Molecular Targets for the Treatment of Testicular Germ Cell Tumors

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Abstract: In the last decade novel therapeutic approaches for the treatment of cancer have been proposed: inhibitors of serine/threonine and tyrosine kinases, angiogenesis inhibitors, gene therapy approaches and others. In some cases the clinical trials have confirmed the efficacy of these approaches. Here, we will review the discovered molecular targets for the treatment of testicular germ cell tumors.

Key Words: Testis, testicular cancer, Aurora kinases, tyrosine kinases, angiogenesis, kinases inhibitors, gene therapy.

1. INTRODUCTION

Testicular germ cells tumors (TCGTs) are the most frequent solid malignant tumor in men 20–40 years of age, accounting for up to 60% of all malignancies diagnosed at this age. Despite a high cure rate, they represent the most frequente cause of death from solid tumors in this age group [1,2].

TGCTs are a heterogeneous group of neoplasms seen mainly in young men [2]. They are classified as seminomatous (SE-TGCT) and non-seminomatous (NSE-TGCT) tumors, both of which appear to arise from intratubular germ cell neoplasias (ITGCN) [3,4]. The former is constituted by neoplastic germ cells that retain the morphology of spermatogonial germ cells, whereas NSE-TGCT display primitive zygotic (embryonal carcinomas), embryonal-like somatically differentiated (teratomasas) and extra-embrionally differentiated (choriocarcinomas, yolk sac tumours) patterns [3,4]. TGCTs are frequently associated with ITGCN that, often, progresses to invasive cancer (Fig. **1**) [5,6].

The molecular basis of germ cell malignant transformation is poorly understood. The most common genetic alterations detected in TGCT and ITGCN are a triploid/tetraploid chromosomal complement and an increased copy number of 12p, which results in hyperexpression of the product of the CCND2 gene, that is, G1 cyclin D2 [6]. In addition, deficiencies in the short arms of chromosomes 1, 3, and 11 are concurrent with TGCTs [2], implicating the presence of potential TGCT suppressor genes in these deficiency regions. TGCTs are often accompanied by the hyper-expression of autocrine and/or paracrine growth and angiogenic factors such as glial cell line-derived neurotrophic factor (GDNF), and vascular endothelial growth factor (VEGF) [7,8]. Recently, it has been shown that Aurora B overexpression is associated to human seminomas [9]. It was recently shown that loss of the tumor suppressor gene *PTEN* plays a crucial role in the pathogenesis of TGCTs [10].

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Seminomas are highly sensitive to both radiation and chemotherapy, with a good prognosis, non-seminomas are sensitive to platinum-based combination chemotherapy and are less susceptible to radiation, with the exception of teratomas. The different therapeutic outcome might be explained by inherent properties of the cells from which testicular neoplasia originate. The unique treatment sensitivity of TGCTs is unexplained so far, but it is likely to be related to intrinsic molecular characteristics of the PGCs/gonocytes, from which these tumors originate. Conversely, up to 30% of patients diagnosed with metastatic non-seminomas will not achieve a durable remission after initial treatment. Patients with an extragonadal seminoma have a long-term chance of cure similar to patients with a testicular primary tumor, but patients with a mediastinal non-seminoma show a significantly inferior outcome. Mature teratomas do not share the general chemosensitivity of TGCTs to cisplatin-based combination chemotherapy. The chemoresistance of mature teratomas compared with the other histological elements of TGCTs might be due to their intrinsic capacity to respond to DNA damage [11].

These data indicate that novel strategies are required for the treatment of testicular cancer in order to achieve a better control of the disease.

The review will focus on the molecular alterations identified in TGCTs and on novel targeted antineoplastic strategies that could contribute to the cure of chemotherapy resistant TGCTs.

2. THERAPY OF TESTICULAR GERM CELL TUMORS

Seminomas are radio- and chemo-sensitive tumors, virtually completely curable [12]. Non-seminomatous tumors are usually treated with surgery and chemotherapy, with different cure rates depending on the disease stage [13]. The cure rate reaches up to 99% in the early stages of non seminomatous tumors. In advanced disease, 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 [13,14] and metastatic disease can be treated only palliatively with modest results. The rapid growth and pro-

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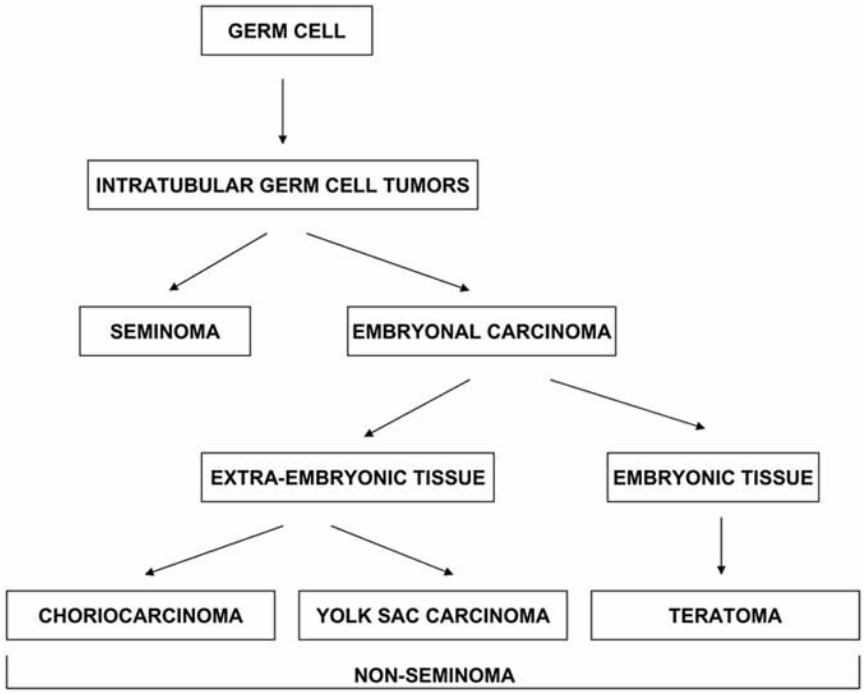

Fig. (1). A scheme illustrating current understanding of the pathogenesis of Testicular Germ Cell Tumors.

gression of TGCTs cause early lymph node metastases and/or distant metastases. At the time of diagnosis about 25% of seminoma patients and up to 60% of the non-seminoma patients suffers from metastatic disease [15-18]. 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 tumor relapse.

It is well known that most types of normal spermatogonia are sensitive to apoptotic stimuli being programmed cell death a costant feature of spermatogenesis. Moreover, spermatogonia are sensitive to DNA damaging agents such as cisplatin or radiations and the intrinsic susceptibility of germ cells to apoptosis plays a crucial role in the sensitivity of TGCTs to treatment with DNA damaging agents drugs [19]. Therefore, cisplatin, etoposide and bleomycin, are the main drugs used in testis cancer treatment [20], however, a significant minority of TGCTs is resistant to chemotherapy either at first presentation or as a relapse. It is conceivable that specific molecular characteristics of these tumors are responsible for the atypical clinical behaviour.

3. AURORA KINASES INHIBITORS

Errors in mitosis can provide a source of the genomic instability that is typically associated with tumorigenesis. Many mitotic regulators are aberrantly expressed in tumor cells. The kinases Aurora-A, B, and C represent a family of protein well conserved throughout eukaryotic evolution and members of this family have been extensively studied in a range of different model organisms [21-23]. All three mammalian members of this family are overexpressed in human cancer cells [9, 24-26]. Although the catalytic domains of the Auroras are highly conserved, these proteins show different subcellular localizations. Aurora-A (STK-15) localizes to the duplicated centrosomes and to the spindle poles in mitosis. It has been implicated in several processes required for building a bipolar spindle apparatus, including centrosome maturation and separation. Aurora A has been found to be overexpressed in the meiotic testicular cells [27]. It is interesting to note the aneuploidy of human testicular germ cell tumors is associated with amplification of centrosomes [27]. Aurora-B (AIM-1) is a chromosomal passenger protein. Aurora-B binds three other chromosome passenger proteins-inner centromere protein (INCENP), survivin and borealin [28,29]. During mitosis, Aurora-B is required for phosphorylation of histone H3 on serine 10, and this might be important for chromosome condensation [30]. Aurora-B clearly regulates kinetochore function, as it is required for correct chromosome alignment and segregation. Aurora-B is also required for spindle-checkpoint function and cytokinesis [31]. Aurora-A and Aurora-B are overexpressed in primary breast and colon tumor samples [32-34]. Aurora-A is localized (20q13) to an amplicon associated with poor prognosis in patients with breast and colon tumors [33]. Many studies have identified other tumor types, in which Aurora-A was amplified or overexpressed [35,36]. Aurora-C (STK-13) is also overexpressed in colorectal cancers [37].

The distribution and the expression of Aurora B were investigated in neoplasms derived from germ cells showing high percentage of Aurora B positive cells (51%) and the expression of Aurora B was significantly related to the MIB-1 proliferation marker [9]. These data demonstrate that the expression of Aurora B is a consistent feature of human seminomas and suggest that Aurora B is a potential target in the therapy of seminomas [9]. Three Aurora-kinase inhibitors have recently been described targeting the enzymatic activity of the Aurora kinase and in particular blocking Aurora B activity: ZM447439, Hesperadin 8 and VX-680

[38-40]. All these molecules act by inhibiting phosphorylation of histone H3 on serine 10 and consequently blocking cell division [30,31].

4. RECEPTOR AND NON-RECEPTOR TYROSINE KINASES INHIBITORS

Protein phosphorylation plays key roles in many physiological processes and is often deregulated in neoplastic lesions. Current understanding of how protein kinases and phosphatases orchestrate the phosphorylation changes that control cellular functions, has made these enzymes potential drug targets for the treatment of different types of cancer. Recently, receptor and non-receptor tyrosine kinases (TKs) have emerged as clinically useful drug target molecules for treating cancer [41].

Imatinib mesilate (STI-571) was primarily designed to inhibit bcr-abl TK activity and to treat chronic myeloid leukaemia [42,43]. STI-571 is also an inhibitor of c-kit receptor TK, and is currently the drug of choice for the therapy of metastatic gastrointestinal stromal tumors (GISTs), which frequently express constitutively activated forms of the c-kit-receptor [41].

Platelet Derived Growth Factor Receptor-α (PDGFR-α), and c-kit are expressed at high levels in TGCTs [44-47].

The c-kit/stem cell factor system is a signalling pathway for migration and survival of primordial germ cells [48]. C-Kit is a tyrosine kinase receptor for the stem cell factor, ligand binding leads to the c-Kit receptor heterodimerization and tyrosine kinase activity and the downstream signal involves both apoptosis and cell cycle progression [49]. Activating mutations of c-kit have recently been found in 93% of bilateral TGCTs, albeit in less of 2% of unilateral TGCTs [50]. These mutations affect codon 816 of *c-kit* gene resulting in a constitutional kinase active, in a manner similar to other receptorial tyrosine kinase activating mutations [50]. However, the mutation in exon 17 is not inhibited by the tyrosine kinase inhibitor imatinib mesylate [51,52].

The success of the tyrosine kinase inhibitors in the treatment of some cancers has invigorated the development of kinase inhibitors as anti-cancer drugs and a large number of these compounds are currently undergoing clinical trials and it is likely that molecules capable to inhibit exon 17*c-kit* activating mutations will be identified contributing to the development of molecular targeted therapies.

5. ANGIOGENESIS INHIBITORS

Tumors require access to blood vessels for the supply of oxygen and to maintain growth. The development and the growth of new vessels (angiogenesis) are essential for tumor growth and progression. Judah Folkman in the early 1970s proposed the inhibition of tumor blood vessel as a therapeutic approach for treating cancer patients [53].

The blood vessel growth in normal tissues is regulated through a balance between the action of pro-angiogenic factors, such as vascular endothelial growth factor (i.e.VEGF) [54,55] the action of angiogenic inhibitors (i.e. thrombospondin-1) [56,57].

In neoplastic lesions the angiogenic balance is shifted toward the pro-angiogenic factors, and irregular and uncoordinated tumor vessel growth is the result. VEGFR tyrosine kinase, p53, cyclooxygenase-2 (COX-2), and matrix metalloproteinases (MMPs) all directly and/or indirectly influence the pro angiogenic switch [56,57].

More than five inhibitors of the VEGF pathway have entered clinical phase I-III trials. Bevacizumab (Avastin (TM)), an antibody against VEGF, was shown to prolong survival in a phase III clinical trial in renal cell cancer and was efficient in two randomized clinical trials investigating the treatment of metastatic colorectal cancer [58,59].

ZD6474 is an orally bioavailable inhibitor of VEGF receptor-2 tyrosine kinase activity that in preclinical studies has been shown to inhibit both VEGF-induced signalling in endothelial cells and tumour-induced angiogenesis. ZD6474 produced significant broad-spectrum antitumour activity in a panel of human tumour xenografts [60,61]. The results obtained so far with inhibitors of angiogenesis suggest that these or novel molecules, currently in development could be useful for the treatment of chemoteraputic resistant TGCTs and to increase patients survival.

Members of the Akt/protein kinase B (PKB) family (Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ) regulate diverse cellular processes including apoptosis [62]. Akt proteins are activated by association through their pleckstrin homology (PH) domain with phosphoinositide second messengers of the phosphatidylinositol-3-kinase (PI-3-K), and by phosphorylation on residues threonine 308/309/305 and serine 473/474/472 (Akt1/2/3, respectively) [63].

The T-cell leukemia/lymphoma 1 (TCL1) protein is a novel Akt activator. TCL1 heterodimerizes with the PH domain of Akt [64-66]. The *TCL1* gene is constitutively activated by chromosome inversions and translocations in chronic and mature T-cell leukemias [67]. *TCL1* is a member of a multigene family that includes *TCL1b* and *MTCP1*. The region of the TCL1 protein required for interaction with Akt is highly conserved between TCL1 family members [68], and appears to be substantial redundancy in interactions between members of the TCL1 and Akt families [64,65,68]. TCL1 is expressed in normal and malignant lymphoid tissue at an early stage of differentiation, in the developing embryo, and in a high proportion of testicular seminomas of germ cell origin. Mouse embryos *lacking TCL1* gene do not progress beyond the four-to eight-cell stage, thus indicating a requirement for TCL1 in blastomere proliferation. The pattern of TCL1 expression, as well as its absence from hematopoietic stem cells, indicates its potential to serve as a highly specific drug target in malignancies of germ-cell origin.

6. GENE THERAPY

Gene therapy is a new approach to treat human diseases based on the transfer of genetic material to the cells. The transferred genetic material is commonly a gene or a chimaeric gene. To facilitate cell transduction, the genetic material is packaged into vectors, of viral or non-viral nature. Several approaches have been developed for transferring genes to human tissues. Plasmidic DNA can be transferred either directly, or attached to cell specific ligands, or embedded in lipidic formulations (liposomes) [69]. The trans-

gene(s) can be incorporated into defective viral particles to facilitate the entry into the cells. Viral vectors are, in fact, the most efficient vehicles for gene transfer. Different viruses have served to construct gene therapy vectors, including adenoviruses, [70], retroviruses (including lentivirus) [71], Adeno Associated Viruses (AAV), [72] and others. The list of viral vectors is still expanding and modifications of already existing systems will increase the number of potential applications of gene therapy. Different gene therapy based approaches have been tested to treat cancer including replacement of functional tumour suppressor genes, inhibition of oncogenes, transference to tumoral cells of genes conferring sensitisation to a specific prodrug ("suicide genes"), stimulation of antitumoral immunity, and inhibition of the formation of tumoral neovessels. TP53 is a tumour suppressor gene, which protein has a dual role in stress response. It trans-activates a number of genes including p21Waf1/Cip1 (p21), Mdm2, Bax, Fas and Apaf-1 [73] that co-ordinately direct cells into either cell cycle arrest or apoptosis.

Although p53 is mutated and therefore inactivated in more than 50% of human cancers, mutations of p53 have not frequently been identified in TGTCs [74] although several reports indicate that p53 protein is functionally inactive in murine teratocarcinoma cells [74,75]. In most TGCTs a lack p21 protein expression was observed [76,77] and in some studies mdm2 gene amplification was reported [78,79].

These observations support the hypothesis that the p53 pathway is functionally inactive in a percentage of TGTCs. Due to the importance of p53 in apoptosis induction, different strategies have been developed to exploit this function. Genetic reintroduction of wt-p53 into p53-deficient cancer cells leads to suppression of tumor growth, and synergistic effects with conventional chemotherapy have been demonstrated in several studies. However, non replicating viral vectors, mostly adenovirus, are not able to express the transgene for a long period of time and reach only a fraction of neoplastic cells. Hepatotoxicity associated with systemic application of adenoviral vectors is another drawback. Another intriguing adenoviral strategy was devised to specifically target p53-deficient tumor cells using replication compent oncolytic viruses, which represent a novel therapeutic approach. These viruses harness the ability of viruses to infect cells, multiply within them, and cause cell death, with released mature viral particles infecting neighbouring cells [80,81]. The first replication-competent adenoviral mutant described, *dl* 1520 (Onyx-015), contains a deletion of E1B-55K, which inhibits p53 and prevents apoptosis [82]. *dl* 1520 was expected to replicate selectively in a high percentage of human cancers being the p53 pathway non functional in about 50% of human neoplasia [83]. However, E1B-55K-mediates late-viral RNA transport, therefore the loss of E1B-55K restricts the viral replication to tumor cells capable of taking over the RNA export function of the viral gene product [84]. An antitumoral activity of *dl* 1520 has been demonstrated in several clinical trials and recent results from a phase III clinical trial have confirmed the ability of an oncolytic adenovirus (H101 bearing a E1B-55kDa gene deletion similar to that present in *dl* 1520) to increase the response rate of nasopharyngeal carcinoma in combination with cisplatin-based chemotherapy. However, objective responses with E1B mutant virus as a single agent are limited to date (tumor regression in only 15% of the cases) highlighting a need for oncolytic adenoviruses with higher replication efficiency within tumor cells [85]. This approach could be used to develop novel therapeutic strategies of TGTCs.

CONCLUSION

A deeper understanding of the molecular mechanisms underlying the development of TGCTs may provide new tools to specifically target neoplastic cells and could contribute to overcome acquired and intrinsic chemotherapy resistance. Promising molecules capable to selectively target neoplastic cells, i.e. the Aurora serine-threonine kinases, TKs, and proangiogenic factors inhibitors, already under clinical evaluation will open a new scenario for TGCTs treatment.

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