

The role of E2F transcription factors family in Urothelial Carcinoma

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Abstract

E2F transcription factor family is a group of 8 proteins that interact with pocket proteins and bind to target DNA in order to modulate cell cycle progression. Thus, their role is pivotal to cancer development and progression. Bladder cancer, a prominent cancer that affects both sexes, is also affected by E2F. E2F-1, E2F-3 and E2F-4 and -5 have a distinct impact on bladder development, progress and prognosis. However the role of the remaining E2Fs remains obscure, awaiting newer data that could provide useful therapeutic targets.

Key words: E2F transcription factor, cancer, bladder, apoptosis, regulation

Introduction

Urothelial carcinoma, also known as transitional cell carcinoma (TCC) or just plain bladder cancer is by far the most common type of bladder cancer, accounting for almost 90% of all cases of bladder cancer. It arises from the urothelium of the bladder, but since urothelial cells line other parts of the urinary tract, it is not uncommon that patients with bladder cancer sometimes also present tumours to the renal pelvis, the ureters and the urethra [1]. It affects both men and women (3:1 ratio) being most prevalent to Caucasians than to Asians or Blacks. In 2012, bladder cancer was the ninth most frequent cancer worldwide (in both sexes) and the 13th most common cause of cancer death [2].

Bladder cancer is a chronic disease, affected by both genetic and environmental parameters, and some of the risk factors associated with the development of the initial tumour may influence its progress and final outcome. Smoking and chronic inflammation are the most studied environmental

factors. Chronic infection with *Schistosoma haematobium*, following exposure to infested water, particularly in Africa and the Middle East, is a probable cause, while exposure to industrial chemicals such as metalworking fluids is also implicated in bladder cancer [3].

E2F is a group of genes, spread throughout the genome, encoding for basic helix-loop-helix transcription factors that control expression of a variety of genes involved in cell cycle progression.

There are several studies on E2F on bladder cancer development and prognosis, however there are no definite conclusions on their individual impact nor any mechanism of their action. This is due partially to the complexity of cancer development and partially to the wide range of action of E2F family. In this short review, we will present all the current knowledge on E2F involvement in bladder cancer development and progression and we will discuss potential therapeutic targets based on these findings.

E2F family of transcription factors

To date, eight E2F genes, producing 10 distinct E2F proteins, have been identified that exhibit varying degrees of sequence and structural differences, but share a very well conserved DNA binding domain [4]. E2F transcription factors typically form heterodimeric complexes with transcription factors (through a partner protein domain) and also have N-terminal DNA binding and dimerization domains (**Fig.1**). E2F transcription factors can act as mediators of transcriptional activation (E2F 1-3) or repression (E2F 4-8) (**Table 1**). Among E2F transcriptional targets are cyclins, cyclin dependent kinases (CDKs), checkpoints regulators, DNA repair and replication proteins. DNA microarray analysis reveals unique sets of target promoters among E2F family members suggesting that each protein has a unique role in the cell cycle, but there is a great deal of redundancy among them [5].

Normally, the Rb pathway constitutes a central molecular module involved in the regulation of cell cycle progression, while in cancer cells, alterations in this pathway promote a faster cell cycle and drive

uncontrolled tumour growth [6, 7]. In non-proliferating cells, Rb protein remains hypophosphorylated in the nucleus and forms Rb-E2F repressor complexes to inhibit the expression of genes that promote S phase entry; additionally, it directly binds to and inhibits the activity of E2F activators (E2F-1, E2F-2, E2F-3). Specifically, during the G0 phase, expressed E2F-4 and E2F-5 limit the expression of E2F-targets which promote entry into the G1 phase of the cell cycle. Mitogenic signalling results into the phosphorylation of Rb protein by cyclin-dependent kinases. This renders Rb protein inactive, directs the release of the E2F repressors and results into the increased expression of the activators E2F -1, -2 and -3 at the end of this phase. The presence and action of these activators on cognate target genes, triggers a transcriptional programme driving cells into S phase; the action of the repressors E2F-6, E2F-7 and E2F-8 brakes the S phase, while E2F-7 and E2F-8 can also specifically mediate the repression of E2F activators. Without this braking mechanism E2F-11 proteins would continue to the activation of genes encoding transcriptional components and aberrant DNA replication [4].

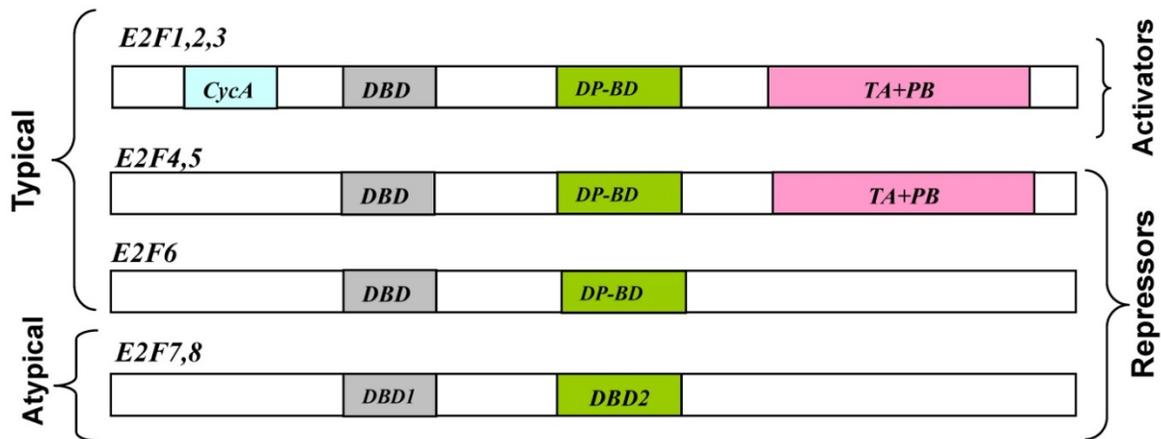


Figure 1. Schematic representation of E2F transcription factors (N-to-C direction). Note that the typical E2Fs have DP-binding domains, which are replaced by a second DBD in the atypical E2Fs [Adapted from [54]]. CycA: cyclin A-binding domain; DBD: DNA-binding domain; DP-BD: Dimerization-binding domain; TA+PB: transactivation and pocket protein-binding domains.

Table 1. Summary of E2F localization and action in urothelial carcinoma [52].

Factor	Localization	Cell cycle	General action	Bladder cancer	Reference
E2F-1 ¹	Nucleus	G1/S-phase	Induction	Overexpression Decreased expression	Hayami et al., 2010
E2F-2 ¹	Nucleus	G1/S-phase	Induction	Overexpression	Hayami et al., 2010
E2F-3a ²	Nucleus	G1/S-phase	Induction	Overexpression	Hurst et al., 2008
E2F-3b ²	Nucleus	G0/G1 phase	Suppression		Hurst et al., 2008
E2F-4 ³	Cytoplasm	G0/G1 phase	Suppression	Translocate to nucleus	Parisi et al., 2009
E2F-5 ⁴	Cytoplasm/	G0/G1 phase	Suppression	Translocate to nucleus	Umamura et al., 2009
E2F-6 ⁵	Nucleus	G1/S phase	Suppression		Yang et al., 2007
E2F-7 ⁶	Nucleus	S-phase	Suppression		Weijts et al., 2012
E2F-8 ⁶	Nucleus	G1/early S-phase	Suppression		Weijts et al., 2012

The inactivation of the retinoblastoma (Rb) pathway is a common occurrence in most human cancers. The main view for carcinogenesis in most human cancers, is that inactivation of Rb results in disassembly of Rb-E2F co-repressor complexes, alleviating the repression of genes that are necessary for progression through the cell cycle. Furthermore, the dissociation of hyperphosphorylated Rb from E2F activators leads to the inappropriate accumulation of free E2F-1, E2F-2 and E2F-3 with unmasked transactivation domains, resulting in additional transactivation of these genes. According to this prevailing model, transcriptional regulation by canonical E2Fs is controlled through association with the pocket proteins, i.e. the retinoblastoma (Rb) family of tumour suppressor proteins (pRb, p107 and p130), in the case of E2F 1-5, or in the case of E2F-6, with the epigenetic silencing polycomb group (PcG) [8]. p130 and p107 mainly interact with E2F-4 and E2F-5, while pRb preferentially binds E2F 1-3 [9]. These associations facilitate recruitment of histone deacetylases and methyltransferases to target promoters and subsequent transcriptional repression. Phosphorylation of Rb by CDK 4/6 results in the disruption of the Rb-E2F repressor complexes, unleashes E2F activity, thus modifying target gene transcription and entry of cells into the S phase [10].

In bladder tumours with complete loss of Rb function, it has been known for some time that the locus of E2F3 is amplified [11]. Similar data have been reproduced in Rb-knockout mice which showed low expression of p53 protein in bladder tumour tissue, with E2F-3 being highly expressed in tumour-tissue of wild-type mice and significantly upregulated in Rb-knockout mice, hinting at the connection between Rb deficiency and E2F-3 expression enhancement, leading to urinary bladder cancer progression [12]. Gene expression profiling of resected bladder tumours, revealed that E2F-1 expression was up-modulated in invasive tumours while expression was low in superficial tumours, suggesting a possible role of E2F-1 as an indicator of progression from the superficial to the invasive phenotype [13]. Data showed that E2F-1 exerts its role through its downstream effector EZH2 that is found overexpressed, along with E2F-1, in invasive bladder cancer [13]. Surprisingly, a study on the potential of CDK4/6 as a therapeutic target within the Rb pathway, where treatment with cyclin CDK4/6 inhibitors PD-0332991 or LY2835219 was tested on ten bladder cancer cell lines, resulted in decreased E2F target gene expression and cell cycle progression from G0/G1 to the S-phase, without affecting apoptosis

[14].

E2F-1 is the prominent and most studied of all E2F factors. E2F-1 drives cell cycle progression at the G1- to S-phase boundary; however, overexpression of E2F-1 can induce apoptosis. E2F-1 is a remarkable example of a network hub as it interacts with many genes, proteins, and other transcription factors, through a variety of regulatory mechanisms. In the context of solid tumours, unbalanced E2F-1 regulation can lead to the emergence of aggressive tumour cells, thus driving fast cancer progression, resistance to therapy and metastasis formation.

It is now known that E2F-1 drives epithelial-mesenchymal transition (EMT), similar to the classical EMT inducer TGF β 1, via signaling pathways that involve non-coding RNAs [15]. EMT is a biologic process that allows a polarized epithelial cell, to 'transform' into a mesenchymal cell phenotype, with enhanced migratory capacity, invasiveness, elevated resistance to apoptosis. As the excessive epithelial cell proliferation and angiogenesis are hallmarks of the initiation and early growth of primary epithelial cancers, the subsequent acquisition of invasiveness, is believed to herald the onset of the last stages for metastatic dissemination, with life-threatening consequences.

Indeed, enforced E2F-1 expression in advanced tumours and metastases of different kinds of cancers correlates with pronounced resistance towards therapy and poor patient prognosis [16] and clinical results indicate that E2F-1 is upregulated in high-grade bladder [16].

Khan et al. (2017) examined the effects of E2F-1 activity on the invasive capacity of patient-derived metastatic bladder tumour cell lines using functional invasion assays, western blotting, and PCR analysis and revealed a clear correlation of E2F-1 expression with the invasive behavior and EMT marker expression in invasive bladder (UM-UC-3, SW1710, J82, T24) vs. low expression in non-invasive or less-invasive epithelial bladder (RT-4, VM-CUB1, HT1197) cell lines [17].

In bladder cancer cell cultures, E2F-1 protein levels increased following treatment with the DNA damaging agents suggesting that E2F-1 may act in p53-independent fashion [18]. Higher expression of both E2F-1 and E2F-2 in bladder tumour tissues than in non-neoplastic tissues has been demonstrated both *in vitro* and *in vivo*, possibly through the mechanism of histone modification of the chromatin [19]. Aristolochic acid, a plant component commonly used in Chinese herbal medicine, has been implicated in urothelial carcinoma, since it increases Rb/E2F-1

complex which prevents the release of E2F transcription factor, leading to accumulation of cells in the G0/G1 phase [20]. However, immunohistochemical detection of nuclear E2F-1 protein in cystectomy cancer samples revealed that E2F-1 was not a significant independent predictor of pelvic recurrence, metastasis nor death [21]. Moreover, in urothelial bladder cancer cell lines, the effectiveness of the chemotherapeutic antimetabolite drug 5-fluorouracil (5-FU) depended, among other factors, on the Rb phosphorylation and E2F-1 expression [22].

E2F-3 encodes two proteins, E2F-3a and E2F-3b, through the use of alternative promoters and different 5'-coding exons. These share DNA binding, heterodimerisation and pocket protein binding domains and differ only in their N-termini. Their function is not entirely clear, but data suggests that they may have partially opposing roles: the E2F-3a is classified as an activator, expressed in proliferating cells with peak levels in late G1; while E2F-3b has been classified as a repressor E2F, and is constitutively expressed throughout the cell cycle [11]. E2F-3a is the predominant family member involved in subsequent G1-to-S phase transitions and has a unique role in centrosome duplication, while E2F-3b has been shown to preferentially bind pRb and repress S-phase genes [23]. Deregulated E2F-3 appears to be an important driver of proliferation in bladder carcinomas, since amplification of E2F-3 is particularly common in this type of cancer. Interestingly, genomic studies have shown that tumours that amplify E2F-3 often also acquire mutations in RB1, suggesting that there may be continuous selection in these tumours for changes that sequentially elevate E2F-3 activity [24]. Amplification and overexpression of the E2F-3 gene at 6p22 in human bladder cancer is associated with increased tumour stage, grade and proliferation index [25].

E2F-4 is ubiquitously expressed throughout cell cycle, accounts for most of the E2F endogenous activity and is primarily localized to the cytoplasm in its free form [26]. E2F-4 and E2F-5 mRNA expression is maximal in mid-G1 phase before E2F-1 expression is detectable, suggesting a contribution of these proteins in the regulation of early G1 events including the G0/G1 transition. When bound to the pocket proteins, in G0/G1 phase, E2F-4 accumulates in the nucleus where it actively represses E2F-target genes [27]. Loss of function of both Rb and E2F-4 has been observed in the urothelium transitional epithelium of both low- and high-grade carcinomas [28]. E2F-4 expression level is a useful predictive marker for the

effectiveness of intravesical BCG therapy that can predict clinical outcomes, including recurrence, progression, and survival in patients with bladder cancer [29]. While E2F-5 protein is more homologous to E2F-4 (72% amino acid identity) than to E2F-1, E2F-2, and E2F-3 (35% amino acid identity), its exact role in bladder cancer is not fully understood. However, overexpression, but not point mutations, of E2F-5 has been found in breast cancer, associated with a basal phenotype and a worse clinical outcome [30].

Overexpression of E2F-6 leads to accumulation of cells in the S-phase and delays re-entry into the cell cycle in quiescent cells, thus attributing an important role for E2F-6 in the control of apoptosis via targeting of BRCA1 [31]. Its role in bladder cancer has not been fully investigated.

E2F-7 and E2F-8 are designated as atypical E2Fs because of their structural differences when compared with canonical E2Fs. E2F-7 and E2F-8, display two tandem DBDs and lack sequences that mediate Rb and DP binding (**Fig. 1**). The mechanisms by which atypical E2Fs regulate gene expression as well as their biological roles are still unclear, although they are able to regulate angiogenesis and lymphangiogenesis in a cell-cycle independent manner and have been implicated into E2F site-dependent transcription independent of Rb.

E2F-7 and E2F-8 share many transcriptional targets and can form homo- and heterodimers at their target promoters. E2F-7 was identified as a direct p53 target potentially induced during oncogene-induced senescence [32]. By repressing mitotic genes, E2F-7 was proposed to cooperate with Rb in limiting oncogenic transformation, although its potential tumour suppressor function has not been further explored [33]. In a separate study, low mRNA levels of E2F-7 were associated with platinum resistance and reduced survival in ovarian cancer patients [34]. Surprisingly, E2F-8 behaved in the opposite direction in the same set of patients, with high levels of E2F-8 transcripts associated with poor prognosis [34]. Finally, given the crucial role of E2F-8 in angiogenesis and lymphangiogenesis [35], further studies to evaluate the contribution of E2F-8 to metastasis might bring new therapeutic opportunities for the future. Since they are the newest members of the family their role in bladder cancer has not been thoroughly investigated.

Since the discovery of microRNAs, as an important modulator for cancer development some research on miRNAs has been conducted in bladder cancer (**Table 2**). Thus, overexpression of miRNA-let-7a (let-7a), a microRNA shown to directly alter cell cycle progression and pro-inflammatory

cytokine production, leads to increased expression of E2F-2 and Rb phosphorylation and inversely decrease of E2F-5 in immune stimulated cells [36]. Moreover, miRNA-125b inhibits the E2F-3-cyclinA2 signalling pathway and suppresses bladder tumour cells growth during G phase via the reduction of E2F-3 expression [37]. E2F-1 expression may induce oncogenic stress and promote premalignant cell apoptosis, thereby inhibiting tumour development, therefore the decrease of E2F-1 by hsa-miR-106b targeting, may be one of the possible mechanisms underlying the development of bladder cancer [38].

Table 2. Control of E2Fs by microRNAs. Note that these are the miRNAs affecting only the E2F family, while others have also been implicated in bladder cancer [53].

miRNA	E2F	Effect
miRNA-let-7a	E2F-2, -5	Up-regulates E2F-2 Downregulates E2F-5
miR-15 family (miR-15, 16 and 195)	E2F-3	Downregulate
miR-106b	E2F-1	Downregulates
miR-125b	E2F-3	Deregulates

Moreover, the novel stratification method, using molecular profiling, has identified three major subtypes of urothelial carcinoma; Urobasal (A and B), Genomically Unstable (GU) and SCC-like (SCCL). UroA and UroB tumours are molecularly similar, but UroB are biologically and clinically more progressed. GU tumours are undifferentiated, highly proliferative, while SCCL tumours show enhanced expression of basal urothelial markers and squamous differentiation. GU tumours are characterized by frequent E2F-3 gene amplifications [39]. Further analysis has revealed that E2F-1, E2F-2, E2F-3, E2F-4 and E2F-7 are highly expressed in GU and SCCL tumours [40].

Conclusions-Further perspectives

E2F family of transcription factors has been mainly implicated in cell cycle progression, classically divided in E2F activators and repressors that operate in a coordinated manner to achieve proper cell cycle progression and/or apoptosis and that disturbance of this balanced interaction can contribute to cancer development and progression. According to this model, E2Fs control the oscillating expression pattern of multiple target genes during the cell cycle: activators E2F-1-3 induce an upswing of E2F targets, essential for the G1-to-S phase transition, whereas E2F-7 and E2F-8, mediate a downswing of the same targets during late S, G2, and M phase. Unfortunately, this simplified approach, mainly stemming from *in*

vitro studies, is challenged in practice, since no unequivocal correlation of effector and result can be easily made. To complicate things further, the plethora of potential E2F targets reflect the fact that E2Fs participate in cellular processes beyond the cell cycle; this poses a major challenge, to identify specific processes in which E2F plays a functional key role and the contexts in which a particular subset of E2F targets dictates a biologic outcome. This is crucial in developing the next generation medication to cancer, which is highly personalized and presents the minimal side-effects.

As the formerly prevailing view that E2F repressors and activators orchestrate proper cell cycle progression and disruptions in this network contribute to cancer, has now been challenged, the delineation of the complexity of E2F function and the cellular context which may dictate the different E2F roles becomes imperative. A number of key questions need to be addressed. The primary, overarching question is whether E2Fs are necessary for the proliferation of all cell types in mammals and other organisms; in case that more components are necessary, research should prioritize on identifying the essential accessories which partner with E2Fs and on how their activities are coordinated to commit cells to proliferation. A second key question is whether E2Fs enact only through their activation or repression functions and whether these activities are tailored to different developmental stages of a cell. The recent findings that E2F-1 also drives epithelial-mesenchymal transition (EMT), i.e. adopts yet another role of an inducer, similar to the classical EMT inducer TGFB1 [15] adds further complexity to the understanding of the diverse role of these proteins, rendering them as cellular mutli-tools which respond to a range of signals and stimuli. Another level of complexity is the differential expression levels of E2Fs which drive variable results. Especially in regard with bladder cancer, the number of available publications does not reflect the level of understanding of these proteins and their specific role in BC initiation or progression. In spite of the acquired knowledge on their activity, control and targets, a clear map of the full span of interactions, levels of control and differential response remains elusive.

There is now substantial evidence that of E2F-1, E2F-3 and E2F-4 and -5 have a distinct impact on bladder cancer development, progress and prognosis; so, what is the present progress in terms of translating findings into potential therapeutics? Efforts in this direction are now surfacing. CG0070 is an oncolytic adenovirus which has been engineered so that the human E2F-1 promoter drives the E1A gene, and the

human GM-CSF gene is inserted. E2F-1 is regulated by the retinoblastoma tumour suppressor protein (Rb), which is commonly mutated in bladder cancer, and a loss of Rb binding results in a transcriptionally active E2F-1. A phase I trial of CG0070, conducted in patients with non-muscle-invasive bladder cancer who did not respond to BCG therapy, gave an overall response rate of 48.6% (17 of 35 recruited patients), which increased to 63.6% (14 of 22 recruited patients) in the multi-dose cohort of subjects. A later randomized phase II/III trial in patients with non-muscle-invasive bladder cancer, where 15 patients received CG0070 and 7 control patients received other standard intravesical therapies (BOND, NCT01438112), demonstrated a better durable response in a subset of high-risk patients (51), while a recent single arm phase III trial that is underway, patients with BCG-refractory non-muscle-invasive bladder cancer are given CG0070 intravesically at a dose of 10^{12} vp weekly for 6 weeks. Patients who achieved a partial or complete response at 6 months after the first intervention are maintained with the same induction cycle every 6 months (BOND2, NCT02365818) [41].

Moreover, the connection of E2F-1 to triggering inflammatory gene expression, gives an added role to this transcription factor, since bladder cancer development is enhanced by inflammatory stimuli [42]. Fucoidan, an algae extract, increases binding of pRb to E2Fs, by blocking phosphorylation of pRb, without marked alteration of E2F-1 and E2F-4 proteins, making it an interesting candidate to anticancer therapy [43]. More recently, Palbociclib PD-0332991, which had been developed as a specific CDK4/6-inhibitor and demonstrated anti-tumour activity in preclinical models of several cancer entities including melanoma [44, 45], breast [46, 47] and ovarian cancer [48], also showed that it can reduce the proliferation of retinoblastoma positive bladder cancer cell lines and act synergistically in combination with cisplatin. Specifically, PD-0332991 or LY2835219 treatment decreased the phosphorylation, total protein and transcript level of retinoblastoma, while the treatment resulted in a decrease in E2F target gene expression (CCNA2 and CCNE2) and cell cycle progression from G0/G1 to the S-phase without affecting apoptosis. These data indicate that CDK4/6 inhibition could be a prospective therapeutic strategy for retinoblastoma positive bladder cancer that probably acts by negatively regulating retinoblastoma transcription [14].

Besides blocking of E2F, the ablation of E2F-4-pRb interaction is currently under investigation for therapeutic use in melanomas [49], rising hopes that

such an approach could be tested in bladder carcinoma as well.

The role of the remaining E2Fs remains elusive or under-investigated, despite the extensive study of all key factors, during the last decade, in urothelial development and differentiation. However, bladder cancer still remains a major health issue, with a 50–70% recurrence and 10–20% progression to invasion. The overall 5-year survival rate for bladder cancer is 77%, and this rate drops to 34% for those with disease that has spread locally beyond the bladder and to 5% for patients with distant metastases. Therefore closer investigation of E2F factors taking into account the newer molecular stratification data as well as miRNAs development should be undertaken. Polycomb group protein Enhancer of Zest Homolog 2 (EZH2) that potentially interacts with E2F-6 is a commonly up-regulated in bladder cancer and its increased expression and activity has been associated with more aggressive forms of this cancer [50]. Besides, as described previously, the possible therapeutic use of Ezh2 inhibitors in the management of bladder cancer is a crucial aspect that merits future research.

Delineating the specific role for each E2F member and their overall contribution to bladder cancer development is a challenging task [51], especially in view of potential E2F targeted therapies. In addition to comprehensive knowledge of the E2F family functions, this necessitates the precise understanding of spatial and temporal alterations of these factors during tumour development [51], to ensure that such therapies will be successful.

Competing Interests

The authors have declared that no competing interest exists.

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