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WJSC covers topics concerning all aspects of stem cells: embryonic, neural, hematopoietic, mesenchymal, tissue-specific, and cancer stem cells; the stem cell niche, stem cell genomics and proteomics, and stem cell techniques and their application in clinical trials.

We encourage authors to submit their manuscripts to *WJSC*. We will give priority to manuscripts that are supported by major national and international foundations and those that are of great basic and clinical significance.

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REVIEW

Therapy-related myeloid neoplasms - what have we learned so far?

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Abstract

Therapy-related myeloid neoplasms are neoplastic processes arising as a result of chemotherapy, radiation therapy, or a combination of these modalities given for a primary condition. The disease biology varies based on the etiology and treatment modalities patients receive for their primary condition. Topoisomerase Ⅱ inhibitor therapy results in balanced translocations. Alkylating agents, characteristically, give rise to more complex karyotypes and mutations in p53. Other etiologies include radiation therapy, high-dose chemotherapy with autologous stem cell transplantation and telomere dysfunction. Poor-risk cytogenetic abnormalities are more prevalent than they are in *de novo* leukemias and the prognosis of these patients is uniformly dismal. Outcome varies according to cytogenetic risk group. Treatment recommendations should be based on performance status and karyotype. An in-depth understanding of risk factors that lead to the development of therapy-related myeloid neoplasms would help developing risk-adapted treatment protocols and monitoring patients after treatment for the primary condition, translating into reduced incidence, early detection and timely treatment.

Key words: Therapy-related acute myeloid leukemia; Therapy-related myelodysplastic syndromes; Ionizing radiation; Alkylating agents; Allogeneic hematopoietic stem cell transplantation; Topoisomerase Ⅱ inhibitors; Therapy-related myeloid neoplasms

Core tip: Therapy-related myeloid neoplasms are becom-

ing an increasing problem as the survival of cancer patients lengthens. The etiology has an important influence on the biological characteristics, time to onset and prognosis of the resultant disease. Although treatment of therapy-related myeloid neoplasms represents a substantial challenge due to prior treatment and comorbidities, cure is possible, especially with allogeneic stem cell transplantation, particularly in those with good-risk karyotype. Ultimately, individual assessment of risk factors may lead to developing risk-adapted therapies to reduce the incidence of this serious complication without affecting therapy for the underlying disorders.

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INTRODUCTION AND EPIDEMIOLOGY

Therapy-related myeloid neoplasms, which include both therapy-related myelodysplastic syndromes (t-MDS) and therapy-related acute myeloid leukemia (t-AML), are well-known sequelae of conventional anticancer chemotherapy and radiotherapy for solid tumors, such as ovarian cancer[1], breast cancer[2], testicular cancer[3] and various sarcomas[4], as well as hematologic malignancies[5-7]. Therapy-related myeloid neoplasms constitute approximately 10%-20% of all cases of AML and MDS[8], with incidence varying depending upon the underlying malignancy, type of cytotoxic agents and/or radiotherapy, and timing of administration and dosage of treatment modalities[9]. Therapy-related myeloid neoplasms can present at any age, but the median age at diagnosis is reported to be approximately 61 years in adults[10,11].

After conventional-dose anticancer chemoradiotherapy, the incidence of t-MDS/AML has been reported between 0.8%-6.3% at 20 years post-treatment, with a median time of 3-5 years from treatment to development of t-MDS/AML[12]. In contrast, the incidence of t-MDS/AML after high-dose chemotherapy and autologous hematopoietic stem cell transplant (auto-HSCT) ranges from 1.1%-24.3% at 5 years post-transplant with a median time to development of only 1-2 years post-transplant[12-16]. Use of etoposide (a topoisomerase Ⅱ inhibitor) priming for stem-cell mobilization and total-body-irradiation (TBI) based conditioning regimens are particularly associated with t-MDS/AML after auto-HSCT[16,17].

According to the World Health Organization classification, therapy-related myeloid neoplasms are broadly categorized into two subtypes: (1) an alkylating agent/radiotherapy-related type; and (2) a topoisomerase Ⅱ inhibitor-related type[18]. The development of t-MDS/AML after alkylating agents/radiotherapy usually occurs after a median latency of 4-7 years, with two-thirds of patients presenting with MDS and one-third presenting with AML[12,19]. There is prominence of peripheral cytopenias and dysplasia of multiple myeloid lineages with frequently observed abnormalities of chromosome 5 [-5/del(5q)] and chromosome 7 [-7/del(7q)][19,20]. Conversely, topoisomerase Ⅱ inhibitor-related t-MDS/AML has a relatively shorter latency between exposure to drugs and onset (median of 2-3 years)[21]. Patients with this subtype often present with overt AML without features of preceding MDS. AML in this subtype shows monocytic predominance[21,22] with a high incidence of balanced translocations involving chromosomal segments 11q23, 17q21 and/or 21q22[21]. While the risk of developing t-MDS/AML after alkylating agents/radiotherapy rises with increasing age, the risk of the same after topoisomerase Ⅱ inhibitors appears to remain constant across all age groups[18,23].

LEUKEMOGENESIS

Therapy-related myeloid neoplasms are clonal hematopoietic stem cell disorders that arise due to iatrogenic somatic mutations after treatment with cytotoxic chemotherapy/radiotherapy. These somatic mutations impart increased proliferative capacity and survival advantage in the affected hematopoietic progenitors[12].

Alkylating agents have established significant clinical applications in virtually all cancer types and were the first chemotherapeutic drugs to be associated with therapy-related myeloid neoplasms[24]. These drugs work by transferring alkyl groups to oxygen and nitrogen atoms on DNA bases, resulting in the formation of highly mutagenic DNA base lesions (such as O6-methylguanine and N3-methylcytosine) and inducing DNA damage[25]. Alkylated DNA-based lesions, specifically O6-methylguanine, cause mispairing during DNA replication, and while this replication error is efficiently repaired by mismatch-repair enzymes, alkylated bases cannot be cleaved by mismatch-repair enzymes, leading to mutagenicity, secondary DNA double-stranded breaks and eventual cytotoxicity[26,27]. Mono-functional alkylating agents, such as nitrosoureas, dacarbazine and temozolomide, have one active moiety and are able to induce such lesions. In contrast, bi-functional alkylators, such as cyclophosphamide, melphalan and chlorambucil, have two active moieties and are able to form crosslinks within and between DNA strands in addition to forming alkylated base lesions[28]. Inter-strand DNA crosslinks halt replication forks during DNA replication, resulting in the formation of double-stranded DNA breaks. These breaks can give rise to chromosomal translocations, insertions, inversions and loss-of-heterozygosity involving several vital cellular genes[29,30].

Drugs targeting DNA topoisomerases are also well-known to cause t-MDS/AML[31]. DNA topoisomerase enzymes mediate the unknotting and relaxing of DNA supercoils, thereby allowing DNA replication to occur. These enzymes accomplish this by creating transient single-stranded (DNA topoisomerase Ⅰ) and double-

stranded (DNA topoisomerase Ⅱ) DNA breaks. The release of topoisomerases from the DNA strands is followed by the re-ligating of these transient DNA breaks[32]. Topoisomerase Ⅱ inhibitors, such as epipodophyllotoxins (etoposide and teniposide) and anthracyclines (daunorubicin, doxorubicin, *etc*.) prevent the release of topoisomerase Ⅱ from cleaved DNA, preventing the re-ligation of strands and persistence of double-stranded breaks[26]. These DNA breaks are highly mutagenic and frequently result in translocations involving the genes *MLL* at 11q23, *RUNX1* at 21q22 and *RARA* at 17q21[33-35].

The substantial incidence of various leukemias and myeloid disorders in the survivors of the Hiroshima and Nagasaki nuclear attacks has established a firm causal relationship between ionizing radiation and hematologic malignancies[36-38]. Epidemiological data from several studies involving individuals receiving therapeutic radiation has corroborated its leukemogenicity[3,39-41]. Cellular exposure to ionizing radiations has multiple mechanisms of causing DNA damage and mutations. Energy in each individual photon of radiation is able to disrupt the sugar-phosphate backbone of the DNA molecule, leading to single- and double-strand breaks[28]. In addition to this direct effect, cellular exposure to ionizing radiations results in radiolysis of water molecules leading to the formation of reactive oxygen species (most notably hydrogen peroxide, superoxide and hydroxyl radicals)[42]. These highly reactive molecules are capable of oxidizing and deaminating DNA bases and disruption of the sugar-phosphate backbone. As discussed with alkylating agents and topoisomerase Ⅱ inhibitors earlier in this section, double-stranded breaks are highly mutagenic and contribute to leukemogenesis in therapy-related myeloid neoplasms.

In the context of auto-HSCT, DNA damage is multifactorial, arising as a result of treatment with cytotoxic agents used in induction therapy prior to auto-HSCT, possibly from the transplant process itself (stem cell mobilization, stem cell collection and storage) and from the stress of engraftment and hematopoietic recovery during the post-transplant period[43-46], apart from the chemotherapy agents and TBI used in the conditioning regimen. It is probable that some progenitor cells persist within the patients despite pre-transplant conditioning and acquire mutations overtime, for example from injury caused by the conditioning regimen, leading to t-MDS/AML after auto-HSCT[16]. To scientifically ascertain this hypothesis, future studies may focus on genetically marking the autograft and performing assays of t-MDS/AML clones in patients who develop this complication post-transplant to ascertain whether progenitor cells persisting in the patient after pre-transplant conditioning give rise to t-MDS/AML or is it the rescuing hematopoietic progenitors that give rise to t-MDS/AML. Currently, the ongoing Center for International Blood and Marrow Transplant Research study LE14-01 is the largest retrospective study to date (to the best of our knowledge) on t-MDS/AML after auto-HSCT[47]. The results of this study may provide deeper insight into t-MDS/AML in patients receiving auto-HSCT.

The *p53* gene plays a crucial role in DNA damage response pathways, DNA repair mechanisms, cell cycle control and apoptosis. Abnormalities affecting *p53* hinder the cell's ability to repair damaged DNA and results in genomic instability and accumulation of various genetic lesions that contribute to leukemogenesis[12]. It is noteworthy that less than 10% of patients with *de novo* MDS and AML harbor *p53* mutations, whereas 27%-50% of patients with t-MDS/AML demonstrate *p53* mutations[48-50]. These are non-germline mutations that are often seen as a late adverse effect of therapy with alkylating agents and often occur simultaneously with chromosome 5 [-5/del(5q)] and chromosome 7 [-7/del(7q)] losses[12,50].

Telomeres are repeat sequences of non-coding DNA that flank the 3' ends of linear chromosomes, permitting the replication of 3' chromosomal ends and are vital for preventing dicentric fusion and chromosomal abnormalities[51]. Each mitotic division results in fractional loss of telomeric DNA, with cumulative telomeric loss leading to cellular senescence, a process by which normal cells lose their ability to divide after a specific number of cell divisions. In addition, loss of telomeric DNA also leads to genomic instability and somatic mutations[52,53]. Exposure to chemotherapeutic agents places proliferative stress on the bone marrow to allow for hematopoietic recovery after/in between cycles of chemotherapy[54]. The increased proliferative rates accelerate the loss of telomeric DNA, which would otherwise be conserved by the telomerase enzyme under physiologic conditions[52]. It is evident that telomere shortening is associated with the development of myeloid malignancies, such as MDS and AML, in both *de novo*[55] and therapy-related settings[43,56,57]. The nested case-control study by Chakraborty *et al*[57] showed that after auto-HSCT, those patients who developed t-MDS/AML showed a substantial increase in the rate of telomeric shortening after day +100 in comparison to the control group who did not develop t-MDS/AML. Other studies[43,56] also demonstrated similar observations. These findings corroborate that increased telomeric loss and telomere dysfunction contributes to leukemogenesis and likely precedes the development of t-MDS/AML in premalignant cells.

TREATMENT AND OUTCOMES

Conventional chemotherapy

Intensive chemotherapy is one of the established therapeutic approaches to t-MDS/AML and its role has been investigated in earlier studies. In a retrospective study of 122 patients with t-MDS/AML at the MD Anderson Cancer Center, intensive chemotherapy with cytarabine yielded a complete remission (CR) rate of 37%[58]. In the same study, pooled data of 496 patients from 13 different studies revealed a cumulative CR rate of 27%[58]. No doubt, CRs have been achieved in this and other early studies on t-MDS/AML, but these rates are lower and short-lived in comparison to *de novo* MDS/

AML[11,59,60]. The fatal course of t-MDS/AML is due to profound and persistent cytopenias due to ineffective hematopoiesis regardless of the fraction of immature blasts accumulating in the bone marrow[61]. In contrast, a subsequent study reported a surprisingly high CR rate of 82% for t-MDS/AML treated with high-dose cytarabine + mitoxantrone[62].

For therapy-related acute promyelocytic leukemia (t-APL) and t-AML with good-risk cytogenetics, specifically inv(16) and t(8;21), induction chemotherapy is recommended, similar to the treatment guidelines for their *de novo* counterparts[28]. For t-APL, outcomes are encouraging with regimens containing all-trans retinoic acid, as evidenced by two large European studies[63,64]. One study reported a CR rate of 87%[64]. The other study reported a CR rate of 80% with actuarial survival of 59% at 8 years[63]. Since outcomes with non-transplant strategies are encouraging in t-APL, this allows patients to be spared from the toxicities associated with allogeneic hematopoietic stem cell transplant (allo-HSCT). However, recent evidence does not favor the same recommendations for t-AML with inv(16) and t(8;21) as these patients have shown shorter event-free and overall survival in comparison to patients with *de novo* AML exhibiting inv(16) and t(8;21)[65-67]. This suggests that these patients may also require allo-HSCT for a durable cure, as is the case with t-MDS/AML with intermediate- and poor-risk cytogenetics[12,61,68]. The general conclusion drawn from literature on the subject is that outcomes of t-MDS/AML treated with conventional chemotherapy are generally poor, with median survival as low as only 6 mo[12].

Role of hypomethylating agents in therapy-related myeloid neoplasms

With suboptimal survival rates for t-MDS/AML after allo-HSCT and even lower with conventional chemotherapy, exploration of alternative treatments and novel therapies is highly warranted to improve survival in this subset of patients. Azacitidine has shown promising efficacy in the treatment of high-risk MDS and AML[69,70] with a limited side effect profile and impressive tolerability, especially in patients with poor performance status and comorbidities[71]. Several recent retrospective studies suggested notable activity of azacitidine against t-MDS/AML, with overall response rates ranging from 39%-43% and median overall survival from 14.5-21 mo[72-74]. Azacitidine yielded the most benefit and better overall survival when used as first-line therapy[74] and detailed analysis of these studies showed similar outcomes between patients with *de novo* MDS/AML and those with t-MDS/AML[72,73]. A recent retrospective account of patients treated with azacitidine at the Memorial Sloan-Kettering Cancer Center and patients treated with decitabine in two industry-sponsored clinical trials (D0007[75] and DACO-020[76]) was published by Klimek *et al*[77]. In a cohort of 42 patients with t-MDS, this account reported an overall response rate (CR + marrow CR + hematologic response) of 38%[77]. However, a multi-center retrospective case series published in 2015 reported relatively inferior outcomes compared to the aforementioned studies (overall survival: 9.6 mo; overall response rate: 35.7%)[78].

Prebet *et al*[79] recently reported results of the E1905 study, a phase Ⅱ randomized trial comparing the effects of combination therapy with azacitidine and the histone deacetylase inhibitor, entinostat, against monotherapy with azacitidine. The results showed lower hematologic normalization rates (17% *vs* 46% in the monotherapy arm), shorter overall survival (6 mo *vs* 13 mo in the monotherapy arm) and increased toxicity in the combination arm, recommending against the use of the azacitidine + entinostat combination for t-MDS/AML[79]. A predecessor of the same study demonstrated pharmacologic antagonism of entinostat when added to azacitidine[80]. However, the same study showed that prolonged administration of azacitidine alone increased the rate of hematologic responses when compared to standard dosing, representing an area of future research interest[80].

Allogeneic hematopoietic stem cell transplant

The standard approach for most patients with t-MDS/AML is allo-HSCT, which has consistently been shown to be a potential curative option for t-MDS/AML[12,61,68]. Outcomes of patients with t-MDS/AML after allo-HSCT, albeit limited and mostly based on retrospective studies, are still uniformly poor due to the high-intensity and transplant-related complications associated with the procedure and the refractory nature of the disease. For example, an account of 13 patients receiving allo-HSCT for t-MDS/AML after auto-HSCT reported that all patients died of either transplant-related complications (11 patients) or relapse (2 patients) with a median overall survival of only 1.8 mo[81]. One study reporting outcomes of 461 patients estimated a 35% overall survival 3 years after allo-HSCT[82]. Another large study involving 306 patients reported a median survival of only 8-10 mo and a 5 year overall survival of less than 10%[35]. Other studies have also reported poor outcomes[68,83-86], with non-relapse mortality ranging between 54%-58%[86-88]. Since most clinical trials in the AML or MDS arena have usually excluded t-AML/MDS, to our knowledge, prospective phase Ⅲ randomized data evaluating the role of allo-HSCT in t-MDS/AML is lacking.

Some studies have described notable influences of conditioning regimens on survival rates. In a large study by Witherspoon *et al*[88], the 5-year disease-free survival for patients receiving conditioning with busulfan (BU) targeted to 600-900 ng/mL steady-state plasma concentration with cyclophosphamide (CY) [(t-BU/CY)] was 30%, the highest in the patient cohort. Survival rates were significantly lower for other regimens (standard BU/CY: 19%; chemotherapy/TBI: 8%) in comparison to t-BU/CY (P = 0.006). In the same report, the 5-year cumulative non-relapse mortality was lowest for t-BU/CY (42%) *vs* that for standard BU/CY and chemotherapy/

TBI regimens (52% and 58%, respectively); (P = 0.02)[88]. Subsequently, an even larger study (including 251 patients) also showed a greater 5-year disease-free survival for patients conditioned with t-BU/CY (BU targeted to 800-900 ng/mL steady-state plasma concentration) of 43% *vs* that for standard BU/CY, fludarabine (Flu)/BU, Flu/TBI and high-dose TBI/CY (28%, 24%, 23%, 18%, respectively); (P = 0.001)[87]. This study also showed the lowest 5-year cumulative non-relapse mortality for the t-BU/CY regimen (28%) *vs* high-dose TBI/CY, Flu/TBI and standard BU/CY (53%, 54% and 61%, respectively); (P < 0.001)[87].

Factors affecting outcomes

The dismal outlook of these patients is likely multifactorial, resulting from relapse-related and/or non-relapse-related mortality. The likelihood of relapse significantly correlates with disease stage. For example, a report from the Fred Hutchinson Cancer Research Center showed varying rates of relapse among their patient cohort (no relapses in the refractory anemia/refractory anemia with ringed sideroblasts group; 22% relapse in the refractory anemia with excess blasts group; and 36% relapse in the refractory anemia with excess blasts in transformation/AML group)[85]. Another study reported similar findings[88]. Likewise, disease karyotype also correlates with relapse rate. The impact of karyotype on outcomes in both *de novo* and t-MDS/AML were compared in large prospective studies which showed disease karyotype to be an independent prognostic factor in both groups, with poor-risk cytogenetic abnormalities more common in the t-MDS/AML group[84,89]. An optimized, 3-group cytogenetic classification proposed by Armand *et al*[90] was found to be the strongest predictor of overall survival in t-MDS/AML by its impact on relapse risk after allo-HSCT. Through this classification, cytogenetic abnormalities in these patients were divided into good-risk [normal, -5, (del)20q or -Y], poor-risk (chromosome 7 abnormalities, complex karyotype) and intermediate-risk (all others)[90]. Also, relapses are less likely with unrelated donor transplants, likely due to a more potent graft *vs* leukemia effect[12,91] and lower peripheral blood blast count (correlating with early-stage disease and low disease burden)[92].

Other outcome parameters after allo-HSCT have been scrutinized. Patient performance status strongly influences survival[79]. Treatment for the primary malignancy causes injury to various organ systems and depletion of normal hematopoietic progenitors, diminishing the patients' ability to withstand the intensive nature and toxicities associated with allo-HSCT. In addition, damage to bone marrow stromal elements from prior therapy (especially radiotherapy) alters the bone marrow microenvironment, making hematopoietic regeneration more difficult[61]. Younger patients (children, adolescents, young adults) have a better bone marrow reserve and better ability to withstand the toxicities associated with multiple treatments (both for the primary disease and allo-HSCT)[4], hence it would be expected that survival is better in this group in contrast to elderly. Since therapy-related myeloid neoplasms are relatively uncommon in young age groups[8,9], there is paucity of literature concerning the prognostic factors and survival in younger patients. This is a potential area of research interest. Future studies are warranted to ascertain if different prognostic factors confer survival advantage in younger patients with therapy-related myeloid neoplasms, or if the dismal outcomes in elderly are just a result of sheer fact of age.

Patients are also immunocompromised from prior treatment regimens and hence often acquire life-threatening infections, a well-known and feared cause of mortality after allo-HSCT. Additionally, relapse of the primary malignancy, especially metastatic cancer or disseminated lymphoma, carries its own risks of morbidity and mortality[61]. Also, the timing of allo-HSCT affects the outlook of patients, as a recent study demonstrated that those who received allo-HSCT later than 6 mo after diagnosis have inferior survival rates[93]. Thus it is imperative to refer a newly diagnosed case of t-MDS/AML to a transplant center early.

In addition to disease stage and karyotype, somatic mutations of specific genes may also have implications on prognostication. For example, frame-shift mutations of the nucleophosmin gene, internal tandem duplications of the fms-like tyrosine kinase 3 gene and double mutations in the *CEBPA* gene are now routinely assessed in the workup of AML patients and incorporated into therapeutic algorithms[94]. They have also been observed in t-MDS/AML[95,96]. While these (and perhaps other specific gene mutations) may have impact on t-MDS/AML prognosis, these mutations usually occur and have prognostic value in cases with normal cytogenetics[94], a karyotype which is relatively rare in t-MDS/AML, making their prognostic utility uncertain in cases of t-MDS/AML.

When taking only t-MDS into account, the International Prognostic Scoring System, a cornerstone in the prognostication of patients with MDS, has shown unsatisfactory ability to predict the outcome of patients after treatment[81]. Instead, an alternative prediction model utilizes the following four factors to gauge survival for patients with t-MDS and t-AML after allo-HSCT: (1) age greater than 35 years; (2) poor-risk cytogenetics; (3) advanced-stage t-MDS or t-AML not in CR after allo-HSCT; and (4) donor other than an HLA-identical sibling or a matched or partially-matched unrelated donor[68]. Five-year overall survival varies with the number of these factors present: None (50%), 1 (26%), 2 (21%), 3 (10%) and 4 (4%)[68]. Male sex has also been indicative of poor outcomes[86]. A proposed algorithmic approach to patients with therapy-related myeloid neoplasms is elaborated in Figure 1.

GAUGING THE RISK OF THERAPY-RELATED MYELOID NEOPLASMS

Keeping in mind the poor outcomes of t-MDS/AML, mea-

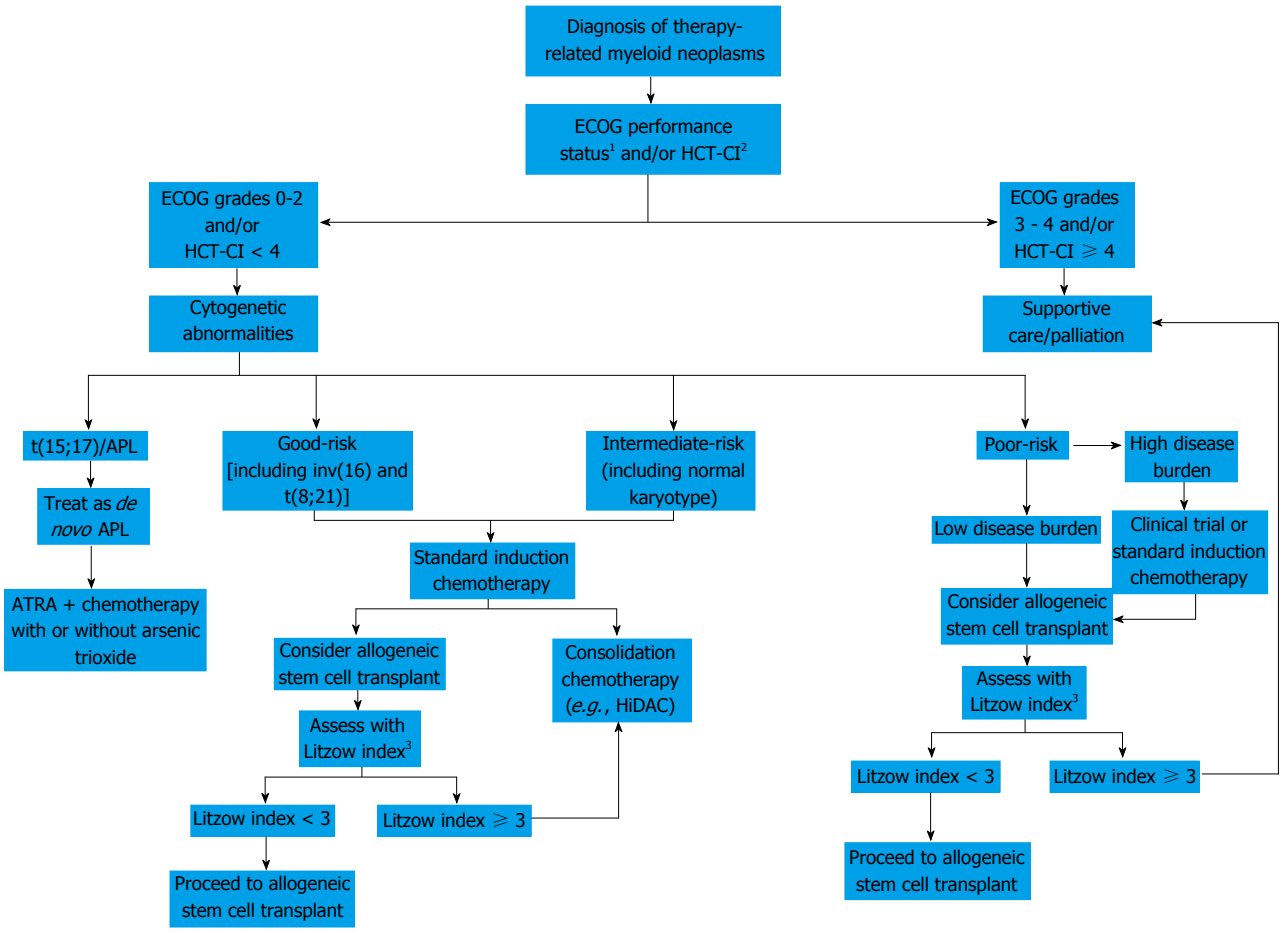

Figure 1 Algorithmic approach to patients with therapy-related myeloid neoplasms. [1]Oken *et al*[109]; [2]Sorror[110]; [3]Litzow *et al*[68]. ECOG: Eastern Cooperative Oncology Group; APL: Acute promyelocytic leukemia; ATRA: All-trans retinoic acid; HiDAC: High-dose cytarabine; HCT-CI: Hematopoietic cell transplant-co-morbidity index.

sures for early detection of this disorder would allow for timely and pre-emptive treatment approaches, such as reduced intensity conditioning allo-HSCT. This approach would yield substantial advantages as opposed to waiting for the development of overt t-MDS/AML, when disease burden is higher and requires more intensive therapy which can have its own risks of morbidity and mortality[28]. In this section we will outline some methods for prediction and/or early detection of t-MDS/AML in patients at risk.

Metaphase cytogenetics and karyotyping analyze actively dividing cells, though the number of cells analyzed is limited (20-30 cells)[44]. It is worthy of note that patients developing t-MDS/AML, for example after auto-HSCT, may not show karyotypic abnormalities before the procedure. Conventional cytogenetics may lack sufficient sensitivity and specificity to efficiently recognize patients with increased predisposition to t-MDS/AML[16,44].

Interphase fluorescence *in situ* hybridization (FISH) offers several advantages over conventional cytogenetics, mainly the lack of need for cells to be actively dividing and the ability to analyze a greater number of cells (several hundreds)[44]. FISH is also able to detect abnormal clones prior to auto-HSCT. For example, in one report, FISH was able to detect clonal abnormalities in 9 out of 12 patients (75%) who later developed t-MDS/AML after auto-HSCT[97]. In another study, FISH identified abnormal cell clones in 20 out of 20 patients who went on to develop t-MDS/AML[98]. Identification of clonal abnormalities in a high percentage of cells may indicate proliferative and survival advantages and foreshadows development of t-MDS/AML[44]. However, the locus specificity of FISH requires prior selection of multiple markers for adequate analysis and its labor- and time-intensive methodology are notable limitations[44].

Loss of heterozygosity (LOH) employs a polymerase chain reaction (PCR) analysis of a selected sample to detect loss of one allele at a specific locus and large chromosomal deletions. This technique is also labor- and time-intensive and is a population-based assay that requires prior selection of loci to be analyzed. In addition, its sensitivity is poor, unable to detect less than 20% cells for LOH of a selected locus[44]. Nevertheless, it may have impressive specificity, as a positive result suggests an abnormal cell clone. Thus, LOH may prove to be a viable "rule-in" test in this context and may be followed by more sensitive techniques, such as high-throughput analysis and next-generation sequencing (NGS)[44,99]. However, prospective studies with large numbers of

patient samples are needed to ascertain its validity as a predictor of t-MDS/AML.

Clonality assay based on X chromosome-inactivation at the human androgen receptor gene is another useful method. This is a PCR-based technique that does not require information about loci prior to analysis and detects abnormal clones with survival/proliferative advantage over normal polyclonal cells[44]. In a single center study by Mach-Pascual *et al*[100], monoclonal hematopoiesis, as indicated by X-inactivation-based clonality at the human androgen receptor locus, prior to auto-HSCT was predictive of the development of t-MDS/AML. Four out of 10 patients (40%) demonstrating monoclonal hematopoiesis before transplant subsequently developed t-MDS/AML *vs* only 2 out of 53 patients with polyclonal hematopoiesis (P = 0.004)[100]. However, this method is limited by the need for high numbers of monoclonal cells to be present for diagnosis (low sensitivity) and its applicability only to female patients[44]. Altered gene expression in $CD34^+$ progenitors may also be used. A large study by Li *et al*[101] showed that a 38-gene panel analyzing gene expression in peripheral blood $CD34^+$ progenitors showed remarkable ability to distinguish patients who would eventually develop t-MDS/AML from those who would not develop the complication after auto-HSCT. The implication of this study is that development of t-MDS/AML requires the acquisition of mutations in multiple genes as opposed to just one gene[44]. Additionally, due to different kinds and combinations of mutations, patients with this disorder show significant heterogeneity with multiple subtypes. Therefore, characterization of single gene mutations may not have a satisfactory predictive value in identifying patients prone to developing t-MDS/AML[12,28,44].

Significant advances have happened for identification of unique biomarkers associated with leukemias which is mainly driven by gene expression analysis and NGS, which have the potential to significantly improve the diagnostic and prognostic criteria. The utilization of a signature NGS panel for each disease (*e.g.*, AML, ALL, MDS, *etc.*) is increasing worldwide[102,103]. In t-MDS/AML, the impact of NGS panel on long term outcomes are awaited. What we do know is some of clonal mutations with known association with leukemogenesis, *i.e.*, *TET2*, *DNMT3A*, and *ASXL1*[104,105], if found in a patient who is at risk of t-MDS/AML may predict a high likelihood of developing t-MDS/AML. Caution must be exercised with such an approach, as some cases of t-MDS/AML may have germline mutations in cancer susceptibility genes[106], thus a careful family history to discover cancer susceptibility is warranted in at-risk patients.

In summary, when a bone marrow biopsy is being obtained for work up for cytopenias in an at-risk patient (*e.g.*, cancer survivor who received chemotherapy or radiation), obtaining an NGS panel specific for MDS and AML should be considered.

RISK REDUCTION STRATEGIES

Based on our knowledge of the risk factors and pathogenesis of t-MDS/AML, development of risk reduction strategies is a certain possibility. Standardized screening tests, including but not limited to the ones discussed in the previous section, may help identify patients at substantial risk. Accordingly, alterations of chemotherapeutic regimens and treatment modalities may be made under a risk-adapted model, thereby minimizing the risk of t-MDS/AML while providing adequate treatment to the underlying malignancy[12].

In the context of high-dose chemotherapy and auto-HSCT, modifications can be made to stem cell mobilization and harvesting and pre-transplant conditioning regimens, circumventing the use of alkylating agents, topoisomerase inhibitors and radiotherapy, to eliminate as many risk factors as possible. Specific FISH loci, such as 5q-, 7q-, +8, -11 and 20q-, may be screened preemptively to predict outcomes when any specific abnormalities in blood work are being worked up[44]. Alternatively, if the risk of t-MDS/AML is substantial (for example, in the case of hematologic malignancies evidence of cytogenetic or FISH abnormalities prior to transplant and high risk disease), these patients can be offered other therapeutic options, such as pre-emptive work up for allo-HSCT (HLA typing) and non-transplant modalities (emerging novel therapies and targeted agents).

CONCLUSION

There is much needed effort for further exploration and validation of biomarkers specifically for t-MDS/AML to develop a viable risk assessment tool for this subgroup of patients. When it comes to cancer survivorship, we urge the current professional societies, *e.g.*, National Comprehensive Cancer Network, American Society of Clinical Oncology, and European Society for Medical Oncology to consider screening the at-risk population of cancer survivors for t-MDS/AML, at least with a complete blood count with peripheral smear annually, which is a relatively simple and economically feasible option for screening for t-MDS/AML.

Lastly, most of the large randomized studies in the arena of AML and MDS have traditionally excluded t-MDS/AML and thus prospective phase Ⅲ data for t-MDS/AML with regards to outcomes is absent. It is imperative that prospective clinical trials be conducted specifically for t-MDS/AML to delineate optimum treatment options. The cancer community has accomplished a lot in the past five decades in alleviating the burden of cancer by improvements in both radiation and chemotherapy fields, and current efforts on personalized or individualized medicine are looking very promising for further improvements in decreasing cancer mortality. However, as the cancer survivors are living longer[107,108], the incidence of t-MDS/AML continues to increase and currently is one of the fastest growing cancers worldwide. Efforts must be made by clinicians and researchers globally for establishment of risk reduction strategies for this fatal cancer.

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MINIREVIEWS

Targeting stem cells by radiation: From the biological angle to clinical aspects

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Abstract

Radiotherapy is a cornerstone of anticancer treatment. However in spite of technical evolutions, important rates of failure and of toxicity are still reported. Although numerous pre-clinical data have been published, we address the subject of radiotherapy-stem cells interaction from the clinical efficacy and toxicity perspective. On one side, cancer stem cells (CSCs) have been recently evidenced in most of solid tumor primary locations and are thought to drive radio-resistance phenomena. It is particularly suggested in glioblastoma, where CSCs were showed to be housed in the subventricular zone (SVZ). In recent retrospective studies, the radiation dose to SVZ was identified as an independent factor significantly influencing overall survival. On the other side, healthy tissue stem cells radio-destruction has been recently suggested to cause two of the most quality of life-impacting side effects of radiotherapy, namely memory disorders after brain radiotherapy, and xerostomia after head and neck radiotherapy. Recent publications studying the impact of a radiation dose decrease on healthy brain and salivary stem cells niches suggested significantly reduced long term toxicities. Stem cells comprehension should be a high priority for radiation oncologists, as this particular cell population seems able to widely modulate the efficacy/toxicity ratio of radiotherapy in real life patients.

Key words: Cancer; Neoplastic stem cells; Radiation therapy; Efficacy; Toxicity

Core tip: Radiotherapy is a cornerstone of anticancer treatments. However, significant levels of toxicity and recurrences are still reported. On the one hand, cancer stem cells have been recently suggested to be the root of radio-resistance, with strong pre-clinical rational. One the other hand, convincing pre-clinical data suggesting the importance of healthy tissue stem cells radiation-induced destruction in long term side effects of radiotherapy surfaced. This article provides an overview of the available literature analyzing from the clinical efficacy and toxicity perspective the interactions between stem cells and radiation. Significant improvement of radiotherapy toxicity/efficacy ratio is suggested.

Vallard A, Espenel S, Guy JB, Diao P, Xia Y, El Meddeb Hamrouni A, Ben Mrad M, Falk AT, Rodriguez-Lafrasse C, Rancoule C, Magné N. Targeting stem cells by radiation: From the biological angle to clinical aspects. *World J Stem Cells* 2016; 8(8): 243-250 Available from: URL: http://www.wjgnet.com/1948-0210/full/v8/i8/243.htm DOI: http://dx.doi.org/10.4252/wjsc.v8.i8.243

INTRODUCTION

Radiotherapy is a cornerstone of anticancer treatments, since it proved efficacy in various primary tumor location when performed with intent to cure[1-4]. It also proved to be efficient for palliation of bone[5] and brain metastases[6], whatever histologic diagnosis. However, significant rates of failure and of radiation-induced toxicities are still reported in spite of recent technological improvements[1-6]. Radiation resistance seems mainly caused by biological phenomenon driven by cancer stem cells (CSCs)[7]. CSCs have been evidenced in the mid-90s in hematological tumors, but their presence has been proved recently in most of solid tumors (glioblastoma, prostate, breast, rectum, colon, and head and neck cancers)[7]. The CSC is defined by three main characteristics: It can initiate tumorigenesis and endlessly proliferate, it can self-renew, and it can give birth to a high number of progenitor parental cells (Figure 1). Although CSCs account for a very small number of cells considering the whole pool of tumor cells, they are thought to play a leading role in radiation resistance. Pre-clinical data showed that CSCs were able to redirect their cell cycle toward a radiation resistant state (the S-G0 phase), had a considerable capacity of tumor re-population, were not dependant of oxygen, and above it all - possessed hyperactive DNA repair processes[8]. Besides, CSCs seem highly gifted for invasion and migration[9] making them the supposed - main responsible for local and metastatic post-radiotherapy recurrences. Targeting CSCs in order to increase the therapeutic index (efficacy/toxicity ratio) of radiotherapy is a very promising way of research[10]. But from another angle, it might lead to concurrently kill stem cells located in the surrounding healthy tissues, and induce serious radiation-caused toxicities. Ideally, radiotherapy should simultaneously destroy CSCs and spare normal tissue stem cells. Several research approaches actually tried to reach this goal with recent publications regarding the CSC pharmacological targeting[10], the CSC dosimetric targeting, and the healthy tissue stem cells sparing. Interesting potential pharmacological targets have been recently suggested: Wnt/β-caderines pathway inhibitors are currently under clinical investigation[11], with the strong pre-clinical rational that Wnt/β-caderines ex–pression is directly related with radiation-resistance[12], de-differentiation, adhesion, and invasion[13]. Notch-1 (involved in CSC repopulation[14], proliferation and radiation-induced apoptosis resistance[15]), SHH (involved in metastases[16], CSC proliferation, survival, morphogenesis and radioresistance[17]), JAK/STAT (involved in CSC de-differentiation, apoptosis resistance, and proliferation[18]) and PI-3 kinase/Akt (involved in CSC survival after radiation[19]) are pharmacological targets of interest, with inhibitors that are currently tested in pre-clinical studies. Hypoxia is also a major topic of interest, since CSCs are thought to be located in hypoxic niches. In pre-clinical studies, decreasing CSC hypoxia resulted in reduced CSCs self-renewing and multiplication[20,21]. The pharmacological targeting of tumor and vascular stroma (using PDGF inhibitors) seems therefore promising, with the *in vitro* radio-sensitisation of CSCs that were initially radio-resistant[22]. Contrary to pharmacological targeting, the CSC "dosimetric targeting" (*i.e.,* directly targeting stem cells by radiation) is still at its early stages. However, most of the publications consist in clinical studies with already promising outcomes. The sparing of organs at risk stem cells is also a hot topic, since healthy tissue stem cell death was suggested to be directly related to side effects widely impacting patients' quality of life, occurring after both curative and palliative radiotherapy. The present article's objective is to address the radiotherapy/stem cells topic from the clinical efficacy and perspective.

TARGETING CSC WITH RADIATION: EFFICACY DATA

Clinical outcomes: The glioblastoma model

Glioblastoma is a major model of radioresistance since in spite of a multi modal approach (ideally combing surgery, radiotherapy and chemotherapy), the median overall survival time only reaches 12-15 mo, with most of the recurrences located in the radiation fields. The underlying phenomena leading glioblastoma to radioresistance are still misunderstood but it was suggested in animal pre-clinical models that the genesis of glioblastoma was linked to a loss of tumor suppressor gene in neural stem cells (NSCs)[23]. NSCs were shown to be physiologically housed in the subventricular zone (SVZ), an area surrounding the lateral ventricles[24-27]. Therefore, delivering high doses of radiation to niches of "healthy tissue" (*i.e.,* the SVZ) possibly harboring glioblastoma CSCs might allow to

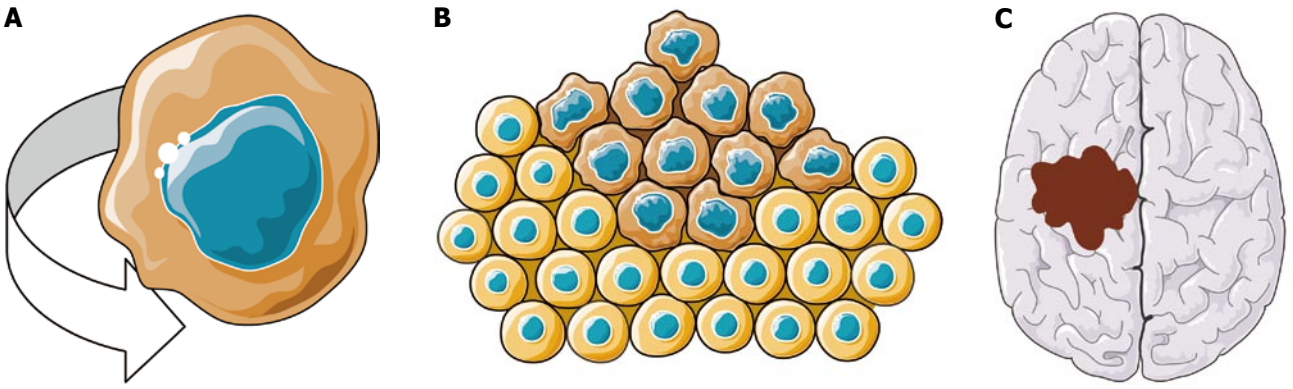

Figure 1 Cancer stem cells main biological characteristics. A: Self-renewal/endless proliferation; B: Giving birth to a high number of progenitor parental cells; C: Tumorigenesis initiation.

overcome its radioresistance. This hypothesis was tested in 2010 by Evers *et al*[28]. Data of 55 patients treated for a glioblastoma between 2003 and 2009 in California, United States, were retrospectively reviewed. Dosimetric data of radiotherapy were analyzed in order to estimate the dose delivered to the supposed CSC niches (*i.e.,* the SVZ), and correlate it with patient global outcomes. Only patients with histopathologically diagnosed anaplastic glioma (grade 3) or glioblastoma (grade 4), with at least 1 mo of follow-up, and who completed the whole planned radiotherapy were included. SVZ was defined based on previous publications, and doses to the volumes of interest could be a posteriori calculated. The authors estimated that the median dose received by the bilateral SVZ was 43 Gy. They then divided the population into a "low dose group" (receiving less than the median dose, n = 27) and a "high dose group" (receiving more than the median dose, n = 28). The two groups were well balanced on all essential prognosis factors (RPA classification, age, Karnofsky performance scale), but one. Complete resection was less achieved in the "high dose" group (n = 6, 21%) than in the "low dose" group (n = 16, 59%). The mean dose received by bilateral SVZ was 50 Gy ± 2 Gy for the "high dose group" and 27 Gy ± 5 Gy for the "low dose group". The median progression free survival (PFS, defined as the time between radiotherapy completion and glioblastoma recurrence) was 15 mo for the "high dose group" and 7.2 mo for the "low dose group". This difference was statistically significant (P = 0.03). Hazard ratio concerning glioblastoma progression was significantly decreased for the "high dose" group (HR = 0.74, 95%CI: 0.567-0.951, P = 0.0019). All other statistical analyses comparing important characteristics could not evidence significant differences, particularly regarding the total dose (P = 0.83), highlighting the high degree of glioblastoma's radioresistance due to CSCs[29]. No correlation was shown between the total dose and dose to SVZ, since SVZ was most of the time outside of the clinical target volume. Therefore, doses per fraction on the SVZ were limited (1.36 Gy CI: 1.2-1.5). The fact that low doses of radiation could result in an increased radio-sensitivity has already been described in glioblastoma[30,31], but not in CSCs[32]. The underlying biological phenomenon is hypothesized to be the non-detection of DNA damages in case of small doses per fractions, while the CSC radio-resistance is supposedly linked with the over-expression of DNA damage checkpoints[33]. However, CSC high sensitivity to low doses must be studied in prospective clinical studies. Interestingly, when statistical analyses were performed regarding the doses received by the ipsilateral periventricular zone only, no significant difference could be evidenced. Linked with the observation that glioblastoma cells can widely migrate within the healthy brain tissue, causing frequent contralateral recurrences[34], it was hypothesized that ipsilateral CSCs could take shelter in contralateral CSC niches. Targeting radio-resistant CSC might therefore be more efficient if all the possible CSC harbors are damaged, but this hypothesis is still to be demonstrated. In 2012, Gupta *et al*[35] published outcomes of 40 glioblastoma patients treated between 2008 and 2010 at the Tata Memorial Centre, India. All patients were treated for histologically proven glioblastoma using standard treatment. Dosimetric data were retrospectively reviewed, and doses to SVZ were a posteriori calculated and linked with global outcomes. Median dose to bilateral SVZ was 56.2 Gy, and patients were divided as previously described into a "high dose group" (n = 20, mean dose to ipsilateral, contralateral and bilateral SVZ of 60.1 Gy, 59.9 Gy and 60 Gy respectively) and a "low dose group" (n = 20, mean dose to ipsilateral, contralateral and bilateral SVZ of 57.5 Gy, 47.4 and 52.5 respectively). Most of known prognosis factors were unfavorably distributed in the "high dose group" *vs* "low dose group": Patients were older (55 yo *vs* 46 yo), with higher RPA class (85% of class Ⅳ-Ⅴ *vs* 55%), with less frequent extensive resection (50% *vs* 70%), and with more frequent MGMT methylation (55% *vs* 40%). At a median follow-up of 15 mo, 25 out of the 40 patients experienced progression, with 21 deaths. Age and RPA class (well known prognosis factors) were significantly linked with survival in univariate analysis, as well as the dose to contralateral SVZ (P = 0.05). A Kaplan-meyer

analysis showed significantly increased overall survival (P = 0.05) and progression free survival (P = 0.02) for patients with the highest doses to contralateral SVZ. In multivariate analysis, RPA class, Karnofsky performance status and dose to ipsilateral SVZ were identified as independent prognosis factors of overall survival (HR = 0.87, 95%CI: 0.77-0.98, P = 0.025). These results corroborate the efficacy of targeting CSCs by radiation in glioblastoma. However, the ideal target (ipsilateral or contralateral SVZ) and the dose threshold (43 Gy? 50 Gy?) are still to be clarified. The brain model is certainly one of the most interesting models for the CSC dosimetric targeting: Due to its anatomical conception, CSC niches are distinct from differentiated cells, making the result of a precisely delivered radiotherapy easier to interpret.

Properly imaging CSC through hypoxia: A necessary condition for an efficient radiotherapy?

These two publications also reflect the need for reliable imaging of CSC niches. The recent development of spectroscopy (identifying the specific metabolic profile of glioblastoma CSCs) is certainly a very promising technique that could allow a precise dosimetric targeting of CSCs in the future[36,37]. Out of the glioblastoma model, the CSC imaging systems are mainly based on hypoxia[38]. Hypoxia is thought to be a cornerstone of radiation resistance since it was clearly proven that the biological effects of conventional radiotherapy (*i.e.,* the DNA damages caused by chain oxidization) are potentiated by oxygen. In case of hypoxia, the efficacy of radiotherapy is *de facto* significantly reduced. It also seems clear that tumor hypoxic niches harbor CSCs (in glioblastoma but also in other solid tumors[39]) and therefore represent a target of interest for radiotherapy: The most radioresistant cells are housed in a micro-environment enhancing radioresistance. Imaging the hypoxic niches and targeting them by radiation might be the key to overcome cancers radioresistance since higher doses could induce the destruction and the re-oxygenation of these niches, initiating a virtuous cycle. The challenge of properly imaging hypoxia is still ongoing. Efficient nitroimidazole-based tracers were developed during the past 30 years, based on the fact that hypoxia induces a transformation of nitro-imidazole intermediates into alkylating agents that bind to cell component[40]. These elements could be then coupled with positron emitting radionuclides (^{18}F, ^{64}Cu, ^{60}Cu) in order to be detected by positron emission tomography (PET) imaging devices. (18F) Fluoromisonidazole and (18F) 1-(5-fluoro-5-deoxy-α-Darabinofuranosyl)-2-nitroimidazole were validated (regarding specificity) by invasive gold standard methods and can be now clinically used. However, sensitivity is still limited due to low tumor-to-plasma ratios and poor spatial resolution of PET imaging systems[38]. Techniques based on magnetic resonance imaging (MRI) have been developed, resolving the issue of spatial resolution (Blood oxygen dependent MRI imaging, Mapping of Oxygen by Imaging Lipid Relaxation Enhancement, and Dynamic-Contrast-Enhanced MRI), but sensitivity issues remained[38]. Moreover, recent data suggested that CSC were not necessarily located in the most hypoxic areas[41], making multi-modal imaging methods absolutely needed (coupled PET-MRI, or imaging techniques detecting CSC surface marker). In this field, nanoparticles are very promising theragnostic tools, since they can be used both as MRI contrast agents, and as radiotherapy targets[42,43]. Finally, the ideal solution might be a radiotherapy technique capable of destroying as well CSC as differentiated cancer cells. Hadrontherapy (carbon or proton-based radiotherapy) seems to fulfill these criteriae, showing *in vitro* the ability to kill with the same efficacy CSCs and conventional cancer cells, thanks to the absence of oxygen effect[44]. However, the high cost of this technique might be a clear drawback to its routine application. Moreover, radio-resistance phenomena have been very recently described *in vitro* and need to be fully investigated to evaluate their possible clinical impact[45].

SPARING NORMAL STEM CELLS DURING RADIOTHERAPY: TOXICITY DATA

Clinical outcomes: The whole brain radiotherapy model

Memory disorders are a well known long term side effect of whole brain radiotherapy (WBRT), performed in case of multiple brain metastases. Radio-damaged neural stem cells (NSCs) located in the subgranular zone of the hippocampal dentate gyrus[46] have been hypothesized to cause the reported cognitive decline following WBRT[47]. Thanks to the development of the intensity modulated radiotherapy (IMRT), Gondi *et al*[48] showed the feasibility of a WBRT avoiding (*i.e.,* reducing the delivered dose of ⩾ 80% to) the hippocampal NSC niches, without impairing the quality of coverage of the remaining brain. IMRT offers the possibility to spare areas that could not be spared with conventional radiotherapy indeed, thanks to highly conformal dose painting (Figure 2). Gondi *et al*[49] published in 2014 the outcomes of an international single-arm phase Ⅱ trial, comparing the results of a WBRT sparing hippocampal NSCs with the results of a 2003 phase Ⅲ trial using conventional WBRT for brain metastasis. Patients treated using WBRT for solid tumor brain metastasis were assessed for standardized cognitive assessments [Hopkins Verbal Learning Test-Revised Delayed Recall (HVLT-R DR)] at baseline, 2-, 4- and 6-mo follow-up, with a primary endpoint being the HVLT-R DR at 4 mo. At 4 mo, the mean relative decline in HVLT-R DR score from baseline was of 30% in the 2003 control trial. In the experimental trial, hippocampal NSC niches definition was standardized and based on MRI fusion with planning computed tomography-scan. Standard (and similar to the control trial) fractionation scheme was delivered, with 30 Gy in 10 fractions. Doses were limited to 9 Gy to the entire hippocampus, with a maximum focal dose of 16 Gy. Between 2011 and 2012, 113 patients were included, with 42 patients being

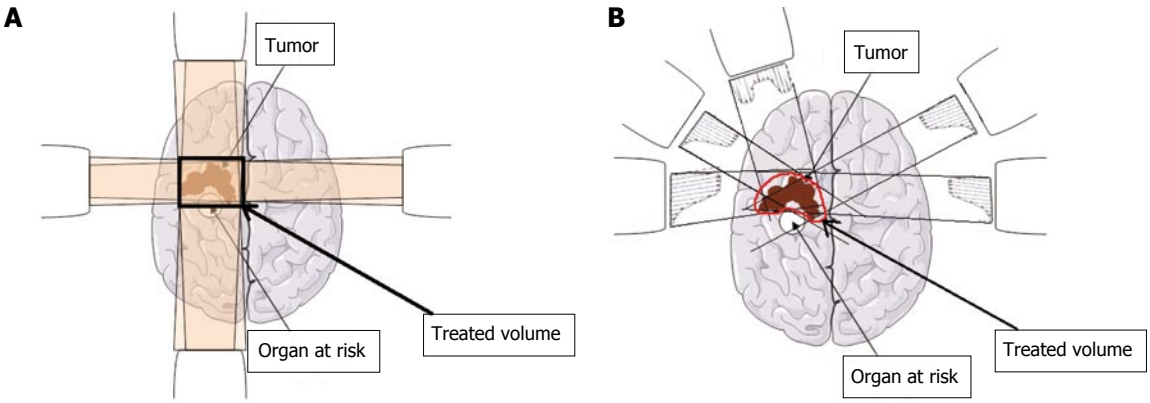

Figure 2 Three dimensional conventional radiotherapy *vs* intensity modulated radiotherapy. A: Three dimensional conventional radiotherapy; B: Intensity modulated radiotherapy.

analyzable for the primary endpoint. At 4 mo, the mean relative HVLT-R DR impairment was significantly lower in the population who experienced the hippocampal NSC protection compared to the population who did not (7% *vs* 30%, $P < 0.001$). Interestingly, if most of patients experienced intracranial progression, with a mean overall survival of 6.8 mo, only 4.5% of patients developed intra-hippocampal progression. The authors concluded that the avoidance of hippocampal NSC was significantly related to memory preservation, bringing a direct clinical evidence that hippocampal NSC niche was implicated in the pathophysiology of radiotherapy-induced memory decline. Of course, the main limitation of this article is the absence of direct control group, but phase Ⅲ trials have been approved and will clarify the place and efficacy of NSC avoidance during WBRT.

Clinical outcomes: The head and neck radiotherapy model

Xerostomia is one of the most quality of life-impacting late side effects of head and neck radiotherapy. Oral dryness frequently ruins patients' everyday life inducing ulcerations, speech, taste and swallowing difficulties. Even with modern radiotherapy techniques minimizing mean dose to salivary glands, important rates of mucosal complications (15% to 40% of treated patients) are still reported[50,51]. It was clearly demonstrated that the xerostomia was linked with the irradiation of salivary glands, because of the high radiosensitivity of stem cells niches located in the salivary glands[50,52,53]. Xerostomia seemed to be proportionally linked with the dose delivered to salivary gland stems cells niches, determining the quantity of post radiotherapy viable salivary stem cells[52,54]. However, the clinically relevant threshold dose of radiotherapy damaging stem cells is still undetermined and only techniques delivering doses as low as reasonably achievable to parotid stem cells-rich regions were tested. Moreover, the exact location of these areas is still debated, the strongest hypothesis being they could be located in the larger excretory ducts[55]. Based on animal models, van Luijk *et al*[56] suggested that the centre of the parotid (containing the major ducts) was certainly rich in stem cells, since its restricted irradiation leaded to long term saliva production collapse. This hypothesis was recently tested in humans[55]. Salivary and dosimetric data of 74 patients treated for a head and neck cancer without salivary gland involvement were retrospectively reviewed. Spatial dose distribution inside the parotid could be correlated to salivary flows 1 year after radiotherapy completion (with a dose-dependent effect relationship), defining a stem cell region located near the dorsal edge of the mandible, at the occurrence of the first branching of Stensen's duct, in concordance with animal stem cells locations. Doses delivered to this area were more predictive of salivary flow than (routinely used) parotid mean dose. Moreover, after radiotherapy, only cells provided by biopsies of these zones could be grown *in vitro*. A feasibility study was performed in 22 patients, showing that the preservation of the parotid stem cell niche seemed feasible with IMRT, even in case of impossible avoidance of the whole parotid. Other areas of parotid have been suggested to house stem cells capable of salivary long-term regeneration. It was suggested in one retrospective cohort derived from an important phase Ⅲ study that sparing the superficial lobe of the two parotid glands could induce a better salivary preservation than complete contralateral parotid gland sparing[57]. These data need to be validated in larger patient cohorts, but might be a significant progress in order to limit radiation-induced xerostomia. The main limitation of these articles (out of their retrospective nature) is that the link between salivary flow and xerostomia is still unclear: The major salivary glands (parotid glands, submandibular glands and sublingual glands) produce 90% of saliva, but minor salivary glands (thousands of small glands located in the oral cavity) secrete the major quantity of mucin, the saliva lubricating agent. Mucin is also secreted for a small account by submandibular glands and sublingual glands. Therefore, only shielding parotids stem cells might insufficient to guarantee the restoration of good quality saliva after radiotherapy. Pre-clinical and clinical data are certainly needed concerning the radio-sensitivity and the location of stem cells in the submandibular and minor salivary glands. Currently, no reliable biological or

imaging markers have been validated to precisely locate salivary stem cells, making progresses difficult to be made.

CONCLUSION

If the interaction between radiotherapy and CSCs is an en vogue topic[58], targeting CSC by radiation is at its early stage of development. Combining radiotherapy with biological drugs targeting CSC could be an efficient mean to overcome local and metastatic recurrences, with various agents that are currently tested based on solid pre-clinical rationales[59]. But directly targeting CSC using radiation is also a promising anticancer therapy with already interesting clinical results. The evolution of modern techniques of radiotherapy might widely depend of the imaging progresses in term of sensitivity. In order to increase the therapeutic index of radiotherapy, sparing stem cells of healthy tissue is also a major topic of interest since significant improvements regarding quality of life-impacting side effects following radiotherapy can be achieved. More than ever, prospective trials with solid methodologies are needed to confirm or infirm the suggested trends. Finally, both cancer and normal tissue stem cells seem to be central elements modulating the toxicity and the efficacy of radiotherapy. A better comprehension of stem cells location and their intrinsic radio-sensitivity is crucial, and permanent return trips between pre-clinical and clinical data are mandatory.

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MINIREVIEWS

Reprogramming of germ cells into pluripotency

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Abstract

Primordial germ cells (PGCs) are precursors of all gametes, and represent the founder cells of the germline. Although developmental potency is restricted to germ-lineage cells, PGCs can be reprogrammed into a pluripotent state. Specifically, PGCs give rise to germ cell tumors, such as testicular teratomas, *in vivo*, and to pluripotent stem cells known as embryonic germ cells *in vitro*. In this review, we highlight the current knowledge on signaling pathways, transcriptional controls, and post-transcriptional controls that govern germ cell differentiation and de-differentiation. These regulatory processes are common in the reprogramming of germ cells and somatic cells, and play a role in the pathogenesis of human germ cell tumors.

Key words: Primordial germ cell; Embryonic germ cell; Germ cell tumor; Reprogramming; Induced pluripotent stem cell; Small molecule compound; Gene; Signal; Transcription factor

Core tip: Primordial germ cells can be reprogrammed into pluripotent stem cells called as embryonic germ cells *in vitro* and into pluripotent germ cell tumors *in vivo*. Germ cell reprogramming can be regulated by signaling pathways, including PI3K/Akt signaling, mitogen-activated protein kinase signaling, transforming growth factor-β signaling, RA signaling. These mechanisms are also involved in somatic cell reprogramming, indicating that there exist common regulatory networks regulating germ and somatic cell reprogramming. On the other hand, regulators for germ cell development prevent germ cell dedifferentiation in unique manners.

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INTRODUCTION

The germ lineage is a privileged cell lineage that transmits genetic and epigenetic information from generation to generation[1]. Primordial germ cells (PGCs) are embryonic germ cell (EGC) precursors that eventually differentiate into sperm or oocytes[2,3]. In mice, a population of proximal epiblast cells in egg cylinder-stage embryos is committed to PGC precursors at embryonic day 6.25 (E6.25). During gastrulation, PGC precursors migrate out of embryos into the extraembryonic region, where a small number of nascent PGCs emerge at E7.0. PGCs return to embryos at E7.75, migrate through the hindgut and dorsal mesentery, and finally colonize the genital ridges until E11.5. PGCs actively proliferate and increase in number from E7.0 to E13.5, being transiently arrested in the G2/M phase at E8.5. In the gonads, PGCs undergo sex-dependent differentiation under the influence of somatic cells. Male germ cells enter into mitotic arrest after E13.5 and retain mitotic quiescence during embryogenesis. After birth, male germline stem cells (GSCs) called spermatogonia resume proliferation and produce sperm *via* meiosis and sperm morphogenesis (spermiogenesis). In contrast, female germ cells enter into meiosis at E13.5, and oocytes mature and are ovulated after birth.

Although totipotency is restored after fertilization, germ-lineage cells differentiate into only sperm or oocytes, but never into somatic cell types, during normal development. However, PGCs can be reprogrammed into pluripotency or can de-differentiate under experimental and pathological conditions as described below. In this review, we present an overview of the molecular mechanisms underlying germ cell preprogramming and germ cell tumor pathology, and discuss the features shared by germ cell and somatic cell reprogramming.

DIFFERENTIATION AND DE-DIFFERENTIATION OF PGCS

PGC differentiation

A number of events take place during PGC specification[2,3]. These include transcriptional activation of germ cell-specific genes [Stella and Deadend-1 (*Dnd1*)], reactivation of pluripotency-related genes (*Sox2* and *Nanog*), and repression of the somatic cell differentiation program. Epigenetic reprogramming occurs concomitantly. DNA methylation is globally erased through two waves by passive and active demethylation mechanisms, and unique genome-wide histone modification patterns are established (acquisition of H3K27me3 and loss of H3K9me2).

Three transcription factors, Blimp1 (Prdm1), Prdm14, and Tfap2c (AP2γ), play central roles in the specification of PGCs from the epiblast. Blimp1 expression commences in PGC precursors, the most proximal layer of the epiblast, at E6.25[4]. Expression of *Prdm14* follows soon after the onset of *Blimp1* expression in the precursors[5]. *Tfap2c* may be a downstream target of Blimp1[6]. In mice lacking these transcription factors, PGC precursors and nascent PGCs have abnormal gene expression patterns and epigenetic status. Gene expression analysis has revealed that Blimp1 represses somatic cell gene expression and Prdm14 activates germline and pluripotency genes[5,7]. Additionally, forced expression of these three transcription factors sufficiently promotes the differentiation of PGC-like cells from embryonic stem cells (ESCs) in culture[8,9].

PGC specification is regulated by interactions with surrounding somatic-lineage cells. Bone morphogenetic protein 4 (BMP4) is secreted from extraembryonic ectoderm, and is critical for the induction of PGC precursors and mesodermal cells from the epiblast *in vivo*[10]. Furthermore, treatment of epiblast explants with BMP4 activates the expression of *Blimp1* and *Prdm14* and induces the formation of PGC-like cells in culture[11], which suggests that BMP4 is an upstream regulator of *Blimp1* and *Prdm14*. Other BMP family proteins, BMP8b and BMP2 (which are secreted from extraembryonic ectoderm and visceral endoderm, respectively), may support PGC specification along with BMP4[11-14]. Wnt3a is also essential for the specification of PGCs and mesodermal cells. Since epiblast explants isolated from *Wnt3a*-deficient mice do not generate PGC-like cells in response to BMP4[11], Wnt3 seems to enable epiblast to respond to BMP4. Finally, the suppression of mitogen-activated protein kinase (MAPK) signaling is critical for the induction of PGC-like cells in the lineage choice between germ and mesodermal cells[15].

Testicular teratomas

Germ cell tumors are classified into two groups: Germinomas (seminomas) and non-germinonatous tumors[16,17]. Testicular teratomas belong to the latter group, and contain a variety of differentiated cells and tissue structures, which belong to the ectoderm, endoderm, and mesoderm lineages. Undifferentiated cells called embryonal carcinoma cells (ECCs) are also found in testicular teratomas[18]. ECC lines can be established from teratomas and maintained indefinitely in culture. However, these cell lines are usually multipotent rather than pluripotent because the cells differentiate into a limited number of cell types *in vitro* and *in vivo*. Teratomas often occur outside of the testis. Non-germinonatous germ cell tumors include yolk sac tumors and choriocarcinomas.

The etiology of testicular teratomas has been extensively studied using the 129/Sv inbred mouse strain, which frequently develops juvenile testicular teratomas[18]. Early teratomatous foci can be detected in E15.5 testes. Seminiferous tubule structures are disorganized, and teratomatous cells are found outside of the tubules thereafter. The foci contain a number of mitotically active cells, suggesting that these cells have failed to enter into mitotic arrest.

Teratoma onset is considered to be at around E12.5

in 129/Sv mice based on two lines of evidence. First, investigation of the sizes of the spontaneous tumors at various embryonic ages has indicated that tumor onset occurs at E12.5[18]. Secondly, when E12.5 gonads of 129/Sv mice were transplanted into the testes of adult 129/Sv mice, about 80% of the grafts developed into teratomas; conversely, the incidence of experimental teratomas was dramatically lower when E13.5 gonads were transplanted[19]. It is noteworthy that testicular teratomas do not develop in other inbred mouse strains both spontaneously and experimentally, suggesting that the genetic background affects the occurrence of teratomas.

The homozygous steel (*Sl*) mutant mouse has been used to show that testicular teratomas originate from germ cells in the gonads[20]. The *Sl* locus encodes a growth factor Kit ligand (KITLG, also known as stem cell factor), which activates the receptor tyrosine kinase c-Kit. c-Kit is expressed in migratory and gonadal PGCs, and its signaling is required for their proliferation and survival *in vivo*. When E12.5 gonads of 129/Sv mice carrying the homozygous *Sl*/*Sl* mutation were transplanted, no grafts developed into experimental teratomas, clearly demonstrating that teratomas are derived from PGCs.

EGCs

Studies that searched for PGC growth factors uncovered methods for reprogramming PGCs into pluripotent EGCs *in vitro*[21,22]. Treatment of PGCs with individual growth factors, such as KITLG, leukemia inhibitory factor (LIF), or basic fibroblast growth factor (bFGF), can promote the proliferation and survival of PGCs in culture. PGCs are responsive to these growth factors for only a few days, and eventually die *via* apoptosis. However, when LIF, KITLG, and bFGF are simultaneously added in culture, PGCs actively proliferate to form ESC-like, dome-shaped colonies (EGC colonies) within 5-7 d. In contrast, PGCs cultured in the presence of KITLG and LIF generate scattered colonies of cells with elongated morphology and do not lead to EGC formation.

After secondary cultures, EGCs can be propagated indefinitely in the presence of LIF, but without KITLG and bFGF[21]. When transplanted into blastocysts, EGCs can be incorporated into development and contribute to the three germ layers and germline in chimeric mice, indicating that EGCs have pluripotency equivalent to ESCs. However, when PGCs are transplanted into blastocysts immediately after isolation without culture, they never contribute to chimeric mice[23]. Thus, stimulation with KITLG, LIF, and bFGF can reprogram germline-committed PGCs into pluripotent EGCs. bFGF can be replaced by retinoic acid (RA) or forskolin[24,25], which increases the intracellular cyclic AMP (cAMP) concentration and leads to the activation of protein kinase A (PKA).

EGC derivation efficiency gradually decreases as germ cell differentiation proceeds. Efficiency is highest in E8.5 migratory PGCs, and sharply declines in E13.5 PGCs[21]. No EGCs can be derived from germ cells after E15.5[26]. In contrast to testicular teratomas, EGCs can be derived not only from 129/Sv mice but also from various other mouse strains. This indicates that PGCs intrinsically have the potential to be reprogrammed, regardless of genetic background, although genetic background has a strong influence on the pathogenesis of testicular teratomas *in vivo*.

PI3K/AKT SIGNALING

PI3K/Akt signaling in germ cell reprogramming

As stimulation with KITLG, LIF, and bFGF is required for the derivation of EGCs, signaling pathways downstream of these growth factors are likely critical for PGC reprogramming. Phosphoinositide-3 kinase (PI3K) is a lipid kinase activated by these growth factors. PI3K produces phosphatidylinositol 3,4,5-triphosphate (PIP3) from phosphatidylinositol 4,5-bisphosphate (PIP2) and transmits signals *via* downstream effector proteins, such as the serine/threonine kinase Akt and the small GTPases Rac1 and Cdc42[27]. Akt promotes physiological and pathological processes, such as proliferation, survival, metabolism, and tumorigenesis, through the phosphorylation of various target proteins[28]. On the other hand, the tumor-suppressor gene product phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a lipid phosphatase that converts PIP3 to PIP2 and antagonizes PI3/Akt signaling.

PGC-specific *Pten*-deficient mice develop juvenile testicular teratomas with a high frequency despite their mixed genetic background[29]. In mutant mice, PGC differentiation appears normal until E13.5, because the expression of germ cell-specific genes such as mouse vasa homolog (Mvh) is activated in mutant PGCs as well as in control PGCs. However, mutant PGCs do not enter into mitotic arrest and a number of PGCs undergo apoptosis after E14.5. Teratomatous foci, which are weakly positive or negative for Mvh, are detected in the E15.5 testes of mutant mice. Additionally, EGC derivation efficiency is much higher in E11.5 PGCs isolated from *Pten* mutant mice than in those from control mice. These findings show that *Pten* is essential for the establishment of the male germ lineage, and suggest that hyperactivation of PI3K reprograms PGCs into pluripotent cells *in vivo* and *in vitro*.

The effects of downstream Akt signaling have been examined using transgenic mice expressing the Akt-Mer fusion protein, which is composed of the myristoylated active form of Akt and mutated ligand-binding domain of estrogen receptor (Mer)[26,30]. The kinase activity of Akt-Mer can be turned on or off by the addition or withdrawal, respectively, of the Mer ligand, 4-hydroxytamoxifen (4OHT). When E11.5 PGCs from transgenic mice are cultured in the presence of KITLG, LIF, and bFGF, EGC derivation efficiency is greatly enhanced by 4OHT treatment. Furthermore, whereas bFGF is essential for EGC derivation, EGCs can be efficiently derived from transgenic PGCs cultured with 4OHT, KITLG, and LIF but without bFGF, showing that Akt hyperactivation can replace bFGF. Thus, the PI3K/Akt signaling axis plays

pivotal roles in PGC reprogramming.

Male GSCs in the testes of postnatal mice also reportedly de-differentiate into pluripotent cells in culture, albeit much less frequently than do PGCs. For example, it has been shown that GSCs, which are established from neonatal mouse testis, spontaneously generate ESC-like colonies during long-term culture[31]. These cells are called multipotential GSCs (mGSCs), and show pluripotency equivalent to ESCs and EGCs. Although both PGCs and GSCs are germ-lineage cells, Akt activation does not enhance the emergence of mGSCs from GSCs[32].

Cellular processes and target molecules in the reprogramming of germ and somatic cells

Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by the introduction of the transcription factors Oct4, Sox2, Klf4, and c-Myc (OSKM)[33,34]. E-Ras is an ESC-specific small GTPase that activates PI3K. Overexpression of E-Ras and downstream active Akt enhance OSKM-induced iPSC derivation efficiency[35,36]. In this section, we discuss the cellular processes and target molecules downstream of PI3K/Akt signaling by comparing the germ cell and somatic cell reprogramming systems.

The tumor suppressor Trp53 is a gatekeeper that checks the balance between proliferation and apoptosis[37]. The amount and activity of Trp53 are regulated transcriptionally and post-transcriptionally by intrinsic and external stimuli that cause DNA damage and oncogenic activation. Mice lacking *Trp53* frequently develop testicular teratomas against the 129/Sv genetic background[38]. Akt activation in cultured PGCs inhibits nuclear accumulation of Trp53 and the phosphorylation required for maximal transcriptional activation of Trp53[26], suggesting that Akt inhibits Trp53 activity in PGCs during reprogramming. Furthermore, deletion of *Trp53* not only enhances the derivation efficiency of EGCs in the presence of KITLG, LIF, and bFGF, but also can replace bFGF[26]. This shows that Trp53 inhibition is a critical event downstream of Akt signaling.

Deletion or knockdown of *Trp53* also greatly enhances iPSC induction[39]. Whereas OSKM introduction and/or culture conditions induce cell cycle arrest in somatic cells during reprogramming, inhibition of Trp53 suppresses cell cycle arrest, promotes cell proliferation, and eventually leads to a high frequency of iPSC production. Moreover, the cell proliferation rate is well-correlated with reprogramming efficiency in iPSC production[40], suggesting the existence of proliferation-dependent reprogramming processes. Likewise, PGC reprogramming also seems to be proliferation-dependent as failure of mitotic arrest in both 129/sv mice and *Pten*-deficient mice *in vivo* leads frequent incidence of PGC dedifferentiation[19,29]. Akt activation enhances proliferation but suppresses apoptosis in cultured PGCs *in vitro*[26,30]. In addition to inhibiting Trp53, Akt is known to promote proliferation through many other target proteins, such as cyclin D and cyclin-dependent protein kinase inhibitors (CDKIs), p21Cip1, and p27Kip1[28,41]. In fact, mutation in *INK4* CDKI promotes incidence of spontaneous testicular teratomas in the absence of *Trp53*[42]. Cell cycle arrest represents a roadblock for reprogramming that can be overridden by higher proliferative activity both in somatic and germ cells.

Metabolic reprogramming, shifting from oxidative phosphorylation to glycolysis, is required for somatic cell reprogramming toward iPSCs[43]. Akt signaling promotes glycolysis by phosphorylation of the Foxo family transcription factors[28,41]. Foxo1 regulates the expression of genes involved in glycogenesis and gluconeogenesis, as well as in proliferation and apoptosis[44]. Akt inhibits the transcriptional activity of Foxo1 through its exclusion from the nuclei, leading to enhanced glycolysis. In fact, forced expression of the dominant-negative form of Foxo1 enhances the derivation efficiency of iPSCs[36]. The mechanistic target of rapamycin complex 1 (mTORC1) is another target of Akt that regulates metabolism[45]. As activation of mTORC1 by Akt inhibits mitophagy, Akt can promote oxidative phosphorylation in mitochondria and thereby antagonize metabolic reprogramming[46,47]. On the other hand, little is known about the metabolic status of PGCs or metabolic changes during germ cell reprogramming.

It has been suggested that only a fraction of cells are randomly selected for reprogramming because of the stochastic nature of the epigenetic reprogramming processes[40]. A number of repressive epigenetic modifications, such as DNA methylation, H3K9me3, and H3K79me2, and their regulators, have been identified as barriers to somatic cell reprogramming[48]. In addition, inhibition of histone deacetylase complex enhances iPSC induction[49,50]. Mbd3 is a component of the nucleosome remodeling deacetylase (NuRD) complex, which is involved in heterochromatin formation. It has been reported that the majority of cells are reprogrammed into iPSCs by knockdown of *Mbd3* in the secondary iPSC induction system[51], showing that the NuRD complex is one of the most important epigenetic roadblocks. In addition, the deletion of *Mbd3* also enhances the efficiency of EGC derivation from PGCs[51]. Gene expression analysis during PGC reprogramming shows that a great number of Mbd3 target genes are affected by Akt activation[52]. Additionally, Akt activation decreases expression of Mbd3 during somatic cell reprogramming. Collectively, the evidence suggests that PI3K/Akt signaling may promote germ and somatic cell reprogramming through multiple pathways, including proliferation, survival, metabolic change, and epigenetic regulation.

PI3K/Akt signaling in human germ cell tumors

Mutants and variants of *KIT* and *KITLG* have been identified as risk factors for human germ cell tumors[17]. A strong association between a variant of *KITLG* and the occurrence of testicular teratomas has been reported. *KIT* mutations, which activate kinase activity in a ligand-independent manner, are found frequently in testicular seminomas but not in testicular teratomas or yolk sac tumors[53,54]. *CBL* mutations have been found in

teratomas, yolk sac tumors, and mixed-type tumors composed of germinomas and non-germinomatous tumors, all of which occur intracranially[54]. Because *CBL* encodes ubiquitin ligase for receptor tyrosine kinases, including KIT, mutations may lead to KIT overexpression.

The PI3K/Akt and MAPK signaling pathways are associated with the occurrence of germ cell tumors. *KRAS* and *NRAS* mutations, which activate both PI3K/Akt and MAPK signaling, are frequently detected in seminomas and teratomas[54]. Single nucleotide polymorphisms (SNPs) of *PTEN* have been identified as risk factors for testicular teratomas[55]. In addition, mutations in *MTOR* and *TRP53* and copy number gains in *AKT1* are frequently observed in intracranial teratomas and yolk sac tumors[54,56]. On the other hand, variants of sprouty-4, encoding a negative regulator for MAPK signaling, are associated with testicular teratomas[53]. Thus, the KIT, PI3K/AKT, and MAPK signaling pathways could be promising therapeutic targets for human germ cell cancers, including testicular teratomas.

REPROGRAMMING BY SMALL MOLECULE COMPOUNDS

In somatic cell reprogramming, reprogramming-inducing transcription factors can be replaced by chemical compounds. For example, the effects of Sox2 and Klf4 can be reproduced by transforming growth factor-β receptor inhibitor (TGFβRi, SB431542 and A83-01)[57-59] or Kempaullone[60], respectively. Kempaullone is an inhibitor of kinases, including glycogen synthase kinase-3 (GSK3) and cyclin-dependent protein kinases. Oct4 can be substituted by forskolin, 2-methyl-5-hydroxytryptamine, and D4476[61]. As forskolin substitutes for bFGF in PGC reprogramming[24], the cAMP/PKA axis mediates cellular reprogramming in both somatic and germ cells.

PGCs are never converted to EGCs when cultured on mouse embryonic fibroblast (MEF) feeder layers with LIF, which is a standard culture condition for ESCs. When post-migratory PGCs at E11.5 are treated with TGFβRi under ESC culture conditions, EGCs can be derived without KITLG and bFGF, showing that TGFβRi can reproduce the effects of KITLG and bFGF[62]. Although Kempaullone alone does not induce EGCs from E11.5 PGCs, simultaneous treatment with TGFβRi and Kempaullone synergistically enhances EGC induction efficiency. In contrast, when E13.5 PGCs are cultured under ESC culture conditions, Kempaullone efficiently induces EGCs, while TGFβRi merely promotes EGC derivation. In addition, the effects of Kempaullone are inhibited completely by TGFβRi in E13.5 PGCs. It remains to be elucidated how PGCs respond differentially to these compounds in a differentiation stage-dependent manner.

ESCs are derived from the epiblast in blastocysts before implantation, whereas epiblast stem cells (EpiSCs) are established from the epiblast in post-implantation stage embryos[63,64]. While mouse ESCs can be propagated in the presence of LIF and form multi-layered colonies, mouse EpiSCs can be expanded and form mono-layered colonies in the presence of bFGF and TGF-β family member activin. These differences may reflect the distinct developmental stages of epiblast. On the other hand, primate ESCs resemble mouse EpiSCs in terms of colony morphology and growth factor requirements. While the pluripotent states of mouse ESCs are called naïve pluripotency, those of mouse EpiSCs and primate ESCs are called primed pluripotency.

Mouse ESCs can be maintained in a more undifferentiated state, so-called "ground-state" pluripotency, when cultured with LIF and two inhibitors (2i), namely inhibitors of MAPK/ERK kinase and GSK3 (PD0325901 and CHIR99021, respectively)[14]. The efficiency of iPSC production is enhanced by treatment with 2i[65,66]. Furthermore, EGCs are derived from migratory PGCs at E8.5 by 2i without KITLG and bFGF[67]. Treatment with 2i also increases EGC derivation efficiency in post-migratory PGCs at E11.5, and the effect is further enhanced by TGFβRi treatment[68].

It has recently been reported that iPSCs can be derived from MEFs by sequential treatment with chemical compounds alone[61,69]. These compounds include TGFβRi (616452), GSK3i (CHIR99021), a cAMP/PKA agonist (forskolin), an RA agonist (AM580), a histone deacetylase complex inhibitor [valproic acid (VPA)], an inhibitor of H3K4 demethylase LSD1 (tranylcypromine), inhibitors of H3K79 methyltransferase DOT1L (EPZ004777 and SGC0946), and a DNA methyltransferase (Dnmt) inhibitor (5-aza-dC). Despite their positive effects on somatic cell reprogramming, VPA and 5-aza-dC inhibit EGC derivation from E11.5 PGCs, indicating differences in epigenetic status between somatic and germ cells[68].

REGULATORS OF GERM CELL DEVELOPMENT

A homozygous Teratoma (*Ter*) mutation dramatically increases the occurrence of testicular teratomas against the 129/Sv genetic background[70,71]. Although germ cells in *Ter*/*Ter* mutant mice appear normal until E13.5, the cells do not enter into mitotic arrest after E14.5, undergo massive apoptosis, and generate early teratomatous foci after E15.5, which are essentially the same phenotype as those of *Pten*-deficient mice. However, the *Ter*/*Ter* mutant mice, against other genetic backgrounds such as C57/BL6, do not develop testicular teratomas but exhibit germ cell deficiency. A homozygous *Ter* mutation causes germ cell death during embryonic development regardless of the genetic background. There exist genetic and epigenetic modifiers required for teratoma formation in the 129/sv genome.

Dnd1 is a gene responsible for *Ter* mutation phenotype[72]. Dnd1 is an evolutionarily conserved RNA-binding protein that counteracts micro RNA (miRNA)-mediated translational inhibition of target mRNAs in zebrafish and mammals[73-75]. The miRNA targets include mRNAs for negative cell cycle regulators (*p27*, *Lats*,

Trp53), pluripotency and germ cell-related genes (*Oct4*, *Sox2*, *Nanos1*) and anti-apoptotic factors (*Bax*, *Bclx*). As translation of these target mRNAs is de-repressed by Dnd1, *Ter* mutation brings about decreased levels of these proteins, which can lead to germ cell deficiency and uncontrolled cell proliferation and survival. Dnd1 is a binding partner of the RNA-binding protein Nanos2, which interacts with the CCR4-NOT deadenylase complex and regulates the stability of mRNAs for germline genes such as *Sycp3*, *Dazl*, *Nanog*, and *Stra8*[76]. Deregulation of RNA metabolism may also be implicated in tumorigenesis in *Ter* mutant germ cells.

Doublesex-related transcription factor (Dmrt1) promotes male differentiation in germ and somatic cells in fetal and neonatal testes. In the absence of *Dmrt1*, testicular germ cells prematurely enter into meiosis and Sertoli cells transdifferentiate into female somatic cells[77-79]. Like *Ter*/*Ter* mutant mice, over 90% of *Dmrt1*-deficient mice develop testicular teratomas against the 129/Sv genetic background, but not other genetic backgrounds. Conditional knockout mice demonstrate that the loss of *Dmrt1* in PGCs, but not in Sertoli cells, leads to teratoma formation[80]. Pluripotency-related genes and Nodal pathway genes are upregulated, whereas the glia-cell derived neurotrophic factor (GDNF) receptor genes including *Ret* and *Gfra1* are downregulated in mutant fetal testes[81]. As deletion of *Gfra1* in 129/Sv mice modestly increases the incidence of testicular teratomas[81], the effects of *Dmrt1* deletion are at least partly mediated by downregulation of GDNF signal. Alternatively, enhanced RA signaling in germ cells lacking *Dmrt1* may drive dedifferentiation, as RA treatment induces PGC reprogramming *in vitro*[25,77,79]. In addition to these effects on fetal germ cells, depletion of *Dmrt1*, together with *Trp53* depletion, increases the efficiency of mGSC derivation from GSCs[82]. It has been reported that SNPs near *DMRT1* are associated with testicular germ cell cancer in humans[83].

The transcription factors Blimp1, Prdm14, and Tfap2c are critical for the specification and differentiation of PGCs. While forced expression of *Blimp1* in ESCs reduces the expression of pluripotency genes, deletion of *Blimp1* in PGCs promotes the derivation of EGCs even in the absence of bFGF[52]. In addition, heterozygous *Tfap2c* mutant mice develop testicular teratomas against the 129/Sv background[84]. *In vitro*, PGC-like cells induced from homozygous *Tfap2c* mutant ESCs show upregulation of cell cycle regulators (*Cdk6*) and pluripotency genes (*Eras*, *Klf4*), but downregulation of germline genes (*Dmrt1*, *Nanos3*)[84]. Furthermore, the susceptibility locus for human testicular germ cell cancer has been found near *PRDM14*[85]. Collectively, these germline genes also function as gatekeepers of PGC dedifferentiation.

CONCLUSION AND PERSPECTIVES

Reprogramming of germ cells and somatic cells is controlled by common signaling pathways, which are activated by PI3K/Akt, MAPK, GSK3, TGFβ, RA, and cAMP/PKA. Therefore, it is critical to understand which downstream effectors are important for reprogramming, and which cellular processes are modulated by these signaling pathways during reprogramming. In contrast, the roles of epigenetic regulators on reprogramming seem to differ to some extent between germ and somatic cells. Furthermore, certain regulators of germ cell differentiation, which are essential for the establishment of the male germline, play critical roles in the prevention of germ cell dedifferentiation.

129/Sv mice frequently develop testicular teratomas. Additionally, mutations in *Dnd1*, *Dmrt1*, and *Tfap2c* lead to testicular teratomas in only the 129/Sv mouse strain. Therefore, it has been suggested that 10-15 susceptibility genes are present in the 129/Sv genome[86,87]. These modifiers include *Ter*, *Trp53,* testicular germ cell tumor 1, and primordial germ cell tumor 1[86-88]. *Ter* mutation increases the incidence of teratomas along with mutations in the genes encoding Trp53, KITLG, the translational regulator Eif2s2 (A^y mutation), and the cytidine deaminase Apobec1[89,90]. Furthermore, the introduction of chromosome 19 from MOLF mice into the 129/Sv background greatly increases the tumor incidence[86]. Investigating the genetic network among susceptibility genes will be necessary to understand the development of germ cell tumors.

Genome-wide association studies have revealed a number of candidate genes for human germ cell tumors. Variants have been found near genes involved in male germ cell development (*DAZL*, *HPGDS*, *SMARCAD1*, *SEPT4*, *TEX14*, *RAD51C*, *PPM1E*, and *TRIM37*), chromosomal segregation (*MAD1L1*, *TEX14*, and *SKA2*), the DNA damage response (*SMARCAD1*, *RFWD3*, and *RAD51C*), and epigenetic regulation (*JMJD1C/KDM3A* and *KDM2A*)[83,85,91]. Mouse models would help to evaluate the roles of these genes in the tumorigenesis of germ cells.

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MINIREVIEWS

Epithelial plasticity in urothelial carcinoma: Current advancements and future challenges

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Abstract

Urothelial carcinoma (UC) of the bladder is characterized by high recurrence rate where a subset of these cells undergoes transition to deadly muscle invasive disease and later metastasizes. Urothelial cancer stem cells (UroCSCs), a tumor subpopulation derived from transformation of urothelial stem cells, are responsible for heterogeneous tumor formation and resistance to systemic treatment in UC of the bladder. Although the precise reason for pathophysiologic spread of tumor is not clear, transcriptome analysis of microdissected cancer cells expressing multiple progenitor/stem cell markers validates the upregulation of genes that derive epithelial-to-mesenchymal transition. Experimental studies on human bladder cancer xenografts describe the mechanistic functions and regulation of epithelial plasticity for its cancer-restraining effects. It has been further examined to be associated with the recruitment of a pool of UroCSCs into cell division in response to damages induced by adjuvant therapies. This paper also discusses the various probable therapeutic approaches to attenuate the progressive manifestation of chemoresistance by co-administration of inhibitors of epithelial plasticity and chemotherapeutic drugs by abrogating the early tumor repopulation as well as killing differentiated cancer cells.

Key words: Cancer stem cells; Clinical management; Cytotoxic effects; Epithelial plasticity; Therapeutic resistance; Urothelial carcinoma; Urothelial stem cells

Core tip: A subset of bladder cancer cells, known as urothelial cancer stem cells, have abilities to self-renew, generate tumor heterogeneity *via* differentiation, and are actually responsible for tumor relapse and metastasis formation. Delineating the mechanistic complexity between epithelial plasticity and cancer stemness in malignant transformation of urothelial carcinoma provides the basis for designing rational therapies. Differentiation and elimination therapies targeting the potential biomarkers could prove to be clinically beneficial by suppressing the cancer stemness and inhibiting epithelial-to-mesenchymal transition phenotype and would provide novel opportunities for targeted therapeutic approaches in the clinical management of patients.

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INTRODUCTION

Urothelial carcinoma (UC) of the bladder, also known as transitional cell carcinoma of the bladder, is the sixth most common cause of cancer-related deaths worldwide[1]. It is the second most frequent cancer of the genitourinary tract where men are at four times greater risk than women. It is caused by the accumulation of genetic or epigenetic changes in the urothelium due to its exposure to multiple risk factors including tobacco and occupational/environmental carcinogens (polycyclic aromatic hydrocarbons). People working in leather, dye, rubber industries, painters, pesticide applicators or those having chronic urinary tract infections are more prone to develop urothelial carcinoma.

UC of the bladder is a heterogeneous disease, which can arise through two different pathways - non-invasive papillary pathway and invasive pathway. It represents a spectrum of neoplasms, including non-muscle invasive bladder cancer (NMIBC), muscle invasive bladder cancer (MIBC) and metastatic lesions. Tumor staging and grading (Tumor Node and Metastasis classification by World Health Organization/International Society of Urology Pathologists, 2004) are the gold standard prognosticators for defining the various entities of UC of the bladder (Figure 1)[2]. Despite the successful treatment of NMIBC through transurethral resection of bladder tumor (TURBT), 70% to 80% of them have a tendency to recur. Hence, there is a need for regular cystoscopy and examination of cytologic and molecular markers in urine, blood or tumor tissues in bladder cancer patients. This intense surveillance after treatment makes this cancer, one of the most costliest cancers to manage. Although in the majority of the cases, these papillary bladder tumors are not lethal, however, 20%-30% of them can progress to more aggressive, invasive and metastatic bladder tumors with an overall survival rate of 5% (Figure 2).

Characterization of molecular and biological mechanisms responsible for distinct bladder tumor phenotypes would facilitate personalization of more effective treatment decisions. Multiple genetic and epigenetic abnormalities are known to be associated with diverse types of urological malignancies. Cancer stem cell theory sheds further light on understanding the biology of the origin of distinct oncological pathways and heterogeneous nature of this disease.

This paper discusses the current concepts on the aberrant activation of epithelial-to-mesenchymal transition (EMT), also known as epithelial plasticity, as one of the primary causes of transformation of urothelial stem cells (UroSCs). Further, recent advancements on the functions of urothelial cancer stem cells (UroCSCs), a tumor subpopulation derived from transformation of UroSCs, in the pathophysiology and its clinical implications in the treatment of UC of the bladder are reviewed.

UROTHELIAL STEM CELLS AND UROTHELIAL CANCER STEM CELLS

The stratified epithelial lining of the urinary bladder wall, also known as urothelium, consists of unilayered polygonal basal cells which are in direct contact with the basement membrane, intermediate cells and umbrella cells. Many recent studies report the existence of a self-renewing unipotent population of slow cycling, label-retaining cells with long life span and high integrin subunit beta 4 expression, also known as urothelial stem cells, as clonal patches among basal cell layer. High nuclear-cytoplasmic ratio and expression of CD44, laminin receptor, cytokeratins (CK-5/14, CK17), β1 and β4 integrins are some of the characteristic features of UroSCs[3]. These cells confer increased regenerative and proliferative potential, lower apoptosis rate and multilineage differentiation at the edge of the basement membrane as compared to other cell types. These cells undergo cellular differentiation to give rise to transit-amplifying cells of intermediate cell layers and later umbrella cells. However, an alternative hypothesis suggests that adult stem cells can give rise to two cell lineages and hence, umbrella cells are formed separately from intermediate/basal cells (Figure 3). Lineage tracing experiments in the murine model of carcinogenesis provide a cellular and genetic basis for the diversity in bladder cancer lesions which could be responsible for their clinical and morphological differences. According to the experimental results of this study, the low grade, non-invasive papillary lesions arise from intermediate cells whereas Keratin 5 expressing basal cells are likely the progenitors of flat carcinoma *in situ*, a flat aggressive lesion, as well as of muscle-invasive lesions depending on the genetic background[1]. A study by Dancik *et al*[4] screened 874 bladder cancer patients in five cohorts for the identification of UroCSCs in muscle invasive tumors and validated the hypothesis of differential origin of non-muscle invasive and muscle invasive tumors from distinct progenitor cells. These results provide a paradigm shift in better understanding the biology of urothelial carcinoma for significant diagnostic and therapeutic implications.

Mutational insults in adult UroSCs and differentiated progenies, help them in acquiring tumorigenic properties and result in the origin of a subpopulation of high tumor-initiating potential cells called UroCSCs. Characterization studies on these cells describe their self-renew ability, clonogenic and proliferative potential. In addition, their capability to conserve cellular heterogeneity *via* differentiation can be explained by the research studies on the regrowth of heterogeneous tumor after *in vivo*

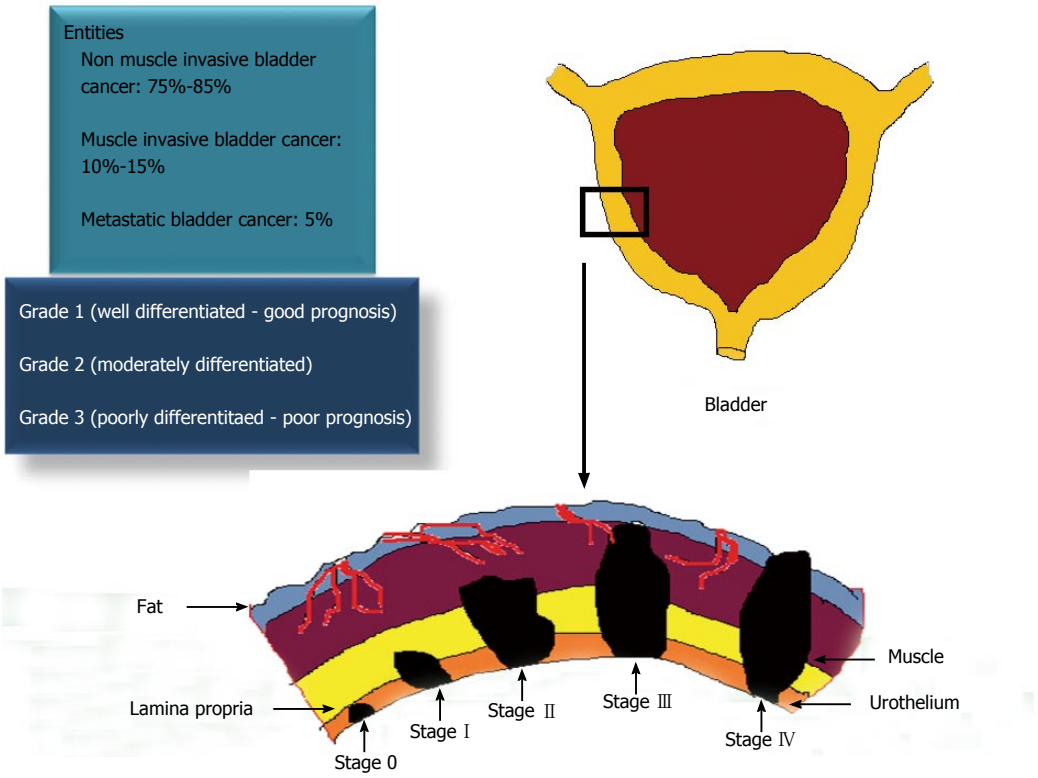

Figure 1 Staging, grading and prognosis of urothelial carcinoma of the bladder.

xenotransplantation of a small number of UroCSCs in immunodeficient mice (Figure 3). These characteristic features of UroCSCs document their large amount of functional resemblance with the normal adult stem cells.

UroCSCs have been examined for the upregulation of various oncogenes which help them to acquire self-renewability. Beta-catenin (β-catenin), signal transducer and activator of transcription 3, glioma associated oncogene 1, B lymphoma Mo-MLV insertion region 1 homolog (BMI1), POU domain, class 5, transcription factor 1/octamer-binding transcription factor 4 (POU5F1/Oct4), sex determining region Y-box 2 (SOX2), Kruppel-like factor 4, v-myc myelocytomatosis viral oncogene homolog (avian) (MYC, formerly C-MYC) and NANOG are the oncogenes/transcription factors that have been observed to be responsible for maintaining the pluripotent properties of stem cells and aggressiveness of tumor invasion[5-7].

Studies on the identification of co-expression of keratin 5 and CD44 markers on UroCSCs distinguish them from differentiated tumor cells and support their basal-like phenotype. Binding of CD47, a marker of tumor-initiating cells, to signal-regulatory protein alpha on macrophages and subsequent inhibition of phagocytosis of tumor cells make it a suitable drug target[8]. Increased expression of POU5F1, an embryonic stem cell marker, and high aldehyde dehydrogenase activity in a fraction of $CD44^+$ tumors correlate with increased clonogenic capacity of UroCSCs, and poor prognosis in UCs[9]. Identification of an extracellular marker, prominin 1 ($PROM1^+$) ($CD133^+$) and intracellular markers $POU5F1^+$, and nestin (NES^+) on putative UroCSCs confer them self-renewal ability and proliferative advantages in clonogenic assays. However, in due course of time, they allow these UroCSCs to lose stem cell phenotype as well as proliferative capacity and initiate the process of differentiation[10]. Differentially expressed cancer stem cell markers CD24/CD44/CD47 in the urothelial cancer cells of bladder cancer patients undergoing radical cystectomy could be of therapeutic value as their presence influenced cancer-specific survival of patients[11]. Many cell surface markers, intracellular proteins and their activities are examined to identify and characterize the putative UroCSCs, however, due to the lack of consensus on these markers, functional assays have been studied to confirm the stem cell phenotype of these tumor cells.

Pumping of DNA-binding dyes, Hoechst 33342 and DyeCycle violet, out of the cells due to overexpression of ABC (ATP-binding cassette) transporters/multidrug resistance (MDR) pumps are considered important features of a side population of urothelial cancer cells, enriched for CSCs. Co-localization of ABC transporters, ABCG2 and ABCB1 (MDR1) and other stem cell markers including POU5F1 and BMI1 further validates their identity and existence[12]. Initiation of tumor formation upon subcutaneous injection of a small number of SP of urothelial cancer cells into immunocompromised mice has been examined by clonogenic assays, and these cells showed rapid cell growth, chemo and radioresistance.

Accumulating evidence suggests that UroCSCs/progenitor cells exhibiting epithelial plasticity are quiescent, show increased DNA damage response, pump drugs out of the cells, reside in difficult-to-reach CSC protective

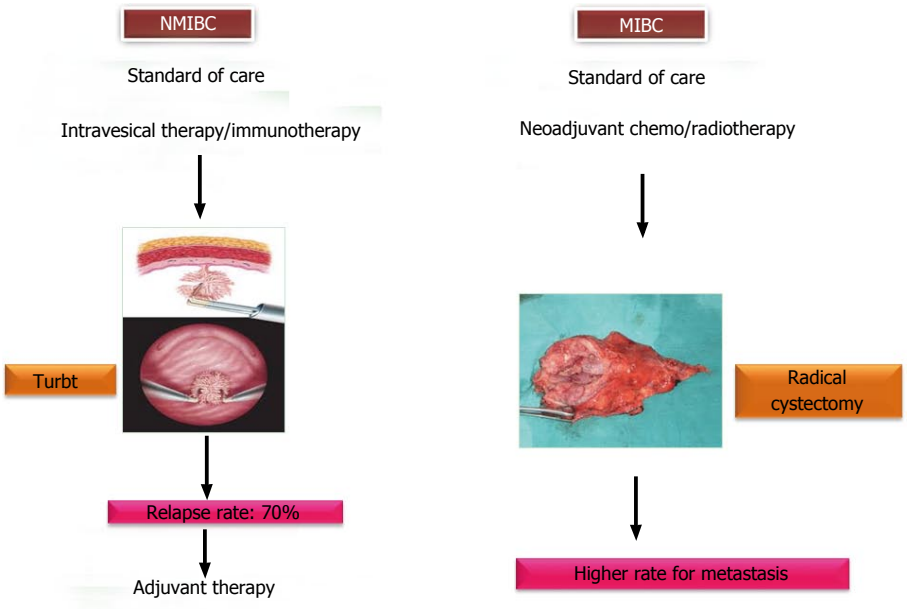

Figure 2 Multimodality approaches for urothelial carcinoma of the bladder. NMIBC: Non-muscle invasive bladder cancer; MIBC: Muscle invasive bladder cancer.

niches and are less affected by antiproliferative therapies.

UROTHELIAL CANCER STEM CELLS AND EPITHELIAL PLASTICITY

During the analysis of transcriptome of microdissected muscle invasive urothelial carcinoma of bladder/MIBC, the cancer cells expressing multiple progenitor/stem cell markers were found to be enriched with elevated levels of genes that derive and regulate EMT[13]. The process of EMT is characterized by the loss of cell polarity and cell-cell adhesion by sessile, epithelial cells and their transition to motile, mesenchymal stem cells with increased migratory and invasive potential. Cells acquire phenotypic or epithelial plasticity when they gain the ability to dynamically switch over between different phenotypic states[14]. EMT helps to establish metastasis by allowing the motile cells to invade the surrounding tissues, intravasate, move to distant sites through bloodstream, extravasate and colonize the target organs. Re-establishment of cancer cells with more epithelial phenotype at metastatic sites can be induced through mesenchymal-to-epithelial transition (MET) (Figure 4).

A study by Franzen *et al*[15] demonstrates the increased expression of several mesenchymal markers, including α-smooth muscle actin, S100A4 and snail, in urothelial cells treated with muscle invasive bladder cancer exosomes (small secreted vesicles that contain proteins, mRNAs and miRNAs and can potentially modulate signaling cascades in recipient cells) as compared with phosphate-buffered saline-treated cells. Moreover, these treated urothelial cells showed loss of epithelial markers, E-cadherin and β-catenin in association with increased migratory and invasive properties.

Loss of E-cadherin, a tumor suppressor gene, and abnormal expression of N and P-cadherin (cadherin switching) have been shown to be key mediators in invasive and malignant phenotype of cancer. In addition, activation of WNT signaling cascade by tumor cells owing to decreased E-cadherin levels, loss of β-catenin expression, its nuclear translocation and increased transcriptional activity have been examined to be associated with epithelial plasticity of tumor cells, disease aggression and metastasis formation. One of the serious implications of cadherin switching include the development of cancer stem cell phenotype and this makes the cadherin cell adhesion molecules and associated pathways, the probable target candidates for inhibition of cancer progression[16].

Tumor stroma/microenvironment has been shown to regulate tumor behavior by maintaining UroCSC population, its properties and EMT. Although the exact mechanism is not known, secretion of stroma-modulating growth factors including basic fibroblast growth factor 2, vascular endothelial growth factor, platelet-derived growth factor, epidermal growth factor receptor (EGFR) ligands, colony stimulating factors, and transforming growth factor-beta; extracellular matrix-degrading proteins, such as matrix metalloproteinases; and chemoattractants result in activation of fibroblasts, inflammatory cells, mesenchymal stem cells, smooth muscle cells, and adipocytes[17,18]. This contributes to angiogenesis, tumor growth, invasion and metastasis formation.

THERAPEUTIC IMPLICATIONS AND CHALLENGES

Intravesical instillations of drugs or adjuvant therapies following TURBT are the standard of care for non-muscle invasive cancer. Similarly neoadjuvant therapies with radiotherapeutic or chemotherapeutic drugs and in some cases radical cystectomy are the standard treatment

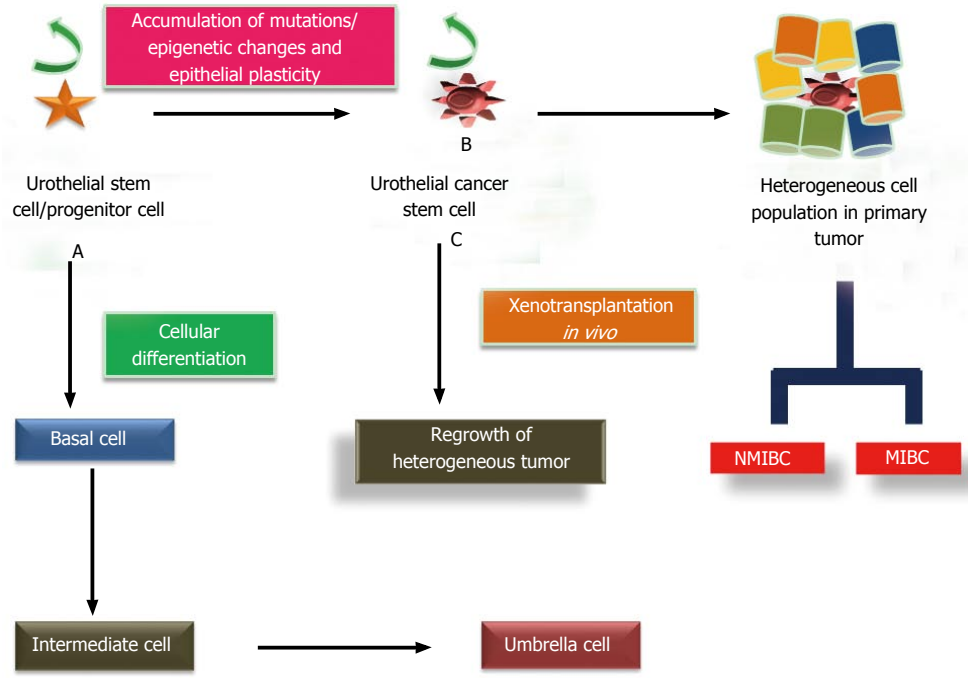

Figure 3 Cellular differentiation and mutational transformation of urothelial stem cells and dual pathways of carcinogenesis. A: Cellular differentiation of UroSCs (exist in the form of clonal patches in basal layer) gives rise to basal cells which further differentiate to intermediate cells and then into single layer of umbrella cells; B: Mutational insults including epithelial plasticity result in malignant transformation of UroSCs into self-renewing UroCSCs which undergo aberrant activation and differentiation to form papillary non muscle invasive and muscle invasive bladder cancer; C: *In vivo* xenotransplantation of a small number of UroCSCs that have the potential for the regrowth of heterogeneous tumor cells. UroCSCs: Urothelial cancer stem cells; NMIBC: Non-muscle invasive bladder cancer; MIBC: Muscle invasive bladder cancer.

options for more aggressive muscle invasive disease[19].

Cytotoxic effects of these drugs can potentially debulk tumor masses initially but tumors progressively develop between or after multiple treatment cycles in due course of time. The SP of tumor cells was found to be enriched for UroCSCs which can possibly contribute to progressive development of therapeutic resistance through enhanced survival. A number of experimental studies on human bladder cancer xenografts provide the probable mechanistic explanation for unexpected proliferative response to repopulate residual tumor cells between chemotherapy cycles. Urothelial carcinoma cell lines were examined for enriching CSCs with CD90 and CK14 expression and the effects of short- and long-term treatment with cisplatin on tumor initiating potential of these separated cells were studied. Substantial phenotypic plasticity as evident by increased expression of EMT markers, an altered pattern of CKs, and WNT-pathway target genes were observed in these sublines and instead of inducing apoptosis, it promoted neighboring CSC repopulation and subsequently the development of clinical resistance to cisplatin[20]. A strong correlation between the existence of CSC-like cells in the population of cisplatin-resistant bladder cancer cells, levels of Bmi1 and Nanog expression and the degree of malignancy of urothelial carcinoma tissues has been observed. This may play a role in the progression and drug resistance of bladder cancer[21].

Recruitment of a quiescent pool of UroCSCs into cell division in response to the cytotoxic effects of clinical drugs, similar to the mobilization of UroSCs during wound repair, reduces the efficacy of existing drugs and dramatically accelerates the pathophysiological spread of more aggressive type of bladder cancer. Combinatorial approaches based on *in vivo* administration of inhibitors of epithelial plasticity could be the probable therapeutic strategy for enhancing chemotherapeutic drug-induced damages by abrogating early tumor repopulation (source of cancer) and killing a bulk of bladder cancer cells, thereby customizing a new method to counter CSC-driven resistance, prevent relapse and improve the survival outcome in the patients with UC of the bladder.

Sox4, a biomarker of UroCSCs and one of the important candidate oncogenes, results in advanced cancer stages and poor survival rate. The results of its knockdown include reduced sphere formation and enriched cell population with high levels of aldehyde dehydrogenase [ALDH (high)]; inhibition of cell migration, colony formation as well as MET; and decreased tumor formation potential of urothelial cancer cells[22]. The essential role of αv integrins has been shown in migration, EMT and maintenance of ALDH activity, tumor growth and metastasis. Therefore, targeting of αv integrins could be a promising therapeutic approach for prevention of metastatic bladder cancer. Treatment with an αv integrin antagonist and its knockdown in the bladder carcinoma cell lines resulted in reduced expression levels of EMT-inducing transcription factors including SNAI2 and self-renewal genes NANOG and BMI1; low ALDH activity; and decreased CDH1 (E-cadherin)/CDH2 (N-cadherin),

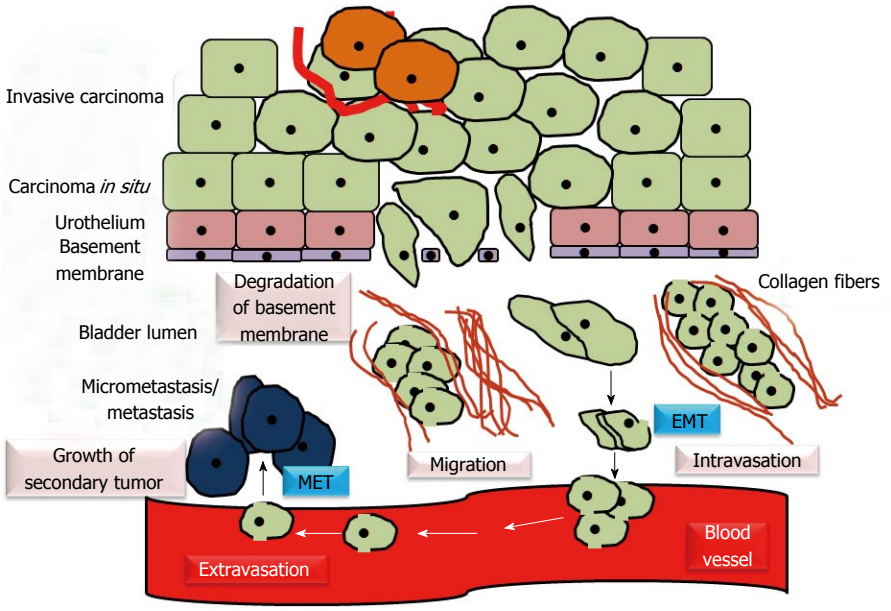

Figure 4 Epithelial mesenchymal transition and mesenchymal epithelial transition in urothelial carcinoma. EMT/epithelial plasticity allows invasive bladder cancer cells to become motile, invade the surrounding tissues and intravasate. Through bloodstream, primary tumor cells move to distant sites, extravasate, colonize the target organs and establish the metastasis. MET induces regrowth and re-establishment of cancer cells with epithelial phenotype at secondary/metastatic sites. EMT: Epithelial mesenchymal transition; MET: Mesenchymal epithelial transition.

indicative of a shift towards epithelial phenotype and decreased proliferative, migratory, clonogenic capacity and metastatic growth[23]. Overexpression of EGFR has been examined to be associated with poor prognosis in epithelial cancers. Hence, targeting cancer cells with an EGFR inhibitor (anti-EGFR antibody, cetuximab) has been shown to increase the expression of CDH1 and confer cancer cells with epithelial phenotypic property[24]. Implications of miRNAs (a class of small non-coding RNA molecules of 21-23 nucleotides in length) in the maintenance of epithelial plasticity, cancer stemness and mediating drug sensitivities make it a potential therapeutic system towards eradication of tumor recurrence and metastasis[25,26]. Forced expression of miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) has been associated with induction of MET in mesenchymal bladder cancer cell lines, which thereby restored EGFR inhibitor sensitivity to attenuate tumor aggressiveness in bladder cancer[25]. Re-expression of miR-23b may be a beneficial therapeutic strategy for the treatment of human bladder cancer by targeting Zeb1, a crucial regulator of EMT, inhibiting cell proliferation and migration and inducing apoptosis[27].

Direct suppression of epithelial plasticity with the use of inhibitors or knocking down EMT markers can also potentially reduce migration, invasion, and survival of cancer cells. Inhibitory effects of prostate-derived E-twenty six (Ets) factor (PDEF), an epithelium-specific member of the Ets family of transcription factors, on the proliferation, invasion, and tumorigenesis have been studied. Its ectopic overexpression in bladder carcinoma cells has been examined to modulate EMT by upregulating E-cadherin expression and downregulating the expression of N-cadherin, SNAIL, SLUG, and vimentin, thereby resulting in lower migration and invasion abilities of cancer cells[28]. Molecular mechanisms for ERK1/2 inhibitor to exert its antiproliferative effects in bladder cancer have been investigated. Treatment of SV-HUC-1 cells with ERK1/2 inhibitor (U0126) significantly reduced the expression of EMT markers including Snail, β-catenin, Vimentin, and MMP-2[29].

Besides inhibiting epithelial plasticity which can check dissemination and migration of invasive cells, it is also important to attenuate the reestablishment of cancer cells at distant sites through MET mechanism. In addition, elimination therapies are required to modulate the properties of UroCSCs, hence facilitate their chemosensitivity and apoptosis. This can be achieved by the application of inhibitors to target ABC transporters and drug-detoxifying enzymes. Cracking the difficult-to-reach protective niche of UroCSCs and creating an inhospitable microenvironment for them as well as for heterogeneous cancer cells at primary and distant sites may provide a basis for developing improved and effective therapeutic strategies for selective elimination of tumor cells. One of the recent studies identify the possible role of connexins, gap junction proteins found in the smooth muscles of detrusor muscle, in bladder tumorigenesis. Preliminary assessment detects the upregulation of connexin 43 in human urothelial carcinomas. Its functions in enhancing the adherence of tumor cells to stroma, increased migration potential as well as dissemination of cancer cells make it a promising target for genetic therapeutic approaches[30].

Long-term follow-up of patients and definite prediction of the biomarkers for patient survival or disease progression are the most important requirements in designing suitable therapies. High-throughput drug

screening for its anticancer effects, reliable methods for detecting the population of UroCSCs, their characterization and validation in appropriate disease models are some of the additional challenges for successful therapies.

Understanding the mechanisms and biology of UroCSCs that can control their proliferation and differentiation allows the possibility of developing effective anti-cancer drugs. Deciphering the connection between epithelial plasticity and cancer stemness paves the way to design rationale therapies for its anti-tumor effects in the clinical management of bladder cancer.

CONCLUSION

Depending upon the genomic integrity and its background, UroCSCs in basal urothelium aggressively colonize a significant region of stratified urothelium to generate histologically different tumor lesions, identical to muscle invasive bladder cancer and carcinoma *in situ*. However, intermediate cells derived from the cellular differentiation of UroSCs can give rise to non-muscle invasive papillary lesions, suggestive of dual pathways of urothelial carcinogenesis. Basal-cell specific markers are examined to be good candidates for enriching UroCSCs in the SP of tumor cells. These cells are characterized by remarkable plasticity, contribute to tumor heterogeneity, relapse, and metastasis, and thereby carry significant information in the clinical management of bladder cancer. Therapeutic applications of EMT inhibitors to reverse the epithelial plasticity may account for inhibitory functions of UroCSCs, reduced migratory and invasive properties of cancer cells and can improve therapeutic planning for better patient management.

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