New treatment method:

targeting hallmarks in testicular cancer

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05-07-2012
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Abstract

Testicular cancer is a common disease in man. Every year, 660 new cases of testicular cancer arise in the Netherlands alone. The curing number is 90%, which is unique for metastatic cancer. Treatment options are chemotherapy, whether or not combined with radiotherapy. The golden standard for chemotherapy is platinum-based chemotherapy. However, 10% of the testicular cancer patients is cisplatin resistant and for these patients there are no good treatment options available. The aim of this literary study is therefore to determine whether mew targets can be identified to cure testicular cancer.
There are a number of proteins which can play a role in the oncogenesis of testicular cancer: k-Ras, Myc (L-Myc, N-Myc and C-Myc), Bcl-2, Mdm2, EGFR and cKit.
k-Ras arise because of a substitution from valin to serine in k-Ras resulting in constitutive proliferation signalling. Myc up-regulation results in stimulation of transcription. Bcl-2 inhibits apoptosis and autophagy. Mdm2 inhibits the functioning of the p53 tumour suppressor gene. The EGFR and cKit stimulate down-stream signalling and therefore proliferation, whereas they both inhibit apoptosis.
Different approaches are possible to target the various hallmarks of testicular cancer, varying from inhibition on the RNA level till dephosphorylation or simply inhibition of the receptor ligand.
k-Ras can be inhibited by the use of oligonucleotides and RNA interference. The Myc oncogene can be inhibited by bromodomain targeting drugs, inhibiting homodimerization or inhibition of down-stream targets. Mdm2 can be inhibited by administrating its antagonist, nutlin 3A. Targeting Bcl-2 can be obtained by using a dephosphorylating agent, PP2A. Also by the use of antibodies, ribozymes and small molecule BH3 mimetics Bcl-2 inhibition is accomplished. For EGFR a lot of targeting methods are possible. Inhibition of EGF and TGF alpha by a monoclonal antibody is one possibility. Also, antibodies with toxic effect, tyrosine kinase inhibitors, antisense RNA, small interfering RNA and ribozymes are methods of inhibiting the EGFR down-stream signalling. cKit can be inhibited by a tyrosine kinase inhibitor. For some treatment methods, like RNA interference, clinical administration is not possible yet, but will be in the nearby future. Other drugs, like the tyrosine kinase inhibitors or antibodies with toxins, have already been clinical administrated. Thus,a wide range of new treatment options can be further explored for the treatment of cisplatin resistant tesicular cancer patients.

Key words: Testicular cancer, hallmarks, treatment, k-Ras, Myc, Bcl-2, Mdm2, EGFR, cKit

1. A short introduction

Testicular cancer is an uncommon disease, but affects every year 660 men in the Netherlands alone. The treatment options are chemotherapy, whether or not combined with radiotherapy. The golden standard at the moment for chemotherapy is platinum-based chemotherapy. The curing rates are promising, 90% of the testicular cancer patients is cured. For the remaining 10% there are no good treatment options available. The aim of this review is therefore to determine whether targeting various hallmarks of testicular cancer can be a new method in curing testicular cancer

1.1 Cancer

Cancer is a common disease and accounts for many deaths. The most common type of cancer is lung cancer and the second most common type is colon cancer. Cancer arises because of mutations. Due to mutations, enhanced cell proliferation, reduced apoptosis and sustained angiogenesis, among other things, occur finally leading to cancer The current hypothesis is that cancer consists of a tumour bulk and a slow dividing, hard to treat, tumour stem cell subpopulation. The tumour stem cell is hard to treat because it can repair its own DNA damage and divides slowly. Medication given to reduce the tumour bulk does not harm the cancer stem cells. The mutations that lead to enhanced proliferation of cancer cells, can be divided in mutations in a tumour suppressor gene or a mutation in a tumour oncogene. A tumour suppressor gene (for example, p53) has a function, like DNA repair or apoptosis induction. When two (recessive) mutations occur in a tumour suppresser gene, one in every allele, it loses its function. A tumour oncogene on the other hand is a gene that has a proliferative or growth function. When one (dominant) mutation occurs in a oncogene, the cell gains a growth dominant function.

1.2 Testicular cancer

In testicular cancer, the same events occur as mentioned above. Testicular cancer is not very common, only 1 to 2 % of the men are affected by this disease (1). However, there has been a major rise in testicular incidence over the last years.(1) Testicular cancer is correlated with age. Three different privileged age groups can be seen. The first privileged age group in boys under the age of 5. The second privileged age group, which makes up for the most cases of testicular cancer, is men around the ages of 30 to 35. Another (smaller) privileged age group can been seen in men around the age of 60.

There are two different types of testicular tumours: germ cell tumours and non germ cell tumours. Non germ cell tumours account for only 5% of all testicular cancers. For example, Leydig or Sertolli cell tumours and gonadoblastomas are types of non germ cell tumours.

Germ cell tumours on the other hand account for 95% of testicular cancer and can be divided into the seminoma germ cell tumours and the non-seminomas germ cell tumours. (1)

60% of the germ cell tumours consist of seminomas. Seminomas originate from immature germ cells. They are slow growing and tend to remain….? for long periods of time. The seminoma normally occurs in men between the age of 30 and 40. Non-seminomas occur around the age of 40. Non-seminomas are also called mixed germ cell tumours, because they contain several cell types: Embryonic carcinoma, Yolk sac carcinoma, Choriocarcinomas and Teratomas. Embryonic carcionoma have an embryonic origin, which means these cells can differentiate in cells of al three germ lines. Yolk sac carcinoma is the most common form of testicular cancer in children. Yolk sac carcinomas are also called endodermal sinus tumours, infantile embryonic carcinoma or orchidoblastoma (1). Choriocarcinoma is a uncommon and aggressive form of testicular cancer in adults. This type of testicular cancer is associated with

a high chance of metastasis. Teratomas consist of cells from all three primordial cell lineages (found in embryonic development).

A feature of all invasive testicular germ cell tumours is gain of material on the short arm of chromosome 12 (2).This is due to an iso-chromosome 12p, which occurs in 80 % of the cases. In about 10% of these cases amplification in the 12p11.2-12.1 region occurs. This is mostly seen in seminomas. In non-seminomas a more heterogeneous cellular pattern can be seen.
Amplification at 12p11.2-p12.1 is associated with an earlier age of presentation and reduced apoptosis compared to seminomas which gain an entire 12p (2).

2. Treatment methods so far.

At this moment, 90% of the testicular cancer is curable. Germ cell tumours are better treatable than the non germ cell tumours. The best treatment option for non-germ cell tumours is chemotherapy (5). Resectomy is also possible. For treatment of non-seminoma germ cell testicular cancer, the best results are obtained by the use of chemotherapy followed by resectomy (3). Seminomas germ cell tumours can be treated with a combination of chemotherapy and, depending on patient characteristics, the kind of tumour and the location, limited radiation. Radiation is not always an option because of side effects (4). Radiotherapy can be used on carefully selected patients, with non bulky tumours (stage I). For stage II, III and IV treatment with radiotherapy should not be used.

2.1 Cisplatin

As mentioned above, chemotherapy can be used in the treatment of both germ cell tumours and non-germ cell tumours. The golden standard for chemotherapy treatment at this moment is the cisplatin based combination (CHT = chemotherapy), existing of cisplatin combined with etoposide. Cisplatin ($PtCl_2(NH_3)_2$) is a drug which elicits DNA cross links and adducts, in combination with the production of oxide radicals. (6) This results in inhibition of DNA synthesis and thus inhibition of replication. Cisplatin contains a platinum core, surrounded by two chloride ions in cis-conformation, and two ammonium ions in cis-conformation. Cisplatin is in its activated state when the two chloride ions dissociate and are replaced by water of hydroxyl related ligands. The platinum core can bind the guanine base on DNA. When it binds two DNA strands it is called an interstrand. When it connects the same strand it is called an intrastrand. DNA intrastrands occur 98 % of the time, interstrands only 2% of the time. By binding to the DNA, template replication is inhibited.(7)
Cisplatin is often administrated in combination with bleomycin. Unfortunately, tumour cells can gain resistance to cisplatin. There are 4 different methods for gaining resistance as shown in figure 1. The first method of gaining cisplatin resistance is due to a mutation. Because of a mutation in a gene, the cell reduces its uptake of cisplatin. Reduced uptake results in reduced formation of intra- and interstrand . Also, when cisplatin is in the cytoplasm, the cell can inactivate cisplatin by binding GSH and methallothionein to it. A third mechanism of cisplatin treatment failure is that enhanced DNA repaire occurs after intra- and interstrand formation. The DNA adducts and cross-links are then removed, which makes replication again possible. A fourth and last mechanism for cisplatin resistance is that the cell becomes resistant to apoptosis. Replication is not possible, but the cell can still be part of the tumour. This also creates extra reparation time.

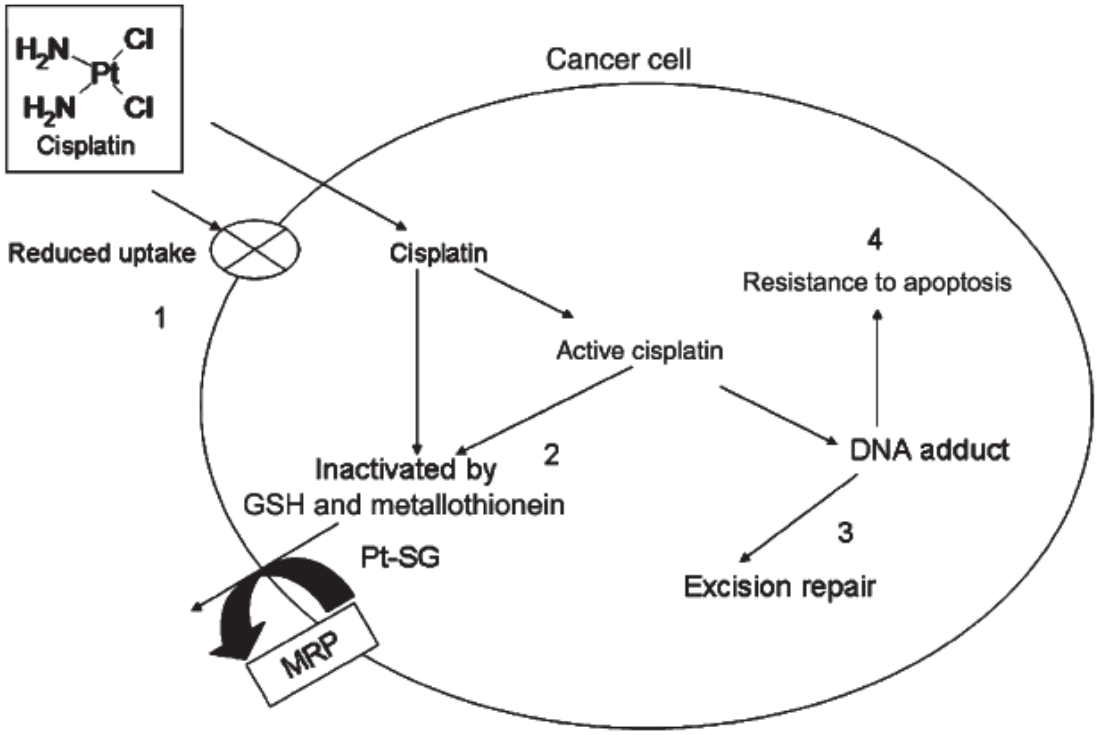

Figure 2: Four mechanisms of resistance. (7)

When cisplatin resistance occurs there is no standard treatment. Clinically, new drugs like gemcitabine and paclitaxel are being tested (8)

2.2 Radiotherapy

For seminoma germ cell tumours there is another treatment option: radiotherapy. The long term prognosis for treatment with radiotherapy is very good (8). The effectiveness of radiotherapy is that the field of radiation is not only the testical itself but also the surrounding tissues (like the para-aortic and common iliac vessel). The radiation of these additional areas results in a low relapse chance. (8)

2.3 Relapse

Treatment of a relapse depends on a number of parameters, such as the initial treatment, the localization of the tumour, the time passed since the previous treatment, and the subsequent response. When first radiotherapy has been used, it is (almost) certain that the cancer is located outside the previous treatment area. The best option then is the use of cisplatin based combination chemotherapy. When there's a relapse after three months after treatment with CHT, the disease is most likely to react on a platinum based CHT salvage treatment (6). Relapse after CHT treatment is very rare, but can occur. This is the 10% of testicular cancer patients observed.

3. Why are new treatment options important in testicular cancer?

Testicular cancer has a curing rate of 90%. Even metastatic cancer is well treatable. The goal of chemotherapy in germ cell tumours is never palliation or prolongation of survival but always curing.(9) Testicular cancer is well diagnosable and treatment specific, because of a number of serum markers.

The first serum marker is Alphafetoprotein (AFP). When the levels of AFP are elevated, a pure seminoma germ cell tumour cannot be present.(10) The second serum marker is the human chorionic gonadotropin (hCG). This is an important maker for making important treatment related decisions. With a hCG beta kit the hCG levels are measured. Also, the free beta-subunit is measured. When the levels of hCG and it's subunit are elevated, a seminoma is indicated.(10)

Due to cell necrosis, a short lasting increase in hCG and AFP is possible during treatment. In that case, hCG and AFP markers are not significant for tumour progression. High hCG levels in the beginning of treatment indicate a poor prognosis. (10)

The third marker is lactate dehydrogenese (LDH). When LDH is elevated, there is a tumour present or recurrence occurred. Iso-enzyme LDH1 is an easy to determine enzyme, and it shows elevation in presence of testicular cancer, even when the total amount of LDH is normal. LDH1 is therefore a more specific marker.(10)

In most cancer types, treatment is difficult. Mostly, this is caused by a mutation of p53.
Because p53 is mutated, it does not respond in the normal way. DNA damage is induced with most chemotherapy. P53 is activated by the DNA damage and results in cell cycle arrest allowing DNA repair or when the cell is too damaged, apoptosis. When p53 is mutated, it is not activated by DNA damage, so the chemotherapy has limited effect. Testicular cancer responds well to treatment, because in testicular cancer p53 is almost never mutated.

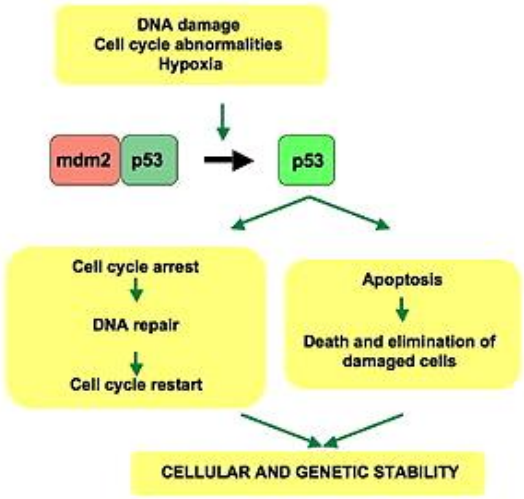

Figure 3: p53 pathway

For the treatment of testicular cancer, as mentioned before, chemotherapy plays an important role. For the non-responder group of patients, there are no good treatment options available. Proteins that are possible hallmarks of testicular cancer and could be new targets to treat the chemotherapy resistant testicular cancer patients are discussed in this review.

4. Hallmarks in testicular cancer

An oncogene is a gene that alters cell growth and proliferation (9). The gene normally regulates cell growth and proliferation. Because of a mutation, amplification or a fused gene, the over-expressed gene is constantly switched on. Cell growth and proliferation is then stimulated.

The consequence of the mutation is that the cell gains a growth dominant function. Therefore, a mutation in an oncogene is also called a gain of function mutation. Because of the constant stimulation of growth but the rate of apoptosis remains normal (or decreases because of a different mutation), there is an increase of cell number. One well known oncogene is RAS, which will be used as an example to explain the function of an oncogene.

4.1 K-Rasoncogene

k-Ras was the first oncogene ever to be discovered and is a member of the Ras family. The oncogene is located on chromosome 12. The Ras family consists of three family members, namely k-Ras-2, n-Ras and h-Ras-1. The mutations occur in codons 12, 13 or 61. There are three properties visible when there is a Ras mutation at codon 12 or 13.

First, colonies are formed with an increased density. Second, anchorage independent growth is visible and third, there is a reduced ability of apoptosis (24).

In testicular cancer, (mostly) K-ras is mutated at codon 12. (11) A base substitution from valine to serine is occurs. As a result of the substitution, the Ras oncogen is switched on constantly. A K-ras mutation is most common in seminoma tumours, but only occurs in 8 % of all testicular cancer patients. An increase in mutated Ras proteins is visible when the tumour metastasises (12).

4.2 Myc oncogene (C-Myc, L-Myc, N-Myc)

The Myc-gene is a transcription factor and regulates many different genes. It regulates these genes by binding to enhancer box sequences (E-boxes), followed by recruitment of histonacetyltransferase. Myc is activated by a number of signal routes, like Wnt signalling and activation by the epidermal growth factor receptor (EGFR) (13).

Myc amplifications occur in 55% of the testicular cancer patients (14).

4.3 Mdm2

Murin double minute (Mdm2) is a member of the Mdm2 family, which consists of Mdm1, Mdm2, Mdm3 and Mdm4 (also called Mdmx). Mdm2 is known for suppressing the tumour suppressor gene p53 function. P53 is a tumour suppressor gene which, when activated, results in cell growth arrest and DNA repair or (when the cell is too damaged) in apoptosis. When Mdm2 is active, it binds and blocks the N-terminal activation domain of the p53 gene, suppressing its function (14)

Mdm2 can also acts as an E3 ubiquitin ligase, which results in degradation by ubiquitinilation of both p53 and Mdm2 itself. (16) P53 on the other hand is also a transcription activator of Mdm2. (15) When p53 is stabilized because of a reduced demolition or when p53 is produced by transcription, transcription of Mdm2 is also induced, resulting in higher Mdm2 levels. This results in p53 inhibition. This is an important negative feedback mechanism, resulting in normal p53 levels. P53 can be activated, for example by DNA damage. After DNA damaged activation of p53, phosphorylation of Mdm2 occurs, which inhibits the suppressor function of Mdm2 and activates p53. (16) Mdm2 is a gene which often is amplified in cancer. Because of higher expression levels of Mdm2, P53 can not be activated and therefore cell cycle arrest of apoptosis can not be induced. This will result in cell proliferation.

Over-expression of Mdm2 is caused by genetic lesions. Mdm2 is over-expressed in more then 40% of all testicular cancer patients (17).

4.4 BCL-2

B-cell lymphoma 2 (BCL-2) is a member of the Bcl-2 family and is an anti-apoptotic protein (18). The name Bcl-2 is derived from B-cell lymphoma 2, because it is the second protein to be discovered in a range of proteins involved in the translocation of chromosomes 18 and 14. (19) There are two different isoforms of Bcl-2: Isoform 1 (1G5M) and Isoform 2 (1G5O/1GJH). The structural topology and electrostatic potential of the binding groove differs. These differences suggest differences in anti-apoptotic activity for the two isoforms (protein data bank) (19).

58% of the testicular cancer patients are positive for Bcl-2 over-expression. Bcl-2 mutations occur more often in non seminomas compared to seminomas. Also, when a tumour metastasises, the Bcl-2 expression becomes higher (20) .

4.5 EGF receptor

The EGF receptor (also ErbB or HER receptor called) exists on the cell membrane of cells. The EGF (HER) family consists of the HER1, HER2, HER3 and HER4 receptor. The EGFR family can be activated by epidermal growth factor (EGF) and the transforming growth factor alpha (TGF). After activation, the receptor homo- or heterodimerizes with another family member, followed by phosphorylation and down-stream signalling. Down-stream signalling results in cell growth, proliferation and migration. (21)

EGFR is over-expressed in 23,7 % of the non seminoma testicular cancer patients. 10,9 % of the yolk sac carcinoma patients has a EGFR mutation. In teratomas, EGFR is mutated in 50% of all patients (22).

4.6 cKit oncogene

cKit is also known as stem cell growth factor receptor (SCFR), tyrosine protein kinase Kit or CD117.(23) It is encoded by the KIT gene. cKit can bind to stem cell factors, followed by the formation of a dimer. The dimer activates the intrinsic tyrosine kinase that phosphorylates and activates signal transduction. Activation of cKit results in cell survival, proliferation and differentiation.

In seminomas, cKit is mutated. There is a mutation in exon 17 of the CD117 gene. Also, the gene is often over-expressed and amplified (23)

cKit is mutated in 48% of all testicular cancer patients. In teratomas and secondary malignaties it is mutated in 62 % of all testicular cancer patients. In late relapses, cKit is mutated in 80% of all testicular cancer patients (24).

5. Signal transduction pathways

5.1 Ras

As shown in figure 4, Ras is activated by the activated receptor. When Ras is activated, it activates its down-stream target Raf. Raf can be activated by Ras but also by phosphorylation by different cytokines. P2A is a cytokine which can remove various phosphorylated sites from Raf. This can lead to activation or inactivation of Raf, subordinated to the location of phosphorylation.

Raf activation leads to the phosphorylation and thereby activation of MEK. In turn, MEK phosphorylates and activates ERK. ERK then activates a number of transcription factors which regulate gene transcription of genes which regulate proliferation and apoptosis. (25)

Another pathway is shown in this figure, the PI3K/PDK/AKT pathway. This pathway interacts with the RAF pathway. P85 PI3K subunit can bind to Ras, which results in

activation of PI3K. PI3K then activates PDK and AKT. AKT can phosphorylate transcription factors such as ETS. (25)
Of the three Ras genes, k-Ras is the most frequently mutated in testicular tumours. (26)
A mutation increases resistance to apoptosis by AKT activation. Mutated k-Ras results in a aggressive tumour, not by changing cell morphology or proliferative capacity but by altering the threshold of apoptosis induction (26) This is a common pathway in testicular cancer.

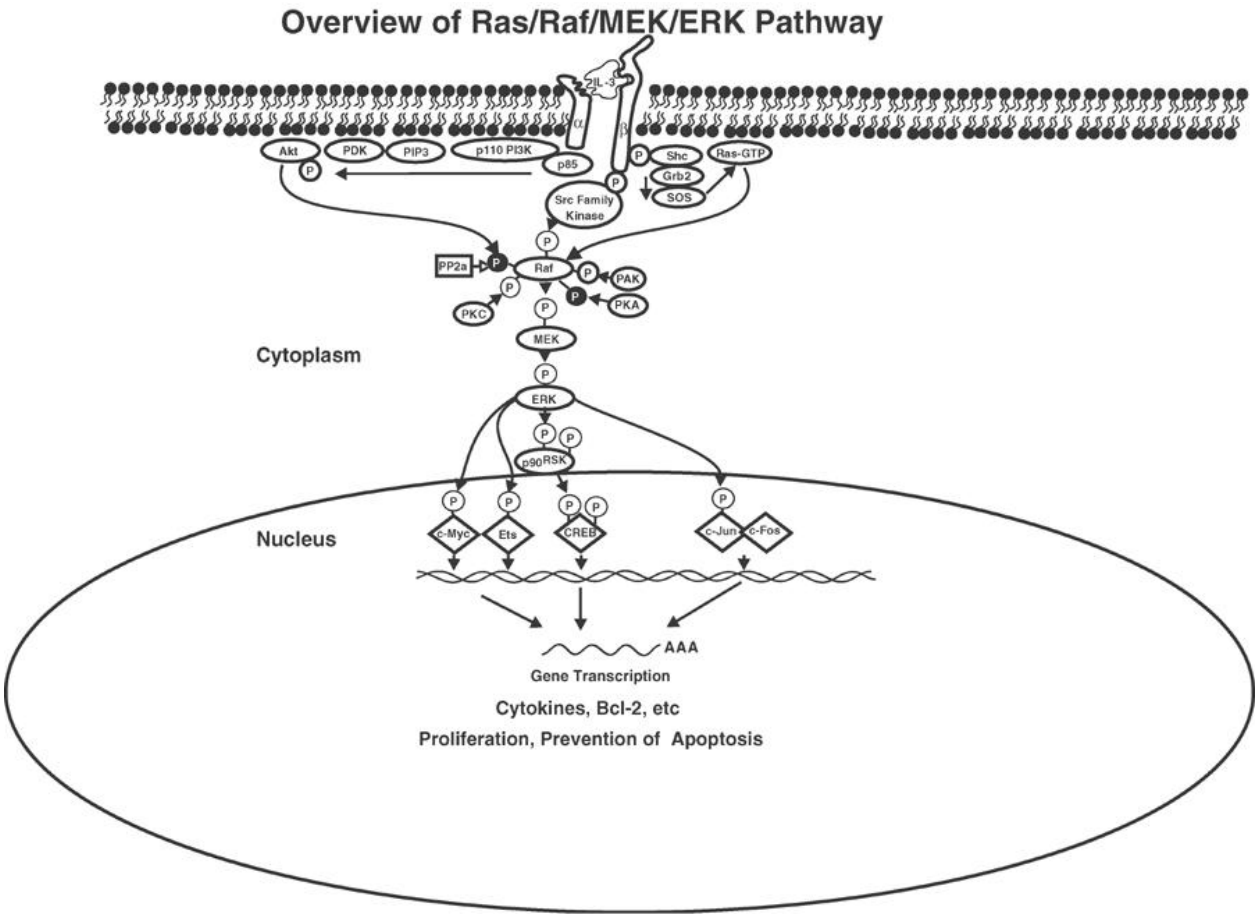

Figure 4: The Ras/Raf/MEK/ERK pathway (25)
Down stream signalling of Ras is visible. Ras is activated followd by phosphorylation of Raf, MEK and ERK. This results in gene transcription

5.2 Myc

Myc interacts with two transcription factors, Sp1 and Miz1. These transcription factors bind to the core of promoters. Sp1 and Miz1 stimulate transcription when they bind DNA in the absence of Myc. When Myc binds these transcription factors, transcription is inhibited.
Both proteins are present at the promoter of many Myc-repressed genes. (27)
Miz1 induces transcription by recruitment of co-activators like p300 or NPM. Myc inhibits the interaction between Mitz1 and the co-activator. Myc on the other hand recruits histon acetylase HDAC3, which results in de-actylation of histon 4 and leads to inhibition. A third mechanism of transcription inhibition is recruitment of DNA methylase Dnmt3a, which methylates DNA and leads to transcriptional repression. A fourth and last mechanism is the formation of a heterotrimeric Myc/Miz1/Arf complex. This complex increases the trimethylation of histone H3, leading to transcriptional repression.(27)

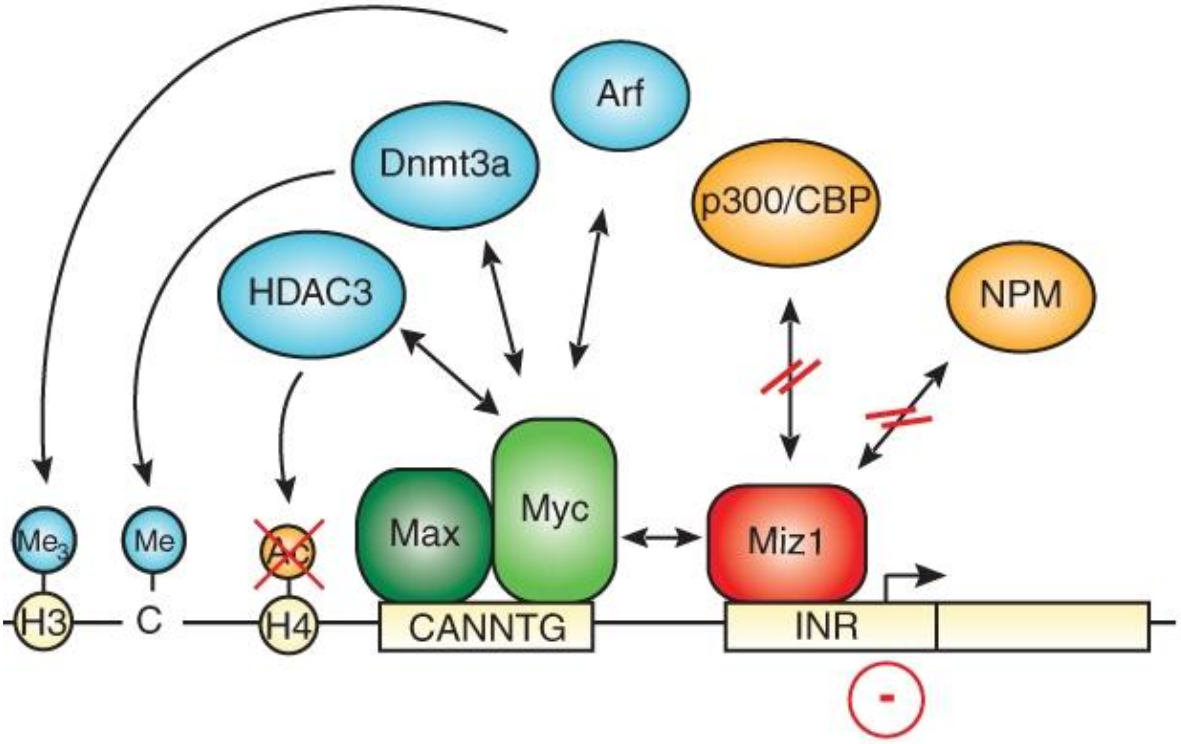

Figure 5: Method of Myc inhibiting transcription (27)
This figure shows the methods whereby Myc interacts with different co-activators to inhibit transcription.

Genes that are repressed by Myc encode negative regulators of cell proliferation, for example, the EBPA genes. The EBPA genes normally promote differentiation. Other genes, like the Cdkn family, inhibit the progression of the cell cycle. A third group of transcription factors that is being repressed by Myc, are the NDRG transcription factors. When the NDRG transcription factors are not inhibited, the suppress growth.(27)
Another group of genes that is repressed by Myc encodes proteins involved in cell adhesion, like Itgb1. Itgb1 encodes integrin β1, a part of the multiple heterodimeric integrin complex. Because of the binding of multiple ligands, like collagen or fibronectin, these complexes mediate cell-cell interactions. Also, they mediate anchoring to the extracellular matrix.
When Itgb1 is inhibited, no complexes can be formed. The consequence is that cells are not tightly bound to the extracellular matrix, which enables metastases. (27)

Transcription repression is not only mediated by Myc inhibited Miz1, but also by other repressor proteins. One of these repressor proteins is Bcl-6, which is an onco-protein. Blc-6 contains at its amino-terminus, just like Miz1, a POZ/BTB domain. This domain is used to heterodimerize with Miz1 and represses transcription. The same POZ domain can be found on transcription factor Zbtb4, which also heterdimerizes with Mit1.
A different mechanism can be seen at the Gfi-1 repressor. The Gfi-1 repressor forms a complex with Myc and Miz1 and represses transcription.

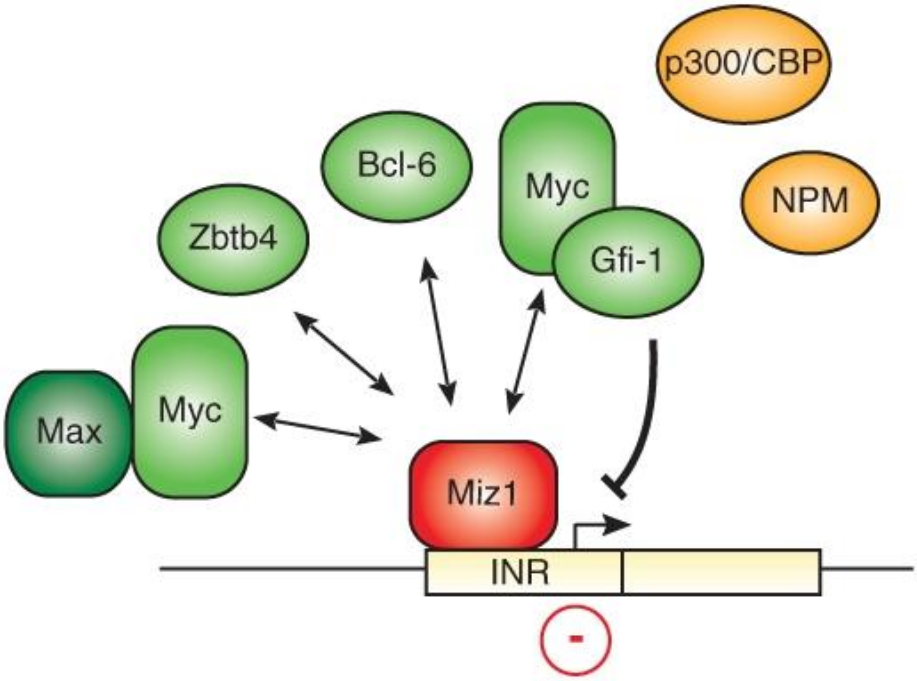

Figure 6: Miz1 functioning (27)
Miz1 acts as a platform for recruitment of repressor complexes, like repressor proteins Bcl-6, Zbtb4, or Gfi-1.

Although Myc is mostly known as a transcriptional repressor, Myc can also promote transcription (28). Myc activates genes involved in cell metabolism and cell proliferation. Myc binds to the concensus sequences (also called E-box sequences) and recruits histon acetyl transferase. Histon acetyl transferase binds lysine amino acids on a histone protein. Histon aceylation is linked to transcriptional activation and is associated with euchromatin, the open state of DNA (28). This pathway is less common in testicular cancer, compared to the two pathways mentioned above (28).

5.3 Mdm2

Mdm2 contains evolutionary conserved acid domains resembling motifs on other transcription factors. These conserved motifs are localization signals for travelling to the nucleus.
Mdm2 is known to bind to the tumour suppressor gene p53 and thereby inhibiting the p53 function. An over-expression of Mdm2 has been seen in human testicular tumour tissue. Mdm2 binds to the TFSDLW domain of p53, located between the amino acids 18 till 23. The TFSDLW region is exactly the region that normally recruits the basal transcriptional machinery, which is necessary for trans-activation of target genes containing p53 responsive elements(29).
P53 is known for activating transcription genes containing a p53 responsive element. Mdm2 contains a p53 responsive element. Up-regulation of p53 therefore, results in up-regulation of Mdm2 (only when p53 is not mutated, in many types of cancer, excluding testicular cancer p53 contains mutations and this mechanism does not apply) (29).
Normally, p53 allows DNA repair. Because of over-expression of Myc, p53 can not be activated, so no DNA and therefore no genes are repaired. More lesions will therefore arise.

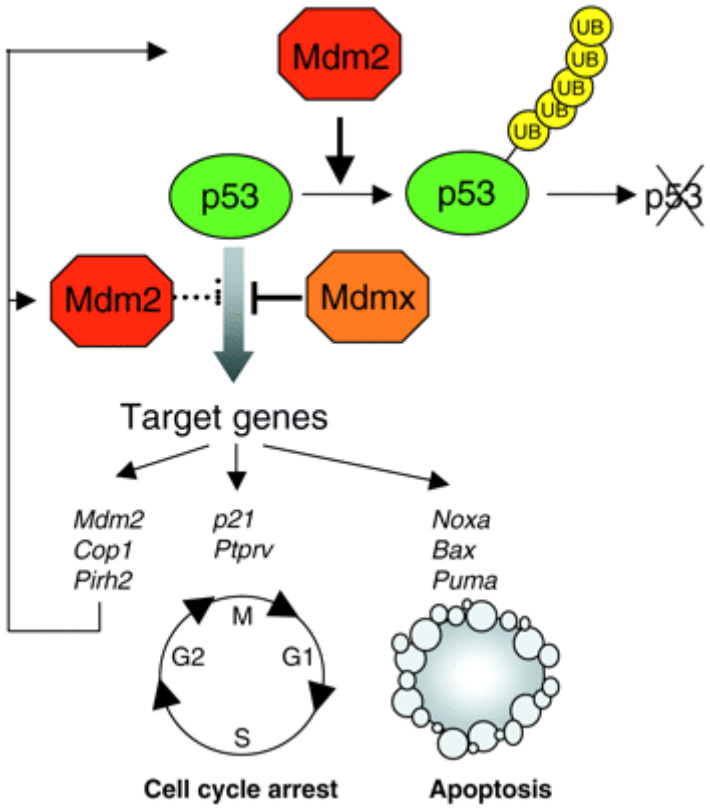

Figure 7: the Mdm2 pathway (29)
Mdm2 inhibits by unbquitinilation the activity of p53

5.4 Bcl-2

Bcl-2 and the other family members (Bcl-xL and BCL-1) are known to have an anti-apoptotic function. Bcl-2 has an anti-apoptotic effect by inhibiting Bax and Bak. Bax and Bak are known for their intrinsic apoptotic pathway. When a cell is damaged, Bak and Bax are activated on the membrane of the mitochondria. They then release cytochrome C and the intrinsic apoptotic pathway is activated. (30) When Bcl-2 is present, it antagonizes the function of Bax and Bak and thus prevents apoptosis. This is a common effect of Bcl-2 in testicular cancer.

Beclin 1, is an initiator of autophagy. Beclin 1 recruits autophagic proteins.(30) These autophagic proteins bundle to a pre-autophagosomal structure. The pre-autophagosomal structure consists of Beclin 1, Vps34, and Vps15. Beclin-1 can interact with anti-apoptotic Bcl-2 family members, through its BH3 domain. The interaction between Beclin-1 and the anti-apoptotic Bcl-2 prevents Beclin-1 from assembling a pre-autophagosomal structure. In this way, autophagy is inhibited. (30) This pathway is less common in testicular cancer. Usually, Bcl-2 is associated with Bax.

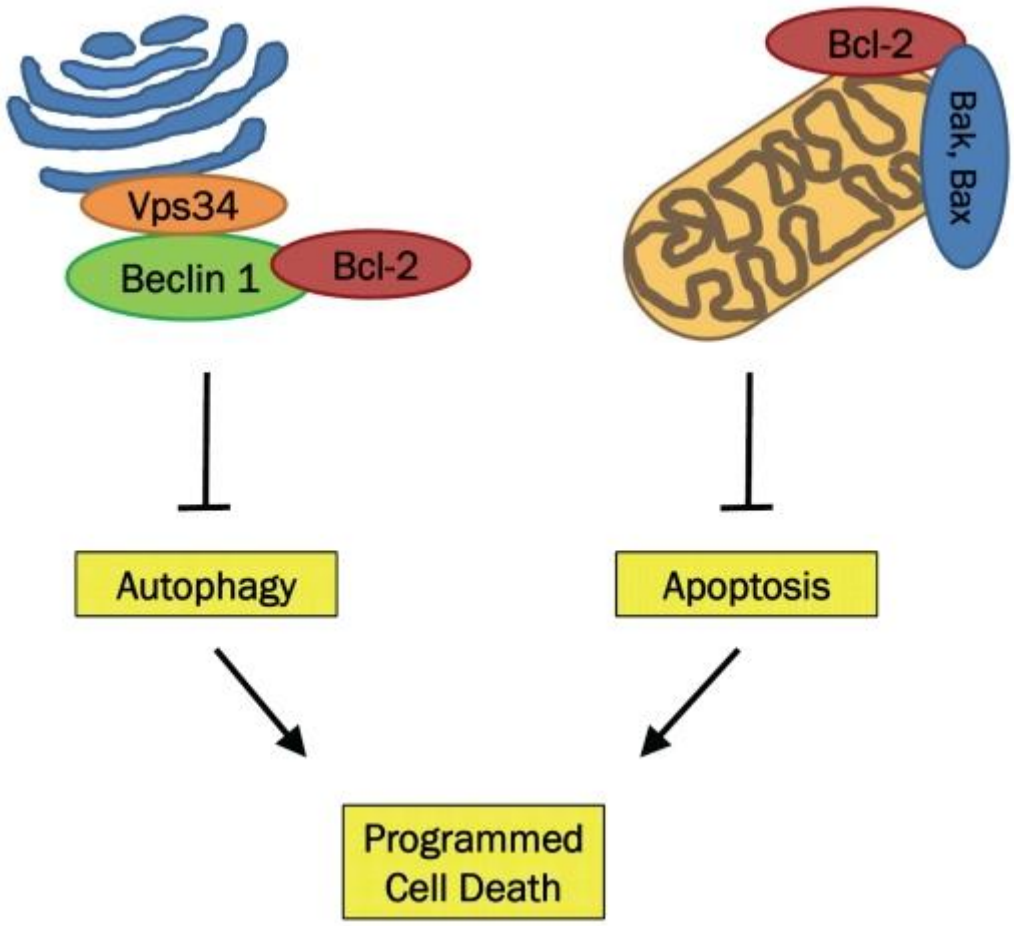

Figure 8: Method of Bcl-2 inhibition (30)
Bcl-2 inhibits autophagy and apoptosis by interaction with other factors

5.5 EGFR

The EGFR receptor is a growth receptor, located at the membrane of cells. It can be activated by its ligands, EGF or TGFalpha The encoding gene for the EGFR receptor is located on chromosome 7p12-22. In cancer, the gene is activated by two different mechanisms.(31)

The first mechanism is an increase in gen copy number. More genes result in more transcription, which in the end results in more EGF receptors. A second and last mechanism is the acquiring of (activating) mutations. (31) When the EGFR receptor is activated, it results in down-stream signalling by two different pathways: the phosphatidylinositol 3-kinase (PI3K) and the previous discussed Ras-pathway(2).

When EGFR receptor activates the PI3K, this in turn activates Akt and PKC. PKC results in cell proliferation. After Akt activation, Akt activates BAD, which inhibits apoptosis. Akt activates at the same time p70s6k, which also induces cell proliferation.(32) This is a common pathway in testicular cancer.

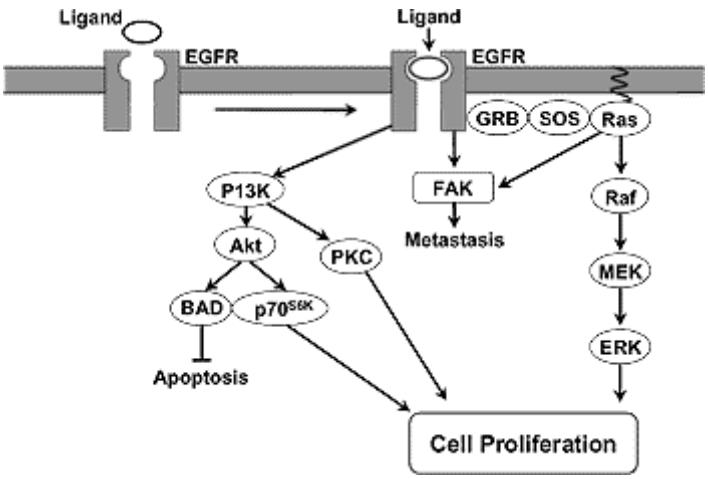

Figure 9: EGFR down-stream signalling (32)

5.6 cKit

cKit is a member of the tyrosine kinase family and is usually auto-inhibited or inactivated, unless a ligand activates the receptor. (33) Activation is followed by dimerization, which results in trans-activation of the cytosolic kinase domain and further down stream signalling in the previously discussed PI3K pathway. cKit activation is also possible in the absence of a ligand. When there is a mutation present, dimerization and autophosphorylation can also take place without SCF. As a result cell growth and proliferation occur. In most types of cancer cKit is over-expressed and amplified.(33) This is a common pathway in testicular cancer.

6. Treatment of oncogenes

6.1 K-Ras

Strategies to inhibit k-Ras protein expression vary from silencing protein expression through the use of antisense oligonucleotides to silencing with the use of RNA interference. Antisense oligonucleotides (ODN) are short (15-17 bases) stretches of synthetic DNA. It is complementary to a specific region of cellular mRNA or DNA. ODN blocks post-translational modifications with farnesyltransferase inhibitors (FTI). Also, downstream targets are inhibited (34).

RNA interference (RNAi) is another way of silencing genes. RNAi is a double-stranded RNA, which is homologue in sequence to the targeted gene. RNAi generates small interfering RNA (siRNA) through RNaseIII endonuclease dicer. The siRNA is the size of 21-23 nucleotides and mediates the degradation of their complementary RNA (34).

Targeted siRNA silencing can be selective against mutant k-Ras. In this way, only tumour growth is inhibited. Good results are accomplished when siRNA is used in combination with gemcitabine-based chemotherapy (34).

6.2 Myc

Myc transcription is associated with an increase in histone lysine side-chain acetylation. This is a modification of chromatin that is associated with transcriptional activation. Histon acetylation results in the assembly of transcriptional complexes (35). This is accomplished by recruiting proteins with one or more acetyl-lysine binding modules or proteins with bromodomains. Bromodomains increase the efficiency of transcriptional activators. Bromodomains therefore are a good target for inhibiting Myc-dependent transcription. JQ1 is

a molecule which can inhibit the bromodomain on Myc. JQ1 molecule enhances the inhibitions of the bromodomain by binding in the actyl-lysine recognition pocket (35).

Myc activating transcription is a good target for modulation. For Myc activation, heterodimerization is required with Max, its partner protein. Dimerization is essential for the oncogenic function. This makes homodimerization a good target for modulating Myc (36). A therapeutic agent which can inhibit dimerization is Omomyc. Omomyc is a Myc gene with four substituted amino acids. It thereby facilitates dimerization with all the other Myc oncogenes (like c-Myc, n-Myc and L-Myc) and Myc therefore can no longer bind Max (36). Because Myc cannot bind the Myc-Max-E-box consensus recognition element, it blocks Myc dependent transcriptional activity (36).

VX-680 is a small-molecule inhibitor, which inhibits the aurora family of mitotic kinases (37). One of the targets of VX-680 is aurora-B kinase. This is a catalytic subunit of the chromosomal passenger protein complex, also called CPCC. This complex is involved in a number of facets of cell division, for example spindle checkpoint, cytokinesis and chromosome segregation (37). VX-680 inhibits aurora-B kinase and therefore the CPCC misses its catalytic subunit and does not work properly. There is also an interaction between Myc and VX-680, which results in apoptosis of the cells, because of a combination of apoptosis and lethal autophagy. VX-680 kills cells that over-express Myc, and spares cells with no over-expression (37).

Down-stream signaling can also be targeted. One example is inhibition of the down-stream proteins Chk1 and Chk2. Excessive Myc expression stimulates a replication associated DNA damage response through the PI3K transduction pathway, as mentioned above. Myc activates DNA damage inducers Chk1 and Chk2. Chk2 deficiency induces slow growth, the cells are viable and protected against DNA damage (38) inhibition of both Chk1 and Chk1 by AZD7762 also induces cell death and in this matter delays disease progression (36). PARP is activated when DNA damage is induced. PARP then travels to the site of the damage and induces repair of the sites of DNA breaks. The combination of Chk2 and PARP inhibition is lethal for cells over-expressing Myc (36) (38).

6.3 Mdm2

Because Mdm2 inhibits the functioning of p53, the goal of anti-Mdm2 therapy is mainly re-activation of the p53 tumour suppressor protein, so down-stream signalling, growth arrest, DNA repair or apoptosis can be induced (39). A antagonist of Mdm2 is Nutlin 3A, which has been shown to activate wild type p53. Nutlins are small-molecules that antagonize the functioning of Mdm2, by binding to Mdm2 in the p53 binding pocket (40). Therefore, Mdm2 cannot bind p53, and p53 can become functionally cative, while the negative feedback by Mdm2 is lost (40).

6.4 Bcl-2

Protein phosphatase 2A, in short PP2A, is a protein that participates in signalling pathways in many processes (41). PP2A is a heterotrimer and consists of PP2A/A,PP2A/B and PP2A/C. The complex also contains a phosphatase activity. Activation of Bcl-2 occurs after phosphorylation by Bcl-2 kinase. Bcl-2 phosphatase (PP2A) on the other hand can dephosphorylate Bcl-2 (41). PP2A binds to the BH4 domain in BCl-2 and therefore results in dephosphorylation. The dephosphorylation facilitates the binding of p53 to Bcl-2 and therefore, negatively influences the Bcl-2 survival function (41).

Also, ribozymes can be used in the treatment of Bcl-2 (41). An example of ribozymes is the Bak BH3 peptide, which binds Bcl-2 in the BH3 pocket and thus inhibits its function. A downside of ribozymes is the lack of stability and efficiency of delivery. A method for stabilization is stapling. A stapled peptide, called stabilized alfa-helix of Bcl-2 domains, is a helical and protease resistant and cell-permeable molecule. It can bind with an increased affinity to the Bcl-2 pocket. Because of the increased affinity, it has a better apoptotic potential then a non-stapled peptide (41).

Bcl-2 can also be inactivated by the use of antibodies directed against Bcl-2 (42). A intracellular anti-Bcl-2 single chain antibody increases the drug- induced cytotoxity in cells.
Recent studies have shown that intracellular antibodies have a high efficiency and selectivity for the targets and have little or no effect on cells that don't over-express Bcl-2. Therefore, this is a drug with great potential (43) .

ABT-737 is a Bcl-2 antagonist (44). It is a BH3 mimetic, also called a BH3 mimetic small molecule inhibitors (SMI). ABT-737 binds Bcl-2 with a high affinity in the BH3 pocket, which is normally associated with Beclin-1 binding. Because Beclin-1 can no longer bind Bcl-2, autophagy is no longer inhibited (44). Because the Bcl-2 pocket is bound by ABT-737, Bcl-2 can also no longer bind and inhibit Bak and Bax. Therefore, more apoptosis occur. Another Bcl-2 antagonist which achieves its effects in the same way is Obatoclax. Because the association of testicular cancer with Bcl-2 – Beclin pathway is less common than the association of Bcl-2-Bax, its administration should be carefully considered.

6.5 EGFR

Inhibition of the EGFR receptor ligands Heregulin and TGF alpha is one method for inactivating the signal transduction pathway. Heregulin and TGF alpha can be inhibited by the use of monoclonal antibodies. Because Hereguline and TGF are bound by an antibodies, they can no longer bind the receptor. Activation of the receptor and down-stream signalling therefore, are inhibited. Most antibodies that bind TGF are still in clinical trials. (45)

Erlotinib is a tyrosine kinase inhibitors and inhibits therefore the EGF receptor (45). Erlotinib binds to the ATP binding site of the EGF receptor. ATP then cannot bind, so after homodimerization of the EGF receptors, no cross phosphorylation is possible and signal transduction is interrupted (45). The use of Erlotinib unfortunately, often leads to resistance.
There are two most common mechanisms of acquiring resistance to TKIs (45). The first method is the acquisition of a secondary peptide, T790M EGFR mutation. This increases the affinity of mutant EGFR for ATP (45). A seconds mechanism is activation of mesenchym to epithelial transition (MET). This is a mechanism where a more differentiated tissue dedifferentiates to a less differentiated tissue. This mechanism offsets the loss of EGFR signalling (45).

Ligand dependent activation of the EGF receptor itself can also be inhibited. By binding of an antibody (also called a antagonist) directly to the receptor, the ligand can no longer bind. Activation of the receptor and down-stream signalling is no longer possible. One example of the antibodies that can bind the EGF receptor is Cetuximab (45).

There are several other method to inhibit the EGFR signal transduction pathway (46). One of these methods is binding of antibodies to the EGFR carrying a toxic load. This can be toxins or radioisotopes (46). A example of such a antibody is MAb 425, labelled with isotope I125. This can be administrated in combination with radiotherapy. This results in a good

affinity, stability and cytotoxity (46). A different treatment method is the use of a ligand as a vector. An example is TP-38, which is a fused protein composed of TGF alfa (ligand) coupled to a mutated pseudomonas exo-toxin (PE-38) (46). Newer treatment methods aim mostly at RNA level (46). One example of a new treatment method is antisense RNA. This hybridized with sense mRNA. In this way it inhibits translation and protein synthesis of the EGFR pathway. It can be administrated locally during surgery or intravenous.(46) Also, small interfering RNA (siRNA) can be used. Double stranded RNA is then processed in to small interfering RNA (siRNA). siRNA suppresses homologues genes and induces sequence specific mRNA degradation (46).

There are a number of problems with siRNA. One problem is the administration. The administration should be locally, but in case there is no surgery, local administration is not possible. Systematic administration of siRNA is not possible yet, the siRNA is not stable enough. Luckily, nanocapsules are developed at the moment. The nanocapsules will make a targeted administration possible (47). A second problem with siRNA is that siRNA almost always also repress other genes then the targeted genes. This can accomplish unwanted side effects. More studies are needed before administrating siRNA clinically (47).

The last method for targeting the EGF receptor down-stream signalling is by using ribozymes (RNA enzyme). Ribozymes are small RNA molecules that cleave other mRNA molecules, so they can no longer be translated. Ribozymes can be delivered to a tumour cell by means of a viral vector. A viral vector does not only deliver the ribozyme to the tumour but also integrates the ribozyme into the chromosome(45). Because of integration, the specifity of a ribzyme is enlarged.

6.6 cKit

Imatinib (Glivec, STI571) is a tyrosine kinase inhibitor, capable of inhibiting cKit. Imatinib binds the tyrosine kinase (TK) active site in cKit. ATP therefore cannot bind the active site. The results is a decreased cKit activity (48).
Resistance to Imatinib is caused by a mutation, D816V, which is located in the activation loop. Because of structural changes at the enzymatic pocket, the activation site is therefore not accessible for Imatinib. Nilonotinib (AMN107) is a different tyrosine kinase inhibitor (48). AMN107 displays equipotent activity to Imatinib and can still access the cKit mutated activation pocket incontrast to Imatinib (48).

7. Conclusion

The aim of this review was to determine whether targeting hallmarks of testicular cancer can be a new method in curing testicular cancer. A great variety of proteins have been confirmed to play an important role in the development of testicular cancer: the k-Ras oncogene, the Myc oncogene, Bcl-2, Mdm2, the EGF receptor and the cKit oncogene. In addition, many of these proteins have proven to be drugable. This creates a good opportunity for the treatment of cisplatin resistant testicular cancer patients.
A different study should be executed to investigate whether there are good markers available to determine which protein is mutated, amplified or overexpressed in a particular testicular cancer patient. One possibility for determining which genes are mutated is genome sequencing. If every testicular cancer patient undergoes genome sequencing, it can exactly be seen which (onco)genes are mutated. This makes a targeted and personalized approach possible.

Concluding, for treating cisplatin resistant testicular cancer patients several treatment options targeting various hallmarks of testicular cancer are possible. This offers a chabce ti unorive survival for those patients who have no good prognosis at this moment

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