and mud along the river, where they used to dance.*

(Bruno Romero, History of Tango 2009)

13 Het leven kan heel anders worden door een komma: dit begrip was al bij de Romeinen bekend. Ibis et redibis (,) non (,) morieris in bello- Cumeaen Sybil.

(Virgilio, Eneide, VI 45-46)

Promotores:	**Prof. dr. E.G.E. de Vries**
	Prof.dr. J.A. Gietema
Copromotor:	**Dr. S. de Jong**
Beoordelingscommissie:	**Prof. dr. E. Vellenga**
	Prof. dr. R.M.W. Hofstra
	Prof. dr. A.J. Moshage

Printing of this thesis was
financially supported by 'Stichting
Werkgroep Interne Oncologie'
Groningen

A Mamily e Papily,
luce serena
nei miei giorni bui;

a Paky e Cristiana,
stelle fulgide
nelle notti tempestose;

alle Nonne,
richiamo costante
alla saggezza antica

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Chapter 1

Introduction

Testicular Germ Cell Tumours (TGCTs) are a heterogeneous group of neoplasms, which mainly affect young men, between 20 and 40 years of age. The incidence of TGCTs is increasing worldwide [1]. In the Netherlands, the incidence is raised from 4.1 per 100 000 person-years in 1989, to 6 per 100 000 in 2003.

The real breakthrough in systemic TGCTs treatment has been the introduction of the heavy metal platinum compound, cisplatin (PVB combination - **C**isplatin, **V**inblastine and **B**leomycin) in the mid seventies. Several years later, etoposide was found to have an antitumour activity against germ cell tumours and to exhibit a more favourable toxicity profile compared to vinblastine. Four courses of BEP (**B**leomycin, **E**toposide and **C**isplatin) resulted in a higher survival rates than four courses of PVB. This made the BEP the standard treatment for metastatic testicular cancer [2, 3]. Depending on the stage of the disease classified by the International Germ Cell Consensus Conference Group (IGCCCG) prognosis group, TGCT patients now achieve with the combination surgery and chemotherapy, cure rates ranging from 90% to 80% and 50% in respectively advanced disease with 'good', 'intermediate' and 'poor' prognostic criteria [4, 5].

This has raised interest in the role of cisplatin in TGCTs response to chemotherapy and in the mechanisms under-laying this unique drug sensitivity for a solid tumour. Despite TGCTs high chance of cure after cisplatin-based chemotherapy, still about 20-30% of all the patients diagnosed with metastatic testicular cancer will be intrinsic or acquired resistant to cisplatin-based chemotherapy and about 10% of all patients will eventually die from this disease. These clinical observations make this tumour type an interesting paradigm for studying mechanisms of cisplatin sensitivity and resistance in human solid malignancies.

In vitro models have shown that cisplatin reacts with the N7 position of G and A residues in DNA, to form a variety of monofunctional and bifunctional adducts causing inter- and intra-strand lesions. When not repaired, these DNA lesions will eventually result in the onset of the apoptosis cascade in cisplatin-damaged cells. Further *in vitro* studies have elucidated that cisplatin resistance in TGCTs cell lines can be either due to too low intracellular levels of cisplatin as a consequence of reduction in uptake, increased drug export

or intracellular detoxification and\or to an enhanced efficiency of DNA repair systems, which slowly remove cisplatin from genomic DNA [6]. Furthermore, a number of cellular proteins (p53, p21 waf[1]/cip[1]) did emerge as important factors for cisplatin response.

The tumour suppressor gene p53 seems to play a crucial role in determining TGCTs sensitivity to cisplatin. P53 plays a dual role in stress response as it regulates, as a transcriptional activator, a number of genes that co-ordinately force cells into either cell cycle arrest (via p21) or apoptosis (via extrinsic membrane pathway or via mitochondria). This depends on the possibility to repair the DNA damage or not. In contrast to other solid tumours, in which p53 is the most frequently mutated gene [7-9], p53 mutations are hardly found in TGCTs [10]. The fact that most TGCTs show a wild type (wt) p53 over-expression is regarded as the biological explanation for their chemo-sensitivity [11, 12], at least in mice models [13]. One of the most important cellular functions of wt-p53 concerns the preservation of DNA integrity during the cell cycle. Despite the increasing knowledge about the role of the p53 protein in cell growth and division, the precise effect of wt-p53 and mt-p53 on drug sensitivity of human tumours is still not precisely defined [14, 15].

Apoptosis, also known as programmed cell death, is an active cellular process, characterised by biochemical and morphological changes (Fig.1). Apoptosis is involved in normal tissue development and homeostasis [16]. A central component of apoptotic machinery is a proteolytic system that involves caspases, a family of proteases which eventually leads to the cleavage of a downstream set of proteins, resulting in the overall disassembly of the cell (Fig.1). In normal testis apoptosis is present as a selection mechanism for those cells, which will not achieve a complete maturation during normal spermatogenesis. In addition human TGCT cell lines are extremely sensitive to cisplatin resulting in massive apoptosis (Fig.1). Taken together these data suggest a pivotal role of apoptosis in the intrinsic sensitivity of TGCTs to treatment by cisplatin-based treatments. In this view, disturbances at any level in the apoptotic pathways might represent an important category of resistance in TGCTs to cytotoxic treatment.

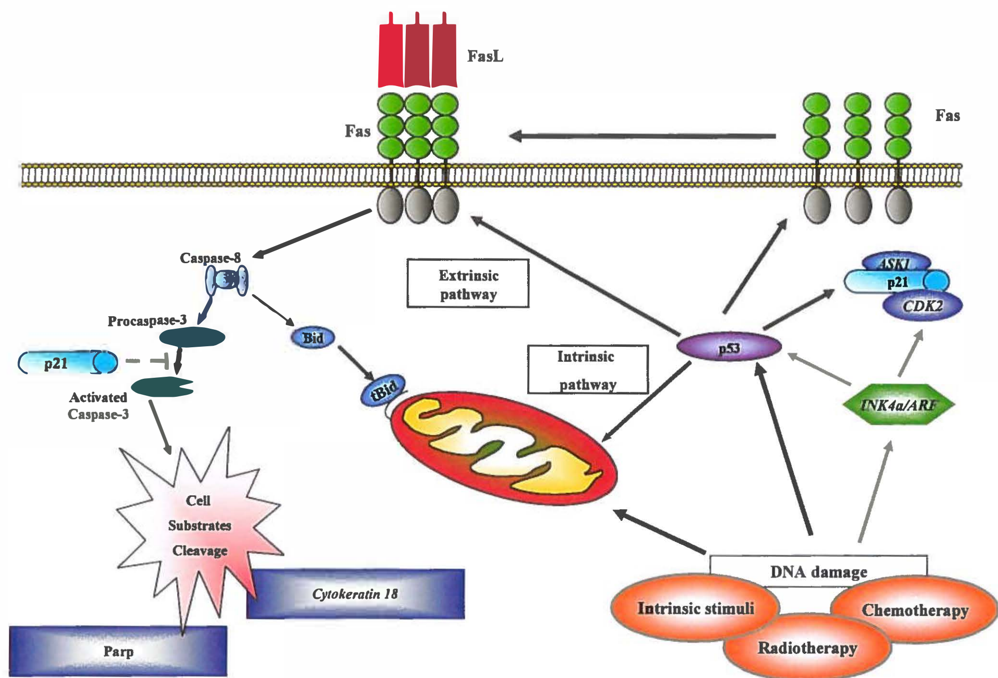

Figure 1

Apoptosis is an active cellular process, characterised by biochemical and morphological changes. Activated by several intrinsic and extrinsic stimuli causing DNA damage, p53 leads to the activation of caspases. This proteolytic enzymes family is responsible for cell substrates cleavage. Activated caspase 3 cleaves cellular proteins, among these PARP and cytokeratin 18. Black arrows indicate pathways, which are known to be functional in TGCTs. Grey arrows indicate general cell pathways, not yet investigated in TGCTs. In *italics* proteins not yet investigated in TGCTs are indicated.

The aim of the present thesis is dual; a) to investigate which molecular mechanisms are responsible for the unique sensitivity of TGCTs to cisplatin-based chemotherapy, and b) to study the presence and the applicability of apoptosis serum markers in a group of patients with advanced TGCTs.

Multiple cellular pathways may be responsible for sensitivity to cisplatin-based treatment in TGCTs. In **chapter 2** an overview of these pathways based on a review of the literature is presented, especially focussing on p53-dependent pathways, such as cell cycle arrest and apoptosis.

Several reports have been studying chemo-sensitivity of human TGCTs cell lines in relation to p53 expression, however with contrasting results. Therefore we performed a study presented in **chapter 3** to determine in detail the role of p53-dependent and p53-independent apoptotic pathways in cisplatin sensitivity and resistance in TGCT cell lines. Especially the role of p53 and p53 dependent-proteins, such as $p21^{Waf1/Cip1}$ (p21) and Mdm2, have been analysed. In a panel of cisplatin sensitive TGCT cell lines (833KE and Tera), a subline with cisplatin-acquired resistance (Tera-CP) and an intrinsic cisplatin resistant TGCT cell line (Scha), all expressing wt-p53, p53 was down-regulated by short interfering (si)RNA, in order to study whether and how p53 suppression affects apoptosis. Importance of p21 in Scha was further demonstrated by p21 siRNA, after cisplatin treatment.

Recent observations indicate that gamma-irradiation in contrast to cisplatin treatment strongly enhance cytoplasmic p21 expression in wt-p53 expressing TGCTs cells without inducing apoptosis. We aimed to better define the role of the p21 levels and above all of the cytoplasmatic localisation of p21 with regard to cisplatin sensitivity in **chapter 4**. We stably transduced Tera with a viral construct containing either p21-ΔNLS and GFP, or GFP only (Mock). P21 localisation was demonstrated by immune fluorescence microscopy. In Tera-p21-ΔNLS and Scha cell lines, we investigated the influence of down-regulation of p21 by siRNA on apoptosis after cisplatin treatment. Using immunoprecipitation we aimed to identify possible cytoplasmatic p21 targets, such as the pro-apoptotic Apoptosis Signalling Kinase 1 (ASK1) and cyclin dependent kinase 2 (CDK2). As cytoplasmic localisation of p21 in TGCT cells emerged as depending on the phosphorylation of the NLS-site (Thr145) of

p21, we inhibited p21 phosphorylation using the PI3K inhibitor LY294002 or the specific p-Akt inhibitor Triciribine, in order to investigate the role of p21 relocation towards the nucleus on apoptosis.

Besides p21, another important regulator of the G1/S transition is the CDK-I INK4 family member, p16. P16 might have a different action with respect to p21, although they both exert a control on the same phase of the cell cycle. P16 is not constantly expressed during the cell cycle, but its promoter is activated by DNA stressing events. In order to investigate whether the imbalance between p21 and p16 affects cisplatin sensitivity in TGCT the expression of p16 was inhibited by siRNA in two TGCT cell lines, the cisplatin resistant Scha and the cisplatin sensitive 833KE cell line. Scha and 833KE cell lines were treated with cisplatin or γ-radiation and changes in expression of p21 and p16 were monitored. The role of p16 was further investigated by p16 suppression with siRNA technique. In addition, in order to analyse whether the p21 and p16 imbalance is actually present *in vivo*, expression of these proteins have been studied in human (**chapter 5**). Primary tumour histological samples from 34 patients belonging to different IGCCCG prognosis group and affected from different histological tumour subtype with immunohistochemistry.

In **chapter 6** we studied whether changes in apoptosis markers as well as patterns of changes during cisplatin-combination chemotherapy treatment were associated with specific disease outcomes in advanced TGCT patients treated with BEP-chemotherapy. In particular, cytokeratin 18, a protein of the cellular cytoskeleton, is a target of activated caspase-3 during apoptosis. The neo-epitope produced by activated caspase 3 cleavage can be detected by a specific antibody, M30. The blood samples before and during chemotherapy were analysed by ELISA for the presence of cytokeratin 18 and its apoptotic neoepitope in 34 patients with advanced TGCTs.

A summary of this thesis with indication of future perspectives both in English and in Dutch is presented in **chapters 7** and **8,** respectively.

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Chapter 2

Testicular Germ Cell Tumours: The paradigm of chemo-sensitive solid tumours

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Int J Biochem Cell Biol. 2005;37(12):2437-56

Abstract

Testicular germ cell tumours (TGCTs) are the most frequent solid malignant tumour in men 20-40 years of age and the most frequent cause of death from solid tumours in this age group. Up to 50% of the patients suffer from metastatic disease at diagnosis. The majority of metastatic testicular cancer patients, in contrast to most other metastatic solid tumours, can be cured with highly effective cisplatin-based chemotherapy. From a genetic point of view, almost all TGCTs in contrast to solid tumours are characterised by the presence of wild type p53. High p53 expression levels are associated with elevated Mdm2 levels and a loss of $p21^{Waf1/Cip1}$ expression suggesting a changed functionality of p53. Expression levels of other proteins involved in the regulation of cell cycle progression indicate a deregulated G1–S phase checkpoint in TGCTs. After cisplatin-induced DNA damage, the increasing levels of p53 lead to the trans-activation of a number of genes but not of $p21^{Waf1/Cip1}$, preferentially directing TGCT cells into apoptosis or programmed cell death, both via the mitochondrial and the death receptor apoptosis pathway. The sensitivity of TGCTs to chemotherapeutic drugs may lay in the susceptibility of germ cells to apoptosis. Taken together, this provides TGCT as a tumour type model to investigate and understand the molecular determinants of chemotherapy sensitivity of solid tumours. This review aims to summarise the current knowledge on the biological basis of cisplatin-induced apoptosis and response to chemotherapy in TGCTs.

Introduction

Germ Cells Tumours (CGTs) are the most frequent solid malignant tumour in men 20 to 40 years of age, accounting for up to 60 % of all malignancies diagnosed at this age [Pottern, 1998], with a still increasing incidence of 6 to 7 per 100.000 men. Despite a high cure rate, they represent the most frequent cause of death from solid tumours in this age group. The most important risk factors identified for GCT development are cryptorchidism, a personal or a family history of GCT, Klinefelter's syndrome or spermatic and testicular dysgenesis [United Kingdom Testicular Cancer Study Group, 1994]. Genetic aberrations of the SRY gene, responsible of testis development from the primordial gonad, seem to be the major cause for testicular GCTs (TGCTs) in these syndromes [Chemes et al., 2003].

A study on genealogical trees of affected patients has suggested the idea of a low penetrante model of inheritance [Heimdal et al., 1997;Holzik et al., 2004]. So far, the linkage analysis study in positive families has shown only a linkage with a number of chromosome regions, among which Xq27, 3p, 12q, 16p, 18q [Holzik et al., 2004;The International Testicular Cancer Linkage Consortium, 1998;Hemminki and Li, 2004]. GCTs are a heterogeneous group of neoplasms derived from germ cell lineage [Skakkebaek et al., 1987;Weissbach and Bussar-Maatz, 1996], which usually occur within the gonads. Only 5% of GCTs develop at extragonadal locations along the midline of the body [Heimdal et al., 1997;The International Testicular Cancer Linkage Consortium, 1998]. Based on histological and biochemical characteristics, GCTs are divided into seminomas and non-seminomas [Ulbright, 2005]. Seminomas are composed of uniform cells, resembling primordial germ cells/gonocytes [Jorgensen et al., 1995]. Non-seminomas contain one or more histological subtypes, representing various differentiation lineages and stages of embryonic development. Embryonal carcinoma cells form the stem cell component and are able to differentiate towards extra-embryonic tissues (yolk-sac carcinoma and choriocarcinoma) or embryonic tissues (immature and mature teratocarcinoma) [Looijenga and Oosterhuis, 1999;Chaganti and Houldsworth, 2000].

The rapid growth and progression of GCTs cause early lymph node metastases and/or distant metastases. At the time of diagnosis about 25% of seminoma patients [Porcaro et al., 2002] and up to 60 % of the non-seminoma patients suffers from metastatic disease [Peckham, 1988;Perrotti et al., 2004;Al Ghamdi and Jewett, 2005]. Because of highly effective

combinations of chemotherapeutic drugs, metastatic testicular cancer, in contrast to most other metastatic solid tumours, can be cured with chemotherapy. Seminomas are radio- and chemo-sensitive tumours, virtually completely curable at each stage [Classen et al., 2001]. Non-seminomatous tumours are usually treated with surgery and chemotherapy, with different cure rates depending on the disease stage [Classen et al., 2001;Shelley et al., 2002]. The cure rate reaches up to 99% in the early stages of non-seminomatous tumours. In advanced disease, however, it decreases to 90% in patients with good prognostic criteria, to 75%-80% in patients with intermediate and to 50% in patients with poor prognostic criteria, evaluated according to the IGCCG classification [Schmoll et al., 2004;Shelley et al., 2002]. Thus, despite the general success of TGCTs treatment, 10-20% of patients diagnosed with metastatic disease will not achieve a durable complete remission after initial treatment, either due to incomplete response or a tumour relapse [Jones and Vasey, 2003a;Oosterhof and Verlind, 2004;Gori et al., 2005]. The growing teratoma syndrome is a rare metastatic complication of GCTs, defined as a detection of an enlarged mass during or after chemotherapy treatment [Amsalem et al., 2004]. Mature teratomas are histologically non-malignant tumours that remain present in 1.9 to 7.6 % of residual lesions after chemotherapeutic treatment of non-seminomatous GCTs [Jeffery et al., 1991;Amsalem et al., 2004]. Despite the same genetically constitution, they do not share the general chemo-sensitivity of GCTs.

Three DNA damaging agents, cisplatin, etoposide and bleomycin, are the main drugs responsible for successful outcome in testis cancer treatment [Jones and Vasey, 2003b]. Consistent with clinical results, most human TGCT cell lines show an extraordinary sensitivity to treatment with cisplatin [Huddart et al., 1995;Chresta et al., 1996;Johnstone et al., 2002;Masters and Koberle, 2003]. Understanding the molecular determinants of chemotherapy efficacy in testicular cancer treatment may provide novel strategies to increase the sensitivity of other metastatic tumours as well as to improve the therapeutic outcome of TGCTs with poor prognosis. Nowadays it is well accepted that the susceptibility of germ cells to programmed cell death, named apoptosis, plays a pivotal role in the intrinsic sensitivity of TGCTs to treatment by chemotherapeutic drugs [Spierings et al., 2003a]. This review therefore will focus on the current knowledge on the biological basis of apoptosis

induction in normal testis and treatment response in GCTs, in particular on apoptosis induced by cisplatin, the most important drug in the systemic treatment of GCTs.

Apoptosis in normal testis

Spermatogenesis is a dynamic process of maturation of stem spermatogonia into mature spermatozoa, continuously occurring during the reproductive lifetime of the individual [Russel, 1990]. As not all germ cells starting the maturation process will eventually achieve maturity, apoptosis is a constant feature of normal spermatogenesis in a variety of mammalian species [Schmelz et al., 2005;Liu, 2005;Sinha Hikim et al., 2003;Koji, 2001;Sinha Hikim and Swerdloff, 1999]. Both spontaneous and increased cell death due to triggering stimuli (deprivation of gonadotropins and intratesticular testosterone, local heating, Sertoli cell toxicants, chemotherapeutic drugs) occurs via apoptosis (for a review on apoptosis see [Zimmermann et al., 2001] (Fig 1). Most types of spermatogonia are sensitive to DNA damaging agents such as cisplatin and irradiation [Beumer et al., 1997;Zhang et al., 2001]. Murine models showed that hormonal deprivation and physical factors induce different stage-specific activation of apoptosis [Hikim et al., 2003], both involving the death receptor (so called extrinsic) and mitochondrial (so called intrinsic) apoptotic pathway.

Engagement of the Tumor Necrosis Factor family member Fas ligand (FasL) to the Fas death receptor triggers apoptosis, both via extrinsic and intrinsic pathways. In normal testis, the Fas-FasL system maintains the immune-privileged nature of testis barrier [Bellgrau et al., 1995]. In addition, the Fas-FasL system regulates the degree of germ cell apoptosis, either by a paracrine loop between FasL positive Sertoli cells and Fas positive germ cells or an autocrine loop of Fas-FasL positive germ cells [Richburg, 2000;Spierings et al., 2003a;Lee et al., 1997;Lee et al., 1999;Pentikainen et al., 1999;Francavilla et al., 2002;Yin et al., 2002]. Several reports show an association between up-regulation of Fas expression and germ cell apoptosis caused by different damaging agents (ionising radiation, ischemia/reperfusion, chemical toxicants, cryptorchidism) [Lee et al., 1997;Lee et al., 1999;Yin et al., 2002;Richburg et al., 2000]. Moreover *in vivo* and *in vitro* experiments reveal that blocking FasL expression or FasL activity increases germ cell survival. I*n vitro* cultured germ cells are susceptible to apoptosis induced by an anti-Fas agonistic monoclonal antibody. In normal testis, Bcl-2 family members are essential for male germ cell homeostasis [Print and Loveland, 2000;Spierings et al., 2003a;Rodriguez et al., 1997;Beumer et al., 1997].

The sensitivity of normal testis cells to apoptosis stimuli suggests that the unique responsiveness of TGCT to cisplatin-based chemotherapy may be implied in inherited biological characteristics of TGCT cells.

TGCTs genome

TGCTs originate from a precursor lesion, carcinoma *in situ* (CIS), in which a series of genomic, genetic and epigenetic alterations lead to progression towards seminoma [Honecker et al., 2004;Rajpert-De Meyts et al., 2003]. Seminoma can further develop into a non-seminomatous tumour [Oosterhuis et al., 1989;Honorio et al., 2003]. A carcinoma in situ originates from a diploid primordial germ cell during foetal life [Skakkebaek et al., 1987;Dieckmann and Skakkebaek, 1999]. Later on, the aneuploidic events and the extensive chromosome instability lead to the further transformation into invasive TGCT, of both seminomatous and non-seminomatous type (Fig.2). Albeit the same regional genomic disruptions are observed, seminomas and non-seminomas show not only morphological differences, but also different epigenomes and consequently different gene expression profiles [Honorio et al., 2003;Liao et al., 2004]. Seminomas, though resembling CIS cells, are not confined to the tubules, as they are continuously proliferating. Non-seminomas develop through pluripotent embryonal carcinoma stage, which may further differentiate into embryonic and extra-embryonic tissues at different stages of differentiation [Jewett, 1977;Ulbright, 2005]. The analysis of familiar clustering and the frequency of bilateral disease has drawn the attention on several genomic regions (3q2-ter; 5q14-22; 12q24.3 and 18q12) [von Eyben, 2004;The International Testicular Cancer Linkage Consortium, 1998]. Matched analysis of hereditary and sporadic groups of patients has, however, failed in showing any difference between the two groups [Skotheim and Lothe, 2003], thus suggesting a common disrupted molecular pathway in both types of cancer [Skotheim et al., 2001]. The main genetic characteristic observed in more than 80% of the TGCTs is the presence of isochromosome 12 (i12p) [Bosl et al., 1989;Skotheim and Lothe, 2003]. In addition, TGCTs lacking i12p harbour amplifications of 12p genetic material. The gain of 12p seems to be associated with the invasive phenotype of TGCT [Rodriguez et al., 1992;Suijkerbuijk et al., 1993]. Loss of heterozygosity and allelic imbalance studies have identified the FHIT

(Fragile-Histidine Triad) gene at 3p14 locus, which provides a wide set of aberrant transcripts and reduced protein expression in TGCTs [Kraggerud et al., 2002]. In 2002, Mayer has demonstrated an association between microsatellite instability in human TGCTs and resistance to cytotoxic systemic treatment (see below) [Mayer et al., 2002]. Furthermore, the pattern of genomic gains/losses is not random, as genes located in particular region are more prone to genomic changes. For instance, c-myc over-expression, frequently observed in TGCTs, induces chromosomal and extra-chromosomal instability of *ccnd2* at 12p13 [Mai et al., 1999]. The *ccnd2* gene encodes cyclin D2, which is one of the main activators of cell cycle G1-S transition in testicular germ cells [Bartkova et al., 1999].

Although K-*ras* and N-*ras* genes, located at 12p, are mutated in only 11% of TGCTs [Olie et al., 1995], their mutations seem to be important in germ cell carcinogenesis [Roelofs et al., 2000;Oosterhuis, 1997], but not for their therapy response[Skotheim and Lothe, 2003]. Ras-effector pathways involve also Platelet Derived Growth Factor Receptor α (PDGFRα), c-Kit as well as cyclin-D2, all expressed at high levels in TGCTs [Palumbo et al., 2002;Rapley et al., 2004]. The c-kit/stem cell factor system is an important pathway *in vivo* for the appropriate migration and survival of primordial germ cells [Kemmer et al., 2004;Shamblott et al., 1998]. In addition, *in vitro* studies showed an important role for c-Kit in the survival of mouse and human primordial germ cells and human seminomas[Olie et al., 1996;Shamblott et al., 1998]. c-Kit is a subclass III tyrosine kinase receptor. Binding its ligand, the stem cell factor, c-kit leads to the receptor heterodimerization and tyrosine kinase activity. The downstream signal proceeding from this activation involves both apoptosis and cell cycle progression regulation [Heinrich et al., 2002]. The c-kit activating mutations, previously described in only one TGCT [Tian et al., 1999], have recently been found in 93% of bilateral TGCTs but only in 1.3% of unilateral TGCTs [Looijenga et al., 2003]. All these mutations affect codon 816 of exon 17 of *c-kit* gene resulting in a constitutional kinase active protein by the phosphorylation of tyrosine residues [Looijenga et al., 2003]. The association of these mutations with the development of bilateral disease suggests that, although somatic in origin, they occur during or before the migration of primordial germ cells to the genital ridge [Looijenga et al., 2003]. In TGCTs active c-kit due to a mutation in exon 17, instead of exons 9 or 11, characteristic for gastrointestinal stromal tumours [Corless et al., 2004], is not

inhibited by the tyrosine kinase inhibitor STI571, imatinib mesylate (Gleevec®) [Madani et al., 2003].

So far the spectrum of TGCTs genetic characteristics is broad. Except for i12p, it thus remains difficult to define other key points of the TGCT genetic background.

Mechanisms of response to cisplatin in TGCTs

Although cisplatin is one of the most effective chemotherapeutic drugs in the treatment of cancer, the precise mechanism of its cytoxicity is still not known. Cisplatin causes DNA inter-strand and intra-strand cross-links, which, when not repaired, may eventually lead to apoptosis. The comparison between cisplatin-sensitive TGCT cell lines and cisplatin-resistant TGCT cell lines has revealed a number of mechanisms which might explain cisplatin sensitivity and resistance of TGCTs [reviewed in [Spierings et al., 2003a]. Resistance to cisplatin in TGCTs can be due to the unsuccessful achievement of adequate intracellular levels of cisplatin. Mechanisms involved are a reduction in drug uptake, an increase in drug export or an increase in intracellular detoxification, as has been demonstrated in several human TGCT cell lines among which SuSa, 833K, GCT27, GH and 1618H [Gosland et al., 1996;Masters et al., 1996]. Resistance can also be caused by an enhanced DNA repairing capacity, among which Nucleotide Excision Repair (NER) system seems to be the most important against cisplatin-induced DNA damage [Wang et al., 2004] in NSCLC [Rosell et al., 2003] and in some ovarian carcinoma cell lines [Selvakumaran et al., 2003]. In general, however, cisplatin is slowly removed from genomic DNA as was found in the NT2/D1, 2102EP, NCCIT, 833K, GCT27, GH, SuSa, 1618K and Tera-1 human TGCT cell lines, indicating a low capacity of the NER repair system. The intrinsic low ability of NER repair system in TGCT cells seems to be due to low levels of Xeroderma Pigmentosum group A (XPA) [Koberle et al., 1996;Koberle et al., 1997;Honecker et al., 2003;Welsh et al., 2004]. Alternatively, it can be due to testis–specific high mobility group (HMG)-box proteins, which can hide DNA adducts and prevent damage detection by NER factors as was found in F9 and nulliSCC1 murine testicular teratocarcinoma cell lines [Zamble et al., 2002]. In addition to the NER system, the Base Excision Repair system, which eliminates small base alterations without distorting the DNA helix, seems to be important for the repair of

irradiation or bleomycin-induced DNA damage in 833K and NT2/D1 cells [Evans et al., 2000;Robertson et al., 2001].

Recently, the Fanconi Anemia-BRCA pathway has drawn much attention, regarding cisplatin sensitivity [Waisfisz et al., 2002;Ferrer et al., 2003;Taniguchi et al., 2003]. The FANC-BRCA proteins complex is involved in repairing double stranded breaks, classically caused by γ-radiation and by DNA-cross linking agents, including cisplatin [Wong et al., 2003]. The FANC-BRCA pathway impairment in ovarian cancer is related to cisplatin response [Taniguchi et al., 2003]. Koul *et al.* investigated the involvement of the FANC-BRCA pathway in the in-vitro development of cisplatin resistance in human TGCT cell lines. In 833K and 240A, low levels of FANCF mRNA expression were detected. Cisplatin resistant subclones derived from both cell lines even showed a decreased FANCF expression suggesting a lack of involvement of FANCF in cisplatin resistance [Koul et al., 2004].

In addition to their repair capacity, some DNA-repair pathways are also capable of initiating apoptotic cascade [Bernstein et al., 2002]. Losses or defects in the mismatch repair system (MMR), measured by microsatellite instability, can confer resistance to cisplatin and alkylating drugs. In several studies, GCTs have been found to be microsatellite stable [Lothe et al., 1995;Devouassoux-Shisheboran et al., 2001]. These findings have been confirmed in a series of 100 unselected GCTs, among which 6% showed microsatellite instability in at most one out of eight investigated loci [Mayer et al., 2002]. This suggests that the MMR system is functional in most GCTs.

Resistance to cisplatin can be due to inactivation at genetic and protein level of the tumor suppressor p53, a trans-activator and a cellular gate-keeper for cell growth and division [Lane, 1994]. In contrast to most solid tumours, however, *p53* mutations are hardly found in TGCTs. The presence of wild-type p53 over-expression in TGCTs has been regarded as the biological explanation for their chemo-sensitivity [Lowe et al., 1993]. A p53 dose-response relationship to DNA damage was demonstrated in an isogenic P19 murine teratomacarcinoma cell line model [Lutzker et al., 2001]. Still, the effect of wild-type p53 and mutant p53 on drug sensitivity of human TGCTs cell lines is not clear [Chresta et al., 1996;Burger et al., 1999a;Burger et al., 1999b;Schweyer et al., 2004]. In a panel of six non-isogenic human TGCT cell lines, no relation between wild-type p53 and cisplatin-induced

apoptosis was observed. The wild-type p53 expressing cell lines NT2/D1, 833K, and TERA-1, the p53 protein negative cell line S2, and the mutant p53 expressing cell line NCCIT were sensitive to cisplatin-induced apoptosis, while the wild-type p53 expressing cell line 2102EP appeared to be resistant [Burger et al., 1999b;Burger et al., 1997].

Mueller *et al.* demonstrated a decreased susceptibility for apoptosis in human TGCT cell lines in response to cisplatin-mediated damage using the intrinsic cisplatin-resistant 141HP cell line compared to the cisplatin-sensitive 2102EP and H12.1 cell lines [Mueller et al., 2003]. The higher threshold necessary to induce apoptosis in the resistant cell line explains the much higher amount of DNA-cisplatin adducts required to initiate apoptosis. Under this condition, the cisplatin-resistant 141HP cells failed in activating caspase-9 as initial step of apoptosis, while caspase-2 and caspase-3 activation was still detected. In an isogenic model for acquired cisplatin resistance consisting of the cisplatin-sensitive cell line NT2/D1 (Tera) and its cisplatin-resistant subclone Tera-CP, we showed that the cisplatin sensitivity in human TGCT cells is dependent on the activation of the Fas apoptosis pathway [Spierings et al., 2003b]. Loss of cisplatin-induced activation of this Fas signalling pathway may result in resistance to cisplatin. At higher cisplatin doses the cisplatin-resistant subline became apoptotic in a caspase-9 dependent manner [Spierings et al., 2003b].

Although these results suggest that cisplatin-induced apoptosis can explain the cisplatin sensitivity of TGCTs, a deeper knowledge of the consequently disrupted signalling pathways is needed to understand the cellular mechanisms of sensitivity in TGCTs.

The p53 pathway

a. p53 circuitry

TP53 is a tumour suppressor gene, which protein has a dual role in stress response. It trans-activates a number of genes including p21$^{Waf1/Cip1}$ (p21), Mdm2, Bax, Fas and Apaf-1 [Janus et al., 1999] that co-ordinately direct cells into either cell cycle arrest or apoptosis. In turn, the p53 function is regulated by several mechanisms, acting not only at the transcription and translation level, but also influencing the stability of the p53 protein as well as its post-translational modifications and its sub-cellular localisation [O'Brate and Giannakakou, 2003]. Several studies have demonstrated that tumours with cytoplasmatic sequestration of p53 are less responsive to therapy (radiation and drug) induced damage [Swamy et al., 2003;Gariboldi et al., 2003]. Furthermore, cytoplasmic p53 accumulation is an independent negative prognostic factor in different types of cancer [Riou et al., 1993;Bosari et al., 1995;Schlamp et al., 1997;Lilling et al., 2002;Sembritzki et al., 2002].

DNA damage activates the ATM/ATR and DNA-PK family protein-kinases, which in turn lead to the post-translational stabilisation of the normally labile p53 protein [Giaccia and Kastan, 1998]. The elevated p53 levels mediate cell cycle arrest via an increased trans-activation of p21. The p21-mediated cell cycle block in G1 phase allows the DNA repair apparatus to repair damage. Afterwards the cell can re-enter the cell cycle. If the damage is not suitable for repair or the stressing stimuli exceed the homeostatic capacity in the cell, p53 activates the intrinsic apoptosis pathway. This can be caused by transcriptional activation of pro-apoptotic Bcl-2 family members and Apaf-1 or a direct p53 protein interaction with pro-apoptotic Bcl-2 family members [Yin et al., 1999;Moroni et al., 2001].

Together with p53, proteins of the Mdm2 family, Mdm2 and MdmX, serve as a major integrator of the signals generated by genotoxic and oncogenic stresses. The relation between p53 and Mdm2 is closely controlled by a complex process of post-translational modifications, which involves stability and activity of p53 and Mdm2 [Alarcon-Vargas and Ronai, 2002]. P53 activates *mdm2* transcription and Mdm2 in turn destabilises p53 by binding to the p53 protein as a negative feedback mechanism. Mdm2 can ubiquitinate itself or p53 in order to increase degradation of the p53 protein via the ubiquitin-proteasome pathway. In this loop the co-compartmentalisation of p53 and Mdm2 seems to be important

[Xirodimas et al., 2001;Gottifredi and Prives, 2001]. Mdm2 is a nuclear cytoplasmatic shuttling protein [Zhang and Xiong, 2001]. Mdm2 function strongly depends on the cellular environment, specifically on the expression levels of MdmX and p14/Arf [Stad et al., 2001]. Unlike Mdm2, MdmX does not target p53 for degradation, but rather suppresses p53 trans-activating function [Jackson and Berberich, 2000], by the sequestration of p53 inside the nucleolus [Jackson et al., 2001]. Furthermore, MdmX inhibits the nucleoplasmatic shuttling of Mdm2, when Mdm2 is bound to p14/Arf [Weber et al., 1999]. The p14/Arf protein directly binds and inactivates Mdm2 [Bothner et al., 2001;Pomerantz et al., 1998;Honda and Yasuda, 1999;Kamijo et al., 1998] without affecting Mdm2 self-ubiquitination [Xirodimas et al., 2001]. Under stressful conditions, p14/Arf accumulation in the nucleolus mobilises Mdm2 to this compartment [Weber et al., 1999;Sherr, 2001]. In this way, p14/Arf segregates Mdm2 from p53, which can thus trigger cell cycle arrest or apoptosis [Lowe and Sherr, 2003]. Furthermore it is noteworthy that the tumour suppression function of p14/Arf is dependent on the presence of wild-type p53.

b. p53 circuitry in TGCTs

To explain the lack of *p53* mutations in TGCTs, several reports indicate that p53 protein is functionally inactive in murine teratocarcinoma cells [Lutzker and Levine, 1996]. P53 becomes only activated after etoposide-induced DNA damaged, suggesting the importance of both covalent post-translational modifications, i.e. phosphorylation and acetylation of p53, and non-covalent interactions with proteins such as Mdm2. Other reports, however, suggest the presence of a transcriptionally active wild-type p53 in TGCTs, since wild-type p53 levels correlate with the *mdm2* gene over-expression [Riou et al., 1995;Datta et al., 2001]. The occurrence of p53 mutations in some human TGC carcinomas in situ and their related tumours as well as in a subgroup of cisplatin-resistant tumours supports the presence of functional p53. Surprisingly, most TGCTs lack p21 protein expression [Guillou et al., 1996;Bartkova et al., 2000;Datta et al., 2001]. Following etoposide treatment, p21 protein levels in the wild-type p53 expressing GH, GCT27 and 833K human TGCT cell lines were still very low as compared to p21 protein levels in wild-type p53 expressing human bladder cancer cell lines [Chresta et al., 1996]. Irradiation, however, resulted in a strong p21 mRNA induction in the wild-type p53 expressing NT2/D1 and 2102EP human TGCT cell lines. In contrast, no p21 induction was observed in the mutant p53 expressing NCCIT cell line but

also not in S2, which has no p53 protein despite the presence of wild-type p53 alleles [Burger et al., 1997]. Recently, we observed that p21 mRNA and protein expression levels in NT2/D1, Scha and 833K were massively elevated following irradiation but almost absent following cisplatin [Spierings et al., 2004]. Inactivation of p53 completely abolished irradiation-induced up-regulation of p21 in NT2/D1 [Burger et al., 1999a]. This suggests that p53 is a transcriptional activator of p21 in human TGCT cells depending on the type of DNA damage. Only a few studies have focussed on the role of Mdm2 in TGCTs [Kersemaekers et al., 2002;Eid et al., 1999;Riou et al., 1995;Fleischhacker et al., 1994;Richie, 2003]. No definitive results are available, as only three out of 65 TGCTs harboured *mdm2* gene amplification [Riou et al., 1995;Fleischhacker et al., 1994]. Inactivation of p53 by Mdm2 could not explain chemoresistance in TGCTs [Kersemaekers et al., 2002]. The inactivation of *p14/arf* locus, so far investigated only in intracranial GCTs [Iwato et al., 2000], and the Mdm2 or MdmX expression represent a new research field in TGCTs, as these proteins are involved in p53 fine regulation after DNA damaging stresses.

The complex DNA damage-induced p53 activation pathway and the feedback mechanisms controlling this pathway seem to play a crucial role in TGCTs chemotherapy response.

P53-family members and p53 co-activators

The recent discovery of two p53 homologues, *p63* (3q2. 7-9) (for a review see: [Mills et al., 1999;Hibi et al., 2000]and *p73* (1p3. 6), has given new insight and new enigmas in cancer understanding [Yang and McKeon, 2000]. A striking sequence similarity and the conservation of functional domains exist among the p53 members. Many cytotoxic drugs activate apoptosis through one or more of the p53 family members, thus suggesting that molecular genetic changes inhibiting signalling by one of these p53 family members can be a mechanism for acquired resistance to cytotoxic drugs [Fojo, 2002]. Unlike the *p53* gene, which encodes one major transcript, both *p63* and *p73* genes contain two separate promoters resulting in transcripts encoding two fundamentally different classes of protein [Yang et al., 1998;Kaghad et al., 1997]. The TAp63/p73 forms, marked by an acid N terminus with homology to p53 trans-activation domain, have two classic p53 activities: trans-activation of p53 target genes (p21, GADD45, Bax) and apoptosis induction capability [Pozniak et al.,

2000;Liefer et al., 2000]. The second promoter gives rise to N-terminally truncated proteins, which lack the TA domain (ΔN forms) and can act as dominant negative against p53 activity.

In normal testis TA-p63 is present and ΔNp63 absent [Dellavalle et al., 2001]. In different squamous cell carcinoma cell lines [Ratovitski et al., 2001] a physical and functional relation between wt-p53 and ΔNp63, mediated by the DNA binding domains of both p53 and p63 (p40 domain) has been recently described. So far, this aspect is not studied in TGCTs.

More is known about *p73*, which, together with c-*abl*, has a specific role in TGCTs response to radiation therapy [Hamer et al., 2001]. The ΔNp73 forms are negative inhibitors of the TA variants and of wild-type p53 [Petrenko et al., 2003;Zaika et al., 2002]. It is noteworthy that ΔNp73 isoforms are inducible by cisplatin [Gong et al., 1999] and doxorubicin [Costanzo et al., 2002] as well as by other clinically used drugs (camptothecin, melphalan, taxanes) [Bergamaschi et al., 2003;Irwin et al., 2003] but not by UV radiation [Ikawa et al., 1999]. A selective induction of ΔNp73 was actually observed in cisplatin-treated NT2/D1 and Scha cells, while no change in expression of this protein in these cells was observed upon irradiation [Spierings et al., 2004]. The cisplatin-induced stabilisation of ΔNp73α, possibly further enhanced by c-Abl [Tsai and Yuan, 2003], and a concomitant inhibition of p53 may contribute to the reduced p21 protein levels in TGCTs.

The nature of the cellular response to DNA damage is also controlled by the so-called p53 co-activating proteins [Samuels-Lev et al., 2001;Gasco and Crook, 2003;D'Orazi et al., 2002;Hofmann et al., 2002;Kim et al., 2002;Shimodaira et al., 2003].

The influence of TA and ΔN-forms of p53 homologues and, on the other hand, of p53 co-activating proteins on the DNA damage-induced p53 pathway still needs to be further clarified in TGCTs. Investigations in this field might help to understand the absence of p53 mutations in TGCTs as well as the TGCTs response to systemic treatment.

P21 and Fas-mediated apoptosis

The dual regulation of p21 and of pro-apoptotic death receptor pathways, among which Fas, can provide the explanation of TGCTs sensitivity to cisplatin. Up-regulation of the cyclin

dependent kinase-inhibitor (CDKI) p21, induced by p53-dependent and p53-independent mechanisms, can cause cell cycle arrest. P21 inhibits a broad range of cyclin-cyclin dependent kinase (CDK) complexes with a preference for those containing CDK2 [Harper et al., 1995]. P21 may, therefore, be a determinant for different responses to chemotherapy [Javelaud et al., 2000;Javelaud and Besancon, 2002;Schepers et al., 2003]. Besides facilitating DNA repair after chemotherapy damage, p21 also inhibits drug-induced apoptosis. According to the current knowledge, nuclear localisation of p21 is responsible for cell cycle progression inhibition, while p21 cytoplasmatic localisation inhibits both intrinsic and extrinsic apoptosis [Asada et al., 1999;Geller et al., 2004]. Interaction of p21 with caspase 3 in the cytoplasm leads to Fas-mediated apoptosis resistance [Suzuki et al., 1998] and to the stabilisation of the apoptotis inhibitor c-IAP [Steinman and Johnson, 2000]. Cytoplasmatic p21 is also able to associate with the Apoptosis-Signal regulating Kinase (ASK-1) [Takeda et al., 2003] thereby inhibiting Fas-induced ASK-1 mediated apoptosis [Asada et al., 1999] (Fig.3). In addition, repression of p53-dependent induction of p21 or elevated caspase 3 cleavage of p21 usually increases apoptosis [Zhang et al., 1999]. We recently demonstrated that after irradiation NT2/D1 cells remained resistant to Fas-mediated apoptosis in contrast to the cisplatin treated NT2/D1 cells that were highly sensitive to Fas-mediated apoptosis. A major difference between irradiation and cisplatin treatment was the massive induction of p21 and higher Fas surface expression in NT2/D1 cells after irradiation [Spierings et al., 2004]. The elevated p21 levels after irradiation did not result in a cell cycle arrest, which may be due to the cytoplasmatic localisation of p21. Down-regulation of p21 by short interfering-RNA efficiently restored sensitivity to Fas-mediated apoptosis induction in irradiated NT2/D1 cells [Spierings et al., 2004].

Several cytotoxic drugs including cisplatin induce FasL and Fas receptor expression. Wild-type p53 is responsible for Fas transport from the cytoplasm to the cell membrane [Muller et al., 1997;Muller et al., 1998;Miyake et al., 1998;Bennett et al., 1998]. In addition, wild-type p53 but also mutant p53 can be an important mediator of *Fas* gene activation in response to DNA damage [Munsch et al., 2000]. In several cancer cell lines it was demonstrated that the Fas death pathway is an important effector of chemotherapeutic treatment [Friesen et al., 1999;Petak and Houghton, 2001;Meli et al., 2004;Beurel et al., 2004;Stumm et al., 2004]. Recently, we demonstrated that cisplatin-induced apoptosis was depending on activation of

the Fas apoptosis pathway in the cisplatin-sensitive NT2/D1 and 833K cells [Spierings et al., 2003b]. Interaction of Fas with FasL was responsible for the activation of the extrinsic pathway in these TGCT cell lines [Spierings et al., 2003b]. In the cisplatin-resistant subline of NT2/D1, Tera-CP, and in the intrinsic resistant cell line Scha no cisplatin-induced activation of the Fas apoptosis pathway was observed, which confirms the involvement of the Fas apoptosis pathway in the cisplatin-sensitivity of TGCT cells [Spierings et al., 2003b]. Results on Fas and FasL expression in human TGCTs are conflicting. The co-expression of Fas and FasL in many TGCTs [Sugihara et al., 1997] suggests that they can bypass the requirement of FasL by Sertoli cells, and may be able to induce apoptosis after cisplatin treatment, via an autocrine or paracrine activation of the Fas death pathway by FasL. In another study, however, 73% of TGCTs containing seminomatous elements express Fas and FasL, but only 56% and 11% of TGCTs without seminomatous elements express Fas and FasL, respectively [Hara et al., 2001]. In a recent study in 25 chemotherapy naive non-seminomatous TGCTs no association was observed between the rate of FasL or Fas positive cells and the apoptotic index [Schmelz et al., 2002]. Activation of the Fas apoptosis pathway after cisplatin treatment, however, could not be excluded.

Taken together these results suggest that high expression of p21, either constitutive or irradiation-induced, protects TGCT cells against DNA-damage induced activation of the Fas apoptosis pathway.

Cell cycle control

a. Cell cycle control in normal and neoplastic cells

In response to DNA damage, accumulating p53 protein trans-activates several genes among which *p21*, which can inhibit several cyclin-CDK complexes, causing a hypophosphorylation of retinoblastoma protein family (pRb, p107 and p130) [Dyson, 1998;Nevins, 1998]. Hypophosphorylated pRb prevents the release of E2F [Dyson, 1998;Nevins, 1998;Chellappan et al., 1991]. This leads to the repression of E2F dependent transcription and to a G0/G1 arrest [Agarwal et al., 1995;Gaubatz et al., 2000] (Fig.4). Hypophosphorylated pRb can have another function in preventing deamidation of the anti-apoptotic protein Bcl-XL. Deverman *et al.* reported that increased deamidation of Bcl-XL

results in decreased anti-apoptotic activity of Bcl-XL in response to DNA damage. Inhibition of the cell cycle progression via p21-mediated inhibition of CDKs results in higher levels of hypophosphorylated pRb levels. Thus, Bcl-XL will become less deamidated and will therefore have more anti-apoptotic activity [Deverman et al., 2002].

The cell cycle clock itself can participate in triggering the apoptotic response as inactivation of one step in the regulatory pRb/E2F pathway can result in deregulation of E2F-1 activity and in p53-mediated apoptosis induction (Fig. 4). The continuous expression of E2F-1 during the phase S triggers apoptosis [Field et al., 1996], both via p73 [Lissy et al., 2000;Irwin et al., 2000] and via p53. The p53-dependent apoptotic pathway may occur via E2F-induced expression of p14/Arf, which in turn inhibits Mdm2 leading to further up-regulation of p53 [Sherr, 2001] (Fig. 4). Cross-talk between the cell cycle control and p53 pathway occurs not only via p21 but also involves the INK4/ARF locus, which encodes the CDKI proteins p16 and p14/Arf. INK4a, encoding p16,is one of the four INK4 family members of CDKI proteins, all acting as inhibitors of CDK4 and CDK6 [Quelle et al., 1995]. INK4a and p14/Arf have their own separate promoters and, although sharing two out of three exons (p16: 1α, 2, 3; p14: 1β, 2, 3), they produce different transcripts, using different open-reading frames [Quelle et al., 1995;Duro et al., 1995;Mao et al., 1995]. The INK4/ARF locus is a sensor of oncogenic stresses, as p16 and p14/Arf are usually present at extremely low levels in normal tissues. They do not restrain proliferation continuously but only under appropriate signalling as is transduced by active oncogenic Ras [Serrano, 2000] (Fig.4).

b. Cell cycle control in TGCTs

The main negative control of G1/S transition provided by the pRb pathway is differently altered in TGCTs [Strohmeyer et al., 1991] as compared to other tumour types. In seminomas and non-seminomas, Strohmeyer *et al.* demonstrated a decreased mRNA expression without any gross alteration of the *pRB* gene [Strohmeyer et al., 1991]. This suggests that changes at the transcriptional level rather than gene mutations could be responsible for the pRb absence or decrease in TGCTs. In this view, Bartkova suggests that the lack of pRb in CIS as well as in seminomas and embryonal carcinomas reflects the origin of TGCTs from foetal gonocytes. Expression of pRb is normally silenced in foetal gonocytes and de-repressed in adult

spermatogenesis. De-repression of pRb is absent or extremely rare at the CIS stage, but occurs in TGCTs occasionally in subsets of seminoma and embryonal carcinoma cells [Bartkova et al., 2003]. The pRb pathway also involves many upstream regulators such as cyclin D-CDK4/6 kinases and INK4 family members. They regulate the function of pRb family proteins by changing their phosphorylation state. TGCTs harbour an over-expression of cyclin D2, rather than cyclin D1. Furthermore, a loss or a down regulation of p18INK4a, rarely encountered in other tumours, and a universal absence of p19INK4d is found in TGCTs [Bartkova et al., 2003]. Inactivation of INK4/ARF locus by deletion or methylation is a rare event in TGCTs [Chaubert et al., 1997;Hatta et al., 1995;Franklin et al., 1998;Ruas and Peters, 1998;Roussel, 1999], but frequently occurs in extragonadic GCTs [Iwato et al., 2000] as well as in many solid [Enders, 2003;Piepkorn, 2000] and haematopoietic tumours [Kubo et al., 2002;Krug et al., 2002]. TGCTs show a paradoxical over-expression of p27 [Bartkova et al., 2003], due to decreased proteolytic degradation [Moller, 2000], but almost undetectable p21 levels. In normal cells, p27 acts as a balance in cyclin D-CDK4/6 formation and activity. However, p27 is usually lost in human solid tumours so that cyclin D-CDK4/6 complex continuously forces cells from G1 phase into S phase. In TGCTs, in contrast, p27 is over-expressed, which may be explained as the functional attempt of the cyclin D-over-expressing tumour cell to balance the high levels of cell progression promoting complexes [Kukoski et al., 2003]. But at least one other explanation can be proposed. Because the locus for *p27* gene is localised on 12p, the *p27* gene could also be the target for a genetic amplification or co-amplification together with cyclin D gene. In this view, also the new data about the emerging role of p27 protein as a promoter of cyclin D-CDK4/6 formation could find an explanation [Coqueret, 2003]. The increasing levels of both p27 and cyclin D due to genetic amplification might support the increased formation of cyclin D-CDK4/6 complexes, which may actually promote cell cycle progression from G1 phase into S phase [Bagui et al., 2003].

Although it still has to be clarified whether the over-expression of these proteins is simply due to gene amplification or a molecular balance between proteins acting in the same pathway, the reported data suggest a loss of cell cycle control in TGCTs.

Conclusions

The high sensitivity of TGCTs to cisplatin containing therapies seems to be an inherited characteristic of the cell of origin, as normal germ cells are very sensitive to apoptotic stimuli, including DNA damaging stresses. We hypothesise that, despite the presence of wild-type p53, the functionality of p53-dependent pathway has changed in TGCTs. This is reflected in an induction of p53 but not of p21 following cisplatin treatment. In contrast, irradiation induces p53 and in a p53-dependent manner also p21. These results suggest that p53-induced trans-activation of p21 is depending on the type of DNA damage. Following cisplatin treatment, the Fas apoptosis pathway and the intrinsic apoptosis pathway are activated, causing a rapid and efficient induction of apoptosis in TGCTs. Elevated levels of p21 after irradiation protect TGCT cells against Fas-mediated apoptosis. However, p21 is predominantly localised in the cytoplasm and does not induce a cell cycle arrest. Unfortunately, only a few data are available about the role of p21 and Fas apoptosis pathway in the response of TGCTs to chemotherapy treatment.

The reduced levels of p21 and the changed expression of proteins involved in the cell cycle regulation also indicate a deregulated G1–S phase checkpoint in TGCTs. It is tempting to speculate that in TGCTs no cell cycle arrest but only apoptosis pathways are activated in response to DNA damage. More research has to be carried out to identify determinants of activation of the p53 dependent pathway including positive and negative regulators on p53 system, such as p14, Mdm2, MdmX, and ΔN p73/p63 in TGCTs. In addition, the cross-talk between the p53 pathway and cell cycle apparatus in TGCTs has to be studied in more detail to determine its relevance in cisplatin-induced apoptosis.

A deeper understanding of the mechanisms underlying the unique cisplatin sensitivity of TGCTs may provide a mean to increase chemo-sensitivity and to overcome acquired and intrinsic chemotherapy resistance in other human tumours.

Figures and Figures Legends to Chapter 2

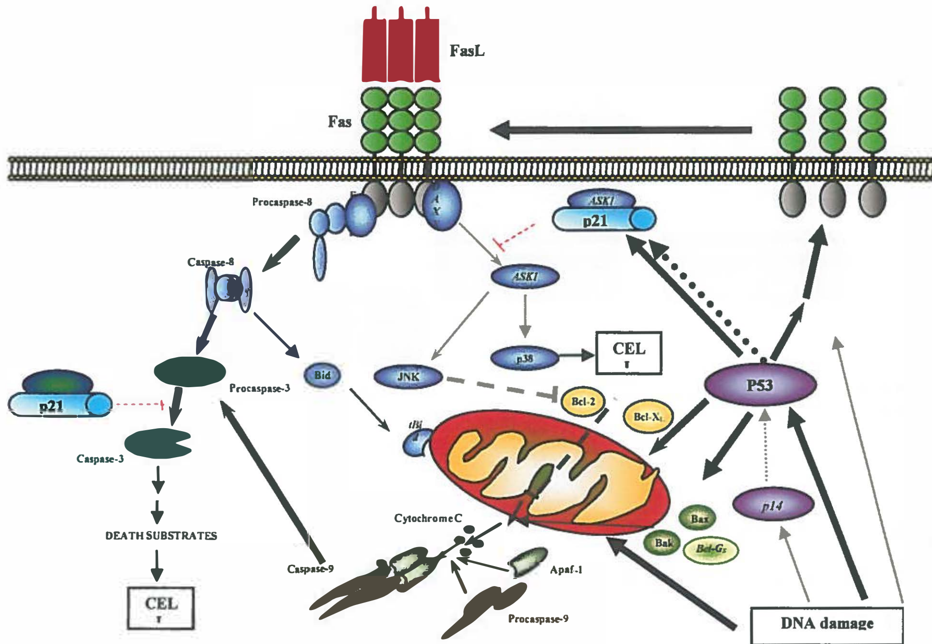

Figure 1

External and internal stimuli are folded to the clock apparatus to determinate the cell fate. DNA damage induced by different stimuli activates p53 protein, which on turn activates both the Fas apoptosis pathway and the mitochondrial apoptosis pathway or forces the cell into cycle arrest, to repair DNA damage (Modified from Spierings DC et al, J.Pathol. 2003;200:137-48). Black arrows indicate pathways, which are known to be functional in TGCTs. Grey arrows indicate general cell pathways, not yet investigated in TGCTs. In italics proteins not yet investigated in TGCTs are indicated.

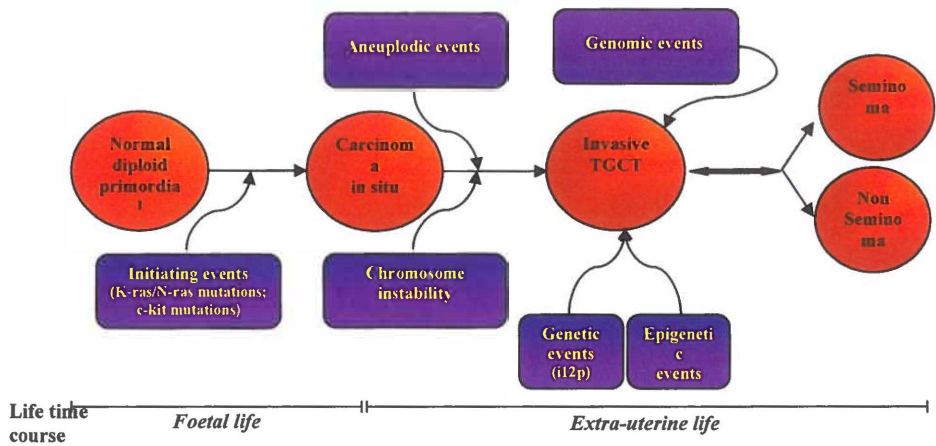

Figure 2

TGCTs are a heterogeneous group of tumours, derived from the germ cell lineage. During the foetal life a diploid primordial germ cell is affected by initiating events, which eventually lead to a precursor lesion, named carcinoma in situ. A series of genomic, genetic and epigenetic alterations in this lesion leads later on during the extra-uterine life to progression towards seminoma and non-seminoma.

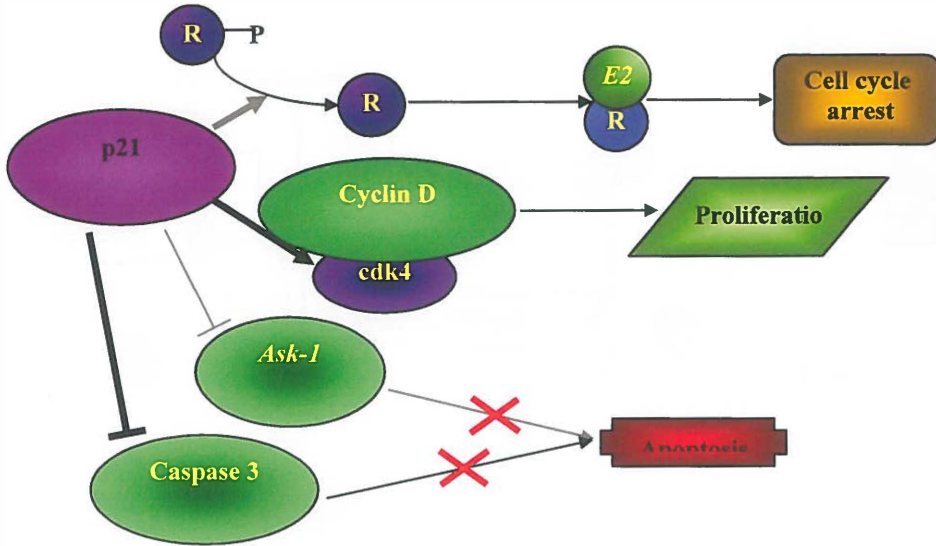

Figure 3

The cyclin dependent kinase inhibitor p21 causes cell cycle arrest by binding and inhibiting a broad spectrum of cyclin-cdk complexes. However p21 is also able to stabilise cyclin D-cdk4 complexes, thus inducing proliferation both in normal and in cancer cells. In this perspective, p21 may confer a protective advantage against apoptosis and furthermore, in cancer cells, it may be responsible for different responses to chemotherapy. Black arrows indicate known p21-regulated pathways, which are functional in TGCTs. Grey arrows indicate general p21-regulated pathways, not yet investigated in TGCTs. In italics proteins not yet investigated in TGCTs are indicated.

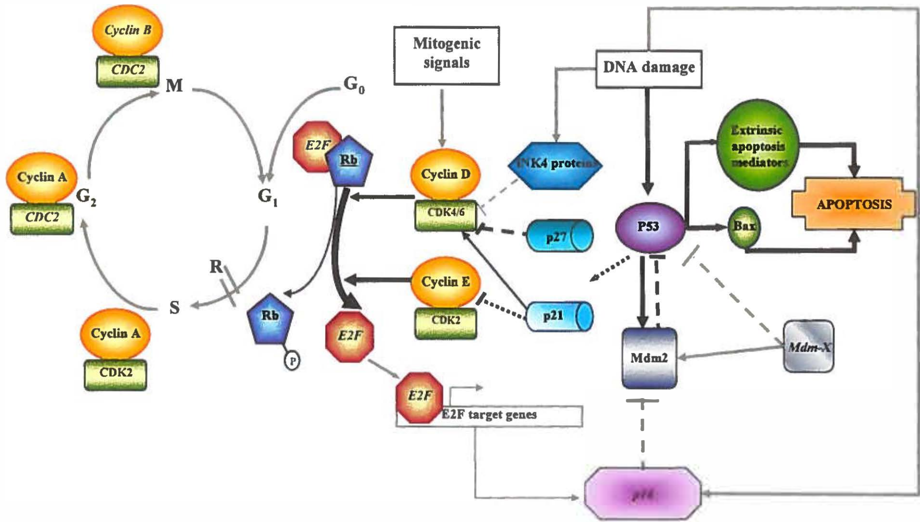

Figure 4

The cell cycle is an ordered sequence of four phases (G1/0, S, G2, M), in which the control of the passage from a phase to another in due to cyclin-cdk complexes. Two families of CDK-inhibitors, INK family and p21-p27 family are responsible for the control of these complexes. The integration between the extra-cellular and intra-cellular signals balance the stimulation and the inhibition on cyclin-cdk complex formation, thus leading to cell cycle progression, to cell cycle arrest or even to apoptosis. For more details see text. (Modified from Spierings DC et al, J.Pathol. 2003;200:137-48). Black arrows indicate known functional pathways in TGCTs. Grey arrows indicate general cellular pathways, not yet investigated in TGCTs. In italics proteins not yet investigated in TGCTs are indicated.

Table 1. Human GCT cell lines

Cell Line	Origin Site	Histology	Characteristics	Reference
833KE	Abdominal Metastasis	NSGCT	EC+ T	[Bronson et al., 1980]
GCT27	Primary	NSGCT	EC	[Pera et al., 1987]
GH	Primary	NSGCT	EC	[Wang et al., 1997]
SuSa	Primary	NSGCT	EC+ T	[Hogan et al., 1977]
1618K	Primary	NSGCT	EC+ ChC+S	[Vogelzang et al., 1983]
Tera-1	Lung Metastasis	NSGCT	EC	[Fogh, 1978]
Tera-2 NT2/D1*	Lung Metastasis	NSGCT	Pluripotent EC	[Fogh, 1978;Andrews et al., 1984]
H12.1	Primary	NSGCT	T	[Hill et al., 1994]
2102EP	Primary	NSGCT	EC	[Wang et al., 1980;Andrews et al., 1982]
1411HP	Primary	NSGCT	EC+YS	[Vogelzang et al., 1985]
NCCIT	Primary mediastinal	NSGCT	EC+S	[Damjanov et al., 1993]
S2		SGCT	S	[von Keitz et al., 1994]

Legend: *NSGCT*= Non-Seminomatous Germ Cell Tumour; *EC*= Ebryonal Cell Carcinoma;*T*= Teratoma;*ChC*=Choriocarcinoma; *YS*=Yolk Sac Carcinoma: *S*= Seminoma; * = Subclone derived from the original line Tera-2

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Chapter 3

The p53-pathway affects cisplatin-induced apoptosis in human testicular germ cell tumour cell lines depending on the cellular context.

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Submitted for Publication

Abstract

The role of wild-type p53 in the unique cisplatin sensitivity of human testicular germ cell tumour (TGCT) was analysed. A panel of cisplatin sensitive TGCT cell lines (833KE and Tera), a subline with cisplatin-acquired resistance (Tera-CP) and an intrinsic cisplatin resistant TGCT cell line (Scha), all expressing wild type p53 were used. Basal p53 and p53-transcriptional target Mdm2 expression was present in all lines but the p53-transcriptional target $p21^{Waf1/Cip1}$ only in Scha. P53 expression increased already after 6 h and further enhanced after 24 h cisplatin exposure, together with Mdm2 and apoptosis induction, whereas minimal $p21^{Waf1/Cip1}$ induction occurred. Previous observations showed, however, that irradiation of TGCT cells induced high levels of $p21^{Waf1/Cip1}$. P53 suppression by p53 short interfering (si)RNA lowered apoptosis in Tera, related to a diminished Fas membrane expression, but had no effect on apoptosis induction in 833KE nor in Tera-CP. In contrast, p53 down-regulation, concomitantly suppressing $p21^{Waf1/Cip1}$ expression, increased cisplatin-induced apoptosis in Scha. Importance of $p21^{Waf1/Cip1}$ in Scha was further demonstrated by $p21^{Waf1/Cip1}$ siRNA, which resulted in elevated apoptosis after cisplatin. Our results suggest a dual role for p53 in transactivation and cisplatin-induced apoptosis in TGCT cells depending on cellular context of p53. Low $p21^{Waf1/Cip1}$ levels under cisplatin-induced p53 activation may explain hypersensitivity of TGCT cells to cisplatin-induced apoptosis.

Introduction

Germ Cell Tumours (GCTs) represent the most frequent solid malignant tumour in men 20-40 years of age [Pottern M.L. et al., 1998] and the most frequent cause of death from solid tumours in this age group. In the last 40 years, the incidence of testicular cancer has been arising world-wide [Einhorn, 2002a]. Despite the rising incidence of testicular cancer and the presence of metastatic disease up to 50% at the time of diagnosis [Xu et al., 2007], a decrease in mortality has been observed thanks to highly effective chemotherapy schemes [Einhorn, 2002b;Einhorn, 2007]. In general, TGCTs are successfully treated; however, about 20% of patients diagnosed with metastatic disease will not achieve a durable complete remission after initial treatment, either due to incomplete response or relapse, and will eventually die from this disease. Understanding the molecular determinants of chemotherapy sensitivity and resistance in testicular cancer treatment may provide a way to improve chemotherapy sensitivity in other solid tumours.

Two DNA damaging agents, cisplatin and etoposide, are mainly responsible for the successful outcome in testis cancer treatment [Varuni Kondagunta et al., 2004]. Cisplatin is an extremely active drug for the treatment of patients with TGCTs. So far the analysis of potentially relevant parameters in cisplatin response (detoxification mechanisms, DNA platination and repair, p53 status, Bcl-2 family status) have not elucidated the determinants of TGCTs sensitivity [Timmer-Bosscha et al., 1993b;Koberle et al., 1996;Koberle et al., 1997;Burger et al., 1998d;Koberle et al., 1999]. Most human TGCT cell lines, however, show a characteristic hypersensitivity to apoptosis-induction by chemotherapeutic drugs [Huddart et al., 1995;Chresta et al., 1996a;Burger et al., 1998b;Burger et al., 1999c].

A major role for p53 in the response to chemotherapeutic drugs and the execution of apoptosis has been described [Lowe et al., 1993;Levine, 1997;Cheng et al., 1999b;Wahl and Carr, 2001;Johnstone et al., 2002]. P53 is a tumour suppressor gene with a dual role in stress response, regulating a number of genes that co-ordinately force cells into either cell cycle arrest (via trans-activation of p21) allowing time for DNA repair or apoptosis. In turn, the function of p53 is regulated by several mechanisms, acting not only at the transcriptional and translational level, but also influencing the stability of p53 as well as its post-translational modifications and subcellular localisation [O'Brate and Giannakakou, 2003]. P53 is the most frequently mutated gene in human cancers [Greenblatt et al., 1994b], but surprisingly, in human TGCTs almost no p53 mutations occur, while the p53 protein is expressed at high levels in the majority of TGCTs [Heidenreich et al., 1998]. In human ovarian germ cell tumours, also extremely sensitive to cisplatin-containing chemotherapy,

no p53 mutations were detected [Liu et al., 1995]. Despite the increasing knowledge about the p53 protein as transactivator and cellular gatekeeper for cell growth and division, the effect of wild-type p53 and mutated p53 on drug sensitivity of human tumours is still not clear. Several reports have studied chemosensitivity of human TGCT cell lines in relation to p53 expression, with contrasting results [Chresta et al., 1996b;Houldsworth et al., 1998c;Burger et al., 1997c;Burger et al., 1998a;Burger et al., 1999a;Arriola et al., 1999a;Arriola et al., 1999b].

In order to determine extensively the role of the p53-dependent and p53-independent apoptotic pathway in cisplatin sensitivity and resistance in a setting closely related to the clinic, a well-defined panel of human TGCT cell lines was used [Timmer-Bosscha et al., 1993a;Sark et al., 1995c;Spierings et al., 2003e]; Spierings *et al*, 2004). We recently reported that p53 was upregulated following cisplatin treatment as well as irradiation. However, cisplatin exposure induced apoptosis in TGCT cells in contrast to irradiation [Spierings et al., 2004i]. Furthermore, p21 was almost not upregulated in TGCT cell lines following cisplatin treatment [Spierings et al., 2004h]. Therefore, we compared p53-dependent cellular and molecular changes with respect to p21 and apoptosis induction by cisplatin in two cisplatin sensitive cell lines (833KE and Tera), a subline of Tera with acquired resistance to cisplatin (Tera-CP) and an intrinsic cisplatin resistant cell line (Scha).

Materials and methods

DNA and Chemicals

RPMI 1640 medium was obtained from Gibco (Paisley, Scotland) and foetal calf serum from Sanbio (Uden, the Netherlands). Cisplatin was purchased from Bristol-Myers Co. (Weesp, the Netherlands). 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (St.Louis, Mo).

Cell Lines

For the cell lines used in this study, origin and pre-treatment were described by Sark *et al* [Sark et al., 1995a].The human germ cell tumour cell lines Tera, Tera-CP, 833KE and Scha and the human ovarian carcinoma cell line A2780, used as a control for different protein, were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum at 37°C in a humidified atmosphere with 5% CO_2. 833KE grew attached and was harvested by treatment with protease XXIV 0.005% for 3 min. Tera, Tera-CP, and Scha were harvested by scraping.

RNA interference

Sequence for p53 small interfering RNA (siRNA) molecules was 5'GCA UGA ACC GGA GGC CCA UdTdT 3' (sense) and 5'AUG GGC CUC CGG UUC AUG CdTdT 3' (anti-sense). Sequence for p21 siRNA was 5'-CUU CGA CUU UGU CAC CGA GdTdT-3' (sense) and 5'-CUU ACG CUG AGU ACU UCG AdTdT-3' (anti-sense). Single-stranded RNA molecules specific for the Luciferase (Luc) gene served as control. The sequence for Luc RNA molecules were 5'-CUU ACG CUG AGU ACU UCG AdTdT-3' (sense) and 5'-UCG AAG UAC GCG UAA GdTdT3' (antisense). Scha and 833KE (0.4×10^6/ well) were transfected in 6 well plates with 10 µl of 20µM siRNA duplexes using Oligofectamine reagent according to the manufacturer's instructions (Invitrogen BV, Breda, the Netherlands). After 24 h, cells were treated with cisplatin. At 6 h or 24 h after the treatment cells were harvested for protein isolation. Alternatively, in order to perform an apoptosis assay, at 24 h after transfection, cell were harvested and plated in 96-well plate. The day after, cells were treated with cisplatin. At 24 h after the treatment, the percentage of apoptotic cells was determined by acridine orange apoptosis assay.

Drug Sensitivity Assay

Drug sensitivity testing was performed with the microculture tetrazolium assay as described previously [Sark et al., 1995b]. The linear relationship of cell number to MTT formazan crystal formation and the exponential growth of cells in the wells were checked. For Tera, Tera-CP, 833KE and Scha 10,000, 10,000, 15,000 and 30,000 cells per well, respectively, were incubated in a total volume of 0.2 ml culture medium in 96-well culture plates. Cytotoxicity for all cell lines was determined after 4 days. Each drug concentration was tested in quadruplicate.

Western Blotting

After 6 hrs or 24 h cisplatin incubation cell were harvested and lysates were examined by Western Blot analysis as described previously [Spierings et al., 2004g]. Immunodetection of p53, p21, Mdm2, poly-(ADP-ribose)-polymerase (PARP) was performed with the following antibodies anti-p53-DO-1 (clone sc-126; Santa Cruz Biotechnology, CA, USA), anti-phospho-p53 (ser15 and ser46; Cell Signaling, MA, USA) mouse anti-Mdm2 (Oncogene Research Products; San Diego, USA), mouse anti-p21 (Oncogene Research Products; San Diego, USA), rabbit anti-Parp (Roche Applied Science), Bcl-2 was detected using monoclonal mouse antisera anti-Bcl-2 (clone: sc-7382) and Bax (clone: sc-493), Bcl-X_L (clone: sc-7195) with polyclonal rabbit antisera, all from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody binding was eventually determined using horseradish peroxidase (HRP)-conjugated secondary antibodies (all from DAKO, Glostrup, Denmark) and visualised with the POD chemoluminescence kit by Roche Applied Science. Chemiluminescence was detected with the BM Chemiluminescence detection kit (Roche, Almere, The Netherlands). Membranes were exposed to Kodak X-OMAT films. Equal protein loading was checked for with Ponceau S and ß-actin staining.

Apoptosis

For apoptosis measurements cells were plated in 24-well culture plates. Cells were continuously incubated with cisplatin at various concentrations. Acridine orange fluorescent staining of nuclei in unfixed cells was used to distinguish apoptotic from vital cells [Timmer-Bosscha et al., 1998]. Staining was performed 6, 24, 48 and 72 h after start

of incubation. Results are expressed as the percentage of apoptotic cells in a culture by counting at least 300 cells per well.

Caspase 3 activity

After 6 hrs or 24 h cisplatin incubation, cells were harvested as usual and centrifuged at 1400 rpm for 8 min. After one wash step with cold PBS 1x, cells were resuspended in 50 µl of chilled Cell Lysis Buffer [10 mM HEPES, 2 mM EDTA, 0.1% CHAPS / NP40, 5 mM DTT, 1 mM PMSF (10 µg/ml pepstatin A) (20 µg/ml leupeptin) (10 µg/ml aprotinin)] and incubated on ice for 10 min. Subsequently cell lysates were centrifuged in a microcentrifuge at 10,000 g for 3 min at 4°C to precipitate cellular debris. The supernatants were then transferred to new microcentrifuge tubes. From each sample, 5 µl were used to perform Bradford. Samples were then diluted to the final concentration of 1 µg/µl for caspase assay, with lysis buffer. 25 µl of 2X Reaction Buffer [31.25% sucrose, 0.3125% CHAPS (3-[3-cholamido-propyl)-dimethammonio]-1propane-sulphonate), HEPES: 312.5 mM (pH 7.5 Mw: 238.31g/l)] (containing DTT and caspase 3/7 substrate DEVD-AFC) were added to each 25 µl sample in a 96-wells plate (Cliniplate flat bottom black) and incubated for 1 h at 37°C. Fluorescent detection of protease activity was performed by means of a fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter. Fold-increase in protease activity was determined by comparing these results with the level of the control.

Detection of Fas membrane expression

Cisplatin-treated or untreated cells were stained with a phycoerythrin (PE)-conjugated Ab against Fas (DX2 from Becton Dickinson, Erembodegem-Aalst, Belgium) for 1 hour at room temperature. Subsequently, cells were washed and analysed by flow cytometry (FACS-Calibur; Becton Dickinson). The mean fluorescence intensity was determined by comparison of the fluorescence intensity of unlabeled cells.

Results

Expression of proteins involved in the p53-dependent pathway

Although all four TGCT cell lines contained wild-type *p53*, p53 protein levels differed considerably. P53 basic levels are highest in 833KE, followed by Scha, Tera and Tera-CP. We also determined the expression of Mdm2, $p21^{Waf1/Cip1}$, Puma and Bax which are p53-dependent transactivated. Expression levels of Mdm2 were higher in 833KE and Scha compared to Tera and Tera-CP, with a trend comparable to the one observed for p53. $P21^{Waf1/Cip1}$ was expressed in Scha, less expressed in 833KE and minimally detectable in Tera and Tera-CP (Figure 1). Bax, a pro-apoptotic Bcl-2 family member, whose expression can be p53-dependent, was also higher expressed in 833KE and Scha. As a positive control for wild-type p53 expressing cells and cisplatin-induced wild-type p53 activation, we used the human A2780 ovarian cancer cell line [Spierings et al., 2004f]. These results suggest that constitutive high expression of wild-type p53 in 833KE and Scha leads to an increased expression of the p53-dependent protein Mdm2 as compared to Tera or Tera-CP

Effect of cisplatin treatment on p53 expression, caspase-3 activation, Parp cleavage, and apoptosis

To determine whether cisplatin treatment induces expression of p53, cells were incubated for 6 h and 24 h with cisplatin concentrations ranging from the ID_{50} of highly sensitive cell lines to more than the ID_{90} of all cell lines used in the present study. Within 6 h a concentration-dependent increase in p53 expression levels was observed in the TGCT cell lines (Figure 2A). Note that the constitutive level of p53 was at least 5-fold higher in 833KE and Scha. Thus, absolute cellular p53 expression levels after cisplatin induction are comparable between the TGCT cell lines. Despite the high induction of p53, no PARP cleavage nor apoptotic cells, defined as cells with condensed and fragmented nuclei, were observed after 6 h (data not shown).

A prolonged incubation with cisplatin for 24 h resulted in a concentration-dependent increase in cellular p53 levels in the TGCT cell lines similar to the wild-type p53 expressing A2780 ovarian cancer cells. The p53 induction levels in the TGCT cell lines after treatment with cisplatin for 24 h were higher than p53 levels after a 6 h treatment (Figure 2B). At lower cisplatin concentration this increase was more pronounced for 833KE than for Scha, while at the higher concentrations the two cell lines contained almost comparable p53 levels. As to the isogenic system, a strong induction of p53 was detectable

after cisplatin treatment in Tera and Tera-CP, while this induction was more pronounced for Tera at lower cisplatin concentrations.

Massive induction of apoptosis, cleavage of PARP and caspase 3 activation in a cisplatin concentration-dependent manner were observed in the TGCT cell lines. However, for Scha and Tera-CP higher cisplatin concentration were needed. The results of the various assays were in accordance with each other showing that Tera and 833KE are the most sensitive cell lines, while Tera-CP and Scha were relatively resistant to cisplatin (Figure 3A-C).

Effect of cisplatin treatment on the expression of p21, Mdm2, Puma and Bax

To determine whether functional wild-type p53 was induced in TGCT cells, we measured the drug induced protein expression of genes, which are transcriptionally activated by wild-type *p53*, i.e. $p21^{Waf1/Cip1}$, Mdm2, Puma and Bax. After a 6 h exposure to cisplatin no changes in expression were found in any of the observed proteins (results not shown). Mdm2 levels increased in the TGCT cells at a similar rate as in A2780 cells after a 24 h treatment with low cisplatin concentrations (Figure 4). At high doses cisplatin, Mdm2 levels fell down in TGCT cells, whereas Mdm2 levels continued to rise in A2780 cells. Despite the high induction of p53 and Mdm2, a minor induction of $p21^{Waf1/Cip1}$ was detectable in the TGCT cell lines as compared to the massive induction of $p21^{Waf1/Cip1}$ in A2780 (Figure 4). At high doses of cisplatin, Mdm2 and $p21^{Waf1/Cip1}$ cleavage products were detected in Tera and Tera-CP cells (Figure 4) as well as 8333KE and Scha (results not shown). Cisplatin treatment, however, had no effect on Bax or Puma in any TGCT cell line (results not shown).

Influence of p53 down-regulation on treatment response

The lack of $p21^{Waf1/Cip1}$ induction following cisplatin treatment suggested that p53 is not functional in TGCT cells. Previously, we, however, showed that irradiation strongly induces $p21^{Waf1/Cip1}$ mRNA and protein expression demonstrating that $p21^{Waf1/Cip1}$ can be induced depending on the type of DNA damage in these TGCT cell lines [Spierings et al., 2004e]. To study the functionality of p53, we suppressed p53 expression with p53 siRNA. Down-regulation of p53 resulted in a strong reduction in Mdm2 expression in Scha and Tera (Figure 5) as well as 833KE, Tera-CP and A2780 (data not shown) and in reduced expression of $p21^{Waf1/Cip1}$ in Scha (Figure 5) and A2780 (data not shown). P53 siRNA however did not affect Puma and Bax levels (data not shown).

When p53 suppressed TCGT cells were treated with cisplatin, different effects on apoptosis were observed (Figure 6A). The down-regulation of p53 sensitised Scha to cisplatin-induced apoptosis as compared to luciferase siRNA treated cells at cytotoxic relevant doses of cisplatin. This sensitisation effect was detectable after 3 h and 6 h of cisplatin treatment (data not shown) and was more pronounced after 24 h. No effect was observed in 833KE and Tera-CP, whereas the down-regulation of p53 makes the extremely sensitive Tera partially resistant to cisplatin-induced apoptosis (Figure 6A). The caspase activity assay confirms the results obtained with the apoptosis assay. A two-fold induction in caspase activity was observed in cisplatin-treated p53 suppressed Scha cells, while a 2-fold decrease in caspase activity was observed in p53 suppressed Tera cells after cisplatin as compared to luciferase siRNA transfected cells treated with cisplatin (Figure 6B). Moreover, down-regulation of p53 does not affect caspase activation in 833KE nor Tera-CP in response to cisplatin.

At the protein level, down-regulation of p53, Mdm2 and $p21^{Waf1/Cip1}$ was still observed in p53 siRNA treated cells 6 h and 24 h after addition of cisplatin. Although treatment of cells transfected with p53 siRNA with a high cisplatin dose induced p53 protein expression as well as Mdm2 and $p21^{Waf1/Cip1}$, these levels still remains lower when compared to the corresponding levels in untransfected or luciferase siRNA transfected cells treated with cisplatin (Figure 5).

These results indicate that p53 is functional in untreated and cisplatin treated TGCT cells. The role of p53 in cisplatin-induced apoptosis in TGCT cells, however, is cell context dependent.

Influence of p53 down-regulation on cisplatin-induced Fas membrane expression

Previously, we have shown that cisplatin-induced apoptosis is depending on activation of the Fas/FasL system in the cisplatin sensitive Tera and 833KE cells, while activation of the Fas/FasL system is inhibited in Tera-CP and Scha [Spierings et al., 2003d]. Therefore, we monitored the effect of p53 siRNA on cisplatin-induced Fas membrane expression. In agreement with our previous results, cisplatin strongly induced Fas membrane expression in Tera and Tera-CP. The upregulation was p53-dependent, since downregulation of p53 with p53 siRNA resulted in a strong inhibition of cisplatin-induced Fas membrane expression in both cell lines. Although, cisplatin only minimally induced Fas membrane expression in 833KE and Scha cells, the effect of p53 siRNA on Fas

expression was still detectable in both cell lines (Figure 7). These results indicate that p53 is causal in the cisplatin-induced upregulation of Fas membrane expression in all cell lines.

Influence of p21 down-regulation on cisplatin-induced apoptosis

The anti-apoptotic function of p53 in Scha may be related to the p53-dependent expression of $p21^{Waf1/Cip1}$. In a previous study we have shown that in Tera, Tera-CP and Scha cells irradiation-induced $p21^{Waf1/Cip1}$ inhibits Fas-mediated apoptosis [Spierings et al., 2004d]. To define the importance of $p21^{Waf1/Cip1}$ in the resistance of Scha to cisplatin-induced apoptosis we used a siRNA strategy. Successful down-regulation of $p21^{Waf1/Cip1}$ was confirmed with Western blot analysis (Figure 8).

The down-regulation of $p21^{Waf1/Cip1}$ led to an increase in apoptosis in Scha, as demonstrated by the apoptosis assay (Figure 8). These results were confirmed by the caspase activity assay, which showed an increase in caspase activation for each concentration of cisplatin in $p21^{Waf1/Cip1}$ suppressed Scha cells (Figure 8). In addition, an increase in PARP cleavage products and caspase 3 activation products was observed in these cells compared to the luciferase siRNA transfected Scha cells. No effect on apoptosis level was observed in 833KE cells after $p21^{Waf1/Cip1}$ down-regulation, nor in Tera or Tera-CP (data not shown). At highly toxic cisplatin concentrations (> 4 µM) the effect of $p21^{Waf1/Cip1}$ down-regulation on cisplatin-induced apoptosis in Scha is less compared to the effect of p53 down-regulation on cisplatin-induced apoptosis (Figure 6 and 8).

DISCUSSION

In the present study we investigated the role of p53 in determining the response to cisplatin treatment in a panel of cisplatin sensitive and resistant human TGCT cell lines. Our results indicate that cisplatin treatment resulted in enhanced wild-type p53 expression and apoptosis induction in all four TGCT cell lines albeit at different cisplatin concentrations. Functionality of p53 was demonstrated by p53 siRNA, which suppressed p53 as well as Mdm2 expression levels and prevented cisplatin-induced upregulation of p53, Mdm2 and Fas cell surface expression in all four TGCT cell lines. Remarkably, inhibition of cisplatin-induced p53 expression with p53 siRNA causes a decrease in apoptosis levels in the cisplatin sensitive Tera cells but an enhanced induction in apoptosis in Scha. These results indicate that p53 is functional in TGCT cell lines, but its involvement in the response to cisplatin, i.e. pro-apoptotic or anti-apoptotic, is cell context dependent.

We demonstrated that higher basal levels of wild-type p53 in 833KE and Scha were accompanied by higher levels of Mdm2 and Bax in comparison to Tera and Tera-CP, which suggests the presence of a transcriptionally active p53 in human TGCT cells. Surprisingly, the basal level of $p21^{Waf1/Cip1}$ in Tera, Tera-CP and 833KE cells was almost undetectable, whereas in Scha $p21^{Waf1/Cip1}$ was clearly detectable albeit at a lower level compared to the wild-type p53 expressing A2780 ovarian cancer cell line. Down-regulation of p53 with siRNA led to a lower expression level of p53-dependent transcriptionally activated factors, like Mdm2, which appeared to be larger in 833KE and Scha than in Tera or Tera-CP. These observations suggest that p53 is functional but has a changed functionality in TGCT cell lines, since an important p53-dependent factor like $p21^{Waf1/Cip1}$ is almost always absent in these cell lines except for Scha. Constitutive expression of $p21^{Waf1/Cip1}$ in Scha could be suppressed with p53 siRNA, demonstrating the p53 dependence of $p21^{Waf1/Cip1}$ expression in Scha cells.

We observed a concentration-dependent induction of p53 induction of Mdm2 in the TGCT cells following cisplatin treatment. A similar induction of p53 and Mdm2 was found in A2780 ovarian cancer cells by cisplatin treatment. A remarkable difference between all TGCT cell lines and A2780 was the absence of a cisplatin concentration-dependent induction of $p21^{Waf1/Cip1}$ in the TGCT cell lines. Induction of $p21^{Waf1/Cip1}$ protein has been observed in human TGCT cell lines after γ-radiation [Burger et al., 1998c;Spierings et al., 2004c] or etoposide treatment (Chresta, Arriola and Hickman, 1996). These observations can be explained by the different activation of p53 after DNA damage induced by either γ-radiation or etoposide versus cisplatin. Moreover, several post-translational modification of

the p53 protein may play a role in determining its functional activity. Phosphorylation of p53 at ser15 blocks p53 binding to MDM2 leading to the accumulation and activation of p53, whereas phosphorylation of p53-ser46 positively regulates apoptosis induction in response to DNA damage (Honda, Tanaka and Yasuda, 1997; Shieh, Taya and Prives, 1997; Chehab *et al*, 1999; Tibbetts *et al*, 1999; Oda *et al*, 2000). We only determined basal levels of p53 phosphorylation but did not find any difference between the cell lines (results not shown). Furthermore, conflicting data about the importance of these modifications have been published (Ashcroft, Kubbutat and Vousden, 1999; Toledo and Wahl, 2006). Other p53 regulatory proteins have been suggested to be more important such as the related proteins Mdm2 and Mdm4 (Vassilev *et al*, 2004; Toledo and Wahl, 2006; Marine, Dyer and Jochemsen, 2007). Until now, no data are available on the expression of Mdm4 in testicular cancer and their involvement in the response to DNA damaging agents of TGCT cells. Moreover, the role of p53-Mdm2 interaction in the response of TGCT is still not conclusive (Kersemaekers *et al*, 2002; Oliver, Shamash and Berney, 2002). Therefore, further studies on the regulatory role of Mdm2/Mdm4 on p53 in cisplatin sensitivity and resistance of TGCT cells are necessary.

In several TGCT studies, the presence of a partially transcriptionally active p53 in vivo (Riou *et al*, 1995) has been largely demonstrated. Although clinical analyses of TGCTs revealed almost no p53 mutations (Greenblatt *et al*, 1994), another report showed that mutant p53 was present in a subset of chemoresistant TGCTs (Houldsworth *et al*, 1998), further supporting the hypothesis of a functional p53 in human TGCTs. In vitro culturing of these tumours displayed a drug resistant phenotype, less apoptosis and no induction of p53-responsive genes, which indicates the relevance of wild-type p53 expression in TGCT cells (Houldsworth *et al*, 1998). A relation between the presence of wild-type p53, the susceptibility for apoptosis induction and the chemosensitivity of a panel of TGCT cell lines has been observed in several studies (Chresta, Arriola and Hickman, 1996; Mueller *et al*, 2003; Spierings *et al*, 2003a), but these results were challenged by another study using a different TGCT cell line panel (Burger *et al*, 1997). Therefore, we have investigated the role of p53 in cisplatin sensitivity in the different TGCTs cell lines by down-regulation of p53 with siRNA. According to our results, the down-regulation of p53 causes different effects in wild-type p53 expressing TGCT cell lines. We found that in 833KE and Tera-CP cell lines, p53 down-regulation did not affect the sensitivity of these two cell lines to cisplatin-induced apoptosis, whereas the down-regulation of p53 led to a

decrease in cisplatin sensitivity in Tera. Surprisingly, down-regulation of p53 enhanced cisplatin sensitivity in Scha. One has to realize that p53 siRNA especially had an effect on cisplatin-induced apoptosis at concentrations relevant for cisplatin-induced cytotoxicity as determined in the survival assay. It has been reported that p53 down-regulation by human papilloma virus type-16 E6 had no effect on the cisplatin sensitivity in Tera (Burger *et al*, 1999), which is in contrast to our p53 siRNA results in this cell line. Besides inactivation of p53, human papilloma virus type-16 E6 can, however, affect other proteins involved in apoptosis or drug sensitivity as well (Massimi *et al*, 1996; Massimi *et al*, 1999; Vikhanskaya *et al*, 1998; Filippova, Parkhurst and Duerksen-Hughes, 2004). Our data are in agreement with a recent micro-array study from Kerley-Hamilton *et al* (Kerley-Hamilton *et al*, 2005) in which the authors demonstrated that in Tera cells p53 is directly involved in cisplatin sensitivity, as the down-regulation of p53 corresponds to the global decrease of several cisplatin-induced genes involved in apoptosis and in particular of genes involved in the extrinsic apoptosis machinery, like Fas, Fas adaptor LRDD and a gene involved in positive Fas regulation, PHLDA3 (Kerley-Hamilton *et al*, 2005). Previously, we have demonstrated that the Fas/FasL system is active and functional in the cisplatin-sensitive Tera and 833KE cell lines but not in the resistant Tera-CP and Scha cells (Spierings *et al*, 2003a). Downregulation of p53 indeed prevented cisplatin-induced Fas surface expression, indicating that in Tera cells p53 is a main determinant of cisplatin-induced apoptosis due to pro-apoptotic signalling via the Fas-FasL system at least at relatively low cisplatin concentration. Although p53 is functional in 833KE and Tera-CP, p53 downregulation had no affect on cisplatin-induced apoptosis levels in these cell lines suggesting the involvement of other, possibly p53-independent, mechanisms in these cell lines. Since 833KE and Tera-CP are differently sensitive to cisplatin-induced apoptosis and cisplatin induces activation of the Fas pathway in 833KE cells only, it is tempting to speculate that p53-independent activation of the Fas pathway is an important mechanism in 833KE cells (Spierings *et al*, 2003a). In Scha, p53 plays an anti-apoptotic role, which probably involves p53 transactivation of $p21^{Waf1/Cip1}$, as p53 down-regulation and to a lesser extent the down-regulation of $p21^{Waf1/Cip1}$ is able to sensitise this cell line to cisplatin induced apoptosis. The importance of $p21^{Waf1/Cip1}$ as an anti-apoptotic factor has been confirmed by a previous study of our group (Spierings *et al*, 2004). We then, demonstrated that γ-radiation treatment was able to massively induce $p21^{Waf1/Cip1}$ in these TGCT cell lines resulting in resistance to anti-Fas induced apoptosis. Taken together, these observations suggest that the lack of induction of $p21^{Waf1/Cip1}$ in TGCT cells after cisplatin treatment could be instrumental in the

cisplatin-induced apoptosis possibly via the Fas pathway. Although drug-induced activation of the Fas-mediated apoptotic pathway is not a general phenomenon in tumour cells (Wieder *et al*, 2001), death-inducing signalling complexes have been observed in human colon carcinoma cells and leukemic cells by cisplatin and doxorubicin treatment, respectively (Micheau *et al*, 1999a; Micheau *et al*, 1999b; Fulda, Meyer and Debatin, 2000).

Recent observations also suggested that $p21^{Waf1/Cip1}$ is involved in cell cycle arrest and suppression of apoptosis induced by drug treatment (Mahyar-Roemer and Roemer, 2001). Disruption of the $p21^{Waf1/Cip1}$ gene by homologous recombination made wild-type p53 tumour cells very sensitive to apoptosis induction by DNA damaging agents (Bunz *et al*, 1999; Mahyar-Roemer and Roemer, 2001). In addition, it was demonstrated that $p21^{Waf1/Cip1}$ binds to procaspase 3, inhibiting its cleavage into the active form and consequently preventing the apoptotic signal (Suzuki *et al*, 2000a; Suzuki *et al*, 2000b), but this observation was not confirmed in a previous study from our group (Spierings *et al*, 2004). An alternative is $p21^{Waf1/Cip1}$ binding to Ask-1, which then no longer can mediate apoptosis (Cheng *et al*, 1999; Schepers *et al*, 2003; Spierings *et al*, 2003b; Di Pietro *et al*, 2005). Therefore, additional studies on the role of $p21^{Waf1/Cip1}$ in cisplatin sensitivity and resistance of TGCT cells are warranted.

In conclusion, these results suggest a dual role for p53 in transactivation and cisplatin-indued apoptosis in TGCT cells depending on the cellular context. The low levels of $p21^{Waf1/Cip1}$ even under cisplatin-induced activation of p53 may partially explain the hypersensitivity of TGCT cells to cisplatin-induced apoptosis.

Figures and Figures Legend to Chapter 3

Table 1

Cell survival after short term and continuous treatment with cisplatin using the MTT assay.

	ID_{50} cisplatin (μM)[1]	
	6 h	continuous
Tera-2	1.6 ± 0.2	0.7 ± 0.1
Tera-CP	5.4 ± 0.9	2.1 ± 0.2
833KE	1.4 ± 0.6	1.0 ± 0.2
Scha	9.9 ± 1.7	3.3 ± 1.0

1) ID_{50} values are expressed as means ± SD of three independent experiments performed in quadruplicate using the MTT assay.

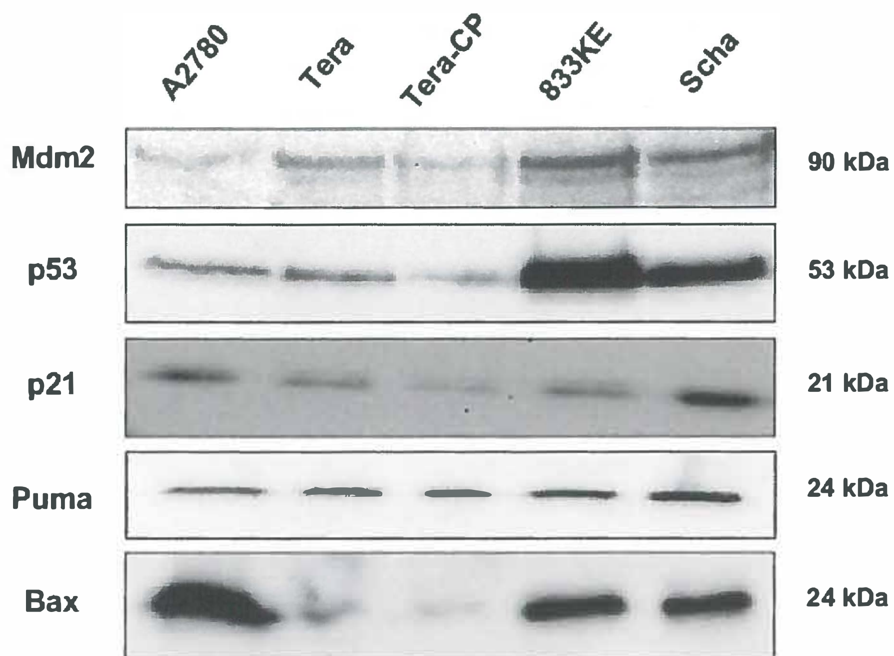


Figure 1

Western blot of proteins in whole-cell lysates of untreated TGCT cells. Protein separation and Western blotting was performed as described in "Material and Methods". Each lane was loaded with 20 µg protein. A representative example of three independent experiments is shown.

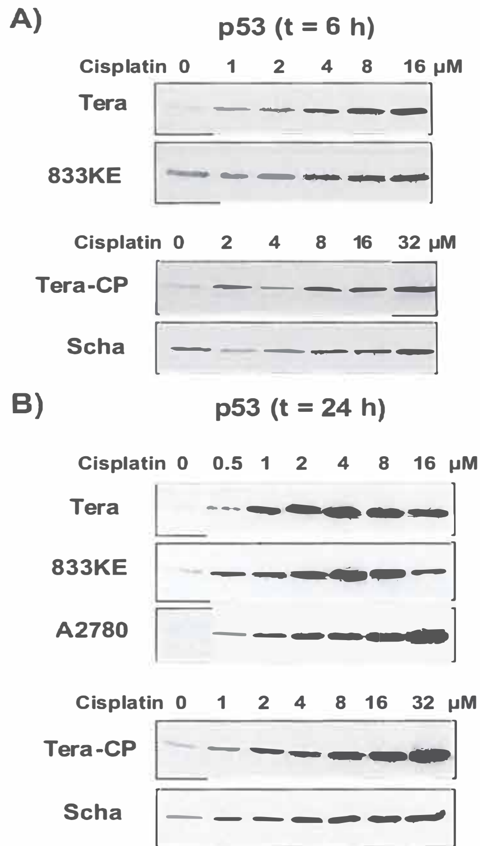


Figure 2

P53 protein induction in whole-cell lysates after 6 h (A) and 24 h (B) of treatment with cisplatin. Protein separation and Western blotting was performed as described in "Material and Methods". Each lane was loaded with 20 µg protein. A representative example of three independent experiments is shown.

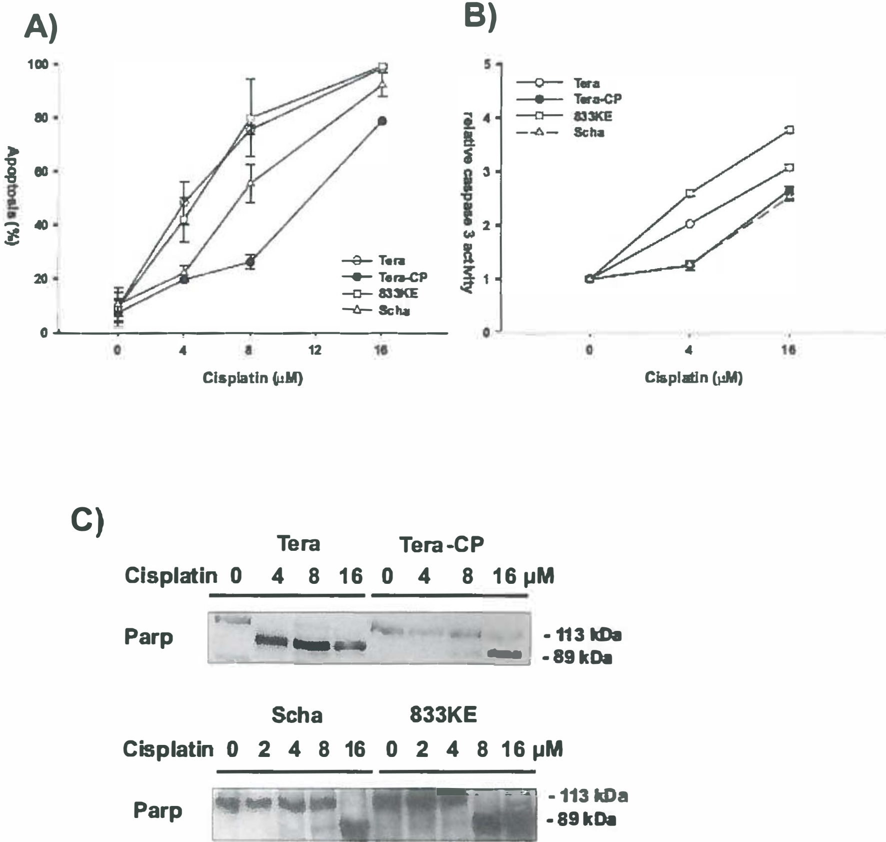


Figure 3

Apoptosis induction, caspase 3 activation, and PARP cleavage in TGCT cell following treatment with cisplatin for 24 h. The percentage of apoptotic cells was determined with fluorescence microscopy on acridine orange stained cells (A), caspase 3 activity was determined in whole cell lysates using a fluorescence assay (B), and PARP cleavage was determined with Western blotting on whole-cell lysates (C) after exposure of the cells to cisplatin. Counting of apoptotic cells, caspase 3 activity assay and Western blotting were performed as described in "Materials and Methods". Values are the mean ± SD of three experiments.

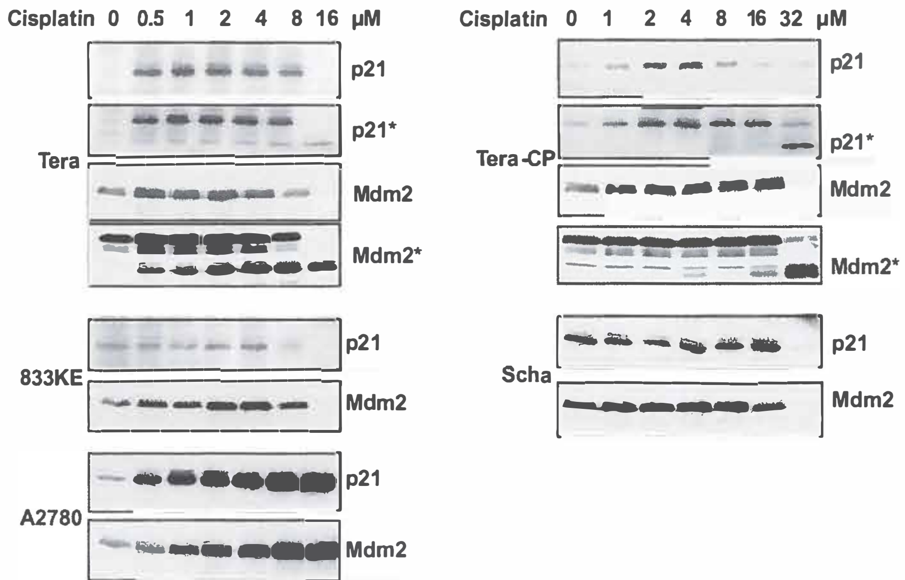
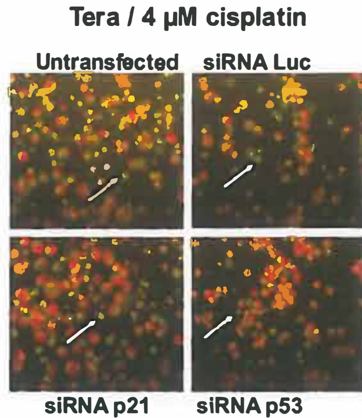
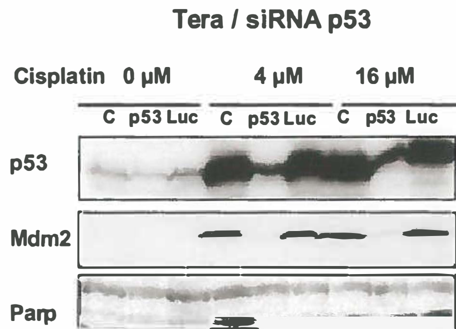
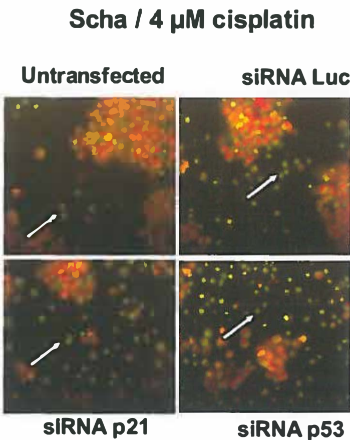


Figure 4

$P21^{Waf1/Cip1}$ (A), MDM2 (B) protein levels after cisplatin treatment for following treatment with cisplatin for 24 h. Expression levels were determined with Western blotting on whole-cell lysates. Protein separation and Western blotting was performed as described in "Material and Methods". Each lane was loaded with 20 µg protein. To detect $p21^{Waf1/Cip1}$ in lysates of Tera, 833KE, and Scha, membranes had to be exposed to X-ray films 10 times longer compared to A2780. $P21^{Waf1/Cip1}$ * and MDM2* indicate cleaved products on blots exposed to X-ray films for a longer time. A representative example of three independent experiments is shown.







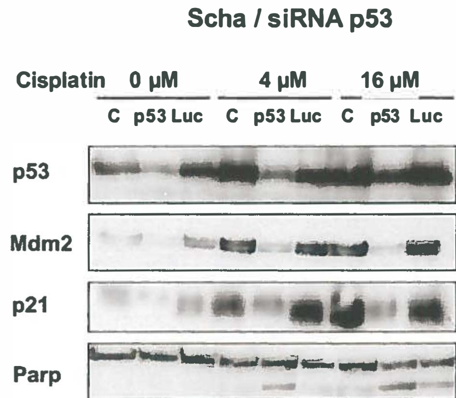


Figure 5

P53 siRNA affects p53, p21$^{Waf1/Cip1}$ and MDM2 expression. Cells were either not treated with siRNA (**C**), with p53 siRNA (**p53**) or luciferase siRNA (**Luc**). Cells were treated with 0, 4 and 16 µM cisplatin. Apoptotic cells, indicated with white arrows, was visualised wiith fluorescence microscopy on acridine orange stained cells. Protein expression was determined in whole-cell lysates with Western blotting. Protein separation and Western blotting was performed as described in "Material and Methods". Each lane was loaded with 10 µg protein. A representative example of three independent experiments is shown.

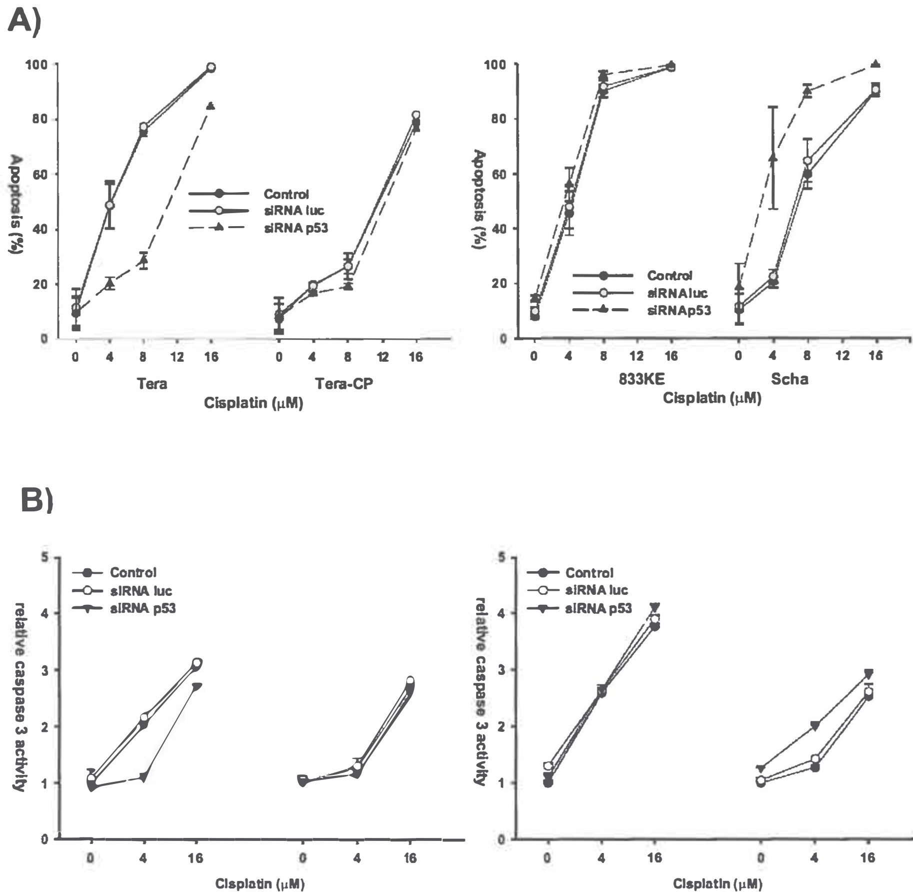


Figure 6

Induction of apoptosis and caspase 3 activation by cisplatin treatment in p53 siRNA treated TGCT cells. Cells were either not treated with siRNA (control), with p53 siRNA (siRNA p53) or luciferase siRNA (siRNA luc). Following siRNA treatment for 24 h, cells were treated with cisplatin for an additional 24 h. Apoptosis was analysed by fluorescence microscopy on acridine orange-stained cells (A) and caspase 3 activation with a fluorescence assay (B). Values are the mean ± SD of three experiments.

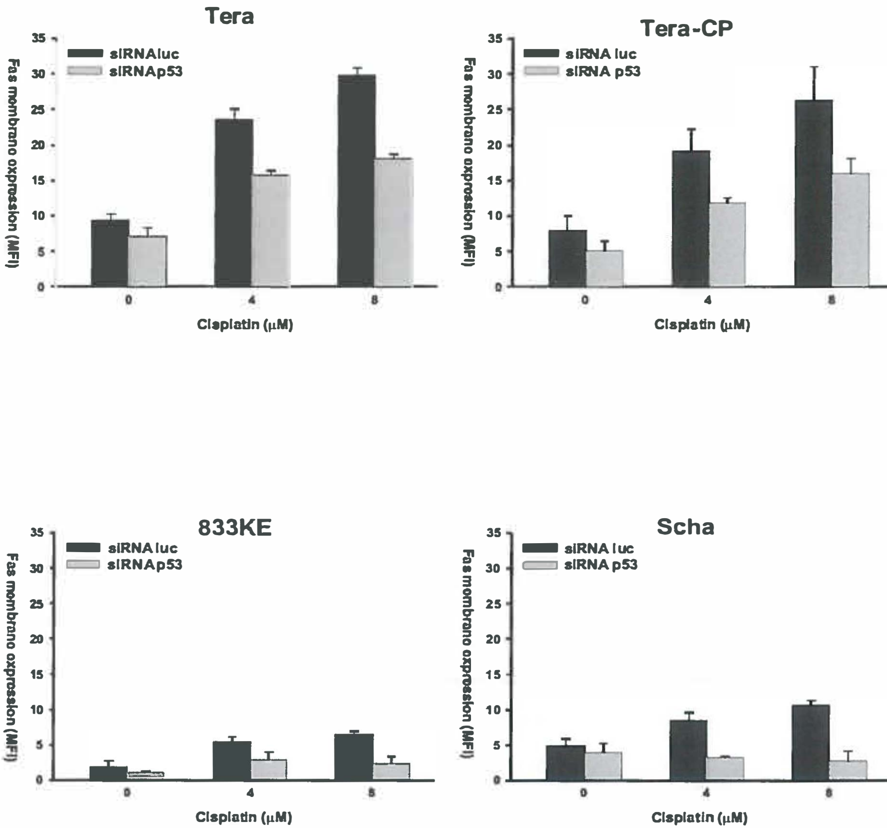
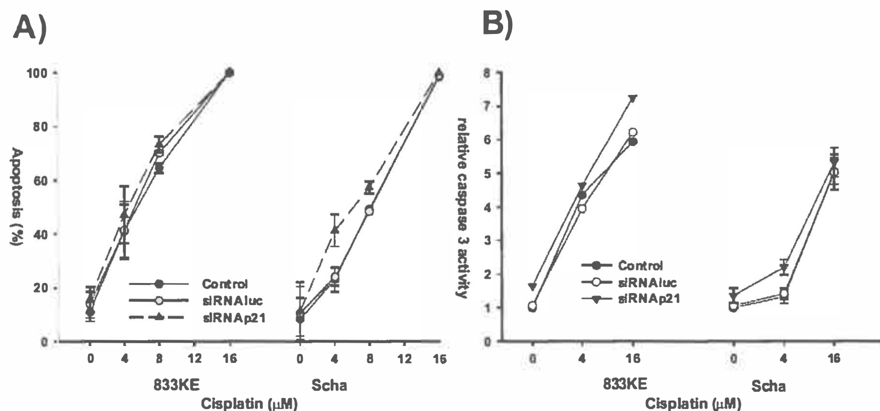


Figure 7

Effect of p53 siRNA on cisplatin-induced Fas membrane expression.

Following siRNA treatment cells were treated with 0, 4 and 8 µM cisplatin for 24 h and the Fas membrane expression was determined with flow cytometry and indicated as mean fluorescence intensity (MFI). Values are the mean ± SD of three experiments.



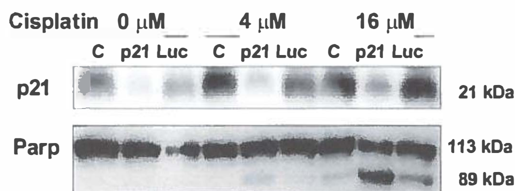


Figure 8

Effect of p21$^{Waf1/Cip1}$ siRNA on cisplatin treated TGCT cells. Following siRNA treatment cells were treated with 0, 4 and 16 µM cisplatin for 24 h. Apoptosis was analysed by fluorescence microscopy on acridine orange-stained cells A) and caspase 3 activation was determined in total cell lysates with a fluorescence assay B), p21$^{Waf1/Cip1}$ expression and Parp cleavage were determined in whole-cell lysates with Western blotting. Cells were either not treated with siRNA (control or C), with p21$^{Waf1/Cip1}$ siRNA (p21) or luciferase siRNA (luc). Values are the mean ± SD of three experiments.

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Chapter 4

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p-Akt dependent cytoplasmic localization of p21$^{cip1/waf1}$ protects human testicular germ cell tumour cells from cisplatin-induced apoptosis

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Submitted for Publication

ABSTRACT

Testicular germ cell tumour (TGCT) cells are extremely sensitive to cisplatin-induced apoptosis. TGCT cells express wild type p53 but almost no $p21^{cip1/waf1}$ (p21). Recent observations indicate that gamma-irradiation in contrast to cisplatin treatment strongly enhance cytoplasmic p21 expression in wild type p53 expressing human testicular germ cell tumour (TGCT) cells without inducing apoptosis. To define the role of p21 and the cytoplasmic localisation of p21 with regard to cisplatin sensitivity, we stably transduced a human TGCTs cell line, Tera, with a viral construct containing either p21-ΔNLS and GFP, or GFP only (Mock). With immune fluorescence microscopy strong cytoplasmic expression of p21 was demonstrated. Treatment with cisplatin induced less apoptosis in Tera-p21-ΔNLS than in Tera-mock. Moreover, downregulation of p21 with p21 siRNA rendered Tera-p21-ΔNLS cells more apoptotic to cisplatin treatment. Similar observations were made with the cisplatin resistant TGCT cell line Scha, in which we observed that p21was mainly expressed in the cytoplasm, also after cisplatin treatment. Downregulation of endogenous p21 sensitized Scha to apoptosis induction by cisplatin. Using immunoprecipitation we identified the pro-apoptotic Apoptosis Signalling Kinase 1 (ASK1) and cyclin dependent kinase 2 (CDK2) as targets of cytoplasmic p21. Cytoplasmic localisation of p21 in TGCT cells was depending on the phosphorylation of the NLS-site (Thr145) of p21. Inhibition of p21 phosphorylation using the PI3K inhibitor LY294002 or the specific p-Akt inhibitor Triciribine resulted in a partial relocation of endogenous cytoplasmic p21 towards the nucleus leading to sensitization for cisplatin or gamma-irradiation induced apoptosis. Combined these results strongly indicate that p-Akt-dependent elevation of cytoplasmic p21 levels functions as an antiapoptotic factor in TGCT cells.

INTRODUCTION

Testicular Germ Cell Tumours (TGCTs) represent the most frequent solid malignant tumour in men 20-40 years of age and the incidence of testicular cancer has been arising world-wide (Einhorn, 2002). Thanks to highly effective chemotherapy schemes a decrease in mortality has been observed even in case of metastatic disease (Einhorn, 2002; Einhorn, 2007). Despite the general success of TGCTs treatment, about 20% of Testicular cancer patients with metastatic disease will not achieve a durable complete remission after initial treatment, either due to incomplete response or a tumour relapse, and will eventually die from this disease.

Cisplatin is foremost responsible for the successful outcome in TGCT treatment (Kondagunta et al., 2004); also in human TGCT cells it is an extremely active drug (Huddart et al., 1995; Burger et al., 1998; Spierings et al., 2003; di Pietro et al., 2008). So far the analysis of potentially relevant parameters in cisplatin response (detoxification mechanisms, DNA platination and repair, p53 status, Bcl-2 family status) have not elucidated the determinants of TGCTs sensitivity (Timmer-Bosscha et al., 1993; Sark et al., 1995; Burger et al., 1997; Timmer-Bosscha et al., 1998; Burger et al., 1998; Koberle et al., 1999).

A major role for p53 in the response to chemotherapeutic drugs and the execution of apoptosis has been described (Lowe et al., 1993; Levine, 1997; Chang et al., 1999; Wahl and Carr, 2001; Johnstone et al., 2002; di Pietro et al., 2008). In turn, the function of p53 is regulated by several mechanisms, acting not only at the transcriptional and translational level, but also influencing the stability of p53 as well as its post-translational modifications and its sub-cellular localisation (O'Brate and Giannakakou, 2003). The p53 gene is the most frequently mutated gene in human cancers (Greenblatt et al., 1994), but surprisingly, in human TGCTs almost no p53 mutations have been detected. P53 is a tumour suppressor gene with a dual role in stress response, regulating a number of genes that co-ordinately force cells into either cell cycle arrest via trans-activation of p21 allowing time for DNA repair or apoptosis. Although the p53 protein is expressed at high levels in the majority of TGCTs (Heidenreich et al., 1998; Spierings et al., 2004; di Pietro et al., 2008) it does not completely explain the hyper-sensitivity for cisplatin. We recently showed that p53 is functional in human TGCT cell lines, although the effect of p53 upon apoptosis is depending on the cellular context (di Pietro et al., 2008). Interestingly, gamma-irradiation induced p53 and Mdm2 protein levels and gave a massive induction of p21 in human TGCT cells without inducing apoptosis. Cisplatin treatment of TGCT cells, in contrast,

resulted in enhanced levels of p53 and Mdm2, while p21 levels were almost not affected (Spierings et al., 2004; di Pietro et al., 2008). Following gamma-irradiation no cell cycle arrest was induced, while p21 was predominantly located in the cytoplasm. Furthermore, downregulation of p21 using siRNA sensitized gamma-irradiated TGCT cells for Fas-induced apoptosis, suggesting that cytoplasmic p21 has anti-apoptotic properties in gamma-irradiated cells (Spierings et al., 2004).

The low levels of p21 often observed in TGCTs, may thus, besides a less efficient inhibition of proliferation, also fail to prevent apoptosis after cisplatin treatment. In order to determine the role of p21 in cisplatin sensitivity and resistance in human TGCT cell lines we studied the sub-cellular localisation of p21 after treatment with cisplatin or gamma-irradiation. Cytoplasmic p21 was introduced in Tera in order to demonstrate the importance of cytoplasmic p21 in the protection against apoptosis induction. In addition, targets of cytoplasmic p21 were identified by co-immunoprecipitation. Finally, strategies to relocate endogenous p21 using PI3K or p-Akt inhibitors were used to sensitize $p21^{cip1/waf1}$ expressing TGCT cells to apoptosis induction by cisplatin or gamma-irradiation.

MATERIALS AND METHODS

Chemicals and Cell Lines

RPMI 1640 medium was obtained from Gibco (Paisley, Scotland) and fetal calf serum from Sanbio (Uden, the Netherlands). Cisplatin was purchased from Bristol-Myers Co. (Weesp, the Netherlands), LY294002 from Cell Signalling and Triciribine from Biomol (Exeter, UK). For the TGCT cell lines used in this study, origin and pre-treatment were described by Sark et al (Sark et al., 1995). The human germ cell tumour cell lines Tera and Scha and the human ovarian carcinoma cell line A2780 and the human breast carcinoma cell line MCF-7, both used as a control for different proteins, were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere with 5% CO2. Tera and Scha were harvested by scraping, A2780 and MCF-7 by treatment with trypsin.

Immunofluorescence

For immunofluorescence cells were seeded in 6 wells plates, grown on with 0.01% poly-L-glycine (Sigma) precoated coverslip. 24h after cisplatin or gamma-irradiation treatment the slides were collected and fixed with 4% paraformaldehyde for 30 min at room temperature and then permeabilized with 0.2% Triton X-100 for 5 min at room temperature. The cells were blocked with 1% bovine serum albumin and 1% normal goat serum in PBS for 30 min at room temperature followed by immunostaining with the corresponding antibodies and counterstained with Alexa-Fluor secondary antibodies (Molecular Probes, Leiden, The Netherlands). Finally, cells were stained with Hoechst 33258 (Molecular Probes, Leiden, The Netherlands) for 5 minutes (1:5000), washed with PBS, and slides were mounted with vectashield (Vector Laboratories, Burlingham, CA, USA).

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting

SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli. After 24 hrs of cisplatin or gamma-irradiation treatment cells were harvested and washed twice with cold phosphate buffered saline (PBS: 6.4 mM Na2HPO4, 1.5 mM KH2PO4, 0.14 mM NaCl, 2.7 mM KCl, pH 7.2). Cells were lysed with standard Western Blot sample buffer (50 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-β-mercaptoethanol) and boiled for 5 minutes. Proteins concentration was determined according to Bradford. All samples were sized fractionated by electrophoresis on SDS-

polyacrylamide gels. Proteins were then transferred from the SDS-acrylamide gel onto PVDF membranes (Millipore, Bedford, United Kingdom) at 250 mA for 1 hr at room temperature using a semi dry-blot system (Ancor, Denmark).

After blocking 1 h in TRIS-buffered saline supplemented with 5% milk powder (Merck, Darmstadt, Germany) and 0.05% Tween-20 (Sigma-Aldrich Chemie BV), immunodetection of p21, p-p21(Thr145), Akt, p-Akt (ser473), poly-(ADP-ribose)-polymerase (Parp), ASK1 and caspase 3 was performed with the following antibodies: mouse anti p21 (F5, Santa Cruz), rabbit anti p21 (H164, Santa Cruz) rabbit anti p-p21(Thr145, Santa Cruz), rabbit anti p-Akt/Akt (Cell signalling) rabbit anti-Parp (Roche Diagnostics), rabbit anti ASKl (H-300, Santa Cruz), mouse anti ASK1 (F9, Santa Cruz) and rabbit anti caspase 3 (cell signalling). The antibody binding was eventually determined using horseradish peroxidase (HRP)-conjugated secondary antibodies (all from DAKO, Glostrup, Denmark) and visualised with the POD chemoluminescence kit by Roche Diagnostics. Chemiluminescence was detected with the BM Chemiluminescence detection kit (Roche, Almere, the Netherlands). Equal protein loading was checked for with Ponceau S and ß-actin staining.

Preparation of fractionated proteins

Irradiated, cisplatin-treated or untreated cells were harvested and washed with ice-cold phosphate-buffered saline (PBS: 6.4 mM Na2HPO4; 1.5 mM KH2PO4; 0.14 mM NaCl; 2.7 mM KCl; pH=7.2) and suspended in 200 µl buffer A (10 mM Hepes/KOH, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF). After incubation on ice for 10 min, 12,5 ml 10% Nonidet P-40 (NP-40) was added and directly vortexed for 10 s. The nuclear-rich fraction was pelleted by centrifugation at 23000 g for 1 min and the supernatant was collected as the cytosol-rich fraction. The pellet was washed with buffer A, dissolved in buffer B (20 mM Hepes/NaOH, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and incubated on ice for 20 min and vortexed every 5 min. After centrifugation at 23 000 g for 5 min, the supernatant was collected as the nuclear-rich fraction. Each fraction was examined by Western blot analysis as described above.

p21-ΔNLS stable cell line

pMSCV-p21-ΔNLS and pMSCV-IGFP were kindly provided by Dr. J.J. Schuringa. For stable transduction of the human TGCT cell line Tera a viral construct containing both p21-

ΔNLS (delta nuclear localization signal) and GFP (Green Fluorescent Protein), or GFP only (Mock) were used. For viral production, 293T cells were transfected with pCL-Ampho and MSCV-IGFP, either with or without p21-ΔNLS. Subsequent virus containing medium was used for transduction of Tera. GFP positive cells were sorted on the Mo-Flow.

Apoptosis

For apoptosis measurements cells were plated in 96-well culture plates. Cells were continuously incubated with cisplatin at various concentrations. Acridine orange fluorescent staining of nuclei in unfixed cells was used to distinguish apoptotic from vital cells (Timmer-Bosscha et al., 1998). Staining was performed 24 hrs after start of incubation. Results are expressed as the percentage of apoptotic cells in a culture by counting at least 300 cells per well.

Caspase 3 activity

After 24 hrs of cisplatin or gamma-irradiation treatment, cells were harvested as usual and centrifuged at 1400 rpm for 8 min. After one wash step with cold PBS, cells were resuspended in 50 μl of chilled Cell Lysis Buffer [10 mM HEPES, 2 mM EDTA, 0.1% CHAPS / NP40, 5 mM DTT, 1 mM PMSF (10 μg/ml pepstatin A) (20 μg/ml leupeptin) (10 μg/ml aprotinin)] and incubated on ice for 10 min. Subsequently cell lysates were centrifuged in a microcentrifuge at 12,000 rpm for 3 min at 4°C to precipitate cellular debris. The supernatants were then transferred to new microcentrifuge tubes. From each sample, 5 μl were used to perform Bradford. Samples were then diluted to the final concentration of 1 μg/μl for caspase assay, with lysis buffer. 25 μl of 2X Reaction Buffer [31.25% sucrose, 0.3125% CHAPS (3-[3-cholamido-propyl)-dimethammonio]-1propane-sulphonate), HEPES: 312.5 mM (pH 7.5 Mw: 238.31g/l)] (containing DTT and caspase 3/7 substrate DEVD-AFC) were added to each 25 μl sample in a 96-wells plate (Cliniplate flat bottom black). Samples were incubated for 1 hr in 37°C incubator. Fluorescent detection of protease activity was performed by means of a fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter. Fold-increase in caspase 3 activity was determined by comparing these results with the level of the control.

RNA interference

Sequence for p21 small interfering RNA (siRNA) molecules was 5'-CUU CGA CUU UGU CAC CGA GdTdT-3'(sense) and 5'-CUU ACG CUG AGU ACU UCG AdTdT-3'(anti-

sense). The Luciferase (Luc) gene served as control. The sequence for Luc RNA molecules were 5'-CUU ACG CUG AGU ACU UCG AdTdT-3' (sense) and 5'-UCG AAG UAC GCG UAA GdTdT3' (antisense). Tera-p21-ΔNLS cells were transfected in 6 well plates with 10 μl of 20μM siRNA duplexes using Oligofectamine reagent according to the manufacturer's instructions (Invitrogen BV, Breda, the Netherlands). After 24 hrs, cells were treated with cisplatin. 24hrs after the treatment cells were harvested for protein isolation. Alternatively, in order to perform an apoptosis assay, at 24hrs after transfection, cell were harvested and plated in 96-well plate. The day after, cells were treated with cisplatin.

Immunoprecipitation

A total of 10^7 Tera-p21-ΔNLS and Scha cells were cultured for 24 hours after cisplatin or gamma-irradiation treatment. Cells were harvested, washed with ice-cold phosphate-buffered saline (PBS), and subsequently lysed in 500 μl lysis buffer (20 mM Tris HCl pH 7.6, 150 mM NaCl, 0.2% NP-40, protease inhibitor COMPLETE®, 1 mM PMSF, 1mM NaF and 1 mM DTT) for 15 minutes on ice. Cell lysates were clarified at 10,000g for 15 minutes; protein concentration was equalized with Bradford, and incubated with 8 μl rabbit anti p21 antibody. After 16 hours rotating at 4°C, protein G Dynabeads® (Invitrogen BV, Breda, the Netherlands) was added and incubated for 2 hours at 4°C. Immunocomplexes were washed 5 times and eluted with 0.5 M Glycine.HCl pH 2.4, mixed 1:1 with standard 2x Western Blot sample buffer and examined by Western blot analysis as described above.

Facs analysis pp21

For FACS analysis of the levels of phosphorylated p21 TGCT cells were seeded in 6 wells plates and 24h after cisplatin or gamma-irradiation treatment the cells were collected, washed with PBS and fixed with Methanol/Aceton for 20 min at room temperature. Fixed cells were washed 2 times with 1% BSA in PBS followed by immunostaining with p-p21 antibody in 1% BSA in PBS and counterstained with Alexa-Fluor secondary antibody. Fluorescence intensity was detected by flow cytometry (FACS-Calibur; Becton Dickinson).

RESULTS

p21 is located in the cytoplasm in TGCT cell lines

The subcellular localization of p21 was investigated with immuno-fluorescence (IF) microscopy and by western blotting (WB) using fractionated protein samples. We stained

the TGCT cell lines Scha and Tera treated with cisplatin or gamma-irradiation and used the breast carcinoma cell line MCF-7 as a control for nuclear staining after chemotherapy treatment (Menendez et al., 2005; Panno et al., 2006). Both untreated TGCT cell lines as well as the breast carcinoma cell line MCF-7 (when untreated) show a cytoplasmic localisation of p21 (top panel Figure 1A-C), although the levels of p21 are much lower in TGCT and almost undetectable in Tera (WB Figure 1C). After cisplatin treatment, p21 remained localized in the cytoplasm of Scha and Tera cells, whereas in MCF-7 cells cisplatin treatment resulted in a more pronounced nuclear localization of p21 (Figure 1A-C, second panel). The cisplatin-resistant TGCT cell line Scha shows higher p21 levels compared to the cisplatin-sensitive TGCT cell line Tera (Figure 1D, 1C). Gamma-irradiation also induced accumulation of p21 in the cytoplasm in Scha and Tera (comparable to cisplatin treated MCF-7), but cellular p21 levels were much higher compared to those after treatment with cisplatin (Figure 1C).

Cytoplasmic p21 protects TGCT cell lines for cisplatin induced apoptosis

To study whether cytoplasmic p21 in TGCT cells is involved in suppression of cisplatin-induced apoptosis we stably transduced human TGCT cell lines Tera with a viral construct containing both p21-ΔNLS and GFP (Green Fluorescent Protein), or GFP only. The p21-ΔNLS protein lacks its bipartite nuclear localization signal and is therefore maintained in the cytoplasm. The cytoplasmic localisation of the p21-ΔNLS protein was confirmed by a positive staining for p21 in the cytoplasmic protein fraction (data not shown) as well as by IF (Figure 2A) and p53 expression and upregulation after cisplatin treatment is considered equal compared to the normal Tera cell line (data not shown).

After treatment with cisplatin (0, 16 and 32 μM, for 24 hours) Tera-p21-ΔNLS is less apoptotic compared to Tera-mock using an acridine orange apoptosis assay (Figure 2B). The same pattern was also observed by caspase 3 activity (Figure 2C) and by staining for active caspase 3 and PARP cleavage (data not shown). Despite the difference in p21 levels (Figure 2B inset) we noticed that the mock transduced cells also became less sensitive for cisplatin compared to non-transduced Tera cells. In order to define the change in sensitivity caused by the p21–ΔNLS we downregulated p21 using a p21 siRNA approach. Downregulation of p21-ΔNLS with siRNA in Tera-p21-ΔNLS rendered cells more apoptotic after cisplatin treatment compared to the same cell lines treated with siRNA against luciferase (Figure 3B). No effect of p21 siRNA on cisplatin-induced apoptosis was observed in Tera-mock (Fig 3A). The same pattern was also observed by caspase 3 activity

and by staining for active caspase 3 (Figure 3C, 3D) and PARP cleavage (data not shown).

P21 in complex with ASK1 (and CDK2)

Previously, we showed that in TGCT cells treated with gamma-irradiation p21 was not in a complex with caspase 3 (Spierings et al., 2004). Similarly, after treatment with cisplatin in Scha, Tera and Tera-p21-ΔNLS no complex formation of p21 and caspase 3 was detected (data not shown). Since it has been shown in cancers of haematopoietic origin that cytoplasmic p21 can associate with ASK1 (Schepers et al., 2003), we performed a coimmunoprecipitation with p21 and stained for ASK1 in Scha, Tera and Tera-p21ΔNLS. As shown in figure 4, ASK1 coprecipitated with the p21 antibody using lysates of Tera, Tera-p21-ΔNLS and Scha, indicating that ASK1 is in complex with p21 when untreated and after treatment with cisplatin or gamma-irradiation (Figure 4). Moreover we performed an IP with ASK1 and we were able to stain p21 in the precipitated fraction (data not shown).

Additionally it has been described that the cleavage of p21 and thereby activating the proapoptotic role of CDK2 plays a role in apoptosis induction. Therefore, we also stained the coimmunoprecipition for presence of CDK2 (Levkau et al., 1998; Jin et al., 2000; Adachi et al., 2001). Furthermore, consistent with the levels of precipitated p21 ASK1 and CDK2 are co-precipitated. In Scha and Tera-p21-ΔNLS, which have higher levels of (precipitated) p21 compared to Tera more ASK1 and CDK2 are co-precipitated, whereas in irradiated Tera and Scha similar levels of p21, ASK1 and CDK2 are precipitated. Moreover, IF shows that ASK1 is present in the cytoplasm, whereas CDK2 is present in both nucleus and cytoplasm in TGCT cells (data not shown).

p-Akt causes the cytoplasmic localisation of p21

The cytoplasmic localization of p21 can be caused by phosphorylation of Thr-145 (or Ser-146) located in the nuclear localization signal (NLS) of the p21 protein (Zhou et al., 2001; Rodriguez-Vilarrupla et al., 2002; Agell et al., 2006); upon phosphorylation of the NLS domain p21 becomes more stable and is translocated to the cytoplasm (Zhou et al., 2001; Harms et al., 2007). Therefore, we stained the TGCT cells for p-p21(Thr-145). In both untreated TGCT cell lines p-p21(Thr-145) is localized in the cytoplasm and the nucleus (data not shown). After cisplatin and irradiation treatment, p21 remained localized in the cytoplasm of Scha and Tera cells, whereas p-p21 is localised in both cytoplasm and nucleus (Figure 5A-C top panel). In order to find out whether phosphorylation of Thr-145 could

occur via p-Akt we stained the TGCT cells for p-Akt and its natural inhibitor PTEN. In both Tera and Scha p-Akt is clearly detectable, whereas PTEN is not expressed in these cells (data not shown). Following treatment with cisplatin or gamma-irradiation of TGCT cells p-Akt is induced (Figure 5C).

Treatment with the PI3K inhibitor LY294002 (10 μM for 24 hours and 50 μM for 2 hours) resulted in dephosphorylation of p-Akt (Figure 5C) and a reduction in phosphorylated p21(Thr145) (Fig 5A-C middle panel, Fig 5D), which is consistent with a more pronounced nuclear localisation of p21 in Scha and Tera (Figure 5A-C), while the levels of total p21 in Tera are lower compared to the levels in Scha (Fig 1D). Moreover the specific p-Akt inhibitor Triciribin (10 μM for 24 hours) resulted in complete blockage of Akt phosphorylation (Figure 5C), less phosphorylated p21 (Thr145) (Figure 5A-C bottom panel, Figure 5D light grey) and a more pronounced nuclear localization of p21 in Tera and Scha (Fig 5A-C). Furthermore, only in Scha the combined treatment with LY294002/Triciribin and cisplatin was accompanied by sensitization to cisplatin, whereas in both Scha and Tera sensitization to gamma-irradiation occurs (Fig 6). In addition, in Tera-p21-ΔNLS, lacking the phosphorylation site Thr145, treatment with the PI3K/p-Akt inhibitors had no effect on the cytoplasmic localisation of p21-ΔNLS and treatment with the inhibitors did not lead to sensitization for cisplatin (data not shown). Finally, we showed that in Scha cells less ASK1 and CDK2 is precipitated after combined treatment with 4 μM cisplatin and 50 μM LY294002, whereas the levels of precipitated p21 stays the same as when treated with cisplatin only (Figure 4).

DISCUSSION

In the present study we have demonstrated that cytoplasmic p21 in contrast to nuclear localized p21 protects TGCT cells against cisplatin and irradiation induced apoptosis. Furthermore, we found that phosphorylation of p21 by p-Akt is essential for p21 localisation in the cytoplasm. Dephosphorylation of Akt shifted p21 to the nucleus resulting in less p21 complex formation with ASK1 and CDK2 and resensitized TGCT cells to cisplatin and gamma-irradiation-induced apoptosis.

The cyclin-dependent kinase inhibitor p21 was first described as a protein controlling both the cell cycle as well as DNA replication. Additionally, p21 is implicated in many other biological processes, including differentiation, tumorogenesis and apoptosis. The functionality of many proteins is thought to be depending on the intracellular localization. Nuclear localisation of p21 has been linked to tumour suppressor activity, whereas cytoplasmic localization is considered oncogenic. Nuclear localized p21 is known to control cell cycle and DNA replication, whereas cytoplasmic p21 has been shown to result in activation of anti-apoptotic and proliferative machinery (Barboule et al., 1995; Erber et al., 1997; Dotto, 2000; Esteve et al., 2003). Recently, we demonstrated that gamma-irradiation in contrast to cisplatin treatment, massively induced p21 in the different TGCT cell lines, which was localized in the cytoplasm and resulted in resistance to anti-Fas induced apoptosis, despite a striking increase in Fas membrane expression. In turn, down-regulation of p21 by siRNA was able to restore the sensitivity of TGCTs to anti-Fas induced apoptosis (Spierings et al., 2004). Furthermore, we showed that cisplatin-induced apoptosis was mediated by activation of the Fas pathway (Spierings et al., 2003). In various other types of cancer cells, subcellular p21 localization has been implicated in preventing the induction of apoptosis following exposure to cytotoxic agents and ionizing radiation.

However, the mechanisms by which cytoplasmic p21 can prevent cells from cisplatin induced apoptosis are not well understood. One mechanism by which cytoplasmic p21 can inhibit apoptosis is by binding to procaspase 3, which consequently inhibits cleavage of procaspase 3 into the active form and preventing further amplification of the apoptotic signal (in HepG2 cells) (Suzuki et al., 1999; Suzuki et al., 2000). In both irradiated and cisplatin treated TGCT cell lines, however, caspase-3 was not coimmunoprecipitated with p21 (Spierings et al., 2004). An alternative explanation, recently demonstrated in haematological tumours, is the association of cytoplasmic p21 with apoptosis signal-regulating kinase 1 (ASK1) and thereby inhibiting ASK1-mediated apoptosis via the intrinsic mitochondrial apoptotic pathway (Asada et al., 1999); (Schepers

et al., 2003). In the present study we not only show complex formation between endogenously expressed p21 and ASK-1 but also show that elevated p21 levels increases ASK1/p21 association. ASK1 is known to activates the Jun N-terminal protein kinase and p38 pathways in response to Fas/FasL ligation, and DNA-damage inducing agents (Ichijo et al., 1997; Chang et al., 1998; Gotoh and Cooper, 1998). Moreover, overexpression of ASK1 triggers for apoptosis (Ichijo et al., 1997), whereas ASK1-deficient cells were resistant to TNF induced apoptosis (Tobiume et al., 2001). This indicates that the inhibition of ASK1 via cytoplasmic p21 may play an important role in cisplatin resistance in TGCT cells with high levels of p21. Another mechanism for p21 mediated apoptosis inhibition is the ability of p21 to bind to and thereby inactivate cyclin A/Cdk2 complexes. Here, we have not only shown complex formation between endogenously expressed p21 and CDK2 but also that elevated p21 levels resulted in enhanced CDK2/p21 association. Moreover, we have found that CDK2 is expressed in both the nucleus and cytoplasm of TGCT cells (data not shown). It has been shown that caspase 3 mediated cleavage of p21 is an important mechanism of cyclin A/Cdk2 activation associated with death in different cell types (Levkau et al., 1998: Jin et al., 2000; Adachi et al., 2001). Apoptosis induction by various stimuli appears to be mediated by caspase 3-induced cleavage of p21 and subsequent up-regulation of cyclin A/Cdk2 activity. The caspase-dependent Cdk2 activity may be involved in death-associated chromatin condensation, cell shrinking, and loss of adhesion to substrate (Harvey et al., 2000). Cdk2 activity is also associated with depolarization of mitochondrial membrane potential during apoptosis (Jin et al., 2003). Furthermore, wildtype HCT116 cells as well as p21-deficient HCT116 cell incubated with the specific CDK(2) inhibitor roscovitine were resistant for ionizing radiation (Sohn et al., 2006). Besides forming a complex with ASK1 or CDK2, cytoplasmic p21 can bind to Rho-kinase (ROCK) and inhibits its activity in vitro and in vivo (Tanaka et al., 2002; Lee and Helfman, 2004). In addition, ROCK is directly downstream of RhoA and both are involved in Fas-induced apoptosis (Subauste et al., 2000). Moreover, RhoA/ROCK modulation of actin enhances Fas/FasL clustering in lipid rafts and Fas receptor capping (Subauste et al., 2000; Gajate and Mollinedo, 2005; Soderstrom et al., 2005). Disruption of lipid rafts and interference with actin cytoskeleton have been shown to prevent Fas clustering and apoptosis (Gajate and Mollinedo, 2005). These data suggest that cytoplasmic p21 might inhibit the formation of Fas into lipid rafts and/or caps, thereby inhibiting Fas death receptor signalling. This would be in agreement with our previous findings in TGCT cells demonstrating that p21 expressing TGCT cells are less sensitive to Fas-mediated apoptosis

(Spierings et al., 2003: Spierings et al, 2004).

We found that inhibition of PI3K by LY294002 and direct inhibition of p-Akt by Triciribin resulted in dephosphorylation of Akt consistent with a more pronounced nuclear localisation of p21 and concomitant sensitization to cisplatin and/or gamma-irradiation in both Scha and Tera. The sensitizing effect of LY294002 and Triciribine was related to their effect on p21 localization, since no effect of these inhibitors was observed in cisplatin treated Tera-p21-ΔNLS cells. Previous studies in other cell types have demonstrated that phosphorylation of Thr-145 in the NLS site of p21 by p-Akt is important for shuttling towards the cytosol (Zhou et al., 2001; Rodriguez-Vilarrupla et al., 2002; Agell et al., 2006). In addition, the importance of p-Akt (and possibly p21) was also shown, by Rasoulpour et al, in mice in normal testis. They showed that germ cell apoptosis was increased 2-fold in Akt1-deficient mouse testes compared to wild-type mice (Rasoulpour et al., 2006). In addition, over expressing Akt in normal primordial germ cells leads to the development of embryonic germ cells and testicular germ cell cancer (Kimura et al., 2008). The presence of activated p-Akt and consequently cytoplasmic localized p21 may be due to the loss of PTEN expression in Tera and Scha. PTEN activity can prevent the phosphorylation of Akt in a variety of human tumours and cell lines (Haas-Kogan et al., 1998 Bruni et al., 2000). Moreover, it has been shown that the inactivation of PTEN leads to the development of testicular germ cell cancer in heterozygous mice (Suzuki et al., 1998; Podsypanina et al., 1999; Kimura et al., 2003; Di Vizio et al., 2005) and normal primordial germ cells (Kimura et al., 2003; Moe-Behrens et al., 2003). In addition, Di Vizio et al. demonstrated that loss of PTEN expression and consistently activated p-Akt is implicated in the development of human TGCT, since normal testis and intratubular germ cell neoplasias intensely express PTEN (Di Vizio et al., 2005). Although both p53 and PTEN are the two most commonly mutated tumour suppressor genes in human cancer it is rare to find them both mutated in the same tumour (Kurose et al., 2002). When PTEN is deleted, mutated or otherwise inactivated, activation of PI3K effectors particularly the activation of Akt can occur in the absence of any exogenous stimulus (Eng, 2003); (Cully et al., 2006). The importance of growth receptor dependent Akt phoshorylation for cytoplasmic localization of p21 in relation to survival has been determined in other types of cancer. These studies reported that cytoplasmic p21 expression was associated with poor survival (Winters et al., 2001); (Winters et al., 2003); (Xia et al., 2004); (Ping et al., 2006); (Perez-Tenorio et al., 2006); (Di Pietro et al., 2007). Whether upregulation or activating mutations in PI3K and or tyrosine kinase membrane receptors like KIT, PDGF, ERBB2 (Palumbo et

al., 2002); (Rapley et al., 2004); (McIntyre et al., 2005); (Goddard et al., 2007) also play a role in the phosporylation of Akt in TGCT needs to be further elucidated.

In conclusion, p-Akt-dependent elevation of cytoplasmic p21 levels functions as an antiapoptotic factor in cisplatin-resistant TGCT cells. Further studies are necessary to identify druggable molecular targets that act upstream of p-Akt.

Figures and Figure Legends to Chapter 4

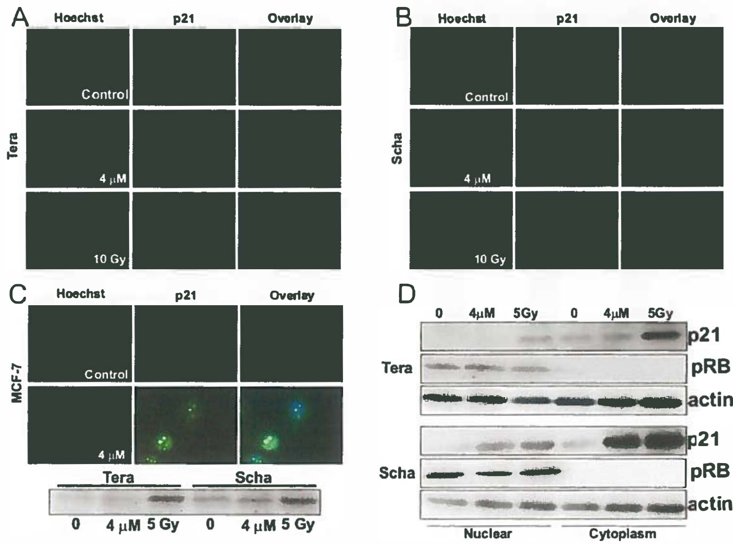

Figure 1

p21 is located in the cytoplasm in TGCT cell lines.

Localisation of p21 was determined using immuno-fluorescence using the MCF-7 cell line as control. After 24 h. cisplatin incubation p21 is localised in the nucleus in MCF-7 cells (C). In both Tera (A) and Scha (B) p21 is localised in the cytoplasm when untreated and after cisplatin/irradiation treatment. After 24 h, nuclear and cytoplasmic proteins were isolated and analysed by WB for expression of p21 using pRB as nuclear control (D) whereas actin is shown as a loading control. A representative example of three independent experiments is shown.

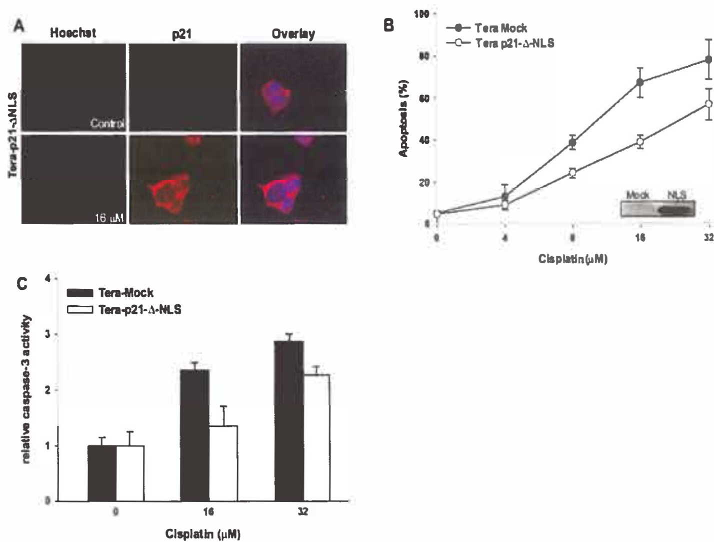

Figure 2

Upregulation of cytoplasmic p21 (ΔNLS) protects against cisplatin induced apoptosis.

p21(-ΔNLS) is localised in the cytoplasm of Tera-p21-ΔNLS cells when untreated or treated with cisplatin (A). Apoptosis induction was analysed by fluorescence microscopy on acridine orange stained cells (B) and caspase-3 activation with a fluorescence assay (C) in Tera Mock and Tera-p21-ΔNLS cells following treatment with cisplatin for 24 h. Values are the mean ± SD of three experiments.

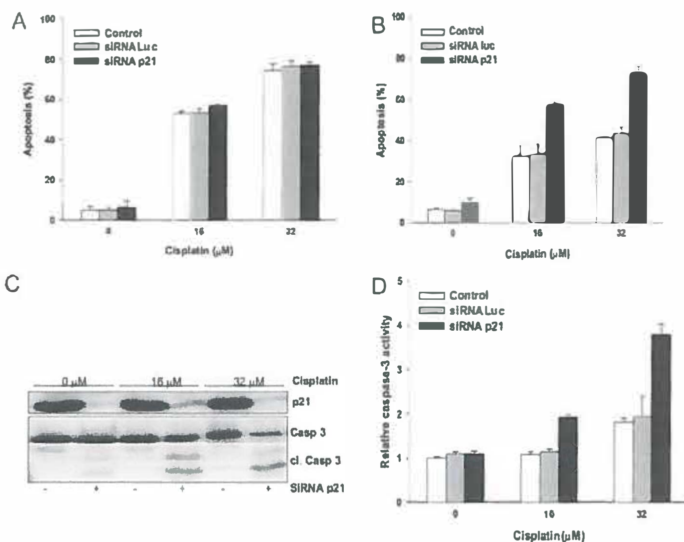

Figure 3

Downregulation of p21-ΔNLS resensitizes for cisplatin induced apoptosis.

Cells were either untreated with siRNA (control), with p21 siRNA (siRNA p21) or luciferase siRNA (siRNA luc). Following siRNA treatment for 24 h, cells were treated with cisplatin for an additional 24 h. Apoptosis induction in Tera Mock (**A**) and apoptosis induction in Tera-p21-ΔNLS (**B**). Western blot analysis showing downregulation of p21-ΔNLS and cleavage of caspase-3 (**C**) and caspase-3 activity in Tera-p21-ΔNLS after treatment with siRNA p21(**D**). Values are the mean ± SD of three experiments.

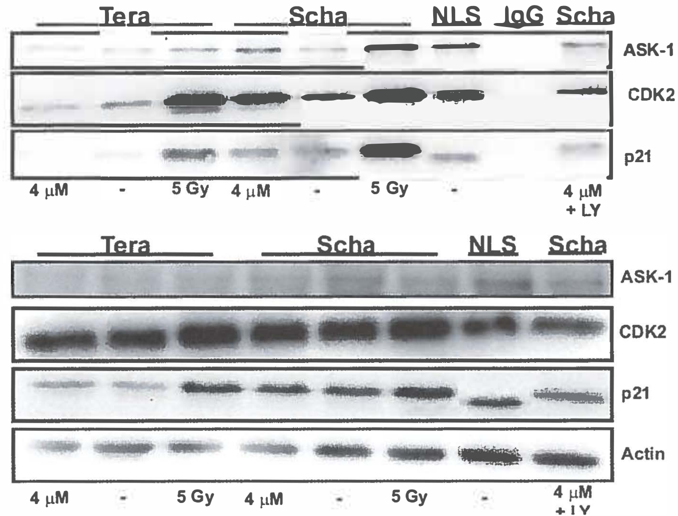

Figure 4

Complex formation of p21 with CDK2 and ASK1.

TGCT cells (10^7) were cultured after treatment with cisplatin or irradiation for 24 h as indicated. Cell lysates were subjected to p21 immunoprecipitation (IP), using a rabbit a-p21 antibody, conjugated to protein G Dynabeads®. Immunoblotting was performed using a-p21, a-CDK2 and a-ASK1 antibodies. The data presented are representative of three individual experiments.

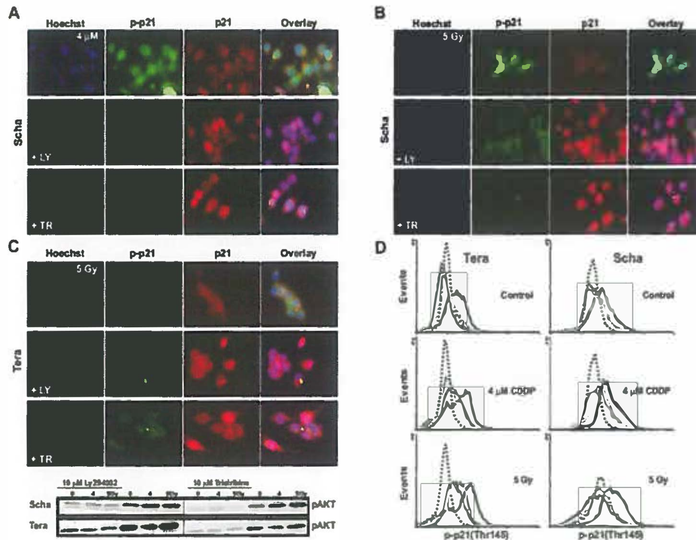

Figure 5

Dehosporylation of p21 and nuclear localisation of p21 after combined treatment with LY294002 or Triciribine.

After 24 hours of treatment with cisplatin or irradiation in combination with either 10 µM LY294002 or 10 µM Triciribine phosphorylated levels of p21(Thr145) decreases, whereas p21 is more pronounced localised in the nucleus of Scha treated with cisplatin (**A**); Scha treated with irradiation (**B**) and Tera treated with irradiation (**C**). Also dephosporylation of pAkt occurred after treatment with Ly294002 or Triciribine in Scha (**C**). FACS analysis of levels of p-p21 (Thr145) show dephosphorylation of Thr145 in both Scha en Tera cell lines after treatment with either Ly294002 (dark grey) or Triciribine (light gray) compared to not treated (black; IgG control in dotted black) (**D**).

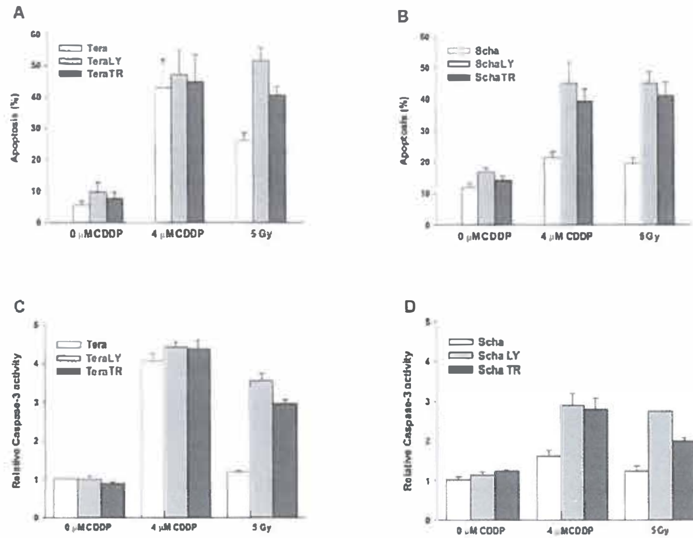

Figure 6

Relocalisation of p21 caused by dephosporyalation of p-p21/pAkt by LY294002 /Triciribine treatment sensitises for DNA-damage induced apoptosis.

After 24 hours of treatment with cisplatin or irradiation in combination with either 10 μM LY294002 or 10 μM Triciribine apoptosis induction was analysed in Tera (**A**) and Scha (**B**). Also Caspase-3 activity was analysed using the same conditions in Tera (**C**) and Scha (**D**). Values are the mean ± SD of three experiments.

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Chapter 5

Role of p16^{ink4} in cisplatin and γ-radiation induced apoptosis in Testicular Germ Cell Tumours

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Abstract

Background: A mutual exclusive role for p16^{ink4} (p16) and p21$^{Waf1/Cip1}$ has been observed in some cancers. Previously, we found that p21$^{Waf1/Cip1}$ has an important function in cisplatin resistance in human testicular germ cell tumour (TGCT) cell lines. In the present study, we investigated if p16 and p21$^{Waf1/Cip1}$ are mutual exclusively expressed using p21$^{Waf1/Cip1}$-positive and p21$^{Waf1/Cip1}$-negative TGCT cell lines and patient samples. Furthermore, we investigated whether p16 is involved in the response to cisplatin and γ-radiation treatment of TGCT cells.

Methods: Human TGCT cell lines Scha (p21$^{Waf1/Cip1}$ -positive and cisplatin-resistant), and 833KE (p21$^{Waf1/Cip1}$-negative and cisplatin-sensitive) were treated with cisplatin or γ-radiation and changes in expression of p16 and p21$^{Waf1/Cip1}$ were monitored. The role of p16 was further investigated by p16 suppression with small interfering (si)RNA. In addition, primary tumour histological samples from 34 testicular cancer patients belonging to different IGCCCG prognosis groups and affected from different histological tumour subtype were stained for p16 and p21$^{Waf1/Cip1}$ using immunohistochemistry.

Results: Expression of p16 was observed in the p21-negative 833KE cell line but not in Scha. Down-regulation of p16 did not affect DNA damage-induced p53 expression or expression of p53-transcription activated proteins, such as p21$^{Waf1/Cip1}$. Moreover, p16 suppression had no effect on cisplatin- or γ-radiation-induced apoptosis in 833KE cells. We found positive staining patterns for p16 in 16/21 embryonal carcinoma, 3/5 yolk sac, 5/6 choriocarcinoma, 16/17 teratoma and 0/7 seminoma components, while p21$^{Waf1/Cip1}$ positive staining was observed in 0/21 embryonal carcinoma, 0/5 yolk sac, 6/6 choriocarcinoma, 17/17 teratoma and 0/7 seminoma components.

Conclusions: The positive staining for p16 and not for p21$^{Waf1/Cip1}$ in embryonal carcinoma and yolk sac components of TGCTs suggests mutually exclusive expression. Suppression of p16, however, had no functional consequence on DNA damage-induced apoptosis in an embryonal carcinoma cell model.

INTRODUCTION

Testicular Germ Cells Tumours (TGCT) are extremely sensitive to chemotherapy. The cure rate reaches up to 99% in the early stages of non-seminomatous tumours. In metastatic disease the cure rate ranges from 90% in patients with good prognostic criteria, to 75%-80% in patients with intermediate and to 50% in patients with poor prognostic criteria [Krege et al., 2008a; Krege et al., 2008b; Schmoll et al., 2004; Shelley et al., 2002]. Despite the general success of TGCTs treatment, 10-20% of patients diagnosed with metastatic disease will not achieve a durable complete remission after initial treatment, either due to an incomplete response to chemotherapy or a tumour relapse [Gori et al., 2005; Jones and Vasey, 2003a; Jones and Vasey, 2003b; Oosterhof and Verlind, 2004]. For this reason research on TGCTs could serve to reveal factors involved in chemotherapy and radiotherapy sensitivity and resistance.

TGCTs are biologically characterised by an imbalance in the G1/S cell cycle transition while they have a wild type (wt)-p53 [Bartkova et al., 2000]. The main regulators of the G1/S cell cycle transition are cyclin-dependent kinase inhibitors (CDKIs) such as $p21^{Waf1/Cip1}$ and $p16^{ink4}$ (p16). In a previous *in vitro* study, we demonstrated the importance of low levels of $p21^{Waf1/Cip1}$ for cisplatin sensitivity (chapter 3 & 4). In TGCT cell lines, cisplatin treatment increased p53 and Mdm2 expression and concomitantly high levels of apoptosis, whereas minimal $p21^{Waf1/Cip1}$ induction occurred. Irradiation of these TGCT cells, however, induced p53 and Mdm2 expression but also high levels of $p21^{Waf1/Cip1}$, whereas almost no apoptosis was found [Spierings et al., 2004]. It might be possible that the lack of $p21^{Waf1/Cip1}$ in basal conditions and after cisplatin treatment is due to mutually exclusive expression of p16 and $p21^{Waf1/Cip1}$, which can be overcome by γ-radiation. P16 belongs to the INK4 family. The INK4/ARF locus encodes for the CDKI proteins p16 and p14/Arf, both affecting cell cycle. P16 acts as inhibitors of CDK4 and CDK6, while p14/Arf inhibits MDM2 which results in p53 activation and thus $p21^{Waf1/Cip1}$ expression [Quelle et al., 1995; Quelle et al., 1997; Sherr, 2001]. *INK4a* and *p14/Arf*, although sharing two out of three exons (p16: 1α, 2, 3; p14: 1β, 2, 3), have separate promoters. They produce different transcripts using different open-reading frames [Duro et al., 1995; Mao et al., 1995; Quelle et al., 1995; Quelle et al., 1997]. The INK4/ARF locus is a sensor of oncogenic stresses, as p16 and p14/Arf are usually present at extremely low levels in normal tissues.

Although p21$^{Waf1/Cip1}$ and p16 exert their control on the same G1/S transition phase of the cell cycle, their actions differ from each other. P16 inhibits cyclinD/CDK4 activity, while p21$^{Waf1/Cip1}$ inhibits cyclin E/CDK2 activity [Serrano, 2000; Sherr, 2001]. Inhibition of the activity of cyclin/CDK complexes reduces Rb phosphorylation, which will result in a Gl arrest. P16 is not constantly expressed during the cell cycle [Sherr, 2001; Voorhoeve and Agami, 2003], but induced by DNA stressing events. A recent study in human cervical cancer cells showed that p16 silencing resulted in an upregulation of p53, p21 and RB. Furthermore, p16 knock-down sensitized the cervical cancer cells to cisplatin-induced apoptosis [Lau et al., 2007]. So far, p16 protein expression represents an aspect still poorly investigated in TGCTs. Cyclin D2 is frequently overexpressed, whereas Rb and p21$^{Waf1/Cip1}$ are almost not expressed in TGCTs [for a review see Di Pietro et al., 2005]. Considering the fact that cisplatin-sensitive TGCT cell lines express very low levels of p21$^{Waf1/Cip1}$ [Spierings et al., 2004], it is of interest to investigate p16 expression in TGCT and p16-related responses in TGCT cell lines after cisplatin and irradiation treatment. In the present study, we have analysed the role of the imbalance between p21$^{Waf1/Cip1}$ and p16 as one of the potential mechanisms responsible for TGCTs sensitivity to cisplatin.

MATERIALS AND METHODS

Culture media and chemicals

RPMI 1640 medium was obtained from Gibco (Paisley, Scotland) and foetal calf serum from Bodinco BV (Alkmaar, the Netherlands). Cisplatin was purchased from Bristol-Myers Squibb (Woerden, The Netherlands). 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (Saint Louis, MO, USA).

Cell lines

For the cell lines used in this study, origin and pre-treatment were described by Sark et al [Sark et al., 1995].The human germ cell tumour cell lines 833KE and Scha and the human ovarian cancer cell line A2780, used as a control for different proteins, were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum at 37°C in a humidified atmosphere with 5% CO_2. 833KE grew attached and was harvested by treatment with protease XXIV 0.005% for 3 min. A2780 and Scha were harvested by scraping. Human HeLa cervical cancer cells were grown at 37°C in a humidified atmosphere with 5% CO2 in 1:1 DMEM/HAM medium supplemented with 10% FCS. Cells were detached with 0.05% trypsin/0.5 mM EDTA in phosphate-buffered saline (PBS, 0.14 mM NaCl, 2.7 mM KCl, 6.4 mM Na2HPO4. 2 H2O, 1.5 mM KH2PO4, pH 7.4).

Cisplatin and γ-radiation treatment

Exponentially growing cell cultures were plated 1:1 in flasks the day before the treatment. Different concentrations of cisplatin were added to the flasks and subsequently cultured for 24 hrs in a humidified atmosphere at 37° C and 5% CO_2. Alternatively, cells were γ-radiation with a ^{137}Cesium γ-ray machine (IBL 637, CIS Bio International Gif/Yvette, France) with a dose rate of 0.9 Gy/min and subsequently cultured for 24 hrs in a humidified atmosphere at 37° C and 5% CO_2.

Western blotting

SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli. After 24 hrs cisplatin incubation cell were harvested and washed twice with cold phosphate buffered saline (PBS: 6.4 mM Na2HPO4; 1.5 mM KH2PO4; 0.14 mM NaCl; 2.7 mM KCl; pH = 7.2). Cells were lysed with standard Western blot sample buffer (50 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-β-mercaptoethanol) and boiled for 5 min. Proteins concentration was determined according to Bradford. All samples were sized fractionated by electrophoresis on SDS-polyacrylamide gels. Proteins were then transferred from the SDS-acrylamide gel onto PVDF membranes (Millipore, Bedford, UK) at 250 mA (150 mA with p21) for 1 hr at room temperature using a semi dry-blot system.

After blocking 1 hr in TRIS-buffered saline supplemented with 5% milk powder (Merck, Darmstadt, Germany) and 0.05% Tween-20 (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands), immunodetection of p53, $p21^{Waf1/Cip1}$, and p16, was performed with the following antibodies: anti-p53-DO-1 (clone sc-126; Santa Crutz Biotechnology, Santa Cruz, CA, USA), mouse anti- $p21^{Waf1/Cip1}$ (Transduction Laboratories, Lexington, Ky USA), mouse anti-$p16^{INK4a}$ Ab-1 (clone: DCS-50.1/A7; Neomarkers, Lab Vision Corporation, Fremont, CA, USA). The Rb protein was detected with mouse monoclonal pRb antisera (IF8) and cyclin D2 with polyclonal rabbit cyclin D2 antisera (C-17) from Santa Cruz Biotechnology (Santa Cruz, Santa Cruz, CA, USA). The antibody binding was determined using horseradish peroxidase (HRP)-conjugated secondary antibodies (all from Dako, Glostrup, Denmark) and visualised with the POD chemoluminescence kit by Roche Applied Science. Chemiluminescence was detected with the BM Chemiluminescence detection kit (Roche Applied Science, Woerden, the Netherlands). Membranes were exposed to Kodak X-OMAT films. Equal protein loading was checked for with Ponceau S and ß-actin staining.

Apoptosis quantified with M30 immunocytochemistry and acridine orange staining

For apoptosis measurements cells were plated in 24-well culture plates. Cells were continuously incubated with cisplatin at 4 µM for 24 hrs or irradiated with 10 Gy and cultured for an additional 24 hrs before cells were harvested. For immunocytochemical analysis of apoptosis induction, the mouse monoclonal M30 antibody was used, which

detects a caspase-cleaved product of cytokeratin 18. Cytospins were fixed with acetone at room temperature for 10 min. Slides were subsequently dried at room temperature. Then, slides were incubated with 0.1% Triton X100 /PBS for 10 min. Subsequently, slides were washed and incubated for 1 hr with the primary anti-M30 antibody (Roche Diagnostic GmbH, Mannheim, Germany). A secondary peroxidase-conjugated rabbit-anti-mouse antibody followed by a tertiary peroxidase-conjugated goat-anti-rabbit antibody (Dako) were used. AEC (3-amino-9-ethylcarbazol) was used as chromagen to visualize peroxidase activity. Counterstaining was performed with haematoxylin. M30 positivity was identified as brown cytoplasmic staining. The positivity was scored as the percentage of positive stained cells on the total number of cells per field. At least 2 different fields were scored and in total 400 cells were counted. In addition, acridine orange fluorescent staining of nuclei in unfixed cells was used to distinguish apoptotic from vital cells [Timmer-Bosscha et al., 1998]. Results are expressed as the percentage of apoptotic cells by counting at least 300 cells per well.

RNA interference

Sequence for p16 small interfering RNA (siRNA) molecules was 5'.CGCACCGAAUAGUUACGGUdTdT.3' (sense) and 5'.ACCGUAACUAUUCGGUGCGdTdT.3' (anti-sense). The double-stranded RNA Negative-control (siRNA-Neg) (Eurogentech S.A., Seraing, Belgium) served as control. Scha and 833KE (0.4 x 10^6/ well) were transfected in 6 well plates with 10 µl of 20 µM siRNA duplexes using Oligofectamine reagent according to the manufacturer's instructions (Invitrogen BV, Breda, the Netherlands). After 24 hrs, cells were treated with cisplatin for 24 hrs. Thereafter the cells were harvested for protein isolation. Alternatively, in order to perform an apoptosis assay, at 24 hrs after transfection, cells were harvested and plated in 96-well plates. The day after, cells were exposed to cisplatin. At 24 hrs after the treatment, the percentage of apoptotic cells was determined by acridine orange apoptosis assay.

Patients

A group of 34 TGCT patients treated with BEP-chemotherapy at the University Medical Center Groningen, the Netherlands between January 2004 and November 2006, was selected [De Haas et al. 2008]. Single component or multi-component primary tumours from these 34 patients with TGCT were used to represent all histological subytypes of the primary tumour (Embryonal Carcinoma = EC: Yolk Sac Tumour = YS; Choriocarcinoma = ChC; Mature Teratoma = MT and Immature Teratoma =IT; Seminoma = S). Furthermore, the 34 TGCTs belonged to each of the three prognosis groups (good, intermediate and poor) according to the IGCCCG classification [Schmoll et al., 2004]. The studies were approved by the medical ethical committee of the UMCG and written informed consent was obtained from each participant.

Immunohistochemical staining for p21$^{Waf1/Cip1}$, p16 and p14/Arf

Representative paraffin-embedded tumour material was collected for each patient,. To perform immunohistochemical staining 3 μm sections were cut and placed on APES-coated slides, deparaffinised and dehydrated. The presence of TGCT tissue was confirmed by two independent investigators (A.J.H.S. and A.d.P.) using standard haematoxylin-eosin staining. For p21$^{Waf1/Cip1}$, p16 and p14/Arf staining, sections were preheated by microwave for 8 min at 700 W in citrate buffer (0.1 M, pH 6.0) followed by blocking of endogenous peroxidase activity (30 min 3% H_2O_2). Slides were afterwards incubated with avidin and biotin blocking solution for 15 min twice (Vector Laboratories, Burlingame, CA, USA). Subsequently, slides were incubated for 1 hr with the primary antibody respectively p16 (BD Pharmingen, Breda, the Netherlands), p14/Arf (Lab Vision Corporation) and p21$^{Waf1/Cip1}$ antibody (Calbiochem, San Diego CA, USA). A secondary peroxidase-conjugated rabbit-anti-mouse antibody followed by a tertiary peroxidase-conjugated goat-anti-rabbit antibody (Dako) was used. Finally, DAB was used as chromagen to visualize peroxidase activity. Counterstaining was performed with haematoxylin. Immunoglobulin-class-matched control sera were used as negative controls. Normal skin and normal colon slides were used as positive control for p21$^{Waf1/Cip1}$. Normal skin slides and paraffin-embedded Scha cells served as negative control for p16 and paraffin-embedded 833KE cells as positive control for p16. Paraffin-embedded 833KE and Scha cells served as

negative controles for p14/Arf and normal prostate slides and paraffin-embedded HeLa cells as positive control for p14/Arf.

Evaluation of staining was performed by two investigators without knowledge of the clinical data. Different tumour component was scored separately for each patient. For all stainings the nuclear and cytoplasmic positivity were scored separately. P21 positivity was identified as brown nuclear and/or cytoplasmic staining. Tumours showing less than 10 positive cells/field were considered negative (-). Samples showing $p21^{Waf1/Cip1}$ positive stained tumour cells were considered + or ++ in the presence of focal or diffuse positivity, respectively. As to p16, positivity was defined as a brown cytoplasmic staining. Tumours showing positivity in less than 10 cells/fields were considered negative (-). A weak diffuse and a strong diffuse cytoplasmic staining were scored as + and ++, respectively. Heterogeneity and pattern of staining regarding each tumour component were also recorded. Morphological characteristics including the presence of apoptotic bodies, nuclear condensation, cytoplasmic shrinkage and membrane blebbing were assessed in the haematoxylin-eosin stained tissue.

RESULTS

$p21^{Waf1/Cip1}$ and p16 expression in TGCT cell models

The humanTGCT cell lines 833KE and Scha contained wild-type *p53*, while basal p53 protein levels were slightly higher in 833KE compared to Scha (Fig. 1). P53 is a transcriptional activator of $p21^{Waf1/Cip1}$, but expression of $p21^{Waf1/Cip1}$ was only observed in Scha cells (Fig. 1). Since $p21^{Waf1/Cip1}$ and p16 are two of the main negative regulators of cell cycle progression, both exerting a control on G1/s transition, we investigated whether $p21^{Waf1/Cip1}$ expression was inversely correlated with p16 expression. Western blot analysis showed that p16 is constitutively present in 833KE but undetectable in Scha (Fig 1). The human ovarian cancer cell line A2780, expressing wild-type p53, was used as control and expressed both $p21^{Waf1/Cip1}$ and p16. Since the INK4/ARF locus encodes both p16 and p14/Arf, we have stained for p14 but no expression was detected in Scha and 833KE, whereas a clear band was visible in HeLa, serving as the positive control (results not shown). Low expression of pRb was observed in 833KE and Scha compared to A2780. A larger band that can be seen in A2780 lysates indicates phosphorylated pRb, which was almost not detectable in the TGCT cells.

Influence of p16 down-regulation on treatment responses

In a previous study, we have demonstrated the inhibitory role of p21 in cisplatin or γ-radiation–induced apoptosis. To investigate the role of p16 in DNA damage response, cells were treated with cisplatin (4 μM) or γ-radiation treatment (10 Gy). Both treatments resulted in enhanced expression of p53 (Fig 2, lanes indicate with no). Cisplatin treatment increased the expression of $p21^{Waf1/Cip1}$ only in Scha. On the contrary, under γ-radiation treatment both cell lines showed an increased expression of $p21^{Waf1/Cip1}$. Neither cisplatin nor γ-radiation treatment induced a change in p16 expression in 833KE cells or Scha cells. In untreated cells, cyclin D2, a cyclin controlling the G1/S cell cycle transition, was exclusively detectable in Scha. Especially γ-radiation treatment induced cyclin D2 expression with the strongest increase observed in Scha. Drug-induced changes in cyclin D2 expression showed a similar pattern as drug-induced changes in $p21^{Waf1/Cip1}$ expression. Since cyclin D2 and $p21^{Waf1/Cip1}$ can form stable complexes resulting in enhanced activity of the cyclinD2/CDK4 complex (Kehn 2004), the drug-induced expression of $p21^{Waf1/Cip1}$ may thus result in concomitant stability of cyclin D2.

Next, we studied the functionality of p16 using a p16 siRNA approach. Successful knock-down of p16 in 833KE was demonstrated with Western blot comparing lanes indicated with scr versus p16 (Fig 2). Down-regulation of p16 did not affect p53 or $p21^{Waf1/Cip1}$expression, while p16 suppression in cells did not have any additional effect on cisplatin or γ-radiation induced p53 or $p21^{Waf1/Cip1}$ expression levels (Fig 2). Following p16 down-regulation, the DNA damage induction of cyclin D2 was slightly less in 833KE cells, suggesting an effect of p16 knock-down on cells in the G1/S transition phase.

When p16 suppressed 833KE cells were treated with cisplatin or γ-radiation, no major effect on apoptosis was observed after 24 hrs using M30 immunocytochemistry (Fig 3). A difference in sensitivity to cisplatin versus γ-radiation was observed in 833KE, which is probably due to anti-apoptotic function of $p21^{Waf1/Cip1}$. Following cisplatin treatment no $p21^{Waf1/Cip1}$ was detectable in 833KE cells, which is in agreement with our previous findings [Spierings et al.; 2004]. In Scha cells, p16 siRNA had no major effect on apoptosis as expected (Fig 2). Similar results were found with the acridine orange assay (results not shown). In addition, we have investigated the effect of p16 knock-down in 833KE cells on apoptosis following treatment with a low concentration of cisplatin (1 μM) for 72 hrs, but still no changes in cisplatin-induced apoptosis were observed (results not shown).

In conclusion, p16 does not have a crucial role in apoptosis regulation and its presence does not affect expression of p21$^{Waf1/Cip1}$.

Quantification of p21$^{Waf1/Cip1}$ and p16 immunohistochemistry

In order to investigate expression patterns of p21$^{Waf1/Cip1}$, p16 and p14/Arf in TGCTs, we stained 933KE and Scha cells and 34 pure or multiple component samples from TGCT patients for these proteines. p21$^{Waf1/Cip1}$ stained positive for Scha cells but not for 833KE cells (results not shown). P16 immunohistochemical staining showed strong positive staining in 833KE cells and not in Scha cells demonstrating the specificity of the p16 staining (Fig 4). In addition, we have attempted to establish a p14/Arf immunohistochemical staining. This resulted in p14/Arf positive staining in paraffin-embedded 833KE and Scha cells, whereas both cell lines were negative for p14/Arf on Western blot (results not shown). Therefore, we only performed p21$^{Waf1/Cip1}$ and p16 staining on TGCT patient samples.

The data for p21$^{Waf1/Cip1}$ and p16 staining are summarized in Table 1. P21$^{Waf1/Cip1}$ positivity, mainly localised in the nucleus, was observed in choriocarcinoma and mature/immature teratomas in both pure and multiple component TGCTs (Fig 4). No staining was detected in seminoma, embryonal carcinoma or yolk sac tumor components. P16 positivity, predominantly localised in the cytoplasm of cells, was observed in all TGCT components except for seminomas. The strongest p16 staining was found in mature/immature teratomas. No relation was found between p21$^{Waf1/Cip1}$ or p16 staining and prognosis group. Taken together, these results show that embryonal carcinoma components are negative for p21$^{Waf1/Cip1}$, while p16 expression was clearly detectable in the majority of embryonal carcinoma components, which may indicate mutual exclusive expression. In contrast, in mature/immature teratomas, strong positive staining of both p21$^{Waf1/Cip1}$ and p16 was observed. In the teratoma component, the presence of these proteins is related to the degree of differentiation.

DISCUSSION

In the present study, we investigated the expression of p16 and $p21^{Waf1/Cip1}$ in TGCT and the mutual exclusive role of p16 and $p21^{Waf1/Cip1}$ in response to cisplatin and γ-radiation treatment in TGCT cell models. Basal expression of $p21^{Waf1/Cip1}$ was observed in the cisplatin-resistant Scha cells, whereas p16 was only expressed in cisplatin-sensitive 833KE cells. Expression of p16, however, had no functional consequence for cisplatin or -radiation-induced apoptosis in an embryonal carcinoma cell model as we demonstrated with an siRNA approach. Moreover no effect of p16 knock-down was seen on $p21^{Waf1/Cip1}$expression. In patient samples, we found positive staining patterns for p16 in embryonal carcinoma components that were negative for $p21^{Waf1/Cip1}$staining, suggesting mutual exclusive expression. However, in other TGCT components such as teratoma, both p16 and $p21^{Waf1/Cip1}$stainings were seen in most cases.

In the present study we found that γ-radiation resulted in a massive induction of $p21^{Waf1/Cip1}$ in both p16-positive 833KE cells and in p16-negative Scha cells, whereas cisplatin treatment minimally induced $p21^{Waf1/Cip1}$ in Scha cells. Cisplatin as well as γ-radiation treatment enhanced p53 expression in both cell lines. These results are in agreement with our previous studies [Spierings, et al., 2004; Chapter 3 & 4]. The strong upregulation of p53 or $p21^{Waf1/Cip1}$ following different treatments allowed us to investigate the relation between p53 or $p21^{Waf1/Cip1}$ expression and p16 expression. However, changes in p53 or $p21^{Waf1/Cip1}$ expression had no effect on p16 expression levels. In addition, p16 knock-down did not have any effect on p53 or $p21^{Waf1/Cip1}$ expression levels either. Moreover, cisplatin and γ-radiation induced apoptosis in TGCT cells was not affected by p16 suppression. Following treatment with cisplatin or γ-radiation, expression of cyclin D2 was induced. Only under these circumstances an effect of p16 knock-down in 833KE cells was observed, showing a diminished increase in cyclin D2 expression. This may reflect an effect of p16 knock-down on cell cycle with less cells accumulating in the G1/S transition phase or an effect of p16 on stability of cyclin D2 in these cells. Because no effect on apoptosis was found, we have not further investigated this finding. Our p16 knock-down results are in contrast with observations in other cancer cell types [Al Mohanna et al., 2007;Lau et al., 2007]. In SiHa cervical cancer cells, the down-regulation of p16 led to an increase in apoptosis levels after cisplatin and UV-irradiation treatment. At the protein level, the down-regulation of p16 corresponded to a strong induction of p53 and its down-stream transcriptional target, $p21^{Waf1/Cip1}$ [Lau et al., 2007]. An important explanation for the minimal effect of p16 knock-down in our study could be the absence or low expression of Rb protein in TGCT cell lines and patient samples [Strohmeyer

et al., 1991; Barthkova et al., 2003]. In a previous study, we found no effect on cell cycle distribution after γ-radiation, despite the massive induction of p21$^{Waf1/Cip1}$, again indicating that CDKIs are not involved in cell cycle control [Spierings et al., 2004]. Since we observed p16 and p21$^{Waf1/Cip1}$ staining predominantly in the cytoplasm of 833KE and Scha cells [present study; chapter 4], respectively, while Rb expression was low, these results suggest that these CDKIs do not play a major role in cell cycle control of TGCT cells.

Previously, we demonstrated that cisplatin-sensitive TGCT cells express very low levels of p21$^{Waf1/Cip1}$, while in cisplatin-resistant TGCT cells enhanced expression of cytoplasmic p21$^{Waf1/Cip1}$ is involved in cisplatin resistance [chapter 3 and 4]. In order to investigate, whether p16 and p21$^{Waf1/Cip1}$ cytoplasmic staining is also present in the clinical setting, we analysed 34 surgical samples from patients with testicular cancer. In these TGCT samples p21$^{Waf1/Cip1}$ was not detectable in embryonal carcinoma components, while teratoma components of TGCT were positive for p21$^{Waf1/Cip1}$ staining. Staining of p21$^{Waf1/Cip1}$ in teratoma was predominantly observed in the nucleus, which is in agreement with previous reports [Bartkova et al., 2000; Mayer et al., 2003, Juric et al., 2005]. Moreover, teratomas in contrast to EC express Rb [Bartkova et al., 2003] suggesting a putative role for p21$^{Waf1/Cip1}$ in cell cycle control in these non/slow growing components. In contrast, p16 was predominantly expressed in the cytoplasm of most embryonal carcinoma, yolk sac and teratoma components with the highest positivity observed in the latter, similar to a previous report [Bartkova et al., 2003] The presence of p16 in the cytoplasm has been described in other cancer cell lines and tumors [Evangelou et al., 2004; Nilsson et al., 2006]. It was shown that phosphorylation of p16 is involved in shuttling to the cytoplasm, where p16 binds to CDK4/6 suggesting functional properties of cytoplasmic p16 [Nilsson et al., 2006].

Our study was not designed to find a correlation between the prognosis group and the pattern of p16 and p21$^{Waf1/Cip1}$ positivity taking in account the few patients belonging to the poor prognosis group and the heterogeneity of the TGCT, i.e. the various components that can be present in a TGCT sample. Remarkably, p21$^{Waf1/Cip1}$ and p16 positive staining was observed in different cellular compartments of teratomas, with p21$^{Waf1/Cip1}$ being nuclear and p16 being cytoplasmic localised . It is tempting to speculate that the presence of p16 in the cytoplasm may prevent the transfer of p21$^{Waf1/Cip1}$ to the cytoplasm, thus indirectly blocking apoptosis inhibition by p21$^{Waf1/Cip1}$, and at the same time maintaining the cells that escaped apoptosis, in cell cycle arrest due to nuclear localized p21$^{Waf1/Cip1}$. This hypothesis should be further investigated in cell lines models and in a larger group of TGCT patients including chemo-resistant TGCT patients.

In conclusion, the positive staining for p16 and not for $p21^{Waf1/Cip1}$ in embryonal carcinoma and yolk sac components of TGCTs suggests mutually exclusive expression. Expression of p16, however, had no functional consequence on cisplatin or γ-radiation-induced apoptosis in an embryonal carcinoma cell model.

Figures an Legends to Chapter 5

Table 1. Histology samples from 34 patients with disseminated TGCT

Single component TGCTs			
Tumor type[$]	**P21 staining (n)**	**P16 staining (n)**	**Prognosis Group[#] (n)**
S	- (1)	- (1)	GP (1)
EC	- (5)	- (2) +(3)	GP (4), IP (1)
YS	- (1)	+ (1)	GP (1)
ChC*	+ (1)	+ (1)	PP (1)
MT /IT	+ (3)	+ (1) ++ (2)	GP (1), IP (1), PP (1)
Multiple component TGCTs			
Tumor component	**P21 staining (n)**	**P16 staining (n)**	**Prognosis Group (n)**
S	- (6)	- (6)	GP (4), IP (1), ,PP (1)
EC	- (16)	- (3) + (13)	GP (11), IP (3), PP (2)
YS	- (4)	- (2) + (2)	GP (2), IP (1), PP (1)
ChC	+ (5)	- (1) + (4)	GP (2), IP (1), PP (2)
MT/IT	+ (14)	- (1) + (4) ++ (9)	GP (10), IP (2), PP(2)

[$] S (seminoma), EC (embryonal carcinoma), YS (yolk-sac), ChC (choriocarcinoma), MT/IT (mature/immature teratoma)
[#] GP (good prognosis), IP (intermediate prognosis), PP (poor prognosis)
[+]Brain metastasis biopsy

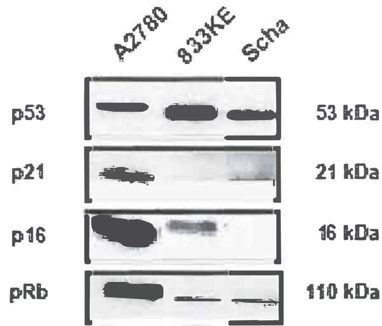

Figure 1

Western blot of proteins in whole-cell lysates of untreated 833KE and Scha testicular cancer cells and A2780 ovarian cancer cells. Protein separation and Western blotting was performed as described in "Material and Methods". A representative example of three independent experiments is shown.

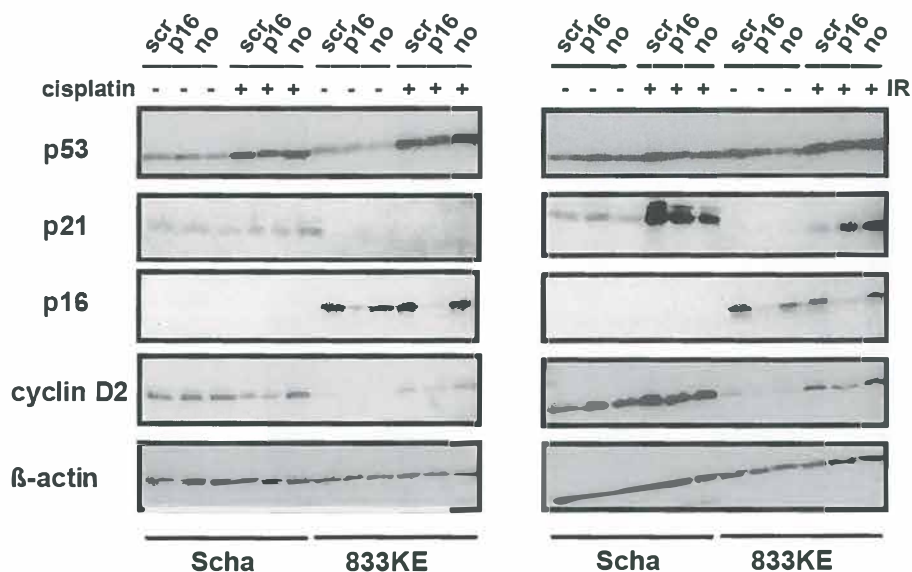

Figure 2

Effect of p16 knock-down on treatment-induced p53, p21$^{Waf1/Cip1}$, p16 and cyclin D2. Cells were treated with cisplatin (4 μM) for 24 hrs or irradiated with 10 Gy and 24 hrs later collected to determine expression levels with Western blotting on whole-cell lysates as described in "Material and Methods". Beta-actin was used as a loading control. Abbreviations: scr (scrambled siRNA), p16 (p16 siRNA), and no (no siRNA treatment).

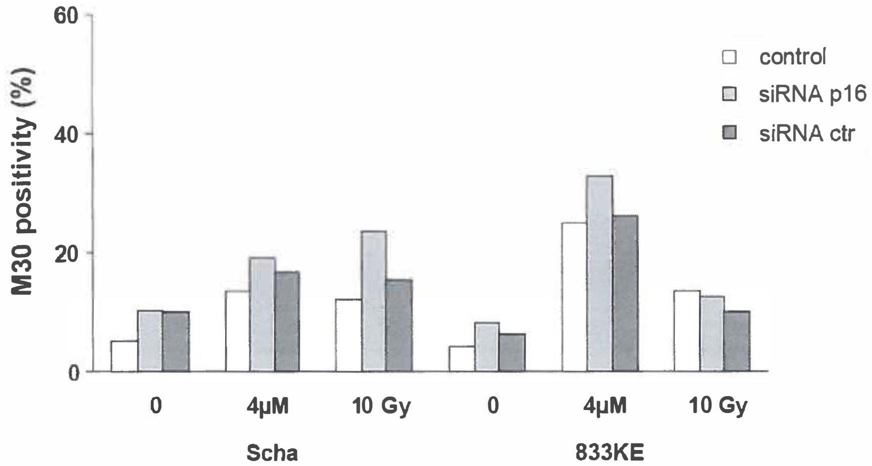

Figure 3

Effect of p16 knock-down on treatment-induced apoptosis in 833KE and Scha cells. Cells were treated with cisplatin (4 µM) for 24 hrs or irradiated with 10 Gy and 24 hrs later collected and apoptosis was measured using M30 immunoreactivity as described in "Material and Methods". The average of two separate experiments was shown. Abbreviations: scr (scrambled siRNA), p16 (p16 siRNA), and no (no siRNA treatment).

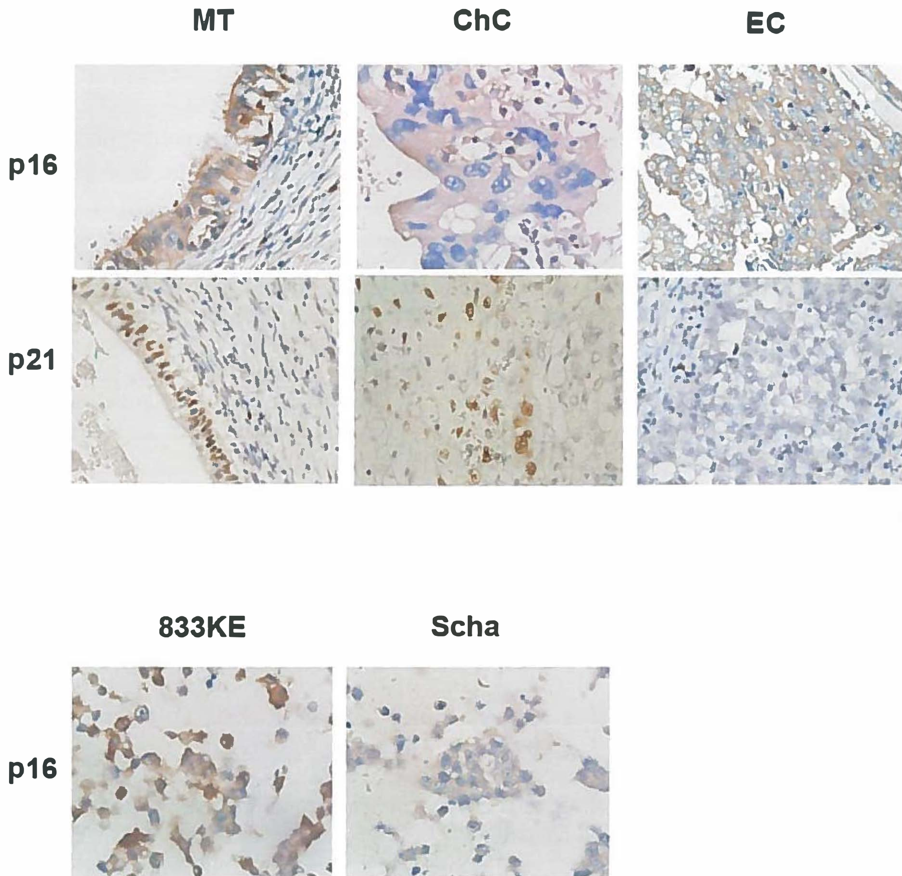

Figure 4

Immunohistochemical staining of p16 and p21$^{Waf1/Cip1}$ in different components in TGCTs. Examples of cytoplasmic p16 expression and nuclear staining of p21$^{Waf1/Cip1}$ are shown for mature teratoma (MT), choriocarcinoma (ChC), and embryonal carcinoma (EC) components of TGCTs. We validated the staining on paraffin embedded p16-positive 833KE and p16-negative Scha cells Representative examples of staining are shown.

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Chapter 6

Clinical Evaluation of M30 and M65 ELISA Cell Death Assays as Circulating Biomarkers in a Drug Sensitive Tumor, Testicular Cancer

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Neoplasia 2008; 10(10): 1041-8

Abstract

Circulating full length and caspase-cleaved cytokeratin 18 (CK18) are considered biomarkers of chemotherapy-induced cell death measured using a combination of the M30 and M65 ELISAs. M30 measures caspase-cleaved CK18 produced during apoptosis and M65 measures the levels of both caspase-cleaved and intact CK18, the latter of which is released from cells undergoing necrosis. Previous studies have highlighted their potential as prognostic, predictive and pharmacological tools in the treatment of cancer. Disseminated testicular germ cell cancer (TC) is a paradigm for a chemosensitive solid malignancy of epithelial origin and has a cure rate of 80-90%. We conducted M30/M65 analyses on 34 TC patients before and during treatment with bleomycin, etoposide and cisplatin (BEP) and showed that pre-chemotherapy serum levels of M65 and M30 antigens are correlated with established TC tumor markers lactate dehydrogenase (LDH), α-fetoprotein (αFP) and β-human chorionic gonadotropin (βHCG), probably reflecting tumor load. Cumulative percentage change of M65 and M30 from baseline to end of study was highest in poor prognosis patients ($P < 0.05$). Moreover, area under the curve (AUC) profiles of M65 and M30 during chemotherapy mirrored dynamic profiles for LDH, αFP and βHCG. Consequently, M65 and M30 levels appear to reflect chemotherapy-induced changes that correlate with changes in markers routinely used in the clinic for management of patients with TC. This is the first clinical study where M65 and M30 antigen levels correlate with established prognostic markers and provides impetus for their exploration in other epithelial cancers where there is a pressing need for informative circulating biomarkers.

List of abbreviations

αFP	α-fetoprotein
AUC	area under the curve
BEP	bleomycin, etoposide and cisplatin
βHCG	β-human chorionic gonadotropin
ChC	choriocarcinoma
CK18	caspase-cleaved cytokeratin 18
EC	embryonal carcinoma
IGCCC	International Germ Cell Consensus Classification
IT	immature teratoma
LDH	lactate dehydrogenase
MT	mature teratoma
NE	neo-epitope
PD	pharmacodynamic
TC	testicular germ cell cancer
YS	yolk sac tumor

Introduction

The development of novel, targeted anticancer therapies coupled with an increased understanding of the molecular processes that occur during cancer progression bring with them an ever more pressing need for the development of clinically robust biomarkers [1, 2]. Ideally these assays may provide information such as optimal patient selection for the design of clinical trials as well as enabling real-time evaluation of treatment efficacy and/or toxicity [3, 4]. These biomarkers may also facilitate no/go, go decision making that is so crucial during the drug discovery process [5].

The M30- and M65-based sandwich ELISAs determine the circulating levels of different forms of the protein cytokeratin 18 (CK18) and are proposed as surrogate biomarkers of drug-induced cancer cell death [6-8]. Cancers of epithelial origin are known to contain relatively large intracellular pools of soluble and insoluble cytokeratins. However, during necrotic and apoptotic cell death CK18 and other cytokeratins are released into the blood in either their intact, or caspase cleaved forms where they remain relatively stable in the circulation of cancer patients [9].

The M30 detection antibody recognizes a neo-epitope (NE) mapped to positions 387-396 of CK18, so called CK18-Asp^{396}, that is only revealed after caspase cleavage of the protein and is postulated as a selective biomarker of apoptosis [10]. The M65 ELISA detects a common epitope present in the full length protein as well as in the caspase-cleaved fragment [8] and is thus believed to measure, in addition to apoptosis, intact CK18 that is released from cells undergoing necrosis [11]. Both assays have now been validated as 'fit for purpose' in the analysis of plasma and serum collected from subjects entered into clinical trials [12-15] and have been extensively applied as pharmacodynamic (PD) biomarkers of chemotherapy-induced cell death in a range of different cancer types treated with different chemotherapeutic agents [6, 8, 16-19].

Testicular germ cell cancer (TC) is the most common malignancy in men between 20 and 35 years old. At the time of diagnosis up to 50% of TC patients have disseminated disease and the current standard chemotherapy regimen consists of treatment with a combination of bleomycin, etoposide and cisplatin (BEP). TC can be considered a paradigm for a chemosensitive solid tumor as the cure rate for disseminated TC is high (80-90%), although about 20-30% of the patients diagnosed with disseminated disease will display intrinsic

resistance or will acquire resistance to first line chemotherapy and about 10% will die eventually from TC.

Approximately 90% of the patients with disseminated TC have elevated levels of one or more of the following serum tumor markers: lactate dehydrogenase (LDH), α-fetoprotein (αFP) and beta-human chorionic gonadotropin (βHCG). Normalization of these tumor markers is the most commonly used criterion for favorable tumor response [20] and levels usually decrease within 5 to 12 days after the start of chemotherapy [21]. When marker levels normalize during or after completion of chemotherapy, patients are considered to have had a complete biochemical tumor response. In the cases where residual disease is present, it will be treated surgically [22, 23]. Upon complete remission, regular measurement of serum tumor markers is an essential component of the follow-up of TC patients [24, 25]. In addition, LDH, αFP and βHCG are used, in combination with the localization of the primary tumor and the presence of non-pulmonary visceral metastases, for prognosis estimation according to the International Germ Cell Consensus Classification (IGCCC). Initial increase in αFP levels after start of chemotherapy appears to be associated with unfavorable outcome [26].
It must be noted however, that initial levels of these serum tumor markers has a limited predictive value for disease outcome in the individual patient. Moreover, up to 10% of patients with disseminated TC do not have elevated tumor markers. Thus, additional serological biomarkers may contribute to a better prediction of response to chemotherapy and disease outcome.

We have conducted an exploratory study where we have investigated changes in serum and plasma concentrations of total CK18 (M65 antigen) and caspase-cleaved CK18 (M30 antigen) in TC patients before and serially after treatment with BEP-chemotherapy, and their association with changes in the clinically used tumor markers LDH, αFP and βHCG, and treatment outcome.

Patients and methods

Patients and collection of blood samples for analysis of CK18 and caspase-cleaved CK18

Blood samples were collected from two groups of patients and analyzed for the acute effects of chemotherapy for disseminated TC. All patients received BEP-chemotherapy, consisting of a combination of bleomycin (30 mg on days 2, 8 and 15 of each course), etoposide (100 mg/m² on days 1-5) and cisplatin (20 mg/m² on days 1-5) for 3 or 4 courses of 3 weeks.

We collected blood samples of 11 consecutive TC patients participating in a prospective study on acute effects of BEP-chemotherapy and treated from May 2006 to November 2006. For this study both serum and heparin plasma were collected on day 1 (before start of chemotherapy) and heparin plasma for days 2 (24 h after start), 3 (48 h after start), 6, 8 and 15 of the first course and days 1, 8 and 15 of the following courses. Samples were stored at -20°C.

In order to extend our exploratory analysis we selected from our serum bank a group of 23 TC patients treated with BEP-chemotherapy at the University Medical Center Groningen, the Netherlands, between January 2004 and May 2006. Selection was based on the availability of serum samples and diversity with respect to the histological composition of the primary tumor, IGCCC prognosis group, disease outcome and levels of tumor markers LDH, αFP and βHCG. From the stored serum samples (at -20°C) we selected samples from before start of chemotherapy and from days 1, 8 and 15 of each chemotherapy course. If no samples were available for days 1, 8 or 15, a sample from one day before or after was analyzed.

The analysis of blood samples was approved by the local medical ethical committee and written informed consent was obtained from each participant.

Immunochemistry for CK18 and caspase-cleaved CK18 in tumor material

Paraffin-embedded testicular tumor material was collected for each patient prior to the start of chemotherapy and analyzed for CK18 and caspase-cleaved CK18. For immunohistochemistry 3 μm sections were cut and placed on APES-coated slides, deparaffinized and dehydrated. The presence of testicular germ cell tumor components was confirmed by a pathologist using standard hematoxylin-eosin staining. For staining of

CK18, endogenous peroxidase was blocked (30 min 0.3% H_2O_2) and in addition, sections were pre-treated with 0.1% protease XXIV for 30 min (Sigma-Aldrich, St. Louis, MO, USA), followed by incubation with avidin and subsequently biotin blocking solution, both for 15 min (Vector Laboratories, Burlingame, CA, USA). For staining of caspase-cleaved CK18, sections were preheated by microwave for 8 min at 700 W in citrate buffer (0.1 M, pH 6.0) followed by blocking of endogenous peroxidase activity as described above.

Subsequently, slides were incubated for 1 h with the primary antibody: CAM5.2 (Becton-Dickinson, San Jose, CA, USA) recognizing both CK18 and CK8, which show a similar tissue distribution (20), or M30 antibody (Boehringer Mannheim GmbH, Mannheim, Germany) to detect CK18-Asp^{396}. A secondary biotinylated rabbit anti-mouse antibody followed by a tertiary StreptAB-complex/HRP and peroxidase-conjugated rabbit-anti-mouse antibody followed by peroxidase-conjugated goat-anti-rabbit antibody (antibodies from DAKO, Glostrup, Denmark) were used, respectively. DAB was used as chromagen to visualize peroxidase activity. Counterstaining was performed with hematoxylin. Immunoglobulin-class-matched control sera were used as negative controls. Normal appendix tissue served as a positive control for CK18 and CK8 and colon carcinoma tissue for caspase-cleaved CK18.

Measurement of CK18 and caspase-cleaved CK18 in tumor material by IHC

Evaluation of staining was performed by a pathologist without knowledge of the clinical data. With respect to CK18/CK8 (recognized by CAM5.2), tumor components showing no positive cells were considered negative (-), while tumor components showing stained tumor cells were considered positive (± in presence of focal positive staining or + in presence of diffuse positive staining). For caspase-cleaved CK18, tumor components showing positivity in less than 5 high power fields (with a field diameter of 0.55 mm) were considered negative (-). Focal positivity for caspase-cleaved CK18 was scored +, while the presence of massive positivity was scored ++. Heterogeneity and pattern of staining regarding each tumor component were also recorded.

Determination of CK18 (M65 ELISA) and caspase-cleaved CK18 (M30 ELISA) in blood

The M30 apoptosense and M65 ELISA kits were both obtained from PEVIVA AB (Bromma, Sweden) and these assays, previously validated for clinical trial use, were

performed under dedicated GCLP conditions as previously described [12-14]. Background variation for M65 and M30 antigens is considered as +/- 30% of the antigen level seen at the start of each treatment cycle as discussed previously [13]. Any peaks or troughs seen in patient antigen levels falling outside of this range may be considered to be as direct result of chemotherapy: either tumor response or toxicity.

Statistics

Data were analyzed statistically with SPSS software package (SPSS Inc., Chicago, IL). Groups were compared for M65 and M30 by using the Mann-Whitney U test, while changes of M65 and M30 over time were tested with the paired Wilcoxon signed rank test. The Kruskal-Wallis nonparametric test was performed to test for significant differences amongst the distributions of the three prognosis groups. Post-hoc pair-wise comparisons were made with Bonferonni correction, when appropriate.

To calculate the cumulative percentage change of M65 and M30 levels over the entire treatment period, values with a 30% threshold from the pre-dose value for each chemotherapy course were judged to be either a 'peak' (increase above threshold), or 'trough' (decrease below threshold), or 'no change' (remaining either above, below or within the 30% threshold in line with the previous reading) [13]. At each peak and trough the absolute values of M30 and M65 were recorded and summed over the entire treatment period to give the cumulative percentage change.

To calculate the area under the curve (AUC) values from the measured concentrations at sequential, specific sampling points, the trapezoidal equation was used as follows:

$$\sum_{n=1}^{n}\left\{\frac{c_n + c_{n+1}}{2} \bullet (t_{n+1} - t_n\right\}$$

Where cn and tn are the concentration and time point respectively for the n'th time point.

To determine the extent to which the AUC values of M65, M30 and the standard tumor markers LDH, αFP and βHCG are proportional to each other, weekly AUC data for all five markers were normalized between 0 and 1 using minmax normalization [27] so that a direct comparison could be made between these markers in terms of the AUC profile over the entire time period. All tests were performed two-sided and P-values < 0.05 were considered statistically significant.

Results

Patient characteristics

General patient characteristics are summarized in Table 1. Among the total group of 34 patients there were 4 patients with refractory disease.

CK18 and caspase-cleaved CK18 (M30) in primary tumor material

For each patient, every testicular germ cell tumor component was scored separately for the expression of CK18/CK8 and caspase-cleaved CK18. All non-seminomatous histological components were positive for CK18/CK8 (all +), with the embryonal carcinoma (EC) component showing most positivity (Figure 1A), followed by the choriocarcinoma (ChC) component. These two components were also most often positive for caspase-cleaved CK18 (EC 22/22 ++; ChC 1/6 + and 5/6 ++), with the variable presence of so-called aponecrosis in EC components. Noteworthy, the yolk sac tumor (YS) component was negative for caspase-cleaved CK18 when it had the reticular pattern, while it showed positivity for caspase-cleaved CK18 with aponecrosis in the solid pattern, which is histologically more similar to the EC pattern (YS 3/6 -, 2/6 + and 1/6 ++). Mature teratoma (MT) and immature teratoma (IT) component showed variable expression of caspase-cleaved CK18 (MT 11/12 - and 1/12 +; IT 3/5 - and 2/5 +) (Figure 1B).

The seminoma (S) component was negative for both CK18/CK8 (7/8 - and 1/8 ±) and caspase-cleaved CK18 (8/8 -; Figure 1C) with the exception of one patient who had a mixed germ cell tumor with focal positivity for CK18/CK8 in the S component.

Circulating CK18 (M65) and caspase-cleaved CK18 (M30) before chemotherapy

Pre-chemotherapy sera were taken and analyzed for M65 and M30 antigen levels from 23 retrospectively sampled patients data were grouped according to IGCCC prognosis group guidelines (good, intermediate or poor). Serum levels of M65 and M30 antigens varied significantly according to prognosis group (Figure 2A). Patients in the poor prognosis group showed the highest median level of M65 (2456 U/l), followed by the intermediate prognosis group (642 U/l) and then good prognosis group (366 U/l). Median M30 levels follow the same pattern (poor prognosis group 648 U/l, intermediate prognosis group 219 U/l and good prognosis group 144 U/l).

Changes in circulating CK18 (M65) and caspase-cleaved CK18 (M30) during the first chemotherapy course

For the 11 patients participating in the prospective study, data were available at early time-points at 24 h, 48 h, 5 days, 7 days, 14 days and 21 days after start of chemotherapy (1st chemotherapy course) (Figure 3). Up to 7 days after start of chemotherapy there was a significant increase in median M65 level (pre-chemotherapy 384 U/l, after 7 days 483 U/l; $p = 0.010$) and median M30 level (pre-chemotherapy 267 U/l, after 7 days 338 U/l; $p = 0.026$). This peak was followed by a significant decrease in M65 levels compared to baseline values resulting in a median level of 294 U/l at 14 days ($p = 0.021$) and 307 U/l ($p = 0.016$) at 21 days after start of chemotherapy. M30 levels were also decreased compared to pre-chemotherapy levels 14 days after start of treatment (median 203 U/l; $p = 0.008$).

Circulating CK18 (M65) and caspase-cleaved CK18 (M30) profiles in patients from the three IGCCC prognosis groups

Typical examples of individual patient profiles from each of the three groups are shown in Figure 4. Arrows indicate the start of each cycle of chemotherapy and error bars at each of these points indicate the 30% signal: noise ratio, thus values out-with this range are considered to be significant [13]. Patients in the good and intermediate prognosis group (Figures 4A and 4B, respectively) displayed repeated drug-induced spikes in M30 and M65 levels throughout the course of therapy, whilst M65 and M30 antigen levels for the patients in the poor prognosis group were initially very high and declined rapidly upon the first chemotherapy course with the absence of clear peaks (Figure 4C). The four patients with refractory disease showed a profile similar to the 30 patients with a favourable tumour response.

Cumulative changes in circulating CK18 (M65) and caspase-cleaved CK18 (M30) over the entire treatment period

Changes in circulating M65 and M30 were analyzed in the 23 patients for whom samples were available throughout their entire treatment regime. Cumulative percentage changes in M65 (good prognosis 166.5%, intermediate prognosis 121.3% and poor prognosis -119.4%) and M30 (good prognosis 90.4%, intermediate prognosis 69.7% and poor prognosis 25.5%) over the entire treatment period differed highly according to IGCCCG prognosis group. Patients in the poor prognosis group showed the largest cumulative decrease in both M65 and M30 (Figure 2B). The median cumulative change in M65 in the

poor prognosis group differed significantly from the good prognosis group (*P*=0.002) and intermediate prognosis group (*P*=0.008), while the median cumulative changes in M30 also differed between the poor prognosis patients and good prognosis patients (*P*=0.011), but not between the poor and intermediate prognosis patients.

Correlation between circulating CK18 (M65), caspase-cleaved CK18 (M30) and standard tumor markers

Pre-chemotherapy serum levels of M65 and M30 were strongly positively correlated with circulating levels of the standard TC tumor markers LDH, βHCG and αFP (Table 2A).

Area Under the Curve (AUC) analysis of median week-by-week changes in M65 (Figure 5A) showed a significant difference according to IGCCC prognosis group during the first two weeks of chemotherapy ($p<0.05$) and was able to distinguish patients in the poor prognosis group during subsequent rounds of treatment ($p<0.05$). Similarly, M30 levels showed significant variation according to prognosis group during the first 2 weeks of treatment ($p<0.05$) (Figure 5B). Of particular note is the finding that AUC analyses of M65 and M30 levels during chemotherapy exhibited profiles that were remarkably similar to those of the standard tumor markers (Figure 5C). Spearman's correlation analysis showed a significant correlation between both M65 and M30 with LDH in all three prognosis groups, βHCG in intermediate and poor prognosis groups and αFP in the poor prognosis group ($p<0.05$; Table 2B).

Discussion

The high levels of intact and caspase-3 cleaved CK18 in the circulation of patients with epithelial malignancies have been attributed to intracellular proteins that have been shed into the blood from dying tumor cells [16, 18, 19]. Similarly, increases in circulating levels of CK18 following chemotherapy have also been reported and thus this protein is considered to be a serological biomarker of tumor cell death [16, 19, 28].

The expression of CK18 in non-seminomatous histological components of TC renders CK18 and its caspase-cleaved fragment CK18-Asp^{396} candidate biomarkers for chemotherapy-induced tumor cell death and cell death mode (apoptosis versus necrosis) in non-seminomatous TC patients. This exploratory study shows that serum levels of CK18 (M65 antigen) and caspase-cleaved CK18 (M30 antigen) before chemotherapy are associated with prognosis group according to IGCCC classification and are correlated with pre-chemotherapy levels of tumor markers LDH, αFP and βHCG. In addition, changes in M65 and M30 are observed during chemotherapy. These changes consist of an overall decrease, combined with peaks during most chemotherapy courses in the good and intermediate prognosis group. AUC analysis shows that changes in M65 and M30 are strongly correlated with chemotherapy-induced changes in LDH, αFP and βHCG.

In our patients CK18 and caspase-cleaved CK18 levels also vary with different histological components of germ cell tumors [29, 30]. It seems conceivable that the large variation in blood levels of M65 and M30 before chemotherapy is partially caused by the present tumor mass(es), their histological composition and the baseline levels of spontaneous tumor cell death. Moreover, the observed association with IGCCC prognosis group and tumor markers LDH, βHCG and αFP, suggests that the height of M65 and M30 levels is primarily influenced by tumor load.

Since CK18-Asp^{396} is expressed during apoptosis and released during loss of cell membrane integrity with further progression of apoptosis or secondary necrosis, we analyzed prospectively changes in serum M65 and M30 in 11 TC patients shortly after start of chemotherapy. An increase in M65 and M30 levels was observed up to 7 days after start of the chemotherapy. In previous studies on the effects of chemotherapy in patients with breast cancer and prostate cancer, increases in M65 and M30 levels were found within 1-3 days after start of chemotherapy [17, 31]. We observed most significant changes 7 days

after start of treatment, which may reflect the cumulative effect of the 5 day dosing scheme of cisplatin and etoposide during BEP-chemotherapy.

During the following courses an overall decrease is observed with the superposition of peaks for both M65 and M30 after start of a new chemotherapy course in good and intermediate prognosis patients. Poor prognosis patients show the most pronounced overall decrease in M65 and M30. The profiles and AUC-change of M65 and M30 appear to mirror the profiles of the standard tumor markers both prior to the start of chemotherapy and throughout treatment. This strong correlation suggests that the overall decrease in M65 and M30 are indicative of treatment response as they appear to reflect a decrease in tumor load due to chemotherapy-induced tumor cell death. A comparable association has been found between docetaxel-induced increases of M30 and baseline levels of PSA, reflecting tumor volume in prostate cancer patients [17]. In addition, serum M30 levels in breast cancer patients correlated with the number of involved organs [18].

In addition, the peaks of M65 and M30 observed after the start of each treatment cycle in good and intermediate prognosis group patients indicate a drug-induced effect, which may reflect tumor response. The fact that these peaks are not observed in patients with poor prognosis is possibly related to high initial levels of M65 and M30. With this exploratory analysis in a small number of selected patients it cannot be proven that M65 and M30 peaks are specific enough for chemotherapy-induced tumor cell death. Neither can it be excluded that these peaks (partially) reflect chemotherapy-induced toxicity to normal epithelial tissue. Recently, elevated serum levels of caspase-cleaved CK18 have been found in for instance patients with an acute myocardial infarction [32].

The 4 TC patients who eventually did not respond to BEP-chemotherapy after an initial decline in tumor markers, showed patterns of M30 and M65 comparable to responding patients, including chemotherapy-induced peaks. In case these peaks are tumor cell death-related, they may represent cell death of chemotherapy-sensitive subpopulations of cells, reflected by initial decrease of tumor markers. However, the number of refractory patients in this study is too small to draw firm conclusions. Consequently, the presence of chemotherapy-induced peaks in M65 and M30 may not exclude future treatment failure. Correspondingly, increases in CK18 have been observed in breast cancer patients with stable disease instead of clinical response to chemotherapy [31].

With regard to the treatment of disseminated TC, the question remains whether determination of M65 and M30 has additional value for monitoring tumor response to chemotherapy. These results suggest that M30 and M65 may reflect drug induced changes in tumor, however, to have a predictive value for the individual patient, serum M65 and M30 need to show distinct patterns for patients with a favorable disease outcome and for those with a non-favorable disease outcome. Fortunately, because the cure rate is so high for disseminated TC (80-90%) a larger study containing more non-responding patients is needed to assess whether changes in M65 and M30 are specific enough for tumor cell response and whether pre-chemotherapy levels and early changes in M65 and M30 are predictive for disease outcome in testicular cancer patients treated with chemotherapy.

This study is the first example in a clinical setting where circulating levels of M30 and M65 antigen correlate with internationally recognized circulating biomarkers that are routinely and successfully used as prognostic indicators and for monitoring treatment response in TC. The correlation between M65/M30 levels and IGCCC prognosis group and their overall agreement with LDH, αFP and βHCG levels suggest that M65/M30 may also have a prognostic value in TC patients. This concordance of M30 and M65 with these prognostic markers of TC adds to the momentum for future exploration of M30 and M65 in other epithelial cancers where informative circulating biomarkers are needed [16, 17, 19, 31].

Acknowledgements

Cancer Research UK (to C.Dive): Grant number C147.

Nynke Zwart and Wytske de Boer are kindly acknowledged for their technical support with the immunochemistry and Alexander H. de Haas for his help with the figures.

Figures and Figure Legends to Chapter 6

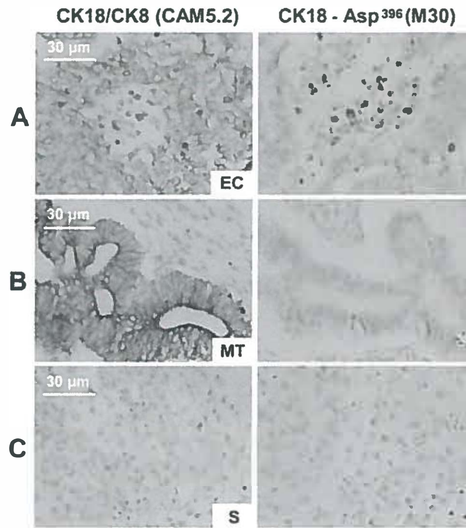


Figure 1

Expression of CK18/CK8 (CAM5.2) and caspase-cleaved CK18 (M30) in different testicular germ cell tumor components. ***A*,** Embryonal Carcinoma (EC): positive for of CK18/CK8 and apoptotic cells are positive for caspase-cleaved CK18. ***B*,** Mature Teratoma (MT): in general positive for CK18/CK8 and negative for caspase-cleaved CK18. ***C*,** Seminoma (S): negative for both CK18/CK8 and caspase-cleaved CK18.

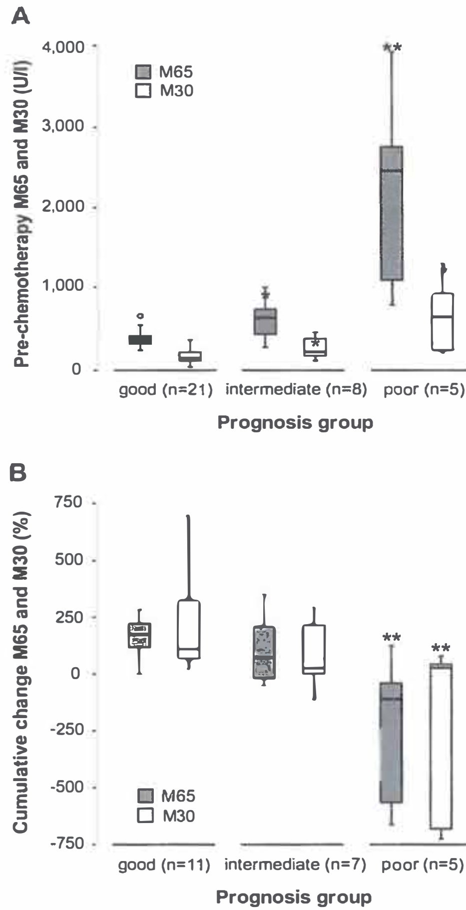


Figure 2

A, Pre-chemotherapy serum levels of total CK18 (M65) and caspase-cleaved CK18 (M30) according to prognosis group (IGCCC) in total group of analyzed patients (n=34). **B,** Cumulative percentage changes in total CK18 (M65) and caspase-cleaved CK18 (M30) levels over entire treatment period grouped by IGCCC prognosis for samples collected from serum bank (n=23). *, significant difference from good prognosis group ($P<0.05$); **, significant difference from good and intermediate prognosis group ($P<0.05$).

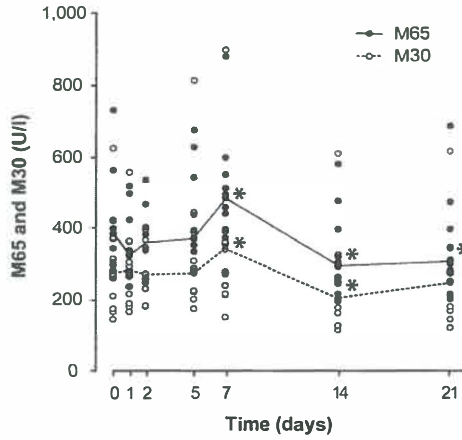
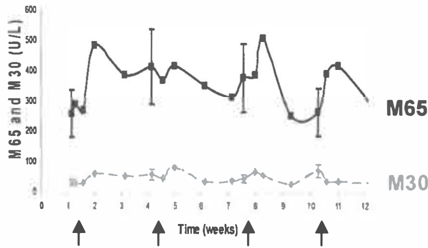


Figure 3

Changes in circulating CK18 (M65) and caspase-cleaved CK18 (M30) in serum of TC patients (n=11) during the first course of BEP chemotherapy. The median values are connected by a line.

* median level significant different from baseline ($P<0.05$).

A



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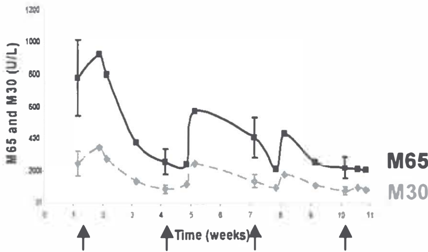

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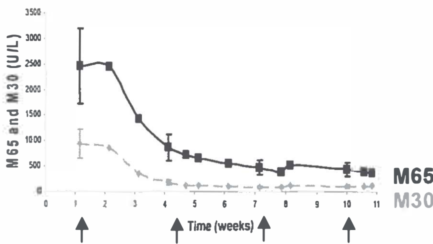

Figure 4

M30 and M65 profiles in a patient from the three IGCCC defined prognosis groups. **A,** good prognosis, **B,** intermediate prognosis, **C,** poor prognosis. Serum from patients receiving standard chemotherapy for TC was analyzed for circulating CK18 (M65) and caspase-cleaved CK18 (M30). Arrows indicate the start of each chemotherapy course and error bars indicate 30% signal:noise for each treatment cycle.

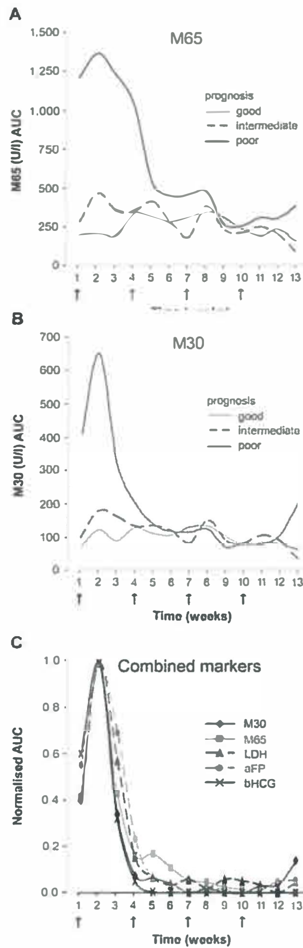

Figure 5

Area under the Curve (AUC) data for **A,** circulating CK18 (M65), and **B**, caspase-cleaved CK18 (M30), according to IGCCC prognosis group. *C*, Levels of M65, M30, and standard tumor markers LDH, αFP and βHCG combined as normalized AUC data. The arrows indicate the start of each chemotherapy course.

Table 1 General patient characteristics

		Prospective group	Serum bank group
Number of patients		11	23
Age (years)	*Median (range)*	35 (19-46)	31 (19-53)
Histology	*N (%)*		
Non-seminoma		10 (91%)	21 (91%)
Seminoma		1 (9%)	2 (9%)
Stage*	*N (%)*		
II		9 (82%)	15 (65%)
III		1 (9%)	1 (4%)
IV		1 (9%)	7 (31%)
Prognosis group†	*N (%)*		
Good		10 (91%)	11 (48%)
Intermediate		1 (9%)	7 (30%)
Poor		0 (0%)	5 (22%)
Chemotherapy regimen	*N (%)*		
4 BEP		2 (18%)	20 (87%)
3 BEP		9 (82%)	3 (13%)
Response to chemotherapy	*N (%)*		
Complete biochemical response		4 (36%)	7 (30%)
Complete biochemical response + surgery		6 (55%)	13 (57%)
Teratoma		*4*	*8*
Necrosis		*2*	*5*
Refractory disease		1 (9 %)	3 (13%)

* Royal Marsden classification

† International Germ Cell Cancer Consensus Classification (IGCCC)

Table 2

Correlation between circulating CK18 (M65), caspase-cleaved CK18 (M30) in serum and standard tumor markers before start of chemotherapy. Values in bold indicate significance ($P<0.05$).

Tumor marker	Correlation coefficient (Spearman's bivariate correlation test)			
	M65	***P***	**M30**	***P***
LDH (n = 34)	**0.698**	0.000	**0.586**	0.000
αFP (n = 20)*	0.129	0.588	**0.475**	0.034
βHCG (n = 22)*	**0.866**	0.000	**0.864**	0.000

* analysis includes patients in whom concerned tumor marker was elevated before start of chemotherapy

Table 3

Correlation between serum levels of CK18 (M65), caspase-cleaved CK18 (M30) in serum and standard tumor markers during chemotherapy, using weekly AUC data. Values in bold indicate significance (P<0.05). Samples were taken from the retrospective study (n=23).

Prognosis group	Correlation coefficient (Spearman's bivariate correlation test)		
	Tumor marker	**M65**	**M30**
Good (n = 11)	LDH (n = 11)	**0.804**	**0.593**
	αFP (n = 5)*	-0.262	0.182
	βHCG (n = 8)*	-0.295	0.092
Intermediate (n = 7)	LDH (n = 7)	**0.654**	**0.579**
	αFP (n = 4)*	0.546	0.547
	βHCG (n = 5)*	**0.561**	**0.579**
Poor (n = 5)	LDH (n = 5)	**0.980**	**0.967**
	αFP (n = 4)*	**0.975**	**0.963**
	βHCG (n = 5)*	**0.963**	**0.973**

- analysis includes patients in whom concerned tumor marker was elevated before start of chemotherapy

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Chapter 7

Summary and Future Perspectives

Summary

Testicular Germ Cell Tumours (TGCTs) mainly occur in men between 20 and 40 years of age. Thanks to the combination of surgery and cisplatin containing chemotherapy, in general consisting of bleomycin-etoposide-cisplatin (BEP regimen), patients with advanced disease achieve cure rates of 90%, 80% and 50%, depending on the stage of the disease classified according the International Germ Cell Consensus Conference Group (IGCCCG) prognosis group [Krege et al., 2008a; Krege et al., 2008b]. However, despite the high chance of cure due to cisplatin-based chemotherapy, about 20-30% of all the patients diagnosed with advanced TGCT will be intrinsic or acquired resistant to chemotherapy and about 10% of all patients will eventually die from this disease [Krege et al., 2008a;Krege et al., 2008b;Schmoll et al., 2004]. The high cure rate induced by chemotherapy in TGCT as well as the resistance to cisplatin-based treatment in a subgroup of TGCTs make this tumour an ideal paradigm to study the mechanisms responsible for cisplatin sensitivity and resistance in human solid malignancies.

The aim of this thesis was to study the molecular mechanisms underlying this unique cisplatin sensitivity in TGCT with a particular focus on the pro- and anti- apoptotic mechanisms leading to cell death after chemotherapy.

In **chapter 2** the multiple cellular pathways possibly responsible for sensitivity to cisplatin-based treatment in TGCT are presented and discussed in a literature review aiming to summarise the current knowledge on the biological basis of cisplatin-induced apoptosis and response to chemotherapy in TGCTs. From a genetic point of view, almost all TGCTs in contrast to solid tumours are characterised by the presence of wild type p53. High p53 expression levels are associated with elevated Mdm2 levels and a loss of $p21^{Waf1/Cip1}$ (p21) expression, suggesting a changed functionality of p53. Expression levels of other proteins involved in the regulation of cell cycle progression indicate a deregulated G1–S phase checkpoint in TGCTs. After cisplatin-induced DNA damage, the increasing levels of p53 lead to the trans-activation of a number of genes but not of p21, preferentially directing TGCT cells into apoptosis or programmed cell death, both via the mitochondrial and the death receptor apoptosis pathway. The sensitivity of TGCTs to chemotherapeutic drugs may be the consequence of the susceptibility of germ cells to apoptosis. Taken

together, this provides TGCT as a tumour model to investigate and understand the molecular determinants of chemotherapy sensitivity of solid tumours.

In **chapter 3,** the role of p53 in the unique cisplatin sensitivity of human TGCT cell lines was analysed. A panel of cisplatin sensitive cell lines (833KE and Tera), a subline with cisplatin-acquired resistance (Tera-CP) and an intrinsic cisplatin resistant cell line (Scha), all expressing wild type p53 were used. Basal p53 and p53-transcriptional target MDM2 expression were present in all lines, whereas p53-transcriptional target p21 was only present in Scha. P53 expression increased already after 6 hours and further raised after 24 hours exposure to cisplatin, together with Mdm2 and apoptosis induction, whereas minimal p21 induction occurred. Previous observations showed, however, that irradiation of TGCT cells induced high levels of p21. P53 suppression by p53 short interfering (si)RNA lowered apoptosis in Tera, related to a diminished Fas membrane expression, but not in the cell lines 833KE and Tera-CP. In contrast, p53 down-regulation, concomitantly suppressing p21 expression, increased cisplatin-induced apoptosis in Scha. Importance of p21 in Scha was further demonstrated by p21 siRNA, which resulted in more apoptotic cells after cisplatin. Our results suggest a dual role for p53 in transactivation and cisplatin-induced apoptosis in TGCT cells depending on cellular context of p53. Low p21 levels under cisplatin-induced p53 activation may explain hypersensitivity of TGCT cells to cisplatin-induced apoptosis.

In **chapter 4** we aimed to better define the anti-apoptotic role of p21 in TGCT cell lines, focussing on p21 suppression of drug-induced apoptosis, when p21 is localised in the cytoplasm [Spierings et al., 2003;Spierings et al., 2004]. To define the role of p21 and the cytoplasmic localisation of p21 with regard to cisplatin sensitivity, we stably transduced Tera, with a viral construct containing either p21-ΔNLS and green fluorescence protein (GFP), or GFP only (Mock). With immune fluorescence microscopy strong cytoplasmic expression of p21 was demonstrated. Treatment with cisplatin induced less apoptosis in Tera-p21-ΔNLS than in Tera-mock. Moreover, downregulation of p21 with p21 siRNA rendered Tera-p21-ΔNLS cells more apoptotic to cisplatin treatment. Similar observations were made with the cisplatin resistant TGCT cell line Scha, in which we observed that p21 was mainly expressed in the cytoplasm, also after cisplatin treatment. Downregulation of endogenous p21 sensitized Scha to apoptosis induction by cisplatin. Using immunoprecipitation we identified the pro-apoptotic Apoptosis Signalling Kinase 1 (ASK1) and cyclin dependent kinase 2 (CDK2) as targets of cytoplasmic p21. Cytoplasmic localisation of p21 in TGCT cells was depending on the phosphorylation of the NLS-site

(Thr145) of p21. Inhibition of p21 phosphorylation using the PI3K inhibitor LY294002 or the specific p-Akt inhibitor triciribine resulted in a partial relocation of endogenous cytoplasmic p21 towards the nucleus leading to sensitisation for cisplatin or gamma-irradiation induced apoptosis. Combined these results strongly indicate that p-Akt-dependent elevation of cytoplasmic p21 levels functions as an antiapoptotic factor in TGCT cells.

Another important regulator of the G1/S transition, besides p21,is p16, an INK4 family member of cyclin dependent kinase inhibitors (CDKIs) [Maddika et al., 2007]. In **chapter 5,** we investigated if p16 and p21 are mutual exclusively expressed in testicular cancer using p21-positive Scha and p21-negative 833KE cells and samples from testicular cancer patients. Expression of p16 was observed in the p21-negative 833KE cells but not in Scha cells. Furthermore, we investigated whether p16 is involved in the response to cisplatin and γ-radiation treatment of TGCT cells. Down-regulation of p16 using p16 siRNA did not affect DNA damage-induced p53 expression or expression of p53-transcription activated proteins, such as p21. Moreover, p16 suppression had no effect on cisplatin- or γ-radiation-induced apoptosis in 833KE cells. Next, primary tumour histological samples from 34 testicular cancer patients belonging to different IGCCCG prognosis groups and affected from different histological tumour subtype were stained for p16 and p21 using immunohistochemistry. We found positive staining patterns for p16 in 16/21 embryonal carcinoma, 3/5 yolk sac, 5/6 choriocarcinoma, 16/17 teratoma and 0/7 seminoma components, while p21 positive staining was observed in 0/21 embryonal carcinoma, 0/5 yolk sac, 6/6 choriocarcinoma, 17/17 teratoma and 0/7 seminoma components. In conclusion, the positive staining for p16 and not for p21 in embryonal carcinoma and yolk sac components of TGCTs suggests mutually exclusive expression. Suppression of p16, however, had no functional consequence on DNA damage-induced apoptosis in an embryonal carcinoma cell model.

As cisplatin induced apoptosis is considered the main mechanism of action in TGCT, serum apoptosis markers could represent a predictive marker of response to chemotherapy in advanced TGCG patients. In **Chapter 6** we describe an exploratory study which we performed in order to investigate, in patients with metastatic testicular cancer treated with BEP-chemotherapy, whether changes in apoptosis markers as well as patterns of changes during the chemotherapy treatment are associated with changes in serum tumour markers (αFP, βHCG, LDH), disease outcome and the occurrence of side effects such as cardiovascular complications and pulmonary toxicity. Circulating full

length and caspase-cleaved cytokeratin 18 (CK18) are considered biomarkers of chemotherapy-induced cell death measured using a combination of the M30 and M65 ELISAs [Cummings et al., 2007;Cummings et al., 2008;Greystoke et al., 2008]. M30 measures caspase-cleaved CK18 produced during apoptosis and M65 measures the levels of both caspase-cleaved and intact CK18, the latter is released from cells undergoing necrosis. Previous studies have highlighted their potential as prognostic, predictive and pharmacological tools in the treatment of cancer [Cummings et al., 2006;Cummings et al., 2007]. We conducted M30/M65 analyses in serum samples of 34 TC patients before and during treatment with bleomycin, etoposide and cisplatin (BEP).Cumulative percentage change of M65 and M30 from baseline to end of treatment was highest in poor prognosis patients ($P < 0.05$). Moreover, area under the curve (AUC) profiles of M65 and M30 during chemotherapy mirrored dynamic profiles for LDH, αFP and βHCG. Consequently, M65 and M30 levels appear to reflect chemotherapy-induced changes that correlate with changes in markers routinely used in the clinic for management of patients with TGCC. This is the first clinical study where M65 and M30 antigen levels correlate with established prognostic markers and provides impetus for their exploration in other epithelial cancers where there is a pressing need for informative circulating biomarkers.

General Discussion and Future Perspectives

The ability to escape apoptosis is defined as a hallmarks of carcinogenesis [Hanahan and Weinberg, 2000] and is regarded as the major factor responsible for resistance to commonly used cytotoxic drugs [Pommier et al., 2004]. In contrast to most solid malignancies, TGCTs are extremely sensitive to cisplatin-induced apoptosis. Based on the studies presented in this thesis the emerging concept is that the most important biological pathway underlying TGCTs cisplatin sensitivity is related to the presence of wild type p53 (**chapter 3**) and to the low levels of expression p21 and its scarce inducibility after chemotherapy treatment (**chapter 4**). Other regulators of the G1/S transition in the cell cycle, such as p16 (**chapter 5**), are not influencing cisplatin sensitivity in TGCTs. Therefore future research focussing on the investigation of the factors influencing p21 expression and localisation (such as Mdm2 and p14 via p53 regulation as well as p-Akt) can further elucidate this aspect and open ways for new treatments in cisplatin resistant tumours

Given the fact that TGCTs are so sensitive for cisplatin based chemotherapy and as apoptosis is the final effect of this sensitivity, it is theoretically possible to find circulating biomarkers, easily measurable in patients' sera. Such biomarkers could give an early prediction of chemotherapy success. The ability to early predict the outcome of the patients on the basis of circulating factors is of interest for the patients belonging to the IGCCCG poor prognosis group. Nowadays still the 50% of the poor prognosis group does not respond to chemotherapy band eventually die from testicular cancer. Unfortunately there are currently no tools yet to predict which patients belonging to the poor prognosis group will not respond to chemotherapy. This is the rational basis to pursue both the research of new circulating markers for early prediction of chemotherapy response and, of apoptosis inducers to be combined with classical chemotherapeutic drug regimens that might enhance the action of cisplatin-based treatment.

At the moment, there are two biomarkers, which are receiving major attention in the literature, i.e. M65 and M30, as possible markers of chemotherapy induced cell death. According to the results obtained in our study (**chapter 6**), circulating levels of M65 and M30 antigen correlate with internationally recognized circulating biomarkers that are established routinely used as prognostic indicators and for monitoring treatment response in TGCC. The correlation between M65/M30 levels and IGCCC prognosis group and their

overall agreement with LDH, αFP and βHCG levels suggest that M65/M30 may also have a prognostic value in TGCC patients.

According to the data available at the moment and those emerged in this thesis, one can hypotheses that a number of molecules are likely useful to be tested as new targeted therapy, such as p53 enhancers and p21 inhibitors as well as inhibitors of p21-downstream molecules, (e.g. flavopiridol and pAkt-inhibitors). Especially TGCTs patients belonging to the poor prognosis group might benefit from the advances in targeted therapy. A major challenge in the future is to translate the molecular knowledge about rational treatments of TGCTs to the treatment of other solid tumours.

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Chapter 8

Hoofdstuk 8

Samevatting en toekomstperspectief

Samevatting

Kiemceltumoren treden bij mannen vooral op in de leeftijdsperiode van 20 en 40 jaar. Dankzij een combinatie van opereren en cisplatine bevattende chemotherapie, die meestal bestaat uit bleomycine-etoposide-cisplatine (BEP regiem), is de kans op genezing bij patiënten met een vergevorderd stadium van deze tumor respectievelijk 90, 80 en 50% afhankelijk van het ziektestadium volgens de International Germ Cell Consensus Conference Group (IGCCCG) prognose groep. Ondanks deze hoge kans op curatie met behulp van deze op cisplatine gebaseerde chemotherapie zijn toch 20 tot 30% van de patiënten met een vergevorderd stadium van kiemceltumor intrinsiek resistent of ontwikkelen resistentie voor chemotherapie. Ongeveer 10% van deze patiënten zullen uiteindelijk aan deze ziekte overlijden. De hoge kans op curatie door chemotherapie bij kiemceltumoren enerzijds en de resistentie voor cisplatine gebaseerde chemotherapie in de subgroep van de kiemceltumor anderzijds, maakt deze tumor een ideaal paradigma om de mechanismen verantwoordelijk voor cisplatine gevoeligheid en ongevoeligheid bij solide tumoren te bestuderen.

Het doel van dit proefschrift was om de moleculaire mechanismen die ten grondslag liggen aan de unieke gevoeligheid van kiemceltumoren voor cisplatine te bestuderen. De focus lag op pro- en anti-apoptotische mechanismen die betrokken zijn bij de regulatie van chemotherapie geïnduceerde celdood.

In **hoofdstuk 2** worden de verschillende cellulaire routes die mogelijk verantwoordelijk zijn voor de gevoeligheid voor cisplatine gebaseerde chemotherapie bij kiemceltumoren beschreven en bediscussieerd. Dit literatuuroverzicht heeft tot doel de kennis op het gebied van de biologische basis voor cisplatine geïnduceerde apoptose en de reactie op chemotherapie in kiemceltumoren samen te vatten. Genetisch gezien worden bijna alle kiemceltumoren in tegenstelling tot andere solide tumoren gekarakteriseerd door de aanwezigheid van wildtype p53. In deze tumoren is hoge expressie van het p53 eiwit geassocieerd met verhoogde Mdm2 expressie, wat een remmer van p53 is, en het ontbreken van $p21^{Waf1/Cip1}$ (p21) expressie, wat een celcyclusremmer is. Dit suggereert dat de functionaliteit van p53 anders is ten opzichte van andere type tumoren met een wildtype p53 . De mate van expressie van andere eiwitten die betrokken zijn bij de regulatie van celcyclusprogressie wijst erop dat er sprake is van een deregulatie van het G1-S fase checkpoint in kiemceltumoren. Nadat cisplatine DNA schade heeft geïnduceerd stijgen p53

spiegels in de cel wat leidt tot meer transcriptie van een aantal genen, maar niet van p21, waardoor kiemceltumorcellen niet in celcyclusstilstand maar in apoptose gaan. Voor dit proces wordt zowel de mitochondriële als de death receptor apoptose route (via de Fas receptor op de celmembraan) benut. De gevoeligheid van kiemceltumoren voor cytostatica kan het gevolg zijn van de intrinsieke gevoeligheid van normale kiemcellen voor apoptose inductie door DNA schade. Op grond van deze eigenschappen vormen kiemceltumoren een interessant en geschikt tumormodel om inzicht te verkrijgen in de moleculaire determinanten die gevoeligheid van solide tumoren voor chemotherapie bepalen.

In **hoofdstuk 3** is de rol die p53 speelt in de unieke gevoeligheid van kiemceltumoren voor cisplatine geanalyseerd. Hiervoor werd een panel gebruikt bestaande uit cisplatine gevoelige cellijnen (833KE en Tera), een sublijn met verworven cisplatine resistentie (Tera-CP) en een intrinsiek cisplatine resistente cellijn (Scha), die allen wildtype p53 bevatten. Basaal p53 en het p53-transcriptionele doeleiwit Mdm2 waren aanwezig in alle cellijnen, terwijl het p53-transcriptionele doeleiwit p21 alleen aanwezig was in de Scha cellijn. Het p53 eiwit ging al na 6 uur omhoog en steeg verder na 24 uur blootstelling aan cisplatine. Op dat moment werd er ook meer Mdm2 eiwit in de cellen gevonden en werd meer apoptose geïnduceerd, terwijl er een minimale toename van p21 eiwit werd gevonden. Eerder onderzoek toonde evenwel aan dat bestralen van kiemceltumorcellen wel leidde tot hogere p21 eiwitspiegels en vrijwel geen apoptose veroorzaakte. P53 onderdrukking met behulp van p53 short interfering (si)RNA, verlaagde de hoeveelheid apoptose in de Tera cellijn. Dit was tevens gerelateerd aan een verminderde Fas membraanexpressie. P53 onderdrukking in de 833KE en Tera-CP cellijnen had echter geen effect op apoptose of op de expressie van Fas op de celmembraan. Daarentegen leidde onderdrukking van p53 expressie tot een verhoogde cisplatine geïnduceerde apoptose in de Scha cellijn. Verlaging van p53 expressie leidde echter ook tot een verlaging van p21 expressie in deze cisplatine resistente cellijn. Het belang van p21 in Scha werd verder aangetoond door het rechtstreeks onderdrukken van de p21 expressie, waarna cisplatine meer apoptose veroorzaakte. Onze resultaten suggereren een duale rol van p53 in kiemcel tumoren, want in cellijnen met lage p21 eiwitexpressie veroorzaakt p53 apoptose en in andere cellijnen leidt p53 juist tot hoge p21 eiwitexpressie en voorkomt zo juist apoptose. Deze resultaten suggereren dat de rol van p53 bij p21 expressie en bij apoptose afhankelijk is van de cellulaire context, waarin p53 functioneert. De lage p21 spiegels tijdens cisplatine geïnduceerde p53 activatie

verklaren mogelijk de hypersensitiviteit van kiemceltumorcellen voor cisplatine geïnduceerde apoptose.

In **hoofdstuk 4** was het doel van de studie om de anti-apoptotische rol van p21 in kiemceltumorcellijnen beter te definiëren. Vroegere resultaten hadden al aangegeven, dat p21 niet in de kern maar vooral in het cytoplasma van de cellen aanwezig was. Ten einde de rol van p21 en de cytoplasmalokalisatie van p21 in relatie tot cisplatine gevoeligheid beter te definiëren, werd de cellijn Tera stabiel getransduceerd met een viraal construct dat p21-ΔNLS bevatte en een groen fluorescerend eiwit (GFP), of alleen GFP (Mock). Met behulp van de immunofluorescentie microscopie werd een sterke cytoplasmatische p21 aankleuring voor p21-ΔNLS aangetoond. Behandeling met cisplatine induceerde minder apoptose in de Tera- p21-ΔNLS dan in Tera-Mock. Bovendien leidde onderdrukking van p21 expressie er toe dat Tera- p21-ΔNLS cellen meer in apoptose gingen op cisplatine. Vergelijkbare observaties werden gedaan in de cisplatine resistente kiemceltumorcellijn Scha. Hier observeerden we dat p21 in het bijzonder tot expressie kwam in het cytoplasma, ook na behandeling met cisplatine. Onderdrukken van p21 maakte Scha cellen gevoeliger voor cisplatine geïnduceerde apoptose. Met behulp van immunoprecipitatie identificeerden we twee pro-apoptotische eiwitten, Apoptosis Signalling Kinase 1 (ASK1) en cycline-afhankelijke kinase 2 (CDK2), waaraan cytoplasmatische p21 bindt. Cytoplasmatische p21 loksalisatie in kiemceltumorcellen was afhankelijk van de fosforylatie van een aminozuur in de nuclear localisation signal-site (Thr145) van p21. Remming van p21 fosforylatie met behulp van de PI3K remmer LY294002 of de specifieke fosfo-Akt remmer triciribine resulteerde in een gedeeltelijke verplaatsing van het endogeen cytoplasmatisch p21 naar de kern en veroorzaakte een verhoogde gevoeligheid voor cisplatine of gammastraling geïnduceerde apoptose. Deze resultaten wijzen er sterk op dat fosfo-Akt-afhankelijke toename van cytoplasmatisch p21 functioneert als een anti-apoptotische factor in kiemceltumorcellen.

Een andere belangrijke regulator van de G1/S transitie, naast p21 , is p16, een INK4 familielid van de cycline afhankelijke kinase remmers (CDKIs). In **hoofdstuk 5** onderzochten we met behulp van de p21 positieve Scha cellen en de p21 negatieve 833KE cellen en tumor samples van patiënten met een kiemceltumor of de expressie van p16 en p21 elkaar wederzijds uitsluiten in kiemceltumoren. We onderzochten bovendien of p16 betrokken is bij de reactie op cisplatine en gammabestraling in kiemceltumorcellen.

Wanneer de p16 expressie werd onderdrukt had dit geen effect op de door DNA-schade geïnduceerde p53 expressie en niet op de expressie van p21. Bovendien beïnvloedde p16-onderdrukking niet de cisplatine of gammabestraling geïnduceerde apoptose in 833KE cellen. Vervolgens werden primaire tumor monsters van 34 kiemceltumorpatiënten die behoorden tot verschillende IGCCCG prognose groepen en afkomstig waren van verschillende histologische tumor subtypen immunohistochemisch gekleurd voor p16 en p21. Wij vonden positieve aankleuring voor p16 in 16 van de 21 embryonaalcelcarcinoom, in 3 van de 5 dooierzaktumor, in 5 van de 6 choriocarcinoom, in 16 van 17 teratoom en in 0 van de 7 seminoom-componenten. P21 aankleuring werd aangetoond in 0 van de 21 embryonaalcelcarcinoom, in 0 van de 5 dooierzaktumor, in 6 van de 6 choriocarcinoom, in 17 van 17 teratoom en 0 van de 7 seminoom-componenten.
Concluderend: de positieve aankleuring van p16 maar niet van p21 in embryonaalcelcarcinoom en dooierzaktumor componenten van kiemceltumoren suggereert dat in deze componenten de aanwezigheid van p16 die van p21 uitsloot. Onderdrukking van p16 had echter geen functionele gevolgen voor p21 expressie en geen effect op DNA-geïnduceerde apoptose in een embryonaalcarcinoom celmodel.

Aangezien cisplatine geïnduceerde apoptose beschouwd wordt als het belangrijkste werkingsmechanisme in kiemceltumoren hebben mogelijk serum apoptosemarkers een voorspellende waarde voor respons op chemotherapie in patiënten met vergevorderde kiemceltumoren. In **hoofdstuk 6** beschrijven we een exploratieve studie. Deze studie had als doel in patiënten met gemetastaseerde kiemceltumoren die BEP chemotherapie kregen te onderzoeken of veranderingen in apoptosemarkers gedurende chemotherapie geassocieerd waren met veranderingen in serumtumormarkers (alfafoetoproteïne, β-HCG, LDH), ziektebeloop en het ontstaan van bijwerkingen, zoals cardiovasculaire complicaties en longtoxiciteit. Circulerende volledige lengte en caspase-geknipt cytokeratine 18 (CK18) worden gezien als biomarkers voor chemotherapiegeïnduceerde celdood en kunnen gemeten worden met behulp van een combinatie van M30 en M65 ELISA's. M30 meet caspasegeknipt CK18 wat ontstaat tijdens apoptose en M65 meet de spiegels van zowel caspasegeknipt als intact CK18. Dat laatste komt vrij uit cellen gedurende necrose. Eerdere studies suggereerden dat deze markers mogelijk een prognostische en predictieve rol hebben voor de behandeling van kanker. Wij analyseerden M30/M65 spiegels in serum monsters van 34 patiënten met een kiemceltumor voor en tijdens behandeling met BEP. Het cumulatieve percentage verandering van M65 en M30 van het begin tot het einde van

de behandeling was het hoogst bij patiënten met een slechte prognose ($P<0.05$). Bovendien weerspiegelde het oppervlak onder de curve profiel van M65 en M30 gedurende chemotherapie het dynamische profiel voor veranderingen in LDH, AFP en β-HCG, de standaard klinisch gebruikte markers bij de behandeling van kiemceltumorpatiënten. Deze veranderingen van M65 en M30 correleerden met veranderingen in LDH, AFP en β-HCG tijdens chemotherapie. Dit is de eerste klinische studie, waarin M65 en M30 antigeen spiegels correleerden met standaard prognostische tumormarkers en het suggereert dat het ook interessant kan zijn deze markers te onderzoeken bij andere epitheliale tumoren.

Algemene discussie en toekomstperspectief

De mogelijkheid om te ontsnappen aan apoptose wordt gezien als een van de typerende eigenschappen van de carcinogenese en wordt beschouwd als een belangrijke factor die verantwoordelijk is voor de ongevoeligheid van tumoren voor cytostatica. In tegenstelling tot de meeste solide tumoren zijn kiemceltumoren extreem gevoelig voor cisplatine geïnduceerde apoptose. Gebaseerd op onderzoek gepresenteerd in dit proefschrift is het idee dat de meest belangrijke biologische route die ten grondslag ligt aan de kiemceltumor-gevoeligheid voor cisplatine gerelateerd is aan de aanwezigheid van wildtype p53 (**hoofdstuk 3**) en aan de lage expressie van p21 en de slechts zeer beperkte induceerbaarheid van p21 met chemotherapie (**hoofdstuk 4**). Andere regulatoren van de G1/S transitie in de celcyclus, zoals p16 (**hoofdstuk 5**) zijn niet van invloed op cisplatine gevoeligheid van kiemceltumoren. In de toekomst zal daarom onderzoek dat zich richt op het uitzoeken van de factoren die p21-expressie en de intracellulaire lokalisatie ervan beïnvloeden (zoals Mdm2 en p14 via p53 regulatie en fosfo-Akt) verder licht moeten werpen op dit aspect. Dit kan mogelijk aanleiding geven tot een verbeterde behandeling van cisplatine resistente tumoren.

Omdat kiemceltumoren zo gevoelig zijn voor cisplatine bevattende therapie en apoptose op cisplatine het uiteindelijke effect is van deze gevoeligheid is het theoretisch mogelijk dat circulerende biomarkers eenvoudig te bepalen zijn in sera van deze patiënten. Zulke biomarkers zouden een vroege voorspelling van het chemotherapie-effect kunnen geven. De mogelijkheid om al in een vroeg stadium de uitkomst van de behandeling voor de patiënten, op basis van circulerende factoren, te bepalen is met name interessant voor die patiënten die behoren tot de IGCCCG groep met een slechte prognose. Op dit moment respondeert 50 % van de patiënten in de poor prognosis groep niet voldoende op chemotherapie en zal uiteindelijk overlijden aan hun kiemceltumor. Helaas zijn er op dit moment geen methoden waarmee al in een vroeg stadium voorspeld kan worden welke patiënt uit de poor prognosis groep uiteindelijk niet zal reageren op zijn therapie. Dit onderzoek, indien het een vroege respons op chemotherapie kan voorspellen, zou dan mogelijk gebruikt kunnen worden voor het selecteren van patiënten die potentieel baat hebben bij het toevoegen van apoptose "inducers" aan klassieke chemotherapeutische medicijnen, om zo het effect van cisplatine gebaseerde therapie te versterken. Op dit moment zijn er twee biomarkers die veel aandacht krijgen in de literatuur, te weten M65 en M30. Dit zijn mogelijke markers van chemotherapiegeïnduceerde celdood. Wij vonden in

onze studie (**hoofdstuk 6**) dat circulerende spiegels van M65 en M30-antigeen correleren met de internationaal erkende circulerende standaardmarkers die gebruikt worden als prognostische indicatoren en voor de monitoring van behandelingsrespons van kiemceltumoren. De correlatie tussen M65/M30-spiegels met de IGCCC prognose groep en de overeenkomst met veranderingen in LDH, AFP en β-HCG-spiegels suggereren dat M65/M30 mogelijk ook van prognostische waarde zijn in patiënten met kiemceltumor.

Gezien de data die uit de literatuur bekend zijn en de data zoals gepresenteerd in dit proefschrift, kan men hypothetiseren dat een aantal medicijnen die gericht zijn op specifiek doelwitten ("targeted therapy"), zinvol zijn om te testen, zoals medicijnen die p53 eiwit spiegels verhogen, die p21 remmen, of die eiwitten die onderdeel zijn van de p21 cellulaire cascade remmen, bijv. flavopiridol en fosfo-Akt remmers. Vooral de kiemceltumorpatiënten die behoren tot de slechte prognose groep zouden mogelijk baat kunnen hebben bij de vooruitgang op het gebied van medicijnen die behoren tot "targeted therapy". Een belangrijke uitdaging voor de toekomst zal zijn om de kennis op het gebied van het celbiologisch gedrag van kiemceltumoren en van rationele behandelingen hiervan te vertalen naar de behandeling van andere solide tumoren.

... per aspera ad astra...

Seneca

Hercules furens, atto II, v. 437

Dankwoord Aknowledgement Ringraziamenti

Mijn liefde voor onderzoek begon in mijn eerste jaar aan de universiteit. Van alle vakken vond ik vanaf het begin de oncologie het meest interessant en boeiend: misschien door het feit dat alles nog te begrijpen is of misschien door het feit dat alle elementen op een bepaalde manier in elkaar moeten zitten om een schade te veroozaken en om de schade zelf te herstellen.

Hoewel ik van karakter nieuwsgierig en niet gauw tevreden ben, zijn een aantal eigenschappen van mijzelf door mijn levenservaring in Nederland versterkt. In 1999, in mijn derde jaar aan de Universiteit, ben ik naar Groningen gekomen voor 'just a summer course of oncology'. Vanaf dat moment wist ik zeker ik verder in de oncologie wilde. De liefde voor de stad Groningen heb ik vanaf het eerste moment gevoeld ('Er gaat niets boven Groningen, toch!!??!). Het studeren en werken in het buitenland was voor mij ook een grote uitdaging. Na de 'summer course of oncology' ben ik terug naar Italië gegaan, maar de grote aantrekkingskracht naar Groningen toe bleef altijd trekken. In 2003 ben ik daarom weer naar Groningen teruggegaan om me verder in de oncologie te verdiepen, in de discipline van karakter, in de kultuur...in de taal....

Het eerste traject van WEA (Working Experience Abroad) en vervolgens het traject van de Italiaanse, European Commitee-sponsored opleiding tot oncoloog met aansluitend het traject tot PhD, hebben mij in contact gebracht met veel mensen. Tijdens mijn PhD opleiding heb ik de kans gekregen om met veel mensen samen te werken. Zij hielpen mij om de wereld van de oncologie vanuit verschillende invalshoeken te beleven. Alle mensen die een bijdrage hebben geleverd aan het schrijven van dit proefschrift wil ik graag bedanken.

Als eerste zou ik van harte mijn promotoren, Prof. dr. E. G. E. de Vries en Prof. dr. J. A. Gietema willen danken.

Beste Liesbeth, voor mij is het echt een voorrecht bij jouw afdeling en onder jouw begeleiding te mogen promoveren. Bedankt voor alles wat jij mij gegeven hebt, voor de mogelijkheid om steeds langer in Nederland te kunnen blijven studeren en voor het wachten totdat ik de Nederlandse taal (ongeveer) onder de knie had. Bedankt voor je steun en voor het nodige commentaar op alle artikelen. Van jou heb ik altijd het gevoel gehad dat een hoger doel altijd te zien en te volgen is. Van jou heb ik geleerd om ondanks soms teleurstellende resultaten uit het lab toch enthousiast te

blijven en om dan doorzettingsvermogen te hebben. Vooral bedankt voor al hetgeen wat ik voorgoed van jou met mij zal meedragen in mijn professionele carrière, zoals altijd nieuwsgierigheid, rationeel onderzoek en kliniek te combineren. Bedankt voor het 'model' van de professional die ik graag zou willen worden en jij mij getoond hebt.

Bedankt aan mijn promoter, Prof. dr. J. A. Gietema. Beste Jourik, het jaar dat wij samengewerkt hebben, vormde voor mij een brug terug naar de kliniek. Dankzij jou heb ik 'smooth' begrepen dat klinisch onderzoek eigenlijk was wat ik wilde doen en dat ik contact met patiënten echt miste. Het schrijven van het M30 artikel is zeker niet gemakkelijk geweest. Toen moest je wel denken 'wat een eigenwijs Italiaantje'. Maar ik geef wel toe dat ik door die moeilijke crisisperiode mijn weg gevonden heb, en wat ik van jou meegenomen heb, is om sterk, scherp, duidelijk en synthetisch in de vergaderingen te zijn.

Aan mijn co-promotor Dr. S. De Jong: beste Steven, ik zal nooit genoeg woorden hebben om mijn dankbaarheid voor jou uit te spreken. Bedankt voor het geduld dat jij aldoor met mij hebt gehad, vooral op de momenten waarbij ik niet kon accepteren dat de meeste experimenten op het lab niet naar wens verliepen. Bedankt voor jouw vermogen om om mij te overtuigen over nut van methode en methoden en belang van gedachten, waarmee ik het niet altijd eens was. Bedankt niet alleen voor het wegwijs op het lab, maar vooral om mij een methode te hebben geleerd die ik nu in de kliniek en het klinische onderzoek aldoor gebruik en probeer te verbeteren. Jij hebt mij, als mijn directe begeleider bijna alles wat ik van de moleculaire biologische technieken ken, geleerd. Jouw grote kennis van de biologie en van de biologische mechanismen over ontelbare en inschatbare routes hebben mij vanaf het begin gefascineerd en mij geïnspireerd om mij in de biologische oncologie te gaan verdiepen.

De leden van de beoordelingcommissie, namelijk Prof. dr. E. Vellenga, Prof. dr. R. M. W. Hofstra, Prof. dr. A. J. Moshage, ben ik zeer erkentelijk voor het beoordelen van dit manuscript..

Alle mede auteurs van de hoofdstukken in dit proefschrift zou ik graag willen bedanken, vooral voor de directe of indirecte steun evenals mijn ex-collega's op het Oncologie lab die mij met de experimenten hebben geholpen en, af en toe, mijn buien getolereerd hebben.

In het bijzonder wil ik Dr. H. Timmer-Bosscha, beste Hetty, en Dr. J. Meijer, beste Coby, bedanken voor 'net op tijd hulp' bieden op het lab wanneer ik me helemaal verloren voelde. Maar vooral bedankt voor het mij tonen de menselijke kant van het leven en het dagelijkse gewone leven.

Bedankt voor het enthousiasme waarmee jullie mijn eerste woorde 'op z'n Nederlands' gevolgd hebben.

Beste Phuong, bedankt voor je enorme geduld tijdens mijn eerste stapjes om cellen te leren kweken en voor het onvermoeid meehelpen toen ik vol met werk zat.

Beste Ninke en Wytske, van harte bedankt. Jullie hebben mij geweldig gevolgd en geholpen met het aankleuren en lezen van coupes. Foto's nemen met dat ingewikkelde apparaat is het moeilijkste geweest, toch?

Beste Gert Jan, bedankt voor het 'smiletje' dat je mij aldoor gegeven hebt door mij dagelijks te "plagen" met het feit dat ik, net zo kort als ik ben, je klein Italiaans 'targetje' was. Dat was goed om mijn humeur weer op te krikken, zeker op sommige zware regende dagen.

Bedankt aan Roelof Koster:beste Roelof, vanaf het begin heb jij mij kleine 'trickies' op het lab geleerd. Jij kon het leven op het lab en buiten het lab met mijn ogen kon zien. Bedankt dat jij mijn vriend en mijn collega ben.

Prof. dr. A.J.H. Suurmeijer, beste Albert, wil ik zeer bedanken, voor wat ik geleerd heb in de halve dagen die wij doorgebracht hebben met coupes bekijken. Dat kritische overzich van jou geleerd genut ik nu voor het interpreteren van de patologie uitslagen in mijn huidige klinische werk.

Verder wil ik de promovendi, analisten en studenten bedanken met wie ik zo prettig op het Laboratorium De Vitrine heb gewerkt en leuk gehad.

Diep wil ik Mevr. Gretha Beuker en Mevr. Bianca Smit bedanken. Jullie hebben voor mij altijd tijd gemaakt om mijn Nederlands 'even' te controleren en mijn CV 'even ' in goedNederlands te schrijven. Bedankt voor het elke keer vragen van: 'zal ik dit even voor je doen?'. Het lopen naar jullie toe was voor mij altijd een moment om 'even' mijn gedachten tot rust te latenkomen.

Mijn dank, een speciale dank uit mijn hart, gaat naar mijn paranimf Edmond de Cuba.

BEDANKT!!!!

Ik zou dit ook met grotere 'HOOFD' letters moeten schrijven. Niet alleen voor het mij stapje voor stapje volgen en meeleven in de laatste periode van dit proefschrift, maar vooral voor het naast mij op het lab te zitten en het feit dat jij mij praktisch alle techniekenhebt geleerd, die ik voor mijn onderzoek nodig had. Voor het aan mij leren van de benamingen van alles op het lab tot, tenminste voor mij, moeilijke technieken op het laboratorium. Maar ik wil je ook bedanken voor je vriendschap, en voor de capaciteit die je mij al zonder woorden kunt begrijpen en mij goede raad geven.

I want to thank my paranymph Prof. dr. S. Agarwala, dear Sanjiv, because of your support especially during the last months of the thesis, when I couldn't find a clue among all the clinical activity, the new scientific activity in melanoma field and the necessity/will to walk up to the end of this education step and life experience which the Netherlands represent to me.

Eventually I would like to thank my chief, dr. Alessandro Testori and my deputy Director. Dr. Pier Francesco Ferrucci, for the patience they had with me for all the last period of the PhD thesis, when I deeply tried to divide myself among thesis, patients, congresses… Posso solo dire: grazie, pieno di stima e di riconoscenza…

Een speciale woord van dank gaat ten slotte aan diegene die mijn familie zijn, die een speciaal plekje in mijn hart hebben en die een belangrijk stuk van mijn leven zijn.

Lieve Oma en Opa, jullie weten hoeveel ik van jullie houd en hoeveel ik van jullie geleerd heb sinds het moment dat ik jullie ontmoette. Leven bracht ons samen, misschien ook dichterbij dan wij ooit mochten denken. Ondanks het verdriet en de moeilijkheden in het begin, is de tijd bij jullie thuis de mooiste periode van mijn leven geworden en steeds vaker voel ik diepe heimwee naar de momenten die wij samen doorbrachten. Jullie weten dat ik jullie als mijn echte Oma en Opa beschouw en dat ik veel dankbaarheid en respect voel voor alles wat jullie mij geleerd hebben over de Nederlandse gewoontes, de Nederlandse taal, de Nederlandse literatuur, poëzie en de goede 'menselijkskennisvolle' adviezen van Opa. Bedankt voor mij op het raam laten tikken als ik aankwam en om mij altijd thuis te laten voelen. Bedankt voor het liefdevolle leven dat jullie mij geschonken hebben tijdens mijn verblijf in Groningen.

Mijn beste Edwin, bedankt voor jouw vriendschap. Wij hebben vele zeer leuke momenten samen doorgebracht, ook samen met de andere jongens van de rest van de groep. Dankzij jouw vriendschap en je grapjes, heb ik me nooit in het buitenland gevoeld. De ochtenden dat wij samen de zonsopgang hebben gezien zitten tussen de meeste gekleurde momenten van mijn herinneringen.

Deeply from my heart thanks to my friend and colleague dr. E. Magni: somebody you really want to meet and know better, because of her humour, because of her culture, because of her wide interests…My dear Elena, thanks because you came to me quite from my beginning back to Italy,

showing me the way among the colleagues, the nurses, the documents, the computer programmes...thanks above all for being my friend outside the hospital, to be able to share with my joy and doubts, happiness and sorrow, coming from daily life...

Mio amato Papily, mia dolce Mamily: non basterebbero tutte le parole della nostra lingua, nè quelle precise ed affascinanti dell'Olandese, nè quelle 'polite' della lingua Inglese per descrivere la gratitudine che vi porto nel cuore, per aver sostenuto ogni mia scelta, ogni mio cammino, per avermi supportato e mai lasciato camminare da sola, anzi! al contrario, esser stati una presenza invisibile, costante, ma discreta, per quella vostra indescrivibile capacità di esser vicini nel cuore, anche quando la distanza fisica e le limitazioni temporali hanno impedito - o, per meglio dire, tutt'ora impediscono- il contatto occhi negli occhi. Ma vi ringrazio per esser capaci di comprendermi, nei silenzi e nelle parole, nei miei giorni di luce e nei mie giorni di notte e, sopratutto, per l'entusiasmo con il quale accogliete ogni mia scelta o novità. Il vostro sorriso, il vostro esempio, l'amore tra voi e per noi, il vostro criticismo, la vostra puntualità e ostinazione nel lavoro, la vostra infaticabile capacità di esser in carriera ed in famiglia allo stesso tempo sono ciò che mi porto sempre nel cuore e ciò verso cui guardo nei miei momenti di smarrimento... grazie, a voi, che siete l'esempio della mia vita.

A mio fratello e mia sorella, 'paraninfi' della mia vita, capaci di camminare insieme a me nei percorsi complessi e, talvolta contorti, della vita lavorativa ed affettiva.

A Paky, grazie: per lo sguardo critico sulla vita e sui fatti di ogni giorno, per quella tua profonda capacità di comprendere persone e circostanze al di là delle apparenze. Grazie per avermi sempre dato il consiglio giusto al momento giusto; grazie per tutte le volte che mi svegli nel cuore della notte e che mi insegni che la vita ha ulteriori aspetti oltre al lavoro. Grazie per l'amore fraterno che mi fai sentire dentro, con le tue e-mail, le tue battute... per quella luce che tu sei capace di gettare su aspetti di me o del mio carattere, che, talvolta, neanche io conosco. Grazie per esser, da sempre, l'uomo giusto al momento giusto. Ti voglio bene, come non serve dirlo con le parole, ma come mi si legge nei mie occhi ogni volta che parlo di te.

Dolce Cristiana, grazie per quel tuo mix impareggiabile di maturità e 'fanciulleria', che per me di grande esempio sono. Grazie per sapermi sempre riportare con i piedi per terra tutte le volte che, abbandonato per un attimo il mio lato cinico e sarcastico, mi addentro in voli pericolosi; grazie per la dolcezza che mi doni in quei miei momenti di acidità acuta, forse non necessari. Grazie per tutti i consigli che ,sin da bambine, tu, mia piccola grande sorella, mi dai: grazie per esser per me l'esempio della 'femme avec le parfaite physique du role', capace di adattarsi affabilmente e con carattere ad ogni persona ed ad ogni circostanza. Ti voglio un bene profondo e ti porto sempre nel

cuore, con orgoglio, come si riflette nel tremolio della mia voce quando pronuncio il tuo nome o parlo di te .

Grazie alle mie Nonne, esempio di donne forti e determinate, donne di altri tempi eppure cosi moderne. Nonna Tina, la nonna-mamma di una volta, capace di tener sotto controllo intere situazioni, pur stando a casa, con un occhio sempre attento, premuroso sull'evolvere e lo svolgersi della vita di noi tre, suoi unici nipoti. Grazie per il sostegno affettivo, telefonico e... serale, che continui a darmi, in modo sempre crescente, ogni giorno. Nonna Morosina, la nonna manager, esempio di modernità ed innovazione, oltre qualsiasi potenziale immaginazione, ma maggiormente esempio di volontà ferma e determinata, di perspicacia e di capacità di donare amore gratuitamente...

So deeply from my heart, just thanks to you all...

e mentre io guardo la tua pace, dorme quello spirto guerrier ch'entro mi rugge

Ugo Foscolo Alla Sera, 1803

Alessandra di Pietro, MD

Curriculum Vitae et Studiorum

General data

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Mother Language: Italian

Spoken Languages: English, Dutch, Spanish; French

Education

- Since June 2007: Assistant at Melanoma and Sarcoma Division, at IEO- European Institute of Oncology, Milan, Italy

- November 2006- November 2009: PhD student Medical Oncology, University Medical Center Groningen (UMCG), Groningen, The Netherlands

- Thesis: '*Role of Apoptotic Pathways in Chemosensitivity in Testicular Germ Cell Tumours*', defensors Prof. dr. Elisabeth G. E. de Vries, Prof. dr. Jourik A. Gietema; co-defensor dr. Steven de Jong, Department of Medical Oncology- Laboratorium De Vitrine, UMCG, Groningen, The Netherlands. To be defended on November, 16th 2009.

- November 2006: Recidency in Medical Oncology – 50/50 Magna Cum Laude at Università Cattolica del Sacro Cuore, Rome, Italy

- Thesis: '*Testicular Germ Cell Tumours, a paradigm of chemosensibility for solid tumours*', defensor Prof. Dr. Achille R.M Cittadini, supervisor Prof. Dr. Elisabeth G.E. de Vries

- July 2002: Graduation in Medicine and Surgery - 110/110 Magna Cum Laude and Notable Mention, at Università Cattolica del Sacro Cuore, Rome, Italy

- Thesis: '*Molecular evaluation of the correlation between susceptibility factors and precocious alterations in superficial bladder cancer, by means of a non-invasive method*', defensor Prof. Dr. Giovanna Flamini, co-defensor Prof. Dr. Achille R. M. Cittadini, Oncology Department

- March 1999-September 2002: Internal student and then internal medical doctor in the Oncology Research Centre Giovanni XXIII - Università Cattolica del Sacro Cuore, Rome, Italy

- 1991-1996: Secondary School Diploma, Benevento (60/60), at the Gymnasium Licaeum "Pietro Giannone", Benevento, Italy

Further education

- November 2007: Course if Basic Life Support-Defibrillator; Cardiological Hospital "Monzino", Milan, Italy

- May 2002: Course of Basic Life Support (BLS) - B category - Theoretic total score 100%, Practical total score 98%, organized by IRC (Italian Resuscitation Council)

Working experience

- June 2007: Melanoma and Sarcomas Division and Medical Oncology Department, European Institute of Oncology, Milan Italy

- January 2004-June 2007: Department of Medical Oncology, University of Groningen Medical Center, The Netherlands: translational research

- July-October 2003: Medical Oncology Research Laboratory, University of Groningen Medical Center, The Netherlands

- October 2001-October 2002: Tutoring and training to medical students at Università Cattolica del Sacro Cuore, Rome, Italy

Other

- May 2007: Command of Dutch language, fourth level- letters faculty RUG University, Groningen, The Netherlands

- January 2007: SPSS Statistics Course, UMCG Groningen, The Netherlands

- November 2005: Automatisation for Physicians, UMCG Groningen, The Netherlands

- July 2005: ` Command of Dutch language NT2 Staatsexaman "Nederlands Als Tweede Taal", for all the four the subparts writing, speaking, listening and reading- IB Groep, Groningen The Netherlands

- May 2005: Command of Dutch language, second level- letters faculty RUG University, Groningen, The Netherlands

- May 2004: Command of the Dutch language, first level - Letters Faculty RUG University, Groningen, The Netherlands

- March 2004: VTM course for Safe Microbilogical Techniques, UMCG Groningen, The Netherlands

- November 2003-now: Collaboration with IMSW society, Paris France, for the translation of medical books from English into Italian

- May 2003: Winner of Residency in Oncology at National Cancer Institute, Milan, Italy

- April 2003: Winner of Residency in Oncology at Università Cattolica del Sacro Cuore, Rome, Italy

- July 1994: Command of the English language at Bootham School of English, York, United Kingdom

Scholarships

- December 2005: WEA (Working Experience Abroad) Scholarship, assigned from Universita' Cattolica "Sacro Cuore"- Rome, Italy

- November 2005: "Foundation for Education in Oncology"(Fondazione per la Formazione Oncologica) scholarship

- October 2005: Doctor Renato Russo Prize, as best young doctor of the year 2005, Ordine dei Medici e degli Odontoiatri, Benevento, Italy

- September 2005: Teaching ESO Oncology Scholarship, to attend the congress on Colorectal cancer, London 1-3 December 2005

- October 2004: Universita'Cattolica Sacro Cuore "Education Abroad" Scholarship

- March 2004: "Foundation for Education in Oncology" (Fondazione per la Formazione Oncologica) scholarship
- November 2002: Leonardo da Vinci scholarship, in relation to the project L-WAY
- October 2002: FIRC (Italian Cancer Research Federation) Scholarship
- April 2002: Best student of the year Scholarship
- October 1996: INPDAP Scholarship

Informatic Skills

- Microsoft Office (Word, Ppt, Excel)
- Access
- Argos
- Sinfonia
- Sygma Plot
- SPSS ver. 12 & 14
- Acrobat Prof
- Reference Manager
- GraphPad
- Media Player/ Mediavideo

Languages

Mother Language **Italian**

Other languages

	Speaking	**Understanding**	**Reading**	**Writing**
English	Good	Good	Good	Good
Dutch	Good	Good	Good	Good
Spanish	Good	Good	Good	Average
French	Basic	Basic	Basic	Basic

Publications

November 2009: Review: 'Heat shock protein peptide complex 96-based vaccine in melanoma. How far we are, haw fare we can get'. A. di Pietro, G. Tosti, P.F. Ferrucci, A. Testori. Human Vaccines Volume 5, Issue 11. *In press*

October 2009: Article "Metronomic administration of pegylated liposomal-doxorubicin in extensively pre-treated metastatic breast cancer patients: a mono-institutional case-series report". E. Munzone, A. di Pietro, A. Goldhirsch, I. Minchella, E. Verri, M. Cossu-Rocca, C. Marenghi, G. Curigliano, D. Radice, L. Adamoli, F. Nolè. The Breast, *Accepted*

June 2009: Abstract: "Circulating endothelial cells (CECs), progenitors (CEPs) and Circulating Tumor Cells (CTCs) for prediction of response in patients with advanced breast cancer (ABC) receiving metronomic oral Vinorelbine (oV). Preliminary Results". F. Nolè, E. Munzone, F. Bertolini, MT. Sandri, G. Petralia, L. Adamoli, D.Radice, D. Cullurà, A. di Pietro, and A. Goldhirsch. ASCO 2009

May 2009: Article "Biology and clinical use of hit shock proteins GP96 in cancer patients". G. Parmiani, A. M. Di Filippo, L. Pilla, C. Castelli, L. Rivoltini, M. Maio, M. Santinami, V. Mazzaferro, A. di Pietro, A. Testori. J Immunotherapy. *Submitted and accepted*

May 2009: Review "HSPPC-96 vaccine (Oncophage®;Vitespen) in metastatic melanoma patients: from the state of the art to a possible future." G. Tosti* , A. di Pietro* (*co-authors), P.F. Ferrucci, A. Testori . Expert Review on Human Vaccine- Future Drugs. *Submitted and accepted*

April 2009: Abstract: "New Apoptosis Markers in Melanoma". A. di Pietro, P.F.F. Ferrucci, A. Testori. AACR 2009. *Winner of BMS prize as best work of Clinical Research session.*

Marzo 2009: Abstract: "Role of the SOS-1-Rac signalling pathway in melanoma progression" A. di Pietro, G. Tosti, S. Gerboth, A. Palamidessi, C. Loise, G. Mazzarol, P. P. Di Fiore, P.F. Ferrucci, A. Testori , G. Scita. EORTC-Melanoma Group Meeting, Bruxelles 2009.

December 2008: Article "Oncophage: step to the future for vaccine therapy in melanoma", A. di Pietro, G. Tosti, P.F. Ferrucci, A. Testori, Expert Opin Biol Ther. 2008 Dec.

October 2008: Article "Clinical evaluation of M30 and M65 ELISA cell death assays as circulating biomarkers in a drug-sensitive tumor, testicular cancer". de Haas EC, di Pietro A, Simpson KL, Meijer C, Suurmeijer AJ, Lancashire LJ, Cummings J, de Jong S, de Vries EG, Dive C, Gietema JA. Neoplasia. 2008 Oct

August 2008: Article. “Basosquamous carcinoma of the thoracic: A case report” J Soteldo, L Jimenez, F Verrecchia, G Mazzarol, F De Lorenzi, F Baldini, A di Pietro, A Testori. Division of Melanoma and Sarcoma, European Institute of Oncology, Milan, Italy. The American Journal of Case Report.

June 2008: Abstract: “A Retrospective Review of Sentinel Node Biopsy for High-Risk Cutaneous Non-Anogenital Squamous Cell Carcinoma”, M.Rastrelli, M.Zonta, J.Soteldo, F.Verrecchia, G.Tosti, G.Spadola, A.di Pietro, A.Testori. Division of Melanoma and Sarcoma, European Institute of Oncology, Milan, Italy. Presented at XXI National Congress of Società Polispecialistica Italiana Giovani Chirurghi

April 2008: Internet Article: “The treatment of cutaneous and subcutaneous lesions with electrochemotherapy with bleomycin” J. Soteldo, A. di Pietro, G. Tosti, M. Mosconi, F. Baldini, M. Rastrelli, G. Spadola, G. F. Verrecchia and A. Testori. Division of Melanoma and Sarcoma, European Institute of Oncology, Milan, Italy

September 2007: Abstract: “New markers of apoptosis: a feasible tool to improve prognostic accuracy and to monitor therapeutic response in melanoma patients” di Pietro A, Zonta M., Jimènez L., Tosti G., Ferrucci P.F., Mazzarol G., Testori A., presented at the 1st World Meeting of Interdisciplinary Melanoma Centers, Barcelona, Spain

September 2007: Abstract: “Safety, feasibility and toxicity profile of a new immunotherapy protocol for metastatic melanoma patients using in vivo Salmonella typhimurium vaccination and treatment.” P. F. Ferrucci, C. Martinoli, A. di Pietro, M. Rastrelli, A. Testori, M. Rescigno, presented at the 1st world Meeting of Interdisciplinary Melanoma Centers, Barcelona, Spain

September 2007: Abstract: “Atypical Spitz nevus and Spitz tumour: a report of 14 cases.” G. Tosti, L. Jimenez, A. di Pietro, A. Testori, G. Mazzarol, presented at the 1st world Meeting of Interdisciplinary Melanoma Centers, Barcelona, Spain

January 2007: Article: “The p53 pathway affects cisplatin-induced apoptosis in human testicular germ cell lines depending on the cellular context” A. di Pietro, R. Koster, W.A. Dam, N.H. Mulder, J. A. Jourik, E.G. E. de Vries, S. de Jong *Submitted*

May 2006: Abstract: “The p53 pathway effects on cisplatin-induced apoptosis in Testicular Germ Cell Tumours cell lines, depending on the cellular context”. A. di Pietro, R. Koster, J. A. Gietema, S. de Jong, presented at the 5th Apoptosis Meeting, “Death between the tulips”, Kerkrade, The Netherlands

April 2006: Abstract: “p21$^{Cip1/Waf1}$ over-expression protects human testicular germ cell tumour cells from cisplatin induced apoptosis”. R. Koster, A. di Pietro, H. Timmer-

Bosscha, H. Schepers, R. Bischoff, J. A. Gietema, S. de Jong-presented at the "97th AACR Annual Meeting, Washington, D.C., USA

June 2005: Review: "Testicular germ cells Tumours: the paradigm of chemo- sensitive solid tumours", di Pietro A, de Vries EGE, Gietema JA, Spierings. DCJ and de Jong, S. - Int J Bio Cell Biol 37 (2005) 2437-2456.

Abstracts

-Abstract RewiewHuman Vaccines Volume 5, Issue 11 2009: 'Heat Shock Proteins(HSPs) are highly conserved, stress-induced proteins and function as chaperones stabilizing and delivering peptides. In several preclinical studies, tumor derived HSP-peptide complexes (HSPPCs) has been shown to be able to induce immunity against several malignancies. HSP-based vaccines, indeed, work across tumor types, bypassing the need for the identification of the single immunogenic peptide, and thus emerging as a class of tumor- and patient-specific vaccines. HSPPC-96-based vaccine Vitespen® (formerly Oncophage®) is the first autologous cancer vaccine made from individual patients' tumors which not only confirmed its activity in different malignancies (e.g., gastric cancer, colorectal cancer, pancreatic cancer, non-Hodgkin's lymphoma and chronic myelogenous leukemia), but was also successfully tested in phase III clinical trials in melanoma and kidney cancer. Even more HSPPC-96- based vaccine demonstrated an excellent safety profile, with almost no toxicity. HSP-based vaccines are emerging as a novel therapeutic approach with a suggestive role in cancer therapy.'

-Abstract ASCO Annual Meeting 2009: Background: The metronomic administration of chemotherapy once or more per week with no extended breaks has been shown to optimize the anti-angiogenic effects of a given drug, leading to growth arrest or apoptosis of endothelial cells in tumour neo-vessels. Preclinical and clinical studies suggest that ultra-low concentrations of several microtubule inhibitors are able to inhibit proliferation or migration of endothelial cells. In a clinical and biomarker Phase II study, ongoing at our institution, we investigated the activity of metronomic administration of oV, in ABC, the kinetics and the predictive potential of CECs, CEPs, CTCs and of other biomarkers of angiogenesis (soluble VEGF, VEGFr2, TSP1, bFGF). Parameters measured by CT perfusion were also assessed. Methods: From February 2008, 47 pts with ABC received oV (50 mg/die TTW). Currently 20 pts are evaluable for both activity and biomarker assessment and their basal levels of biomarkers of angiogenesis were correlated to response. Results are shown in table 1

	BEST RESPONSE	PTS	MEAN ± SD	MEDIAN	MIN,MAX	PVALUE
CECs (N/ml)	PR/SD	10	177.9 ± 45.3	133.6	36.0, 533.5	0.7375
	PD	10	186.3 ± 41.1	163.2	15.5, 378.0	
Apoptotic CECs (N/ml)	PR/SD	10	95.2 ± 19.8	76.3	18.0, 213.4	0.4372
	PD	10	143.4 ± 37.7	119.9	9.3, 355.3	
Apoptotic CECs (%)	PR/SD	10	55.7 ± 4.2	50.0	40.0, 77.0	0.0161
	PD	10	75.2 ± 3.9	78.0	60.0, 94.0	
Viable CECs (N/ml)	PR/SD	10	82.7 ± 28.5	47.5	18.0, 320.1	0.2016
	PD	10	42.8 ± 10.1	35.7	6.2, 110.7	
CEPs (N/ml)	PR/SD	10	222.4 ± 79.6	146.4	39.6, 853.6	0.7375
	PD	10	165.4 ± 42.3	99.6	34.1, 354.8	
CTC (N/7.5ml)	PR/SD	10	6.4 ± 2.7	4.0	0, 25.0	0.2107
	PD	10	73.3 ± 30.3	42.0	0, 229.0	

Conclusions: We found that the baseline value of apoptotic cells (expressed as %) was significantly correlated to outcome. The baseline total, viable, and apoptotic CEC count and CTCs might represent an indirect measure of the angiogenic turnover and an indicator of better response to antiangiogenic therapy, supporting the use of these treatments in patients expressing high levels of baseline CECs. Updated results will be presented together with correlation with perfusion CT scan and levels of CTCs.

-Abstract AACR- Annual Meeting 2009: Background: Although the prevailing dogma states that cytokeratin (CK) intermediate filaments (CIF) are hallmark of epithelial cells, several publications suggest that CK may be expressed by a variety of normal and malignant cells of different embryonic origin, including germ cells tumours and melanomas[1,2]. In vitro studies also demonstrated that the co-expression of vimentin IF, characteristic of melanoma cell lines, together with CK 8/18 IF leads to a more invasive phenotype[1,2]. These *in vitro* data support the clinical observation that vimentin/keratin co-expression is linked to poor patient outcome in cutaneous melanoma[3,4]. Moreover the presence of CK 18 in cutaneous melanoma patients allows the possibility to measure the effect of chemotherapy in terms of apoptosis. The main effect of most chemotherapeutic drugs is the activation of apoptotic pathways inside tumour cells. In particular, active caspase 3 cleaves CK 18 (M65 antigen) at the position Asp396[5]. The neo-epitope produced by the cleavage of CK18 is specifically recognised by a monoclonal antibody (M30), which does not recognize the whole protein nor its necrosis products[5]. This characteristic allows the use of M30 antibody to distinguish apoptotic from both necrotic and viable cells. Furthermore this antibody can be used on tissue samples (Immuno-Histo-Chemistry, IHC), to define the level of intrinsic apoptosis in the single patient. Aim: The correlation between CK18 expressions and poor prognosis was investigated in a group of melanoma patients visited at our institution, in order to define the role of this putative marker. Methods: A pilot study performed on histological samples from 24 patients (12 SNB positive [SNB +] and 12 SNB negative [SNB -]) who underwent surgery at IEO between 1997 and 2007. Primary tumour and SNB samples were collected and stained for CK 18 by IHC. Data relative to disease free survival and overall survival were available at the database of the department. Correlation PFS/OS *vs* positive staining was analysed by *SPSS14*. Results: As to patients' characteristics at the diagnosis, SNB – patients were on average older than SNB+ ones (median age: 51.5 yrs *vs* 64.5 yrs). As to sex and clinical-pathological features, such as SN basin, Clark's level, Breslow's thickness and ulceration, the two groups were quite comparable. Follow-up was slightly longer for SNB - patients (42 months in SNB + vs 57 months in SNB -). As to CK 18 staining, only 2 SNB + patients showed a positive staining for CK18 in the primary tumour; one of them had a positive CK18 staining in SNB too. Conclusion and Future Perspectives: Due to the small number of patients analysed, it was not possible to evaluate the presence of a correlation between CK 18 expressions and prognosis. A case control study is at the moment ongoing (100 SNB + and 100 SNB - patients) in order to determine staining-prognosis correlation. Furthermore, we planned to extent the study patients who underwent lymphadenectomy.

-Abstract EORTC- Melanoma Group 2009: During past the decades, knowledge of melanoma biology has considerably increased. Numerous therapeutic modalities based on this knowledge are currently under investigation. Sos-1 plays a well-

established role in signal transduction from receptor tyrosine kinases (RTK) to the Ras-MapK pathway. Additionally, the DHPH tandem repeat of Sosl displays intrinsic GEF activity towards Rac, which, under resting conditions, is auto-inhibited. This is accounted by the fact that the DH and PH domains are in a closed conformation, preventing a direct interaction of the latter domain with Rac. Two mechanisms of activation have been currently proposed. The former invokes the engagement of Sos-1 into a complex with Eps8 and Abi1, while the latter is mediated by tyrosine phosphorylation of Sosl by ABL kinase. To investigate how tyrosine phosphorylation may regulate Sosl-Rac-GEF activity and its relevance in physiological and pathological conditions, we mapped the tyrosine which becomes phosphorylated by Abl by a combination of phosphopeptide mapping and mass spectrometry analysis. We identified tyrosine 1196 as the major phospho-site. Validation of the relevance of this site was obtained by generating a single point mutant, Sosl Y1196F, which is no longer phosphorylated *in vitro* and in cells by Abl, and fails to promote the exchange of nucleotides on Rac, but not on Ras. This is mirrored by the essential role played by this modification of Sosl in PDGF-mediated Rac activation and actin remodeling in physiological conditions, and in BCR-ABL-mediated transformation in a pathological context. To investigate whether the Sosl-Rac pathway is deregulated in other types of cancer, and in particular in solid tumors, immunohistochemistry (IHC) was performed using a monoclonal antibody raised against the pY1196 of Sosl on a variety of tumor tissues using tissue micro arrays. We found that high levels of tyrosine phosphorylated Sosl were frequently detected in melanomas. In order to further investigate this aspect tissue micro arrays were performed using the anti Sosl pY1196 antibody in a small group of tissue specimens, including 34 melanocytic nevi, 161 primary melanomas and 61 melanoma metastases. Our preliminary observations show that Sosl tyrosine phosphorylation status correlated with different pathologic characteristics of the tumours including the Clark level of invasion and the Breslow thickness, pointing to the relevance of this post translational modification in melanoma progression. These preliminary data must be confirmed by further investigations considering other pathological characteristics of the primary tumor and lymph node metastasis (including presence/absence of ulceration, number of mitosis, presence/absence of histological regression, pattern and burden of lymph node involvement, etc) and clinical and epidemiological information about the melanoma patients included in the present analysis.

-Abstract Article EBOT 2008: Heat shock proteins (HSPs) are a group of proteins whose expression is increased when the cells are exposed to elevated temperatures or other stressful conditions. This increase in expression is transcriptionally regulated. The function of HSPs is similar in virtually all living organisms, from bacteria to humans. Their expression also occur under non-stressful conditions, simply "monitoring" the cell's proteins, ie they carry old proteins to the cell's "recycling bin" and they help newly synthesised proteins fold properly. These activities are part of a cell's own repair system.

HSPs are molecular chaperones for protein molecules. They are usually cytoplasmic proteins and they perform functions in various intra-cellular processes. Tumor derived HSP-peptide complexes (HSPPCs) can be used for vaccination against malignancies. In particular, HSPPC-96. Vitespen (formerly Oncophage®) is a HSP-based vaccine made from individual patients' tumors with a promising role in cancer management. This vaccine has been extensively studied in phase I and II clinical trials, showing activity on different malignancies, including gastric cancer,

colorectal cancer, pancreatic cancer, non-Hodgkin's lymphoma and chronic myelogenous leukaemia. The vaccine has also been studied in phase III clinical trials in melanoma and kidney cancer showing an an excellent safety profile, with essentially no toxicity. Thus, HSP-based vaccines are a novel therapeutic approach with a promising role in cancer management.

-Abstract Article Submitted 2008: The role of p53 in the unique cisplatin sensitivity of human testicular germ cell tumour (TGCT) cell lines was analysed. A panel of cisplatin sensitive cell lines (833KE and Tera), a subline with cisplatin-acquired resistance (Tera-CP) and an intrinsic cisplatin resistant cell line (Scha), all expressing wt-p53 were used. Basal p53 and p53-transcriptional target MDM2 expression was present in all lines, whereas p53-transcriptional target $p21^{Waf1/Cip1}$ only in Scha. P53 expression increased already after 6 h and further enhanced after 24 h cisplatin exposure, together with Mdm2 and apoptosis induction, whereas minimal $p21^{Waf1/Cip1}$ induction occurred. Previous observations showed, however, that irradiation of TGCT cells induced high levels of $p21^{Waf1/Cip1}$. P53 suppression by p53 short interfering (si)RNA lowered apoptosis in Tera, related to a diminished Fas membrane expression, but not in 833KE nor in Tera-CP. In contrast, p53 down-regulation, concomitantly suppressing $p21^{Waf1/Cip1}$ expression, increased cisplatin-induced apoptosis in Scha. Importance of $p21^{Waf1/Cip1}$ in Scha was further demonstrated by $p21^{Waf1/Cip1}$ siRNA, which resulted in elevated apoptosis after cisplatin. Our results suggest a dual role for p53 in transactivation and cisplatin-induced apoptosis in TGCT cells depending on cellular context of p53. Low $p21^{Waf1/Cip1}$ levels under cisplatin-induced p53 activation may explain hypersensitivity of TGCT cells to cisplatin-induced apoptosis.

-Abstract Article October 2008: Circulating full-length and caspase-cleaved cytokeratin 18 (CK18) are considered biomarkers of chemotherapy-induced cell death measured using a combination of the M30 and M65 ELISAs. M30 measures caspase-cleaved CK18 produced during apoptosis and M65 measures the levels of both caspase-cleaved and intact CK18, the latter of which is released from cells undergoing necrosis. Previous studies have highlighted their potential as prognostic, predictive, and pharmacological tools in the treatment of cancer. Disseminated testicular germ cell cancer (TC) is a paradigm for a chemosensitive solid malignancy of epithelial origin and has a cure rate of 80% to 90%. We conducted M30/M65 analyses on 34 patients with TC before and during treatment with bleomycin, etoposide, and cisplatin and showed that prechemotherapy serum levels of M65 and M30 antigens are correlated with established TC tumor markers lactate dehydrogenase, alpha-fetoprotein, and beta-human chorionic gonadotropin, probably reflecting tumor load. Cumulative percentage change of M65 and M30 from baseline to end of study was highest in poor prognosis patients ($P < .05$). Moreover, area under the curve profiles of M65 and M30 during chemotherapy mirrored dynamic profiles for lactate dehydrogenase, alpha-fetoprotein, and beta-human chorionic gonadotropin. Consequently, M65 and M30 levels appear to reflect chemotherapy-induced changes that correlate with changes in markers routinely used in the clinic for management of patients with TC. This is the first clinical study where M65 and M30 antigen levels correlate with established prognostic markers and provides impetus for their exploration in other epithelial cancers where there is a pressing need for informative circulating biomarkers.

-Abstract Article August 2008: Background: Basosquamous carcinoma (BSC) is a rare malignancy with specific histopathological features and high metastatic potential. Case Report: We report the case of basosquamous carcinoma in a patient presenting a widespread ulcer on the thoracic wall. Conclusions: We suggest to give more echo to the peculiar behaviour of BSC among physicians as a first, aggressive treatment can be curative. We encourage physicians to organize multicentric clinical trials to evaluate the usefulness of sentinel node biopsy in BSC.

-Abstract June 2008: Introduction: Certain patients with Cutaneous Squamous Cell Carcinoma (SCC) have much higher rates of regional nodal metastases than is often reported. SCC usually metastasizes through lymphatics with regional nodal metastases developing before distant metastases. Current management of regional nodes for SCC is controversial and Sentinel Node Biopsy (SNB) may have a role in the management of specific patients. This study aims to further validate SNB for SCC and the outcome of these patients following SNB. Methods: 20 patients with high-risk non-anogenital SCC who underwent SNB between 1998 and 2007 were retrospectively reviewed. High-risk tumours included those greater than 2cm or grade 1 differentiation, and recurrent or ulcerating tumours. Patients with clinical or radiological evidence of nodal metastases were excluded. SNB was performed under local or general anaesthesia following Lymphoscintigraphy (LS) and blue dye injection. Results: The mean age of patients was 72 years. The median follow-up from SNB was 24 months. Tumour location included the head and neck (n = 11), extremities (n = 9), and trunk (n = 1). One patient had a positive sentinel node. This patient developed regional recurrence 13 months after refusing a completion neck dissection and is alive with progressive disease after 31 months. Two patients developed regional recurrence after negative SNB (1 is alive and disease-free, the other died of progressive disease). Of the remaining patients, 15 are alive and disease-free, 1 died of another malignancy and 1 was lost to follow-up. Conclusion: This study shows SNB for high-risk SCC is feasible and allows early detection and treatment of nodal metastases. Currently SNB for SCC is not standard treatment and requires further investigation to determine which patients would best benefit from this procedure.

-Abstract September 2007: Although cytokeratin intermediate filaments (IFs) are considered characteristic of epithelial cells, recent publications suggest that these IFs may be expressed by cells of different embryonic origin, including melanoma. Previous studies demonstrated that co-expression of vimentin IF, characteristic of melanocytes and melanoma, and cytokeratin-18 (CK18) IF results in a more invasive phenotype and poorer outcome in melanoma patients. Nevertheless, CK18-positivity allows chemotherapy apoptotic effects to be monitored. Caspases activated during chemotherapy-induced apoptosis cleave cellular substrates including cytoskeleton. Active caspase-3 cleaves CK18, producing M30 neo-epitope. CK18 and M30 antigens can be detected by specific monoclonal antibodies on paraffin samples and ELISA assay can quantitate apoptotic levels in serum. Because many melanoma patients are treated annually at our Institute, we investigated whether CK18 expression correlates with poor prognosis. In a pilot study of 50 patients undergoing surgery between 1997 and 2007, primary tumour samples were stained for CK18 and M30. Data regarding disease-free and overall-survivals were obtained from our database. We will also investigate prospectively the correlation between serum levels of these markers and patient outcome, in those expressing CK18 in the primary tumour.

-Abstract September 2007: A major obstacle for the development of effective immunotherapy is the ability of tumours to escape the immune system. Here we report a conceptually new approach based on *in vivo* infection of tumours and killing of infected tumour cells. Since may 2006, 8 patients affected by not operable stage III or IV M1a metastatic melanoma where enrolled in the trial. Two steps were planned: oral vaccination with Ty21a and intratumoral treatment with Ty21a, in order to stimuli an autologous immune response. Two metastasis were treated and 1 observed in order to evaluate the indirect effect of vaccination. Among 8 enrolled patients, 1 could not be vaccinated due to the evidence of hepatic metastasis during stadiation, 1 had a rapid worsening of the performance status during vaccination while 2 didn't develop adequate anti-ST vaccination titre. Of the remaining 4 patients, 1 completed the first cycle, while the other 3 interrupted the treatment due to progressive disease, important hypersensitivity reaction and a worsening of the hepatic function indexes. At injection, all the patients developed a variable degrease of lumbar pain, nausea/vomiting, shivering, fever, hypotension easily controlled by treatment with antihistaminics, antipyretics, antiemetics without the use of cortisones. Treated metastases showed all the signs of local inflammation and objective dimensional stabilization or reduction, but systemic disease seem not to be affected by treatment.

-Abstract September 2007: "Spitz nevus is considered as a variant of benign melanocytic nevus, which may represent a simulator of melanoma. In some cases, it can be difficult to distinguish between benign and malignant cases because there are lesions in which histological features characteristics of Spitz nevi are mixed to features generally found in melanomas. Such histologically ambiguous cases, mostly referred as "atypical Spitz nevus/ Spitz tumors", represent a frequent source of diagnostic uncertainty and of discordant opinions among pathologists, because of the absence of consistent diagnostic criteria.Several techniques have been assessed for differentiating nevi from melanomas including histochemical (reticulin) and immunohistochemical staining and the expression of proliferation markers such as Ki67 is particulary important for this purpose.We report 14 patients with problematic melanocytic lesions showing histological features characteristics of Spitz nevus mixed to histological features generally referred to as melanoma (in the study, referred to as atypical Spitz nevi and Spitz tumours). Clinical data was reviewed, including patient age; sex; clinical outcome; location and dimension of the primary tumour. Cases were retrieved from the files of the Melanoma Division of the European Institute of Oncology in Milan, Italy, over a period of 7 years (2000-2006). Eleven patients were females, 3 were males. Median age was 31,8 years with age ranging from 9 to 64 years. All patients underwent a wide local excision at the site of the primary lesion. A sentinel lymph node biopsy was performed in 10 patients, all were negative. Median follow-up time was 36 months.

-Abstract Review 2005: Testicular germ cell tumours (TGCTs) are the most frequent solid malignant tumour in men 20-40 years of age and the most frequent cause of death from solid tumours in this age group. Up to 50% of the patients suffer from metastatic disease at diagnosis. The majority of metastatic testicular cancer patients, in contrast to most other metastatic solid tumours, can be cured with highly effective cisplatin-based chemotherapy. From a genetic point of view, almost all TGCTs in contrast to solid tumours are characterised by the presence of wild type p53. High p53 expression levels are associated with elevated Mdm2 levels and a

loss of $p21^{Waf1/Cip1}$ expression suggesting a changed functionality of p53. Expression levels of other proteins involved in the regulation of cell cycle progression indicate a deregulated G1–S phase checkpoint in TGCTs. After cisplatin-induced DNA damage, the increasing levels of p53 lead to the trans-activation of a number of genes but not of $p21^{Waf1/Cip1}$, preferentially directing TGCT cells into apoptosis or programmed cell death, both via the mitochondrial and the death receptor apoptosis pathway. The sensitivity of TGCTs to chemotherapeutic drugs may lay in the susceptibility of germ cells to apoptosis. Taken together, this provides TGCT as a tumour type model to investigate and understand the molecular determinants of chemotherapy sensitivity of solid tumours. This review aims to summarise the current knowledge on the biological basis of cisplatin-induced apoptosis and response to chemotherapy in TGCTs

Speaker/selected poster presentation at conferences

-June 2009: Docent at Ground Rounds for ECM", IEO, Milan Italy: 'Antiangiogenesis: a new pathway in the treatment of Melanoma'

- April 2009: 100th Poster presented at AARC Annual Meeting, Denver, Colorado, USA, organized by the American Association of Cancer Research. Title 'Apoptosis markers: A feasible tool to improve prognosis definition and to define therapy response in melanoma'

- March 2009: Speaker at EORTC-Melanoma Group Meeting, Bruxelles 2009. Title: "Role of the SOS-1-Rac signalling pathway in melanoma progression"

- September 2008: Docent at "Ground Rounds for ECM", IEO, Milan Italy. "Anti-CTLA4 Treatment in metastatic Melanoma: IEO experience".

- June 2008: Speaker at "ICATMM_EADO Meeting 2008", Marseille, France. "Combining chemotherapy and targeted therapy".

- June 2008: Speaker at "ICATMM_EADO Meeting 2008", Marseille, France. "New apoptosis markers: a feasible tool to improve prognosis definition and to define therapy response"

- January 2008: Speaker at "Insieme oltre la malattia (Together beyond the disease)", Belluno Italy

- November 2007: Speaker at "Diagnosis and treatment of malignant melanoma", Caracas, Venezuela

- May 2006: 5th Apoptosis Meeting, "Death between the tulips", Kerkrade, The Netherlands. Selected poster. Title '

- December 2005: ColoRectal Cancer Conference, organized by ESO (European School of Oncology), London, England

- October 2005: Speaker at "Cancers in the Elderly", topic "Bladder cancer", Telese Terme, Benevento, Italy

- July 2004: Evidence Based Oncology", organized by ESO (European School of Oncology), Antwerp, Belgium

- March 2004: 95th AARC Annual Meeting, Orlando, Florida, USA, organized by the American Association of Cancer Research

- October 2002: 2nd Colorectal Cancer Conference, Rome, Italy, organized by the European School of Oncology

  September 2002: Developments in the integrated therapies, La Maddalena (SS), Italy, organized by the European School of Oncology
- September 2003: First National Meeting of Oncology, Fiuggi (FR), Italy, organized by the Italian Oncology Association

- July 2000: New therapies in bone metastasis, San Felice Circeo (LT), Italy, organized by the Oncological Radiotherapy Association

Leisure activities

- June 1996:Classical and Contemporary Dance Diploma, Dance School Academy, Benevento
- Tango and Caribbean Ballet
- Italian Literature
- Greeks and Latin Literatures
- Aerobics and fitness
- Reading, travelling
- Volunteer at the poors reception centres

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'ons hart wordt stil en wit:
zijn wij nu morgen vrij?

Vandaag is het een eind,
morgen een nieuw begin'

Muus Jacobse, Vuur en wind 1945