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Review – Bladder Cancer

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Cancer-associated Fibroblasts in Bladder Cancer: Origin, Biology, and Therapeutic Opportunities

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Abstract

Context: Bladder cancer (BLCA) is a highly prevalent tumour and a health problem worldwide, especially among men. Recent work has highlighted the relevance of the tumour microenvironment (TME) in cancer biology with translational implications. Cancer-associated fibroblasts (CAFs) are a prominent, heterogeneous population of cells in the TME. CAFs have been associated with tumour development, progression, and poor prognosis in several neoplasms. However, their role in BLCA has not yet been exploited deeply.

Objective: To review the role of CAFs in BLCA biology and provide an understanding of CAF origin, subtypes, markers, and phenotypic and functional characteristics to improve patient management.

Evidence acquisition: A PubMed search was performed to review manuscripts published using the terms “cancer associated fibroblast” and “bladder cancer” or “urothelial cancer”. All abstracts were reviewed, and the full content of all relevant manuscripts was analysed. In addition, selected manuscripts on CAFs in other tumours were considered.

Evidence synthesis: CAFs have been studied less extensively in BLCA than in other tumours. Thanks to new techniques, such as single-cell RNA-seq and spatial transcriptomics, it is now possible to accurately map and molecularly define the phenotype of fibroblasts in normal bladder and BLCA. Bulk transcriptomic analyses have revealed the existence of subtypes among both non-muscle-invasive and muscle-invasive BLCA; these subtypes display distinct features regarding their CAF content. We provide a higher-resolution map of the phenotypic diversity of CAFs in these tumour subtypes. Preclinical studies and recent promising clinical trials leverage on this knowledge through the combined targeting of CAFs or their effectors and the immune microenvironment.

Conclusions: Current knowledge of BLCA CAFs and the TME is being increasingly applied to improve BLCA therapy. There is a need to acquire a deeper understanding of CAF biology in BLCA.

Patient summary: Tumour cells are surrounded by nontumoural cells that contribute to the determination of the behaviour of cancers. Among them are cancer-associated fibroblasts. The “neighbourhoods” established through these cellular interactions can

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now be studied with much greater resolution. Understanding these features of tumours will contribute to the designing of more effective therapies, especially in relationship to bladder cancer immunotherapy.

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1. Introduction

Bladder cancer (BLCA) is the tenth most commonly diagnosed cancer in the world, with 573 278 new diagnoses and 212 526 deaths during 2020; 74.7% of the total burden corresponds to men [1]. At diagnosis, 70% of patients present with non-muscle-invasive BLCA (NMIBLCA), while 25% of patients present with muscle-invasive BLCA (MIBLCA, $\geq T2$) and 5% have distant metastases [2,3]. NMIBLCA is one of the tumours with the highest recurrence rate: in the first 5 yr, 40–70% of NMIBLCA will recur and 10–30% will progress to MIBLCA [4–6]. The 5-yr overall survival (OS) rate for patients with metastatic disease is 5% [7,8]. Although new therapies for advanced BLCA have been developed in recent years, including small molecules targeting fibroblast growth factor receptor (FGFR), anti-PD-(L)1 antibodies, and drug-antibody conjugates, first-line treatment remains unchanged.

The main, established, risk factors for BLCA include tobacco, occupational exposures to aromatic amines and polycyclic aromatic hydrocarbons, and genetic variation, among others. The evidence for other factors, such as cystitis, is less strong [9,10].

BLCA develops through two distinct morphogenetic pathways, both of which are associated with a high prevalence of *TERT* mutations and partial or complete loss of chromosome 9: (1) the *hyperplasia/papillary* pathway, characterised by mutations in *FGFR3*, *STAG2*, and *KDM6A*, and (2) the *dysplasia/carcinoma in situ* pathway with a high frequency of alterations in *TP53*, *RB1*, and genes involved in chromatin remodelling.

The past decade has brought a great interest in understanding the role of the tumour microenvironment (TME) in the development and progression of cancer. Simple histology shows that papillary low-grade tumours contain a low proportion of stromal cells, which increases with stage and grade. The TME includes the extracellular matrix, cancer-associated fibroblasts (CAFs), adipocytes, nerves, smooth muscle, and vascular and immune cells. A BLCA transcriptome analysis has revealed a direct association of the abundance ratios of immune and stromal cells with shorter survival in The Cancer Genome Atlas (TCGA) cohort [11–13]. While many studies have addressed the relationship between the immune TME and outcome or response to therapy in BLCA, much less is known about the role of CAFs. Here, we review current knowledge on BLCA CAFs, their heterogeneity, and relevance in tumour biology and therapy.

2. Evidence acquisition

A PubMed search was performed to review manuscripts published using the terms “cancer associated fibroblast”

and “bladder cancer” or “urothelial cancer”. All abstracts were reviewed, and the full content of all relevant manuscripts was analysed. In addition, selected manuscripts on CAFs in other tumours were considered.

3. Evidence synthesis

3.1. CAFs: origin, heterogeneity, and plasticity

In most normal organs, the stroma contains quiescent or resting fibroblasts. Invasion by neoplastic cells initiates a chronic wound healing-like response involving fibroblast activation. This response includes upregulation of collagen and other components of the extracellular matrix (ECM), fibroblast activation protein (FAP), α -smooth muscle actin (α -SMA), and inflammatory cytokines.

CAFs are a heterogeneous population of cells the origin of which is under debate. They can originate from tissue resident cells but also from circulating bone marrow-derived precursor cells that—upon recruitment to the tumour site—differentiate to CAFs [14]. Transdifferentiation of pericytes, and endothelial and epithelial cells can also give rise to a CAF-like phenotype [15–17]. This diversity has fostered major efforts to identify the markers of CAF subtypes and characterise CAF subpopulations. In addition to their origin, functional regulation can profoundly impinge on fibroblast phenotype. A consensus statement has recently been published summarising current knowledge [18]. Despite the broad relevance of CAFs in cancer biology, little work has been done in BLCA.

The markers most commonly used to identify CAFs include α -SMA, Fibroblast-Specific Protein 1 (FSP1; also known as S100A4), vimentin, FAP, desmin, and platelet-derived growth factor receptors (PDGFRs α/β). However, none of these markers is completely exclusive to the fibroblast lineage. For example, macrophages express FSP1, FAP is present in a subset of CD45+ cells [19], and PDGFR β and desmin are expressed in a subset of perivascular cells and smooth muscle cells [20]. The expression of these proteins is dynamically regulated, thus introducing another level of variation [21,22]. Hence, multiple markers as well as spatial distribution need to be considered to distinguish between quiescent and activated fibroblasts. Single-cell RNA-sequencing (scRNA-seq) and spatial transcriptomics have begun to provide a glimpse of the heterogeneity of normal fibroblasts (NFs) and CAFs, and to infer lineage relationships [23].

In cancer, FAP+ cells that are α -SMA-high and produce high levels of ECM components have been named myofibroblastic CAFs (myCAFs). Another α -SMA-low subpopulation of CAFs secreting interleukin (IL)-6, IL-11, and leukaemia inhibitory factor, which can activate the signal

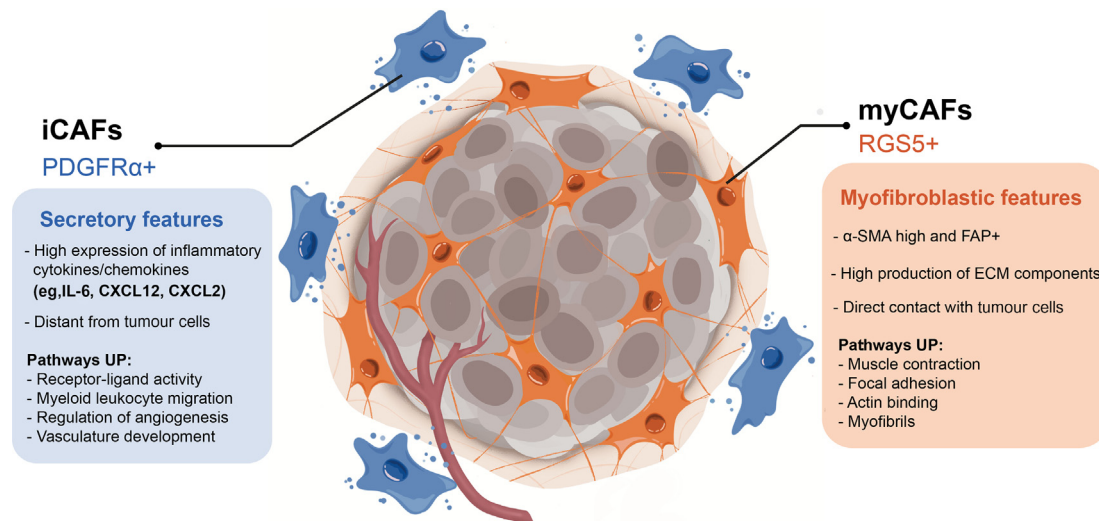


Fig. 1 – Cancer-associated fibroblasts in bladder cancer. Schematic representation of the PDGFR α (iCAF-like) and the RGS5+ (myCAF-like) fibroblast subtypes identified in BLCA. Their essential features are summarised in the boxes. BLCA = bladder cancer; ECM = extracellular matrix; FAP = fibroblast activation protein; iCAF = inflammatory cancer-associated fibroblast; IL = interleukin; PDGFR = platelet-derived growth factor receptor; α -SMA = α -smooth muscle actin.

transducer and activator of transcription 3 in epithelial cells, is referred to as inflammatory CAFs (iCAFs; Fig. 1). Öhlund et al [24] first reported that CAF subtypes display a distinct spatial distribution in mouse and human pancreatic ductal adenocarcinoma (PDAC): myCAFs are in direct proximity to neoplastic cells, forming periglandular rings, whereas iCAFs are more distant from tumour cells. A third CAF subtype with high expression of MHC class II and CD74, designated as antigen-presenting CAFs (ap-CAFs), has also been reported in mouse and human PDAC [25].

Fibroblasts with features of myCAFs and iCAFs have also been identified in BLCA: PDGFR α fibroblasts showed high expression of cytokines and chemokines, such as IL-6, CXCL12, and CXCL2, and are reminiscent of the iCAFs identified in PDAC. These iCAFs were found to have a pro-proliferative effect on tumour cells and can promote angiogenesis by producing vascular endothelial growth factor (VEGF) that acts on endothelial cells. CXCL12 secreted by bladder iCAFs may be responsible for the infiltration of immune cells into the tumour. On the contrary, RGS5+ fibroblasts found in BLCA show high expression of genes coding for focal adhesion and ECM components, similar to PDAC myCAFs [25,26]. Further studies will be needed to determine whether fibroblast subpopulations from different tissues have distinct phenotype and function (Fig. 1). Comparative reports reveal fibroblast heterogeneity across tissues; the transcriptional profiles of fibroblasts display higher similarities with those of other structural cells within the same tissue than with the profiles of fibroblasts from other organs [27,28]. These observations raise questions about the relationship between normal tissue resident fibroblasts and CAFs, and about the molecular mechanisms driving these phenotypes.

Within a given tissue, distinct—spatially delimited—functional fibroblast populations have been identified in the gut, skin, breast, liver, and bladder [29]. Yu et al [30] performed scRNA-seq of normal human and mouse bladder, and identified five clusters of vimentin-expressing stromal

cells: three corresponded to fibroblasts, one to myfibroblasts, and another one to a new class of interstitial cells expressing high levels of α 2A-adrenergic (ADRA2A) and H2 histamine (HRH2) receptors. In normal mouse bladder, Baker et al [31] have also used scRNA-Seq and spatial transcriptomics to identify three topologically organised tissue-resident fibroblast subtypes with distinct transcriptional profiles: Car3+ fibroblasts localise to the suburothelial space, Npy1r+ localise to the lamina propria, and Penk+ fibroblasts are found in the detrusor muscle (Fig. 2A). The Car3+ population resembles the myCAF subtype, while Penk+ cells display iCAF features. As previously reported for myCAFs and iCAFs, transforming growth factor (TGF) β 1 and PDGFR β drive the differentiation of Car3+ and Penk+ NFs, respectively [24,31]. Lineage tracing experiments are required to determine which population of NFs gives rise to the CAF subtypes reported in BLCA.

The simple distinction of CAF subtypes may in fact be an oversimplification, as suggested by scRNA-seq. Based on gene signatures, four subpopulations of CAFs were described in a recent scRNA-seq analysis of human BLCA: resting fibroblast-like (C1), iCAF-like (C2), myCAF-like (C4), and a new subpopulation denominated interferon-regulated CAFs (irCAFs, C3). The irCAF subpopulation characteristically showed higher expression levels of growth factor-related genes such as *NRG1*, *STC1*, and *WNT5A*, and could be identified by immunohistochemistry through the expression of SLC14A1, a membrane urea transporter. This subpopulation of CAFs was shown to be a biomarker of response to therapy and a predictor of shorter survival [32]. In breast cancer (BrC), Pelon et al [33] identified four fibroblast clusters (S1-S4): S1- and S4-CAF were found to have myofibroblast features, and were present in primary cancers and metastatic lymph nodes but not in normal tissue. Both S1-CAF and S4-CAF stimulate BrC cell motility but do so via different mechanisms: S1-CAFs initiate an epithelial-to-mesenchymal transition (EMT) and secrete soluble cancer cell chemoattractants (eg, CXCL12 and TGF β),

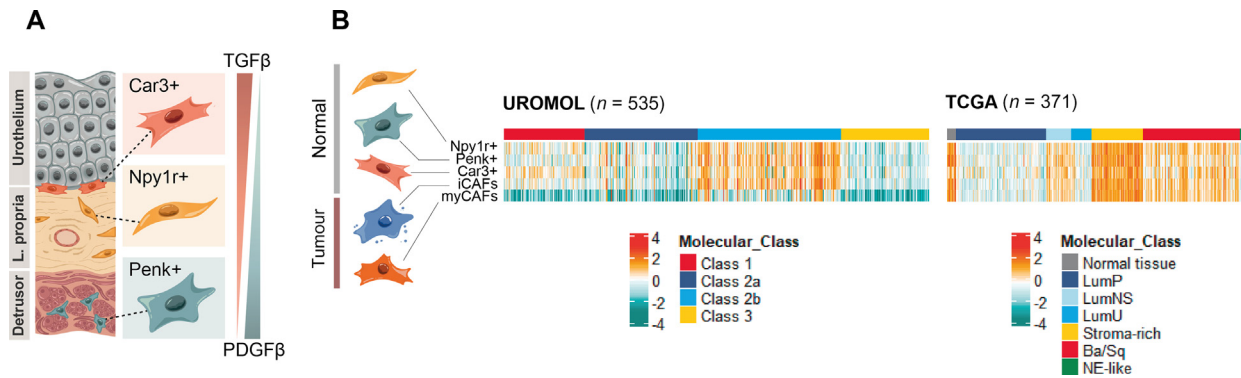


Fig. 2 – Fibroblast heterogeneity in BLCA molecular subtypes. (A) Schematic representation of the three normal mouse bladder fibroblast subtypes identified using scRNA-seq and spatial transcriptomics. Car3+, Npy1r+, and Penk+ fibroblasts display distinct spatial distribution. (B) Heatmaps show Z-score enrichment of fibroblast signatures in samples from the UROMOL (left) and TCGA (right) cohorts using a single-sample gene set enrichment analysis. Ba/Sq = basal/squamous; BLCA = bladder cancer; LumNS = luminal nonspecified; LumP = luminal papillary; LumU = luminal unstable; NE = neuroendocrine; PDGF = platelet-derived growth factor; TCGA = The Cancer Genome Atlas; TGF = transforming growth factor.

while S4-CAFs remodel the matrix and promote cancer cell invasion in three-dimensional cultures by increasing contractility through the NOTCH signalling pathway. A subsequent analysis from the same group has further dissected S1-CAF heterogeneity. Eight clusters with specific transcriptional profiles were found, including those overlapping with “myCAF” and “iCAF” subtypes. S1-CAF clusters 1, 2, and 5 were reminiscent of iCAF (detox-iCAF, IL-iCAF, and IFN γ -iCAF) and clusters 0, 3, 4, 6, and 7 were reminiscent of myCAF (ecm-myCAF, TGF β -myCAF, wound-myCAF, and acto-myCAF) [34]. The relationship between the BLCA fibroblasts described above and those reported in other tumours remains to be determined.

3.2. CAFs and BLCA molecular subtypes

CAF heterogeneity is reflected not only across cancer types, but also across patients, having a variable impact on prognosis and response to therapy. For instance, triple negative BrC contains a higher proportion of iCAFs than luminal A tumours, which contain more myCAFs. Using the scRNA-seq-derived subtype signatures described above, detox-iCAF and IL-iCAF are found to be enriched in triple negative compared with luminal A BrC, whereas ecm-myCAF, TGF β -myCAF, and wound-myCAF are enriched in luminal A versus triple negative tumours [34]. These associations suggest that stromal cells may impact the tumour cell phenotype, or vice-versa.

In the past decade, several BLCA transcriptomic classes have been identified, which differ regarding genetic alterations, dysregulated oncogenic pathways, degree of immune and stromal cell infiltration, and prognosis. In NMIBLCA, four transcriptomic classes have recently been reported (1, 2a, 2b, and 3) [35]. In MIBLCA, a consensus molecular classification identified six transcriptomic subtypes: luminal papillary (LumP), luminal nonspecified (LumNS), luminal unstable (LumU), stroma rich (SR), basal/squamous (Ba/Sq), and neuroendocrine like (NE) [36].

It is still unknown whether the BLCA transcriptomic classes are associated with specific fibroblast subtypes. To explore this question, we interrogated the enrichment of gene signatures of normal bladder fibroblasts (Car3+,

Npy1r+, and Penk+) and from CAFs (iCAFs and myCAFs) in the UROMOL (NMIBLCA) [35] and TCGA (MIBLCA) transcriptomic datasets [36,37]. Each signature contains the genes that are differentially expressed in a given cluster, compared with the others [27,32]. Among NMIBLCA, class 2b tumours, characterised by high immune infiltration and the expression of cancer stem cell markers, exhibit the highest activity of the fibroblast signatures. In contrast, class 3 tumours—characterised by *FGFR3* mutations and immune cell depletion—display the lowest signature activity. When we analysed the expression of fibroblast subtype gene signatures within discrete NMIBLCA subtypes, myCAFs were selectively depleted in all subgroups. The other signatures were equally represented in all the subtypes, except for class 1 tumours where the Car3+ and Npy1r+ signatures were significantly enriched (Fig. 2A). In MIBLCA, in agreement with previous reports, Ba/Sq and SR tumours display the highest activity of the fibroblast signatures. Of the three luminal subtypes, LumNS tumours are more stromal enriched [27,36]. We did not detect a significant increase in the activity of a particular fibroblast signature in any of the MIBLCA molecular subtypes, although myCAF and Npy1r+ were the less represented fibroblast subtypes in LumP, with the highest scores in the Ba/Sq tumours (Fig. 2B).

Overall, our analyses suggest that: (1) specific NF subtypes might be associated with NMIBLCA groups, (2) the predominant CAF phenotype might be different in luminal and Ba/Sq MIBLCAs, and (3) myCAFs may be associated with the progression of BLCA to muscle-invasive disease, consistent with previous histopathological studies [38].

3.3. Role of CAFs in invasion, metastasis, and patient outcome

There has been a long debate on whether CAFs have pro- or antitumour activity. In PDAC, a very desmoplastic cancer, genetic elimination of α -SMA+ CAFs, or suppression of the stroma by deleting *Shh*, resulted in more aggressive tumours [22,39–41]. In BrC, elimination of thymidine kinase-expressing FSP+/S100A4+ stromal cells with ganciclovir did not influence primary tumour growth, but it sup-

pressed metastasis [42]. These findings, together with the heterogeneity described above, indicate that the role of CAFs is complex and context dependent.

In NMIBLCA, overexpression of CXCL1 and IL-6 has been associated with increased recruitment of CAFs and tumour-associated macrophages, low expression of E-cadherin, aggressiveness, and poor prognosis [43]. In another study, IL-6 overexpression in BLCA reduced tumourigenesis and cell invasion associated with a switch to the expression of E-cadherin instead of N-cadherin and vimentin [44]. In vitro, CAFs and BLCA cells can establish cytokine/chemokine-based feedback loops that contribute to enhanced tumour cell migration and invasion [27,45]. Prominent among these cytokines is CAF-derived TGF β 1, which induces EMT and the in vitro invasion of BLCA cells through SMAD2 phosphorylation [46]. Similarly, epidermal growth factor and TGF α induce an EMT in cultured human BLCA cells [47]. Using mouse models of BLCA, Lee et al [48] have shown that CAF-produced collagen I promotes tumour cell invasion in the primary tumour through CD167a (also known as Discoidin Domain Receptor 1, DDR1), a collagen receptor. In turn, CD167a is subsequently used to colonise the lung in a Stat3-dependent manner.

The association of CAF abundance/features with outcome has also been analysed widely. Two recent metadata analyses revealed that high activity of a CAF gene signature, or myCAF marker genes, was associated with significantly shorter OS and disease-free survival of BLCA patients. These reports revealed novel prognostic biomarkers related to CAFs such as Caldesmon 1 gene (*CALD1*) and highlighted their association with immunosuppressive TME [49,50]. Mezheyeuski et al [51] assessed the association of CAF marker expression with prognosis in BLCA. A multivariable analysis showed a strong positive association of CD90, FAP, and PDGFR β levels with tumour stage and grade, with the FAP-dominant patient group having the worst 5-yr OS. The immunosuppressive functions of FAP-expressing fibroblasts and an association with worse prognosis have previously been reported in other cancer types [52–54]. On the contrary, cases with CD90-enriched stroma were associated with a higher CD8 infiltration and more favourable 5-yr OS [51].

Recent studies in PDAC and BrC suggest that CAF subset composition (rather than their sheer number) may be associated with disease progression. Thus, myCAF s appear to be protumoural and have been linked to poor prognosis [24,55]. The data reviewed here, and the transcriptome analyses presented above, suggest that this is also true for BLCA, but further investigations should be carried out to demonstrate the contribution of CAF subsets to BLCA phenotypes and disease progression.

The negative association of CAF abundance/activity with outcome might result from their ability to modulate the growth and metastatic potential of tumours at different levels by: (1) promoting EMT and cancer cell invasion, (2) favouring angiogenesis, (3) suppressing innate and adaptive anti-tumour immune responses, (4) inducing ECM remodelling, and (5) contributing to the metastatic niche [56,57]. These processes can be mediated through the secretion of protumoural and proangiogenic growth factors (eg, TGF β ,

HGF, FGF, and VEGF) and cytokines (eg, CXCL1, CXCL12, CXCL14, and IL-6), secretion of ECM proteins [58], and production of extracellular vesicles [59–61].

3.4. CAFs as predictors of response to BLCA therapies

There is increasing evidence that CAFs can also affect drug efficacy and contribute to drug resistance. A wide variety of mechanisms may be involved, including direct tumour cell-CAF crosstalk, modulation of tumour cell-ECM interactions, and cytokine- or chemokine-mediated signalling [62,63]. CAFs can broadly influence drug pharmacodynamics through the secretion of ECM proteins that assemble to increase interstitial fluid pressure and tumoural stiffness, thus reducing drug uptake [64,65]. They can also impact mechanotransduction signalling (ie, YAP/TAZ) [66]. Consequently, therapeutic targeting of CAFs may enhance antitumour activity. We summarise below current knowledge on the evidence that CAFs can impact the prediction of response to therapy.

3.4.1. Chemotherapy

Cisplatin-based CT remains the most effective and widely used treatment for advanced BLCA, with response rates in the range of 50%. However, 50% of patients cannot receive this drug due to comorbidities or poor performance status. Cisplatin-based CT also constitutes the standard of neoadjuvant treatment, although only 30–40% of patients achieve a pathological complete response and the improvement in survival is modest (OS benefit of 5% at 5 yr). Thus, elucidating the determinants of response and chemoresistance is a priority to personalise BLCA treatment [67,68].

An insulin-like growth factor-oestrogen receptor β (ER β) signalling axis has been shown to promote the activation of BLCA fibroblasts and promote cisplatin resistance through the upregulation of the antiapoptotic protein BCL-2 [69]. The role of ER β in tumour progression, through the upregulation of BCL-2, has also been associated with patient outcome [70,71].

Another scenario where CAFs may play an important role is in the postchemotherapy (post-CT) setting: treatment results in histological changes involving the TME and CAFs and stromal-derived factors may provide a survival benefit to cancer cells, resulting in tumour relapse and progression [72]. In non-small cell lung cancer, the number of CAFs is increased in the tumour bed after CT administration, and there is an inverse relationship between the amount of tumour and stromal cells [73]. In CT-treated colorectal cancer patients, CAF-derived IL-17A promotes tumour cell growth and relapse. Little work has been done on the effect of cisplatin-based CT on the TME in BLCA, although a molecular classification of platinum-based neoadjuvant CT-treated tumours has been identified. Within this classification, the tumours with the wound-healing and scar-like subtype (CC4) had the best prognosis [74]. In addition, in a cohort of patients with MIBLCA treated with platinum-based neoadjuvant CT, a higher percentage of irCAF s has been associated with treatment resistance and worse prognosis [32].

3.4.2. Immunotherapy

In recent years, immune checkpoint inhibitors (ICIs) have become a key component of the standard therapy of patients with metastatic BLCA (mBLCA), with 20–30% response rates and increased long-term survival. Similar promising results have been obtained in the neoadjuvant setting. However, the genetic, molecular, and cellular bases underlying the reason why only a subset of patients respond are not well established. The expression of programmed death ligand 1 (PD-L1) in tumour or immune cells, CD8⁺ T-cell infiltration, immune signatures, tumour mutational burden, and alterations in DNA repair genes have been proposed as predictive markers, but none of these has shown consistency across studies [75–80]. Necchi et al [81] have analysed the bladder of patients who received neoadjuvant pembrolizumab and described a “scar/wound healing” signature in 50% of them. It is tempting to speculate that CAFs contribute to this transcriptomic phenotype. In preclinical models of colorectal cancer and PDAC, CAFs mediate resistance to ICIs [52,54]. In BLCA, the irCAF signature has been associated with worse response to ICIs and poor survival [32].

To date, one of the most robust markers predictive of resistance to ICIs is the transcriptional activity of the TGF β pathway. Both in BLCA and in other tumours, TGF β —mainly produced by CAFs—has been linked to ICI resistance. TGF β signalling promotes EMT and favours an immunosuppressive TME with reduced differentiation/activity of T lymphocytes and other immune cells [82]. A TGF β risk score built with TGF β differentially expressed genes between BLCA and nontumoural tissue samples has been shown to be prognostic. Besides, high TGF β risk scores were positively associated with fibroblast signatures, immune hot TMEs, and the expression of inhibitory immune checkpoint-related proteins [83]. In the IMvigor210 study, where atezolizumab was administered as first-line therapy for mBLCA, three immune tumour phenotypes were described: (1) immune desert, with no infiltrating lymphocytes; (2) immune excluded, with T cells present in the stroma; and (3) immune inflamed, with a large immune infiltrate and PD-L1 expression. The highest TGF β signature activity was found in immune-excluded tumours, where CD8⁺ T cells were absent from tumour cell areas and were trapped in the desmoplastic stroma, conferring a lower response to ICIs [84]. A recent reanalysis of the ABACUS phase 2 trial, with a median follow-up of 25 mo, showed longer relapse-free survival in patients who received neoadjuvant atezolizumab and had higher pretreatment stromal CD8⁺ (hazard ratio [HR] 0.25, 95% confidence interval [CI] 0.09–0.68, $p = 0.007$); post-treatment FAP expression was associated with poor outcome (HR 4.1, 95% CI 1.3–13, $p = 0.01$) [85]. These data point to an active immunosuppressive role of CAFs through TGF β that hampers ICI responses and support the notion that assessing the activity of the stroma could impact patient management.

FGFR3 mutations have been associated with low PD-L1 non-T-cell inflamed tumours [86]. In BrC, *FGFR3* alterations have been associated with decreased T-cell infiltration and resistance to ICIs [87]. However, the analysis of the main clinical trials with ICIs in mBLCA (ie, IMvigor210,

atezolizumab, and CheckMate 275, nivolumab) and real-world experience have failed to show statistically significant differences in response rates between *FGFR3*-mutant and wild-type tumours [88,89].

3.4.3. Radiotherapy

Radiotherapy (RT) has classically had a modest relevance in BLCA treatment, largely in patients who were not candidates for radical cystectomy or as part of palliative care. In the past few years, trimodal therapy consisting of maximal transurethral resection of bladder tumour followed by chemoradiation has become an attractive alternative for selected patients and is currently referred to in the European Association of Urology guidelines [90]. A proposed effect of CAFs on tumour radioresistance is insufficiently established, and the effects of RT on CAFs in other tumours are variable [91]. Therefore, the crosstalk between CAFs and BLCA cells in the context of RT remains underexplored and worthy of additional work.

3.4.4. New therapies

Drugs targeting FGFRs have recently been approved for patients with *FGFR3*-mutant mBLCA. *FGFR* alterations are more common in NMIBLCA and in the papillary MIBLCA subtypes. These tumours have less stroma, and patients have a better outcome, raising the question of whether *FGFR3* mutations have an impact on the composition and activity of the stroma [92,93]. Bioinformatics analyses reveal that tumours with high myCAF gene signature scores display low *FGFR3* mutation frequency [49]. In BLCA, *FGFR3* fusions have been associated with fewer myCAF, which may explain the more favourable prognosis [94].

Other important novel therapies include enfortumab vedotin and sacituzumab govitecan, antibody-drug conjugates targeting nectin-4, and the trophoblast cell-surface antigen 2 (Trop-2), respectively. They have recently been approved for patients with mBLCA with progression to platinum-containing CT and ICIs. There are no reports yet on the putative relevance of CAFs and the stroma in the context of these new therapies.

3.5. Targeting CAFs as a therapeutic opportunity in BLCA

While there are many options for targeting CAFs (Fig. 3), it is conceivable that the greatest therapeutic impact will emerge from combination therapies. Given the association of TGF β with ICI resistance, the simultaneous targeting of the TGF β and PD-1/PD-L1 pathways is one of the most attractive approaches and the main objective of several on-going clinical trials. Preclinical studies have demonstrated that the selective inhibition of TGF β 1 using the fully humanised monoclonal antibody SRK-181, combined with anti-PD-1, can overcome the primary resistance of ICI-refractory BLCA [95,96]. Similar findings were reported in a murine metastatic colon cancer model where the combined inhibition of TGF β receptor signalling, using galunisertib, and the PD-1/PD-L1 axis also led to antitumour responses. In both cases, the concomitant blockade of PD-1/PD-L1 and TGF β was associated with increased infiltration by CD8⁺ T cells [97]. In preclinical models, continuous long-term exposure to galunisertib caused cardiotoxicity, which

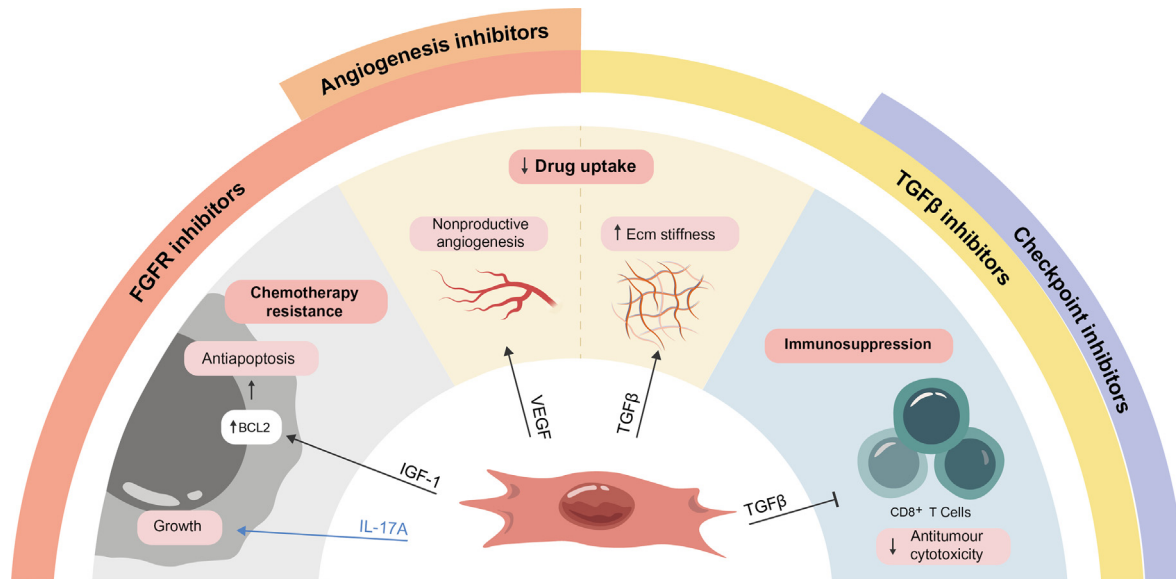


Fig. 3 – Targeting CAFs in BLCA. Scheme displaying some of the mechanisms through which CAFs may impact therapy efficacy and strategies proposed to address CAF-mediated therapeutic failure. Direct tumour cell-CAF crosstalk favours chemotherapy resistance in BLCA through the IGF-1/BCL2 axis. In colon cancer, resistance to chemotherapy is mediated by CAF-secreted IL-17A. CAF-mediated angiogenesis and ECM remodelling may impact drug delivery. CAF-secreted TGF β promotes an immunosuppressive TME, depleted of CD8 $^{+}$ T cells. BLCA = bladder cancer; CAF = cancer-associated fibroblast; ECM = extracellular matrix; FGFR = fibroblast growth factor receptor; IL = interleukin; TGF = transforming growth factor; VEGF = vascular endothelial growth factor.

was solved with an intermittent dosing regimen (14 d on/14 d off, on a 28-d cycle); this dose is currently used in on-going trials, both in monotherapy and in combination [98,99]. These preclinical works have led to an on-going phase 1 trial of SRK-181, alone or in combination with anti-PD-L1, in patients with locally advanced or metastatic solid tumours (NCT04291079) and a phase 2 trial of durvalumab (anti-PD-L1) combined with the TGF β receptor inhibitor vactosertib in patients with advanced or recurrent BLCA refractory to ICIs (NCT04064190) [100]. Vactosertib has already been tested in combination with pembrolizumab in patients with previously treated microsatellite-stable metastatic colorectal cancer with a favourable safety profile but modest activity, with an objective response rate of 15.2%; the median duration of response has not been reached yet [101].

Recent protein engineering strategies have led to the design of bintrafusp alfa, a bifunctional fusion protein that inhibits simultaneously TGF β and PD-L1. When combined with RT, bintrafusp alfa showed a reduction in RT-induced fibrosis and an increase in CD8 $^{+}$ T-cell infiltration in multiple murine tumour models, including PDAC and BrC, among others [102]. In vitro studies showed that bintrafusp alfa rendered BLCA cells more susceptible to NK- and antigen-specific CD8 $^{+}$ T-cell-mediated killing [103]. However, the phase 2 study of bintrafusp alfa in CT- and immunotherapy-refractory BLCA patients was terminated due to safety concerns (NCT04501094).

Losartan, an angiotensin II receptor blocker, has also been tested as a TGF β signalling suppressor with antifibrotic properties, and has demonstrated promising results increasing OS in mice with BrC or PDAC and in a PDAC trial in combination with CT and RT. Losartan is currently being tested in a PDAC clinical trial combined with CT, RT, and ICI (NCT03563248) [104–106].

4. Conclusions

Understanding the TME is the key not only to unravel tumour biology, but also to harness this information to develop/improve novel precision therapy strategies. The BLCA TME is highly heterogeneous, reflecting the diversity of tumour subtypes, and is under-researched.

Future work should aim at discerning how fibroblasts contribute to the composition of the stroma during BLCA progression, their crosstalk with tumour and immune cells, and how these processes can be leveraged to improve therapy. The burgeoning fields of single-cell genomics, spatial transcriptomics, and computational biology—together with the ease of access to BLCA tissue and urine—will undoubtedly contribute to these aims.

The most immediate applicability of these findings is reflected in the role of CAFs as a major source of TGF β , a key mediator of resistance to ICIs. There is much expectation in the on-going clinical trials focused on the combination of anti-TGF β agents with immunotherapy. Challenges ahead include reducing the associated toxicities to optimise combination therapies and discovering new drug combinations.

Author contributions: Francisco X. Real had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Caramelo, Zagorac, Corral, Marqués, Real.

Acquisition of data: Caramelo, Zagorac, Corral, Marqués.

Analysis and interpretation of data: Caramelo, Zagorac, Corral, Marqués, Real.

Drafting of the manuscript: Caramelo, Zagorac, Corral, Marqués, Real.

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