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Curing testicular cancer: Just a matter of breaks?

Almost all solid tumors in adults are incurable once they have spread beyond the primary site. Testicular germ cell tumors (TGCT) represent one of the few exceptions, in which more than 70% of patients with extensive metastatic disease can be cured with chemotherapy. The case of Lance Armstrong, who was diagnosed with an extensive metastatic testicular tumor, treated by surgery and chemotherapy and cured from his cancer, went on to win the Tour de France 7 times. This case clearly highlights one of the major successes of modern oncology, but also raises the question why such success stories are very common in TGCT but rarely seen in other solid tumors. In this essay we would like to address this question by proposing that TGCT cells show major differences in their response to DNA damage as compared to carcinomas. This inadequate DNA damage response (DDR) paired with a low threshold to undergo apoptosis could explain the high chemosensitivity and the success of systemic chemotherapy in TGCT.

Over the past four decades major advances have been made in our understanding of cancer cell biology and this has resulted in a wide range of anticancer drugs beginning with the development of antimetabolites such as methotrexat and nucleoside analogues, followed by DNA targeting drugs like cisplatin and more recently very rationally-designed therapeutic approaches that exploit specific vulnerabilities in the cancer cells such as mutated kinases [1]. Unfortunately, however, despite frequent initial therapy responses, curative treatment through systemic chemotherapy is rarely observed in the majority of metastatic tumors. TGCT are one exception to this dismal rule, since chemotherapy even in advanced stages of the disease is routinely curative.

Overall TGCT are relatively uncommon and make up only 1% of male malignancies. The disease mainly affects young men between 15 and 45 of age and represents the most common malignancy in this age group. Histologically, germ cell tumors can be divided into two major subgroups: pure seminomas and a collective group of non-seminomatous germ cell tumors that include embryonal carcinoma, yolk sac tumor and choriocarcinoma, among others [2]. Despite its rare occurrence, TGCTs have been recognized very early as a fruitful testing ground for novel chemotherapeutics [3], since (a) serum biomarkers, such as alphafetoprotein (AFP) and human chorionic gonadotropin (hCG), were available to monitor treatment response, (b) due to the younger age, patients would tolerate high dose chemotherapies better and (c) early clinical trials indicated very high chemosensitivitity of TGCT. Clinical reports from the 1930s [4] established the radio- and chemosensitivity of TGCT but also demonstrated that a combination of two or more drugs was by far more superior than monotherapy and complete remissions were at this time achieved in 10-20% of patients [5]. Recognizing the potential of a combination drug therapy together with the introduction of novel compounds Lawrence Einhorn started a seminal clinical trial at the Indiana University in 1974 in which he combined cis**p**latin, **v**inblastine and **b**leomycin (PVB) for the treatment of metastatic TGCT. The trial was a major success with 70% of patients showing a complete remission and with minor modification the PVB treatment schema soon became standard of care, changing the cure rates from 5% to more than 60% [3].

The clinical development for new therapeutic approaches for TGCT continued. For patients who initially failed PVB therapy another treatment combination involving **b**leomycin, **e**toposide and cis**p**latin (BEP) was introduced which, as a second line therapy, again dramatically increased the cure rates [6]. Thereafter numerous clinical trials have established the curability of TGCT and today the 10-year disease specific survival rates for patients with intermediate to poor prognosis are between 87% and 66%, respectively [2].

These high cure rates of metastatic TGCT are in strong contrast to what is observed in other solid tumors in which cures are rarely observed and the majority of patients succumb to the disease. This is certainly not due to the lack of clinical trials trying different mono- or combination therapies in other solid tumors, but more likely reflects a tumor cell intrinsic difference between

"common carcinoma" and TGCT, raising the question of the underlying molecular mechanism for this observation.

Interestingly, *in vitro* models of TGCT recapitulate the high chemo- and radiosensitivity of TGCT. It is worth noting, however, that TGCT cell line models only show a high sensitivity to drugs that directly target DNA by inducing double strand breaks (DSBs) or forming DNA adducts such as etoposide, mAMSA, bleomycin and cisplatin, but are significantly less sensitive to drugs that do not directly interfere with DNA integrity such as the spindle poisons colchicine or antimetabolites like methotrexate and 5-fluorouracil [7]. Together with the fact that TGCT are also exquisitely sensitive to ionizing radiation, which also primarily acts by inducing DNA damage, it appears that TGCT exhibit a low tolerance for any type of DNA injury. The observation that TGCT cells recapitulate the high sensitivity to DNA damaging agents *in vitro* furthermore argues for a tumor cell intrinsic vulnerability and not necessarily a tumor-host interaction that contributes to the high chemo- and radiosensitivity.

Overall TGCT cells appear to be 4 to 10 fold more sensitive to the above mentioned DNA damaging drugs as compared to cancer cells from the bladder or prostate [7,8]. Given the narrow therapeutic index of these drugs, even a modest difference in sensitivity can result in a major difference in clinical outcome. Following this logic, one can envision that the maximum tolerated dose of a therapeutic regime could eliminate 100% of tumor cells in a patient with TGCT but the same dose given to a patient with metastatic bladder cancer would only lead to significant reduction in tumor size but not to the complete elimination of all neoplastic cells. This incomplete eradication of tumor cells will inevitably result in disease recurrence – a scenario that is recapitulated in almost all current clinical trial for solid tumors.

DNA damage in the form of DNA adducts, single strand breaks, base oxidation but also DSBs represent a major challenge for a cell. Mammalian cells have therefore developed highly sophisticated recognition and repair systems for such lesions. Interestingly, TGCT harbor multiple alterations in different DNA repair pathways: nucleotide excision repair (NER) for instance plays a major role in removing DNA damaged in the form of inter- and intrastrand adducts – a characteristic DNA lesion induced by cisplatin. Some of the major components of NER such as XPA, ERCC1 and XPF appear to be expressed at low levels in TGCT cell lines [9,10]. It is therefore possible that defects in this specific DNA repair pathway can contribute to the increased sensitivity of TGCT to cisplatin.

TGCT are highly sensitive to drugs like etoposide, mAMSA and doxorubicine that directly or indirectly interfere with the function of class 2 topoisomerases. Topoisomerases are an interesting class of enzymes that can solve topological constraints in the DNA molecule by inducing transient DNA breaks. In the case of class 2 topoisomerases, the enzyme gets covalently bound to the DNA during DSB induction via a 5'-phosphotyrosyl bond. If the catalytic cycle of the enzyme is disrupted for instance by etoposide, reactive oxygen species (ROS) or other agents, the enzyme gets covalently trapped on the DNA. The repair of the resulting DNA lesion requires the removal of the covalently attached topoisomerase. Recently, an enzyme (TDP2/TTRAP) with 5'-tyrosyl DNA phosphodiesterase (5'-TDP) activity was described that can cleave 5'-phosphotyrosyl bonds [11]. TDP2/TTRAP appears to be the major if not the only enzyme with 5'-TDP activity present in mammalian cells. Catalytic activity of this enzyme is important to repair TOP2 induced DSB since cells lacking TDP2 are highly sensitive to etoposide [12]. *In silico* expression analysis revealed, that TDP2 is expressed at very low levels in testis and TGCT which could further explain the high sensitivity of testicular cells to a great variety of agents that target topoisomerases.

DNA DSBs can be induced by a variety of chemotherapeutics but also ionizing radiation. DSB are recognized by a protein complex and eventually lead to the activation of the kinase ATM. ATM is a sensor kinase that plays a key role in the cellular response to DSBs. It is therefore possible to monitor DSB response *in situ* in tissue sections using antibodies that recognize the phosphorylated and therefore active form of kinases of ATM itself or substrates of ATM such as the phosphorylated form of the histone variant H2Ax (γH2Ax). Using these robust markers for DNA double strand break response (DDR) several seminal papers demonstrated that almost all invasive tumors and tumor precursor lesions show activation of DDR [13-15]. TGCT as well as intratubular germ cell neoplasia, unclassified type (IGCNU, the precursor lesion to invasive germ cell tumors in the testis) however show no signs of DSB response (Figure 1, and [15]). To highlight this striking finding we coimmunolabeled whole sections of formalin-fixed paraffin embedded tissue from normal testis, IGCNU, embryonal carcinoma and seminoma with antibodies to γH2Ax as well as OCT4, a specific marker for seminomatous/(dys)germinomatous tumours [16]. Normal testis showed γH2Ax foci formation in maturating spermatides, likely reflecting DNA double strand breaks that are induced during chromatin compaction (Figure 1A) [17]. Preinvasive IGCNUs showed positive staining for OCT4 but no γH2Ax was detectable (Figure 1B). Furthermore, invasive embryonal carcinoma as well as seminoma also showed no γH2Ax foci (Figure 1C,D),

suggesting that DSB repair response is not active in these lesions.

What could explain these differences in DDR between most carcinomas and TGCT? To address this question we would have to consider why solid tumors that are not challenged with any genotoxic stress would show a DDR response. Bartek et al. proposed a very elegant model in which activation of oncogenes leads to a dysregulation of cell cycle check points which then in turn results in replication stress [13,14]. This DNA replication stress is likely the consequence of a depletion of deoxynucleotides and leads to the collapse of the replication forks resulting in DSBs and DDR [18]. The presence of DNA replication stress can be recapitulated in simple model systems in which the introduction of a single oncogene in an untransformed cells can lead to a depletion of the nucleotide pool and the induction of DSBs [18].

Oncogene induced DNA damage appears to represent a natural barrier for tumor progression since an activation of the DDR can induce cellular senesce and apoptosis. Only if the cancer cell can overcome this barrier for instance by mutating master regulators of this response like *TP53,* further neoplastic growth is possible [19]. Interestingly, the tumor suppressor gene *RB* is silenced in a large number of IGCNUs and most seminomas and embryonal carcinomas [20]. *RB* loss on the other hand is associated with DNA replication stress and DDR [18]. The majority of TCGT should therefore experience oncogene induced DNA damage with DSB formation, however no evidence of a canonical DDR involving activation of ATM and phosphorylation of H2Ax is detectable. It is therefore likely that the DNA damage sensing mechanism is corrupted in TGCT.

The lack of this canonical DDR that usually represents a barrier for tumor progression could also explain the low frequency of mutations in *TP53* and other repair associated genes observed in TGCT [21]. p53 response in turn is highly active in TGCT cells [22,23]. This p53 hypersensitivity appears to be independent of active DNA damage signals, since depletion of ATM and ATR and DNA-PK does not prevent p53 accumulation suggesting that the p53 dependent apoptosis observed in TGCT does not require activation of canonical DDR [22].

TGCT harbor major defect in DNA damage sensing and response but show an active p53 mediated apoptosis program (summarized in Figure 2). This generates an interesting scenario in which TGCT cells cannot repair DNA damage due to repair defects on multiple levels but show hyperactive apoptosis. These features appear to be intrinsic to testicular germ cell tumor precursor lesions and invasive tumors and are likely reflecting a linage specific program. There is evidence that TGCT and IGCNUs originate from primordial germ cells and gonocytes [24]. It is worth noting that spermatogenesis, the physiological maturation and differentiation from a primordial germ cell to a mature sperm is characterized by a high frequency of apoptosis [25]. In the primordial germ cell niche, cells that are unfit due to genomic injuries are eliminated by apoptosis rather than subjected to extensive DNA repair.

Taken together TGCTs appear to harbor multiple deficiencies in detecting and repairing DNA injuries as compared to other cancer types. These differences in DDR together with an intrinsic proapoptotic behavior could likely be responsible for the high chemo- and radiosensitivity observed in these TGCT.

The question remains, how these potential vulnerabilities in DDR can be exploited for the treatment of other, more therapy refractory tumors? Since most other solid tumors appear to have a functional and active DNA repair system, which reduces chemosensitivty, a targeted inhibition of key players in this system could theoretically result in chemosensitization and eventually elimination of the tumor. PARP1, a protein involved in base excision repair as wells as DSB repair, has therefore been in the focus of drug development and several highly specific inhibitors were recently described [26] with the idea that PARP inhibition in combination with DNA damaging agents could increase cytotoxicity. Indeed, in several preclinical models PARP inhibition together with cisplatin, carboplatin, and cyclophosphamide, caused regression of established tumors, whereas comparable doses of cytotoxic agents alone only exhibit modest growth inhibition [27]. In early clinical studies a combination of PARP inhibition together with the alkylating chemotherapeutic temozolomide was well tolerated and exhibited clinical activity in patients with metastatic cancers [28-30]. However, the combination of a systemic inhibition of DNA repair coupled with conventional systemic chemotherapy is associated with a high risk of systemic toxicity limiting dose escalation and potential wide spread clinical use.

Very elegant preclinical studies revealed that cancer cells harboring mutations in *BRCA1* and *BRCA2* are highly sensitive to PARP inhibition. These findings suggest that the complex DNA repair system represents a druggable vulnerability in cancer cells [31]. The demonstration of single-agent antitumor activity of PARP inhibitors in BRCA1 and BRCA2 mutation carriers with advanced cancers provides strong evidence for the clinical application of this approach [32,33]. Furthermore, the tumor specific delivery of shRNAs targeting key component of the DNA repair cascade such as DNA-PK represents a highly interesting approach to increase radio- and chemosensitivity in cancer cells. Indeed, in an elegant proof of principle study Ni et al.

demonstrated the feasibility of this approach in a murine xenograft model, which might be readily translatable in clinical trials [35].

These results illustrate how different pathways cooperate to repair DNA damage, and suggest that the targeted inhibition of particular DNA repair pathways may allow the design of specific and less toxic therapies for cancer. With multiple novel, highly potent inhibitory approaches for different DNA repair enzymes available, it might just be a matter of time until cancer type specific vulnerabilities in DDR are translated into clinical success stories.

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