rijksuniversiteit groningen

Bachelor thesis Biomedical Sciences

University of Groningen
Faculty of Science & Engineering

Testicular germ cell tumours: why so sensitive?

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March 2017
Academic year 2016/2017

Abstract

The incidence of testicular cancer, that is the most frequent solid malignant tumour in young men, is still increasing. Despite the rising number of patients diagnosed with this kind of cancer, the curability of this tumour is high due to effective cisplatin-based treatment. Still there is a small group of patients that is resistant to this therapy, but the underlying mechanism(s) behind this resistance is not clear. This thesis will give an overview of the current ideas and research that is done in this field in the past decades till very recently. Ranging from targets and factors upstream or downstream the apoptosis induction pathways in testicular cancer. The keyplayer in this overview is p53, because p53 is shown to be important in intrinsic and extrinsic apoptosis induction after chemotherapeutic treatment. Regulation of p53 by MDM2 is shown to influence this sensitivity and inactivation of p53 decreases this sensitivity. Another possible mechanism for this sensitivity is the mitochondrial priming proximity of the cell. When the cells are highly primed, the pro-apoptotic proteins are more abundantly expressed, causing the cell to go into apoptosis more easily. Resistance to cisplatin treatment can be caused by various reasons, including deficient mismatch repair, induction of differentiation, loss of function of p53, epigenetic alterations, overexpression or activation of the PI3K/pAKT pathway and decreased expression of pluripotency markers like Oct-4 and Noxa. Nowadays BH3 mimetics, that bind the anti-apoptotic factors, are already used in acute myeloid leukemia and may be promising for testicular cancer as well. Besides this, further research and clinical trials need to be done to bring phosphorylating kinases like AKT or PI3K inhibitors or other possible targets that can decrease the resistance to the clinic and to cure all testicular cancer patients.

Contents

Introduction

In the recent years cancer research has significantly improved and therapeutic targets to cure tumours are no longer a rear exception, thereby improving prognosis. However, the incidence of germ cell tumours that are the most frequent solid malignant tumour in men, is still increasing with 6-7 per 100,000 men yearly (*Di Pietro et al., 2005*). Despite the rising number of patients, the good curability of this kind of metastatic solid tumour is due to the use of highly effective combinations of chemotherapeutic drugs. Seminomas, for example, are radio- and chemo-sensitive tumours at each stage (*Classen et al., 2001*), whereas non-seminomatous tumours have different cure rates depending on the disease stage combined with prognostic criteria (*Shelley et al., 2002*).

Most human testicular germ cell tumours (TGCT) show an extraordinary response to cisplatin treatment (*Masters et al., 2003*). In a paper from Ni Chongaile *et al.* the extraordinary clinical response to these drugs may be partially governed by the pre-treatment proximity of tumour cell mitochondria to the apoptotic threshold, a property of the cell called mitochondrial priming. Apoptosis is a form of programmed cell death where unwanted cells are eliminated. It can be initiated via two different pathways: the extrinsic pathway and the intrinsic pathway. In the end both pathways activate the same effector molecules. An important feature is that both pathways are connected to each other. There can be crosstalk and both pathways can activate or inhibit each other. A remarkable difference is seen between cell lines that express different p53 types. Wild type p53 cells are known to be very sensitive for cisplatin therapy, whereas mutated p53 cells show almost no response. (*O'Connor et al., 1997*). However, a lot of tumours don't contain this characteristic and this fact is criticized for a long time. p53 is a tumour suppressor gene, which encodes a transcription factor important in the stress response. Its level is influenced by means of a negative feedback loop with Mouse Double Minute 2 homolog (MDM2) and the other homolog MDM4. Besides this, p53 trans activates other genes that either promote cell cycle arrest and DNA repair or apoptosis.

Koster *et al.* describes in their review that activation of these pathways is crucial in the response to chemotherapeutic drugs. In most cancer types p53 is mutated, causing a malfunctioning in one or both pathways that leads to DNA damage repair or apoptosis induction. But in TGCT the majority of tumours express wild type p53. The presence of p53 is a major determinant of the sensitivity of TGCT for the cisplatin treatment. This thesis will give an overview and try to answer several related questions like: How does this mechanism work? And is it really determined by the p53 tumour suppressor gene, or is it caused by other regulatory factors up- or downstream the pathways important for apoptosis induction?

Apoptosis overview

Apoptosis, or programmed cell death (PCD), is a crucial factor to control cell proliferation. It is needed to maintain a proper tissue homeostasis and to eliminate harmful or unwanted cells and it is marked as one of the hallmarks of cancer. A dysfunction in apoptosis is of great impact on the organism, and causes an accumulation of genetic faults resulting in various pathological disorders like cancer. An aberrant function is a common factor in tumour growth and the development of anticancer drug resistance. The understanding of the pathogenic mechanism may be a key for developing new drugs that target specific apoptotic pathways or genes involved in these pathways. Key factors in these pathways are caspases that function in developmental, inflammatory or apoptotic pathways (*Weyhenmeyer et al, 2012*).

Extrinsic apoptotic pathway

The extrinsic pathway is activated by the binding of death ligands like tumour necrosis factor (TNF), Fas ligand (Fas-L) or TNF-related apoptosis-inducing ligands (TRAIL) to the extracellular part of the death receptors. Upon binding from ligands the intracellular part of the death domains bind to adaptor proteins (Fas associated death domain FADD, or TNF receptor associated death domain TRADD). The adaptor proteins have a death effector domain (DED) that can interact with the DED from the procaspases 8 and 10 as seen in figure 1. The complex that is formed is called the Death Inducing Signalling Complex (DISC). The procaspases are cleaved into their active form. These active caspases can activate effector caspases 3, 6 and 7 which can cause cell death by damaging the nucleus and other intracellular structures (*Jin et al., 2005*).

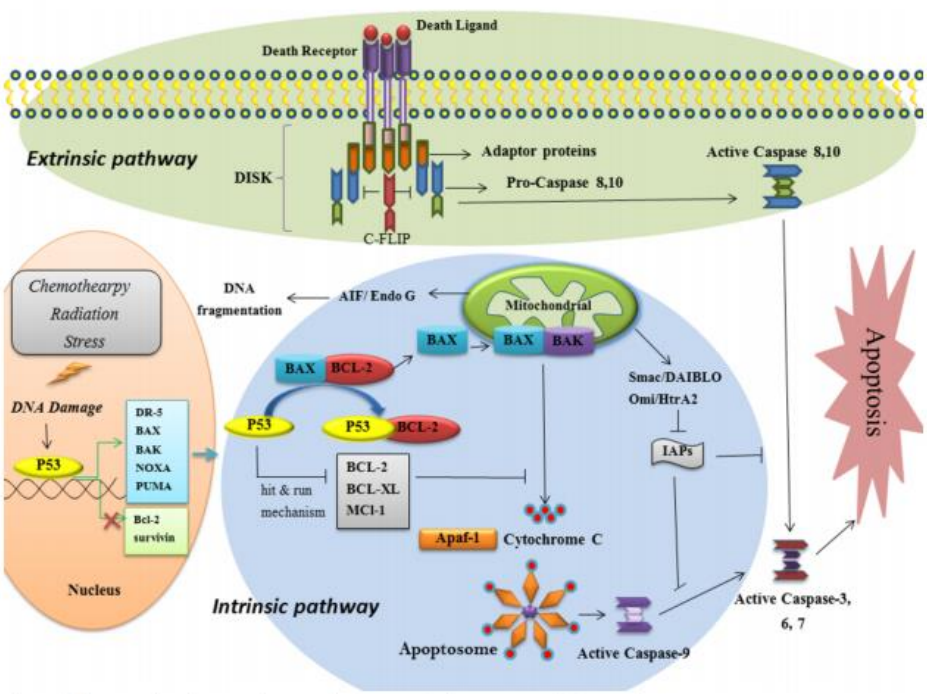


Figure 1. Figure 1. Schematic Diagram of the Intrinsic, Extrinsic Pathways of Apoptosis. Apoptosis is executed via two central apoptotic pathways, the extrinsic and the intrinsic pathways. Both apoptotic pathways converge on the same terminal or execution pathway. The extrinsic pathway begins with the attachment of extracellular ligands to the extracellular domain of transmembrane receptors, whereas mitochondrial apoptosis is initiated by several intracellular stimuli. Initiator caspase of both pathways (caspase 8 and 9, 10) can catalyse the proteolytic maturation of effector caspases (such as caspase 3, 6, 7), which lead to initiation of a caspase cascade and finally result in demolition of the cell. DNA damage triggers apoptosis by activation of the tumor-suppressor protein

p53, which induces the transcription of DR-5, BAX, BAK, NOXA, PUMA and also can inhibits the transcription of anti-apoptotic genes such as Bcl-2 and survivin. The cytoplasmic interaction of p53 with the anti-apoptotic Bcl-2 family proteins in the mitochondria induces the release of apoptogenic factors such as cytochrome c from the mitochondrial outer membrane. In addition, p53 can interact directly with Bak and/or Bax and activate these through a "hit and run" mechanism that prompts the permeabilization of the outer mitochondrial membrane. (Adapted from Goldar et al. 2015)

Intrinsic apoptotic pathway

The intrinsic, or mitochondrial, apoptosis is triggered by the induction of DNA damage by different stressors like chemotherapy, radiation or stress for example. In this case the tumour suppressor gene p53 is activated and this induces the transcription of other pro apoptotic genes like DR-5 (death receptor 5), FAS (CD95), BAX (BCL-2-associated protein), BAK (BCL-2-antagnosist/killer), NOXA (phorbol-12-myristate-13-acetate-induced protein 1) and PUMA (p53 upregulated modulator of apoptosis) and inhibits the transcription of anti-apoptotic genes such as Bcl-2 and survivin. (*Goldar et al., 2015*) Via a cascade of proteins and genes the interaction of p53 and anti-apoptotic Bcl-2 proteins causes the mitochondria to release cytochrome C. Cytochrome C is a factor that has an apoptogenic function. Together with Apaf-1 the apoptosome[1] is formed recruiting procaspase 9. Procaspase 9 is processed to active caspase 9, while activation of this caspase causes activation of caspase 3, that initiates a caspase cascade including effector caspases 6 and 7. This cascade eventually induces apoptosis in the cell as seen in figure 1.

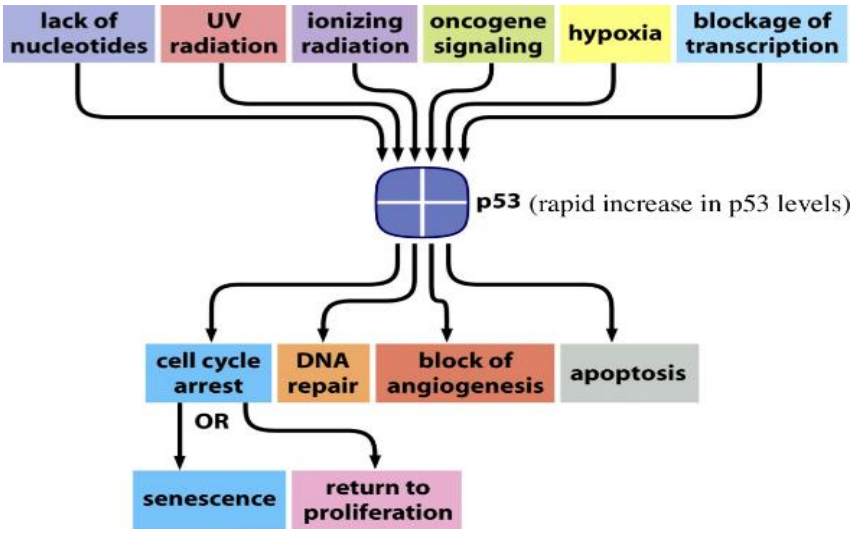
Goldar *et al.* proposes another mechanism to initiate apoptosis by means of the mitochondrial proteins Smac/DIABLO (second mitochondria-derived activator of caspase / direct IAP-binding protein with low PI) and Omi/HtrA2 (high temperature requirement protein A2).
Smac/DIABLO and Omi/HtrA2 interact and antagonize Inhibitor of Apoptosis (IAP) proteins and promote caspase activation (*Gustafsson and Gottlieb, 2008*).

p53, the guardian of apoptosis

p53 is a very important factor in the apoptosis pathway. The presence of the wild type allele results in a protein that causes a suppression in cell proliferation by regulating apoptosis, cell cycle arrest and senescence. p53 mutations are dominated by missense mutation[2] that leads to mutant proteins with full length (*Olivier et al., 2004*). Mutated p53 is not only characterized by loss of tumour suppressive functions, but also by gain-of-function properties like promoting cell proliferation, angiogenesis, migration, invasion, metastasis and chemoresistance. (*Oren M., Rotter V. 2010*). Therefore the gene is seen as a tumour suppressor gene[3].
p53 is highly susceptible for mutation. In approximately 50% of all human tumours p53 is present in its mutant form (*Olivier et al., 2004*). A study from Jacks *et al*. demonstrated that p53 was not a normal tumour suppressor gene. Deletion of both p53 gene copies had no significant effect on the development of the p53 -/- embryos, whereas a normal tumour suppressor gene would have. But

[1] Multiprotein complex that consists of cytochrome c molecules and Apaf-1 and helps to initiate apoptosis by activating procaspase 9 into caspase 9.
[2] Type of mutation with a change in one DNA base pair that results in the substitution of one amino acid for another in the protein made by a gene (U.S. National Library of Medicine, consulted on 20-4-2017).
[3]Any gene whose encoded protein directly or indirectly inhibits progression through the cell cycle and in which a loss-of-function mutation is oncogenic. Inheritance of a single mutant allele of many tumor-suppressor genes (e.g., RB, APC, and BRCA1) greatly increases the risk for developing certain types of cancer. (Molecular Cell Biology 4th edition)

mice lacking both copies had a short life span due to lymphomas and sarcomas (*T. Jacks et al., 1994*). Thus p53 is not a protein that guides the proliferative and anti-proliferative signals, but seemed to be specialized in preventing the accumulation of abnormal cells.



When sensors of the cell note a problem in functioning or detecting damage caused by various factors, they send signals to p53 and its regulators. This results in an increase in p53 levels within a cell. This increase can lead to cell cycle arrest, DNA repair, block of angiogenesis or apoptosis as seen in figure 2.

Figure 2. P53-activating signals and p53's downstream effects. Adopted from Biology of Cancer, Robert A. Weinberg.

MDM2, the other side of the p53 coin

The levels of p53 are regulated by MDM2 in all cells. This is a protein that recognizes p53 as a target. When the p53 level rises, the tetramer of p53 binds to several promotor regions, including the gene that encodes for the MDM2 protein. When this gene is transcribed and translated into a protein, the MDM2 molecules bind to the p53 tetramer preventing p53 to block the cell cycle and go into apoptosis, or it induces a state of senescence[4]. This induces the exportation of the complex to the cytoplasm of the cell and initiation of ubiquitylation. When polyubiquitin side chains are attached to p53, the complex is tagged for degradation and is digested into oligopeptides in cytoplasmic proteasomes. This causes a decrease in p53 quantity, therefore less MDM2 is transcribed because less promotor regions are bound. This system is better known as the negative feedback loop of p53. However, in vivo MDM2 is not monomeric, but forms a heterodimeric complex with its close cousin MDM4 (also known as MDMX). This complex may be responsible for much of the ubiquitylation activity that drives p53 degradation. Pant *et al*. proposed that MDM4 is an important cofactor of MDM2, but is not necessary for regulating p53 and MDM2 stability. MDM4 has no ligase activity towards p53, but is capable to enhance MDM2-mediated ubiquitylation and therefore degradation of p53 (*Stad et al., 2001*). Besides MDM4, p14/ARF protein can bind MDM2 directly (*Bothner et al., 2001*) and therefore inactivates MDM2 functionality by preventing its binding to p53.

When cells are suffering some damage or stress, it is important that p53 can function to suppress proliferation of the cell. This means that they need to be protected from their MDM2 executioner. This protection is most of the time achieved by phosphorylation of p53 that blocks the possibility for MDM2 to bind. Therefore ubiquitylation is not possible and p53 will not be degraded.
Several kinases, like ATM, Chk1 and Chk2, can cause this phosphorylation.

[4] A nongrowing state of cells in which they exhibit distinctive cell phenotypes and remain viable for extended periods of time but are unable to proliferate again. Often arises after extended passaging in vitro.

Besides this, ATM kinase can phosphorylate MDM2 which results in functional inactivation and a decrease in MDM2 stability. Therefore it can't bind to p53 anymore. Via these mechanism, the levels of p53 can rise to a functionally significant level in the cell.
Phosphorylation of p53 via the PI3K pathway and the Ras/MAPK pathway causes increased transcription of the MDM2 gene. This causes p53 protein levels to decrease. (*Biology of Cancer, Robert A. Weinberg*).

Interestingly, in a study from Bartkova *et al.* there was almost no spontaneous activation of the DNA-Damage Response (DDR) in testicular germ cell tumours. At least this means that DNA damage here does not trigger the classical DDR pathway to initiate cell cycle arrest and to repair the faults or cause apoptosis to occur. To support this idea, in a previous study from Bartkova *et al.* in 2005 it is seen that invasive TGCT lack RB expression, a tumour repressor protein important in the inhibition of cell cycle progression when replicative stress is noticed. Whereas in other somatic cells and tumours this leads to replication stress and initiation of the DDR pathway, the initiation is not seen in TGCT (*Bartkova et al., 2005*).

Testicular cancer

Germ cell tumours (GCT) consist of a group of neoplasms that occur in the gonads, both testes and ovaries. In 2005 Oosterhuis and Looijenga developed a classification system based on different detectable variables. It consist of five categories. Type I consist of benign teratomas and malignant yolk sac tumours (occur in testes and ovaries), type II consist of malignant seminomas and non-seminomatous in testes, the third group consists of spermatocytic seminomas (exclusively in testes). Type IV consist of dermoid cysts (created only with maternal chromosomes via nuclear transfer experiments) and type V of hydatidiform mole (created with only paternal chromosomes via nuclear transfer experiments) (*Oosterhuis et al., 2005*). All the different groups have different characteristics, and therefore need specific treatment.
Type II can be further classified depending on histological differences. TGCT can be divided into seminomas and non-seminomas. Seminomas are like primordial germ cells (PGCs) and non-seminomas are undifferentiated (embryonal carcinoma) or differentiated. (*Oosterhuis et al.,2005*). Non-seminomas have a more aggressive character than seminomas, what could explain why this kind manifests at an earlier age.
The non-seminomas can be further characterized by means of different histological elements. The embryonal carcinoma cells can differentiate into teratoma cells, yolk-sac tumour cells, and choriocarcinoma cells. (*Oosterhuis et al., 2005).* Oosterhuis *et al.* also concludes that seminomas are highly sensitive to radiation and chemotherapy, but non-seminomas are not and are able to repair radiation-induced damage. Instead, non-seminomas are very sensitive to platinum-based combination therapy, except the teratomas that are not sensitive to this treatment.

Testicular germ cell tumours

TGCTs are the most common cancer in Caucasian young men aged between 20 and 40 with an incidence of almost 60%. (*Looijenga, 2009*). Overall it is accepted that most of the testicular germ cell tumours have carcinoma in situ (CIS)[5] as precursor form (*Skakkebaek, 1972*). During the development there are initiating and aneuplodic events, causing chromosome instability and when the stage of puberty is reached, there is a huge change of developing into a seminoma or non-seminoma as seen in figure 3 (*A. di Pietro et al., 2005*).

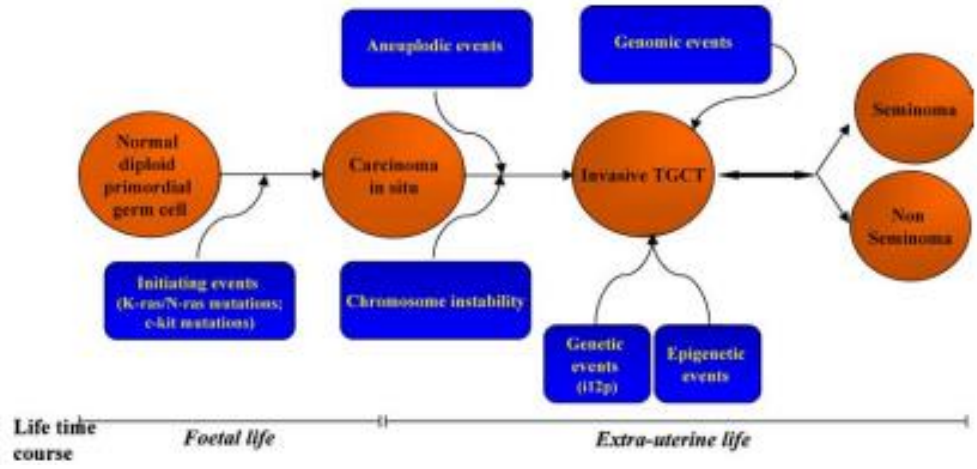


Figure 3. Progression of TGCTs (adopted from di Pietro et al. 2005).

Germ cell tumours are a heterogeneous group depending on histological characteristics. Primary and chemoresistant tumours show highly recurrent chromosome arm level 12p amplification and reciprocal deletions (reciprocal loss of heterozygosity, LOH[6]) (*Weiner et al., 2016*). Different groups investigated whether there are any differences in the molecular pathway in both types. Matched analysis of hereditary found no differences between the groups that developed into seminomas or non-seminomas, which makes it more probable that the same molecular pathway is present (*Skotheim et al.,2003*).

Chemosensitivity in testicular germ cell tumours

TGCTs are very chemosensitive; combination cisplatin- and etoposide-based chemotherapy cures even more than 80% that are diagnosed with a metastatic disease (*Hanna et al., 2014*), but approximately 10% of the patients are resistant to this treatment.

When cancer cells are treated with a platinum based chemotherapeutic drugs, an induction of DNA-damage is seen. The platinum compounds enter the cell and undergo hydrolysis, thereby losing the chloride or oxalate ions resulting in reactive derivatives. The reactive molecules bind to nucleophilic groups containing oxygen, nitrogen or sulfur donors (*Wang et al., 1996*). These groups are broadly present in the RNA or DNA from the cells. The binding to DNA causes a platinum-DNA adduct. These

[5] A group of abnormal cells that remain in the place where they first formed. These abnormal cells may become cancer and spread into nearby normal tissue. Also called stage 0 (*NCI Dictionary of Cancer Terms*).
[6] A genetic event in which one of two alleles at a heterozygous locus is lost; the lost allele may simply be discarded or be replaced with a duplicated copy of the surviving allele. Also called allelic deletion (*Biology of Cancer, Robert. A. Weinberg*).

adducts interfere with the transcription machinery. When DNA polymerases detect an altered base caused by the chemotherapeutic drugs, the replication forks are stalled and single strand DNA is detected. The ssDNA sensors activate ATR kinase, which acts via the Chk1 kinase, to phosphorylate p53, protecting it from degradation.

Besides this, the DNA adducts cause inter- and intrastand DNA crosslinks, unwinding and bending (*Kelland et al., 2007*). Kelland *et al*. also describes that this process activates several pathways in the DNA damage response resulting in cell-cycle arrest, DNA repair and/or apoptosis.
These crosslinks are usually repaired by Nucleotide Excision Repair (NER)[7] , but when this is not sufficient the double-strand breaks activate upstream DDR kinases that target downstream proteins to stop the cell cycle and initiate DNA repair or apoptosis (*Koster et al., 2013).*

But what makes TGCTs so sensitive for cisplatin treatment? Different ideas have been proposed. Spierings *et al*. contributed an important role for p53 in the apoptosis induction caused by cisplatin treatment, with a significant decrease in this cisplatin hypersensitivity when p53 was shut down. Usanova *et al*. showed that testicular cancer has very low levels of a few essential nucleotide excision repair components: ERCC1, XPF and XPA. This is a limiting factor for the repair of especially interstrand crosslinks seen in testicular cancer.
Another possible reason was proposed by Cavallo *et al*. who noticed a reduced homologous recombination activity that correlates with cisplatin sensitivity.

In other tumour types it was shown that, after exposure to cisplatin treatment, p53 was able to activate the extrinsic (via FAS and KILLER/DR5) and intrinsic (via Puma and Noxa) pathway of apoptosis (*Petak et al., 2001 & Fulda et al., 2001*).
In testicular cancer Spierings *et al*. (2003b) showed that cisplatin treatment causes an enhanced Fas death receptor expression, and therefore an activation of the extrinsic apoptotic pathway by the Fas - Fas ligand interaction. Besides this, there was an upregulation of the intrinsic apoptotic pathway. This idea is supported by the cisplatin induced upregulation of p53 seen in TGCTs (*Spierings et al., 2003*) and by downregulation of p53 transactivated genes important in the intrinsic (Puma and Noxa) and extrinsic (FAS, LRDD and PHLDA3) apoptotic pathway (*di Pietro et al, 2012; Kerley-Hamilton et al., 2005*).

Contradictory to these results, Burger *et al*. (1997) found no significant difference in sensitivity to cisplatin treatment of p53 wild type cells compared to p53 mutant cells. Suggesting apoptosis through DNA damage, caused by cisplatin treatment, is not correlated to p53 status. This contradiction can be caused by using different cell lines. Burger *et al.* (1997) used 4 different cell lines: NT2 and 2102EP from non-seminomas, S2 and NCCIT that exhibit seminomatous characteristics cultured in 8,5% CO2 and supplemented with HEPES-buffered RPMI 1640. Serial dilutions cisplatin were added and exposure for 4 days. Spierings *et al.* (2003b) used Tera carcinoma cell line and two other unrelated human TGCT cell lines 833KE and Scha. Cultured in 5% CO2 supplemented with Leibovitz L25- RPMI 1640. Continuous incubation with cisplatin. Besides this, Burger *et al.* (1997) used Human Papilloma Virus E6 (HPV E6) to shut down p53, whereas Spierings *et al.* (2003b) used

[7] A type of DNA repair in which the initial step involves the excision of nucleotides (rather than bases). (*Biology of Cancer, Robert A. Weinberg)*

siRNA. The other biological functions from HPV E6 might contribute to the different outcomes of these studies. So, other cell lines were used and the cells were not treated exactly the same way. Burger *et al.* (1997) used high dose direct addition of cisplatin and Spierings *et al*.(2003b) low dose continuous addition. Besides the different method to target p53 this might cause the different results in these studies.

A more recent publication from Taylor-Weiner *et al.* was performed in clinical TGCT tumours that were characterized by wild type TP53, chromosome arm 12p gain, and additional recurrent copy number changes. The cells were very sensitive to DNA damage induced by chemotherapy, and they contributed it to intact p53 and high mitochondrial priming.

Chemo resistance in testicular germ cell tumours

As already mentioned TGCTs are very sensitive to cisplatin treatment, curing most of the diagnosed patients. But, there is a small group of patients diagnosed with TGCT that are resistant to cisplatin treatment. What is the underlying mechanism why these patients are resistant while others respond very good?

The resistance is believed to be multifactorial (Mayer *et al.,* 2003), but the most important deficiencies are: deficient mismatch repair system (MMR), induction of differentiation, loss of function of p53, epigenetic alterations and overexpression or activation of the PI3K/pAKT pathway. (*Jacobsen and Honecker, 2014*).

It is known that MMR can detect cisplatin-induced DNA lesions that lead to an apoptotic signal (*Vaisman et al., 1998*). This idea is further investigated by Honecker *et al.* in 2009 and they described that cisplatin resistance is characterized by high microsatellite instability (MSI), low or absent MMR proteins and high incidence of BRAF mutation V600E. MMR proteins are able to induce cell cycle arrest in the S-phase and apoptosis (*Mueller et al., 2006*). A deficiency in MMR could therefore interfere with the apoptosis induction.

Koster *et al.* identified another factor contributing to resistance in 2010. They found a correlation between loss of Oct-4 expression and resistance in EC cells. Downregulation of Oct-4 results in downregulation of Noxa and Puma that are important factors in the apoptosis induction. Besides this Koster *et al.* described an upregulation of p21 and a downregulation of miR17/106b resulting in G1 cell cycle arrest and decreased apoptosis induction. But Oct-4 seems not the direct link leading to resistance. In a study from Gutekunst *et al. (*2011) Oct-4 depleted cells showed no decrease in the p53 response to cisplatin, therefore Oct-4 is seen as a factor that ensures a low apoptotic threshold by keeping levels of Noxa high.

As already mentioned p53 is a very important factor in apoptosis induction, and therefore could play a role in resistance to cisplatin treatment. Port *et al*. described that high levels of miR-372 and miR-373, that silence the p53 pathway (*Voorhoeve et al., 2006*), are seen in 2 out of 3 cisplatin resistant TGCT sublines indicating a possible mechanism to silence p53 without an actual p53 mutation (*Port et al., 2011*).

P53 can also be silenced by MDM2, and potent disruptors of this p53-MDM2 interaction, like nutlin-3, are already described. (*Bauer et al., 2010 and Gutekunst et al., 2011*). P53 silencing contributes to loss of p53 functionality and increase in cisplatin resistance.

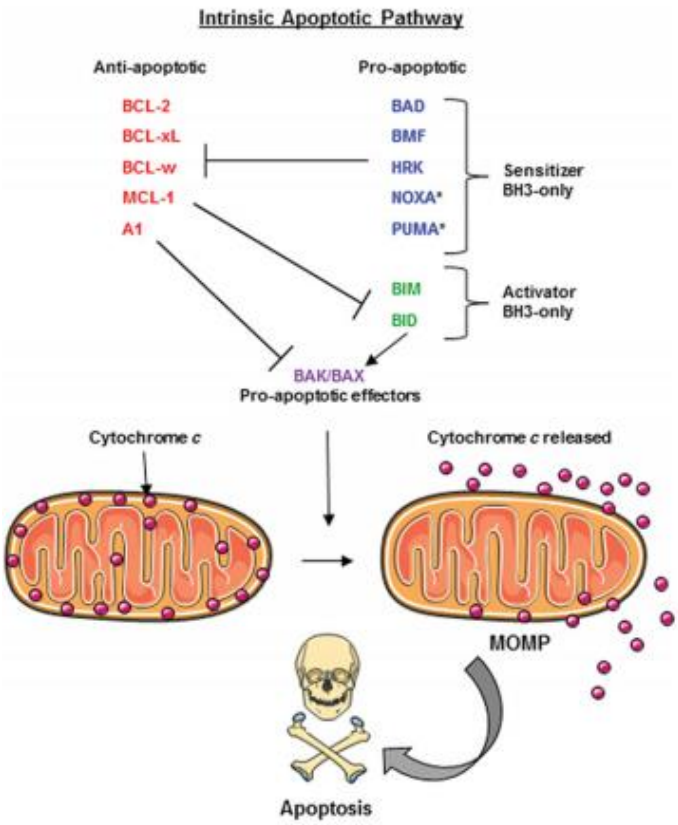
DNA methylation is another factor that can contribute to cisplatin resistance. Koul *et al*. (2004) found that sensitive non-seminomas had different hypermethylated regions then resistant non-seminomas.

5-aza-deoxycytidine and 5-azacytidine are demethylating agents that can be incorporated in the DNA and mediates DNA adduct formation, causing a DDR response. (*Biswal et al., 2012*). Cisplatin resistance can be partially reverted using such demethylating agents combined with cisplatin. (*Jacobsen and Honecker, 2014*). The exact molecular mechanism is not clear and needs further research.
The PI3K/p-AKT pathway seems to play a role in the resistance to cisplatin treatment as well. First evidence for this was identified by Di Vizio *et al*. that showed a loss of PTEN in 50% of GCT. A study from Feldman *et al*. (2014) was the first to describe that mutations within PI3KCA, AKT1 and FGFR3 are seen. When the PI3K is activated, this results in phosphorylation of AKT. Phosphorylated AKT causes phosphorylation of p21, that translocates to the cytoplasm. Besides phosphorylated AKT can cause activation of MDM2, that causes a decrease in p53 activity. Both features can cause cell cycle arrest and failure to induce apoptosis (*Jacobsen & Honecker, 2014*). PTEN is able to inhibit PI3K activity and phosphorylated AKT (*Ramaswamy et al., 1999*), and therefore promote apoptosis.

In a very recent publication from Taylor-Weiner *et al.* (2016) in Nature is it proposed that the pluripotency and apoptosis regulators NANOG and POUF5F1 (better known as Oct3/4), that are expressed in TGCT, are sensitive to cisplatin treatment, are not expressed in the resistant tumours. Besides this, the tumours are associated with continued progression of LOH copy number events and in a further differentiated histology post chemotherapy there is a change in methylation marker.

Mitochondrial priming phenomenon

Mitochondrial outer membrane permeabilization (MOMP) is an event that is regulated by the balance of pro- and anti-apoptotic proteins and is present in the final stages of the intrinsic apoptotic pathway causing the actual apoptosis induction. Several proteins are involved as seen in figure 4. Apoptotic signals, in this case due to chemotherapeutic agents, results in an increase in pro-apoptotic BCL-2 family members: BAD, BMF, HRK, NOXA, PUMA, BIM, BID, BAK/BAX,[8] and/or a decrease in anti-apoptotic proteins: BCL-2, BCL-xL, BCL-w, MCL-1 and A1. [9]



The equilibrium between these proteins shifts to the pro-apoptotic proteins side, that causes release of the pro-apoptotic effectors BAK/BAX that are activated by BIM and BID which leads to MOMP and apoptosis (*Potter & Letai, 2016*).

[8] BAD: BCL-2-associated agonist of cell death, BMF: BCL-2-modifying factor, HRK: hara-kiri, BIM: BCL-2-interacting mediator of cell death, BID: BH3-interacting domain death agonist.
[9] BCL-2: B-cell lymphoma 2, BCL-xL: BCL-2-like protein 1 isoform long, BCL-w: BCL-2-like protein 2, MCL-1: myeloid cell leukemia 1, A1: BCL-2-related protein A1.

Figure 4: intrinsic apoptotic pathway proteins and effectors . Adapted from: Potter and Letai, 2016

The sensitivity to cisplatin treatment has been point for discussion for a time now. Liu *et al*. (2013) have shown that this sensitivity in hESCs is due to expression of wild type p53 combined with another feature of the cell, mitochondrial priming (*Liu et al. 2013*).

A study from Ni Chonghaile *et al*. (2011) demonstrated that the mitochondria were primed to the apoptotic threshold. Meaning that the mitochondrial depolarization is induced by related interacting BH3 peptides.
By performing BH3 profiling[10] and a comparison of these results to clinical responses they found a correlation between superior clinical response and increased mitochondrial priming (*Ni Chonghaile et al., 2011*), thereby demonstrating a mechanism that contributes to the clinical response of chemotherapeutic treatment. Even in a solid tumour, like ovarian cancer, highly primed mitochondria correlated with superior progression-free survival. This correlation was demonstrated in multiple myeloma, AML, ALL and ovarian cancer, suggesting a fundamental relationship between the clinical response to chemotherapeutic agents and mitochondrial priming (*Ni Chonghaile et al. 2011*).

But Ni Chonghaile *et al*. (2011) proposed that the chemosensitivity of tumour cells is not only determined by the priming proximity of these cells, but also by the insensitivity of non-cancerous cells. This idea is supported by Evan *et al*. (2001) that demonstrated that cancer cells are inherently more sensitive to undergo apoptosis, probably because of their oncogenic lesions.

To further support this idea Ni Chonghaile *et al*. (2011) demonstrated that normal human cells that are characterized as relatively chemoresistant were indeed poorly primed, whereas mitochondria from chemosensitive cancers are more primed that those from chemoresistant forms. This supports the idea that mitochondrial priming can be a fundamental basis of the response to chemotherapeutic agents.
This contributes to the idea that testicular germ cell tumours should be highly primed, as it is a chemosensitive, rapidly dividing form of cancer. But whether the previous results are indeed applicable to testicular germ cell cancer is still unclear and needs further research.

Another paper from Liu *et al*. (2013) showed that human embryonic stem cells (hESCs) are primed to undergo apoptosis because of the sensitivity of DNA-damage induced by chemotherapeutic drugs. The sensitivity of the hESCs for DNA-damage induced apoptosis is due to the high mitochondrial priming of these cells. Also these cells were more sensitive than their differentiated progeny, but when the mitochondrial priming of the differentiated cells was increased, the sensitivity to DNA damage increased which ultimately led to apoptosis induction. (Liu *et al., 2013*). These results contribute to the idea of mitochondrial priming to be an important factor in chemotherapeutic sensitivity.

[10] BH3 profiling assay measures the mitochondrial outer membrane permeabilization (MOMP) after the moment that mitochondria are exposed to pro-apoptotic interacting BH3 peptides.

But what leads to mitochondrial priming? Liu *et al*. (2013) demonstrated that p53 was not the determinant of this, because when p53 was silenced no influence on mitochondrial priming was seen. But they found a correlation between priming and pluripotency, because hESCs had higher levels of the pro-apoptotic protein PUMA and lower levels of the anti-apoptotic protein Bcl-2. And differentiated cells had higher levels of the anti-apoptotic protein Mcl-1 (*Liu et al., 2013*).
Disturbance of the balance between pro-apoptotic and anti-apoptotic proteins, shifting it to the pro-apoptotic side, leads to changes in priming and eventually apoptosis induction (*Liu et al., 2013*).

Potter and Letai (2016) argue that mitochondrial priming is a switch-like event, with a difference in cells that are closer to turning on the switch, than other cells types that are closer to turning off the switch. Priming is a relative event, meaning that when the cells are more primed, they have a lower apoptotic threshold. Priming itself can be influenced by various factors like: growth factor deprivation, genetic aberrations or changes in metabolism (*Certo et al., 2006*).

To support this idea Taylor-Weiner *et al.* (2016) propose that the basis of chemosensitivity in TGCT with a wild type TP53 and RLOH background is caused by the apoptotic propensity due to a balance of pro- and anti-apoptotic BCL2 family members.

The balance between the pro-apoptotic and anti-apoptotic proteins is demonstrated to be important in apoptosis induction, and therefore it is a target for developing drugs to increase mitochondrial priming and induce apoptosis more easily. Potter and Letai (2016) demonstrated that this balance is influenced by BH3 mimetics, that bind in the binding site of anti-apoptotic proteins and therefore release pro-apoptotic BCL-2 family members to induce MOMP.

The first BH3 mimetic in preclinical studies ABT-737, developed by AbbVie in 2005, was functionally the same as the pro-apoptotic protein BAD, causing the binding of BCL-2, BCL-xL and BCL-w (*Oltersdorf et al., 2005*). Another more recently developed BH3 mimetic is the clinical derivative from ABT-737 navitoclax (ABT-263) (*Tse et al., 2008*). It was promising in phase I trials, because markers of apoptosis were detected (*Ghandi et al., 2011*). Phase I/II clinical trials showed single-agent efficacy in phase II trials in chronic lymphocytic leukemia (*Roberts et al., 2012*), but had thromotyopenia as a toxic side effect (*Schoenwaelder et al., 2011*).

A more specific BCL-2 agonist was developed to avoid these toxicity. Venetoclax (ABT-199) was developed, that is a BCL-2 selective BH3 mimetic (*Souers et al., 2013*) and is already used in acute myeloid leukaemia.
Whether these mimetics contribute to chemotherapeutic sensitivity was determined by Ni Chonghaile *et al.* ABT-737 caused enhanced sensitivity to doxorubicin, vincristine and etoposide. This suggests that mitochondrial priming is important in determining chemosensitivity and can be regulated by using BH3 mimetics.

Discussion

Testicular germ cell tumours are a form of cancer that frequently occurs in young men and the number is still rising. It is characterized by wild type p53 and sensitivity to cisplatin treatment. At the

moment more than 80% that is diagnosed with a metastasized disease is cured by means of combination of chemotherapy (*Hanna et al., 2014*), but approximately 10% is resistant to this.

How come that a very small group is resistant to cisplatin treatment, whereas the majority is responding very good? Resistance for this therapy is multifactorial. Deficient mismatch repair is characterized by low or absent MMR proteins and high incidence of BRAF mutation V600E (*Mueller et al., 2006*). MMR proteins are able to induce a cell-cycle arrest in the S-phase and induce apoptosis. Deficient MMR can therefore interfere with apoptosis induction.
In embryonal carcinoma (EC) cells the expression of Oct-4 is lost, that causes a downregulation of Noxa and Puma that are both important factors in apoptosis induction (*Koster et al., 2010*). But Gutekunst *et al.* (2011) showed that Oct-4 protein depleted cells had no decrease in the p53 response to cisplatin. Therefore Oct-4 is seen as a factor that lowers the apoptotic threshold by keeping Noxa levels high, instead of being a direct suppressor of apoptosis. Whether this is applicable to TGCT was still unknown. But in a paper from Koster *et al*. (2013) it was demonstrated in a TGCT cell line that high Noxa levels are contributing to the pro-apoptotic side of the balance, inducing apoptosis because of the pro-apoptotic functions of p53. This effect is due to high Oct-4 levels, elevated Noxa and reduced p21. In intrinsic cisplatin-resistant cells a higher expression of p53, MDM2 and p21 and a lower expression of Oct-4 and Noxa is seen, because in this case p53 functions predominantly as a transcriptional activator by phosphorylating p21, that becomes located in the cytoplasm where it inhibits Noxa functionality and therefore inhibits apoptosis induction (*Koster et al., 2013*). Therefore it is promising to specifically inhibit the cytoplasmic localisation of p21 in these tumours, to stop it from inhibiting Noxa functionality and increase cisplatin sensitivity.

P53 is an important factor in apoptosis induction and TGCT are characterized by wild type p53. But the resistance to cisplatin can be caused by silencing of p53 without an actual mutation of p53 itself. (*Voorhoeve et al.,2006; Bauer et al., 2010; Gutekunst et al., 2011*). Silencing of p53 contributes to loss of p53 functionality and increase in cisplatin resistance.
A relatively new idea for cisplatin resistance is the methylation status of DNA. Koul *et al*. (2004) found different hypermethylated regions in sensitive tumours compared to non-sensitive tumours. The partially reverted resistance by demethylating agents seen by Jacobsen and Honecker (2014) is promising, but needs further research.
As the PI3K/p-AKT pathway is fundamental in apoptosis induction, some resistant tumours showed abnormalities in this pathway. Loss of PTEN and mutation within PI3KCA, AKT1 and FGFR3 are seen is some of the resistant cell lines (*Di Vizio et al. 2005; Jacobsen & Honecker, 2014*). Koster *et al.* (2013) concluded that the PI3K-p-AKT pathway is hyperactivated, possibly because of a mutation of PTEN, what causes p27 to be mainly localised in the cytoplasm. Here p27 has gained tumour progression functions and lost the ability to inhibit cell cycle progression. Besides this, hyperactivated Akt causes p21 to be phosphorylated and shuttled to the cytoplasm. Cytoplasmic p21 blocks intrinsic and extrinsic apoptosis induction. Because both pathways are blocked, this is an important target to overcome the resistance to treatment. A possible target for treatment is the prevention of hyperphosphorylation of PIP_2. Possibly by restoring PTEN functionality, or decrease PI3K activity.

The other side of the coin is the group that is sensitive to cisplatin treatment. There are several ideas why this group is so sensitive for cisplatin treatment.
Different groups compared p53 status and functionality. P53 is able to cause an activation of the extrinsic apoptotic pathway by the Fas-Fas ligand interaction (*Spierings et al., 2003a*). Also the intrinsic pathway, via Puma and Noxa, was activated by p53 (*Fulda et al., 2001*). Contradictory, Burger *et al.* (1997) didn't contribute these effects to p53, because no difference in apoptosis

induction after cisplatin treatment was seen between wild type p53 and mutant p53 cells. The mutant p53 cells showed approximately the same level of apoptosis induction after cisplatin treatment. But whether this apoptosis induction is not induced via other mechanisms higher or lower downstream the pathway was not investigated. And whether other dysfunctions besides a direct mutation of p53 are applicable to the outcome of these results is not clear.

These contradictory results can be due to the fact that different cell lines are used, that there was a different way of administering cisplatin and that the origin or pretreatment of these tumours from which the cell lines are derived was different, might explaining the different outcome. Another more recent and promising idea is that mitochondrial priming is pushing cancerous cells to the apoptotic threshold edge. The balance between anti-apoptotic and pro-apoptotic signals is important in apoptosis induction and is functioning like a switch. Mitochondrial depolarization is induced by related interacting pro-apoptotic BH3 peptides. By BH3 profiling Ni Chonghaile *et al.* (2011) demonstrated that high mitochondrial priming was related to superior clinical response in AML, ALL and ovarian cancer. Supportive to this idea, is the demonstration that insensitive cells were indeed poorly primed and that non-cancerous cells that are relatively chemoresistant were also poorly primed (*Ni Chonghaile et al. 2011*).
To take this ideas to the clinic and increase mitochondrial priming Potter and Letai (2016) demonstrated that the balance between pro- and anti-apoptotic factors can be influenced by BH3 mimetics that bind the anti-apoptotic factors and therefore release of pro-apoptotic factors is seen. These factors induce MOMP and also apoptosis. The first developed BH3 mimetic was ABT-737. Another more recently developed one was navitoclax, but this compound caused thromotyopenia as a toxic side effect. To overcome this problem, venetoclax was developed, that is a BCL-2 selective BH3 mimetic. (*Souers et al., 2013*) and this is already in use for acute myeloid leukemia.

Whether these mitochondrial priming features and BH3 mimetics are really applicable in TGCT is still unknown. But these results are very promising for the future to cure all TGCT patients if the defect is downstream of p53 in the pathway of apoptosis induction, because p53 causes upregulation of pro-apoptotic proteins increasing the mitochondrial priming and pushing the balance more to the pro-apoptotic side.

To give patients the best treatment, personalized medicine should be ideal. Individual tumour samples should be screened and malfunctions/mutations should be detected. With these data a treatment plan should be created. Nowadays this is not possible due to financial reasons. In the future with the development of new and probably cheaper methods, this might be achievable.

Till then, platinum based combined therapy is a good method to cure testicular cancer. Further research to BH3 mimetics in testicular cancer, PARP inhibitors, dephosphorylating agents, AKT and PI3K inhibitors needs to be done to improve survival. Some of these are currently in clinical trials, but not already applicable in the clinic.

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