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MINIREVIEWS

WNT/β-catenin signaling in urothelial carcinoma of bladder

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Abstract

Urothelial carcinoma of bladder is the second most prevalent genitourinary disease. It is a highly heterogeneous disease as it represents a spectrum of neoplasms, including non-muscle invasive bladder cancer (NMIBC), muscle invasive bladder cancer (MIBC) and metastatic lesions. Genome-wide approaches and candidate gene analysis suggest that malignant transformation of the bladder is multifactorial and a multitude of genes are involved in the development of MIBC or NMIBC phenotypes. Wnt signaling is being examined to control and maintain balance between stemness and differentiation in adult stem cell niches. Owing to its participation in urothelial development and maintenance of adult urothelial tissue homeostasis, the components of Wnt signaling are reported as an important diagnostic and prognostic markers as well as novel therapeutic targets. Mutations/epigenetic alterations in the key molecules of Wnt/β-catenin canonical pathway have been linked with tumorigenesis, development of drug resistance and enhanced survival. Present review extends our understanding on the functions of key regulatory molecules of canonical Wnt/β-catenin pathway in urothelial tumorigenesis by inducing cancer stem cell phenotype (UCSCs). UCSCs may be responsible for tumor heterogeneity, high recurrence rates and complex biological behavior of bladder cancer. Therefore, understanding the role of UCSCs and the regulatory mechanisms that are responsible for high relapse rates and metastasis could help to develop pathway inhibitors and augment current therapies. Potential implications in the treatment of urothelial carcinoma of bladder by targeting this pathway primarily in UCSCs as well as in bulk tumor population that are responsible for high relapse rates and metastasis may facilitate potential therapeutic avenues and better prognosis.

Key words: Chemoresistance; Therapeutic approaches; Urothelial carcinoma of bladder; Urothelial cancer stem cells; Wnt/β-catenin

Core tip: Wnt/β-catenin signaling pathway plays significant role in maintaining balance between stemness and differentiation in adult stem cell niches. Mutations/epigenetic alterations in the regulatory components of Wnt/β-catenin signaling lead to acquisition of

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urothelial cancer stem cell phenotype, chemoresistance and enhanced survival. Key regulatory molecules of Wnt/β-catenin pathways are being examined as diagnostic/prognostic markers as well as novel therapeutic targets in urothelial tumorigenesis.

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NTRODUCTION

Bladder cancer is the second most prevalent genitourinary disease and ranks ninth most common cause of deaths due to cancer worldwide. Nearly 90% of bladder cancers are diagnosed as urothelial carcinoma of bladder (UCB). Industrial and occupational exposure to carcinogens/aromatic amines, prolonged use of arsenic or chlorine contaminated drinking water, use of tobacco, anatomical, hormonal, genetical, and socio-economical status are considered as major risk factors for bladder cancer[1]. Schistosomiasis infections are identified as the main cause for non-urothelial cancer and are prevalent in many parts of the world including Africa and Middle East.

UCB is highly heterogeneous disease as it represents a spectrum of neoplasms, including non-muscle invasive bladder cancer (NMIBC), muscle invasive bladder cancer (MIBC) and metastatic lesions. Around 80% of UCB are papillary bladder tumors that arise through the non-invasive papillary pathway (primary tumor stage: pTa-T1). Approximately 20%-30% of patients are initially presented with more aggressive muscle invasive disease (primary tumor stage: pT2-T4) which evolves from severe dysplasia or carcinoma *in situ*. Genome-wide approaches and candidate gene analysis suggest that malignant transformation of the bladder is multifactorial and a multitude of genes are involved in the development of MIBC or NMIBC phenotypes[2,3]. Recent experimental studies validate the involvement of oncogenic mutations in telomerase reverse transcriptase, fibroblast growth factor (FGF) receptor, Harvey rat sarcoma viral oncogene, and phosphoinositide 3'-kinase (PI3K); activation of mitogen-activated protein kinases; and increased expression of cyclin D1 in the development of papillary cancer[4,5]. A strong cooperation of Ras pathway activation with dysregulated Wnt/β-catenin signaling to drive UCB in mouse bladder has been reported[6]. Gene expression, whole genome array-comparative genomic hybridization and mutation analyses validate frequent alterations in loss of tumor protein 53 function; increased instability of chromosomes; inactivation of phosphatase tensin homolog; severe disturbances in cell cycle regulators; loss in functions of retinoblastoma and cyclin dependent kinase inhibitor 2A (CDKN2A)/p16INK4A; and the effect of altered DNA methylation on the expression of cell cycle regulators, in muscle invasive disease[4,7]. Castillo-Martin *et al*[8] report the presence of mutations in PI3K, tumor suppressor genes, deleted in bladder cancer, tuberous sclerosis 1, CDKN2A and patched in both papillary and invasive tumors.

Transurethral resection of the bladder tumor is the treatment of choice in NMIBC, however, 50%-90% of patients presented with pTa (primary tumor) suffer frequent recurrences, whereas 20%-25% of patients with pT1 tumors progress to high grade MIBC[9,10]. MIBCs are treated with radical cystectomy, chemo- and radiotherapies. Despite these therapies, about 50% of MIBC patients eventually develop metastasis[5]. Besides, possible toxic effects of adjuvant/neo-adjuvant chemo-radiotherapies may adversely affect the quality of life and overall survival of the patients[11]. Despite of significant advancements in treatment modalities and prognostic methods, bladder cancer continues to be an extremely common disease with high mortality rates and thus this disease still remains a challenge for genito-urinary surgeons.

Recently identified concept of cancer stem cell theory can be explained by the presence of subset of cells with the self-renewal/ differentiation ability into hierarchical cells, known as urothelial stem cells (USCs), and their transformation into malignant counterparts, known as urothelial cancer stem cells (UCSCs). UCSCs may be responsible for tumor heterogeneity, high recurrence rates and complex biological behavior of bladder cancer. Therefore, understanding the role of UCSCs and the regulatory mechanisms that are responsible for high relapse rates and metastasis may facilitate potential therapeutic avenues and better prognosis.

Evolutionarily conserved Wnt signaling initiates and regulates diverse range of cellular activities including stem cell self-renewal, degree of differentiation and tissue homeostasis. Activated Wnt signaling has been linked with tumorigenesis of many cancer types and development of drug resistance. The present review summarizes our understanding on detailed molecular insights of Wnt signaling into the complex biological pathology of UCB. The paper presents an overview on the components of Wnt signaling and their participation in urothelial stemness, tumorigenesis and drug resistance. Understanding the mechanisms of Wnt signaling dysregulation in the development and progression of UCB could help to develop pathway inhibitors and augment current therapies by targeting UCSCs and bulk tumor progenies.

UROTHEILAL STEM CELLS AND UROTHELIAL CANCER STEM CELLS

Epithelial lining of bladder wall/urothelium also known as transitional epithelium, is composed of highly differentiated, specialized, giant, frequently multinucleated single layered umbrella cells towards the bladder lumen and is in direct contact with urinary space. Single layer of small polygonal basal cells with the highest proliferative capacity due to presence of subset of basal stem cells, is organized at the stromal edge of the basement membrane. Variable number of multilayered intermediate cells is organized in between two layers.

Long life span; high regenerative potential; high colony-forming efficiency; high nuclear-cytoplasmic ratio; and low granularity are the properties of slow cycling basal USCs[12-15]. The subset of USCs lying in basal layer is characterized to express CD44, β1/β4 integrins, cytokeratins (CK-5/14, CK-17) and laminin receptor (LR). Nevertheless, studies on p63-null mice that lack basal/intermediate cells support the origin of umbrella cells by another independent pool of USCs[15]. Urothelium provides anatomical and functional microenvironment to USCs and protect them from loss of self-renewal ability, differentiation and apoptosis. Regenerative potential of USCs diminishes with its differentiation into transit-amplifying cells of intermediate cell layer and fully differentiated umbrella cells. Differential/maximal distribution of USCs in the caudal region of the rat bladder may be correlated with relatively higher incidence rate of carcinoma per surface area in this region[15].

Epigenetic changes, genomic instability, altered tumor microenvironment, and mutations in proto-oncogenes/tumor suppressor genes lead to aberrant molecular pathways in either adult stem cells or differentiated tumor progenies and thus transform them into a subset of unique UCSCs. These subsets of cells, also known as side-population, are characterized by their high tumor-initiating, self-renewal, clonogenic and proliferative potential; and enriched ability to conserve cellular heterogeneity by inducing xenograft tumors *in vivo*[16]. These cells are examined to express cell surface markers which are also shared by adult stem cells and mature urothelial cells. USCs specific markers were used to identify and isolate UCSCs for the first time in 2009[16]. Subset of these markers included early progenitor Nestin (NES); low carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6^{low}); 67LR at the tumor-stromal interface (upregulated in 80% of invasive cancer); aldehyde dehydrogenase 1A1 (ALDH1A1^{hi}); CD44, CD44 splice variant 6 (CD44v6); CD133; and cytokeratins (CK5, CK7, CK17). *In vivo* and *in vitro* studies identify upregulation of host of oncogenes including cadherin-associated protein beta 1, also known as β-catenin; B lymphoma Moloney murine leukemia virus insertion region 1 homolog (BMI1); GLI1; NANOG; POU domain class 5 transcription factor 1 (POU5F1)/Oct4; and signal transducer and activator of transcription 3. Efflux of vital dye Hoechst 33342 and DyeCycle violet due to higher expression of ATP-binding cassette transporters and multidrug resistance pumps provide evidences for their survival and preservation of cancer stem cell phenotype[17].

Wnt signaling pathway crosstalks and regulates diverse set of cellular activities including embryonic development, wound healing, stemness maintenance, calcium and tissue homeostasis, cell proliferation, and cell polarity of USCs and UCSCs[18]. Wnt signaling is classified into two intersecting signaling networks: (1) The canonical Wnt (or β-catenin-dependent); and (2) Non-canonical Wnt (or β-catenin-independent) pathways. Present review organizes collection of studies that extends our understanding on canonical Wnt/β-catenin pathway, highlights the functions of major components involved in maintenance of UCSCs properties, and their therapeutic implications in the treatment of UCB.

WNT SIGNALING IN UROTHELIAL CARCINOMA OF BLADDER

Wnt signaling is being examined to play central role in urothelial development as well as in the maintenance of adult urothelial tissue homeostasis[19]. The human Wnt gene family encodes 19 evolutionarily conserved 40 kDa glycoproteins with several glycosylation sites. Besides, Wnt ligands contain 22 or 24 conserved cysteine (Cys) residues that participate in lipidation, impart hydrophobic character to it and maintain their activity. Binding of secreted Wnt ligands to the members of the seven transmembrane family G-protein coupled receptors of proteins with cysteine-rich domains, called Frizzleds (Fzds), and their associated co-receptors, stimulates complex network of events including, non-canonical and canonical pathways.

CANONICAL WNT SIGNALING

Canonical Wnt signaling is initiated with the activation of Dishevelled (DVL) protein, a key cytoplasmic partner of Wnt signaling, which directly interacts with FZD at the cytoplasmic side; promotes the phosphorylation of single-pass transmembrane proteins of the low-density lipoprotein family called LRP5 and LRP6; and facilitates the formation of FZD-LRP5/6 heterotrimeric complex. Recruitment of AXIN to the inner membrane leaflet *via* interactions with DVL and LRP-5/6 disrupts the β-catenin destruction complex. In Wnt "OFF" state, casein kinase 1 phosphorylation at Ser45 primes β-catenin for subsequent phosphorylation by glycogen synthase kinase-3β (GSK-3β) on Thr41, Ser37 and Ser33[20,21]. Phosphorylation at N-terminal serine and threonine residues creates a binding site for the ubiquitin ligase, SCFb-TrCP and targets β-catenin for proteasomal degradation and consequently maintains β-catenin in cytoplasm at a low level[22]. In Wnt "ON" state, binding of receptors to Wnt activates the downstream signaling cascade; initiates the disassembly of the destruction complex consisting of DVL/AXIN/APC (adenomatous polyposis coli)/GSK-3β/CK1; facilitates the release of the stabilized β-catenin to cytoplasm and its translocation to nucleus. Nuclear β-catenin binds with T-cell factor/lymphoid enhancing factors (TCF/LEF) family of DNA-binding factors. It then displaces Groucho to create a transcriptional activator complex (Figure 1). It further recruits co-activators including histone acetyl transferases, cyclic AMP response element binding protein, p300 and the chromatin remodeling factor Brg1. Following chromatin remodeling, many target genes including c-myc, stromelysin, FGF, epidermal growth factor (EGF), cyclin D1, c-Myc, CD44 and ALDH are transactivated[23].

Other receptor proteins with known Wnt-binding domains, single-pass receptor tyrosine kinase–like orphan receptor-1 and -2 (Ror1/Ror2: Structurally distinct from Fz receptors) mediate the Wnt5a signal and inhibit β-catenin-TCF signaling. Another receptor protein, tyrosine kinase, Ryk, with Wnt inhibitory factor (WIF) ligand binding domain, is known to control Wnt3a-mediated canonical Wnt signaling. Wnt/receptor tyrosine kinase signaling is known to be involved in human cancer recurrence and therapeutic resistance, partly through activation of PI3K-AKT signaling[24].

NON-CANONICAL WNT SIGNALING

Two non-canonical pathways (a) planar cell polarity (PCP) pathway and (b) Wnt/Ca^{2+} pathways are best-characterized and do not require the involvement of β-catenin. PCP pathway regulates actin cytoskeletal dynamics and maintains apical-basal polarity of cells or polarity of cells within an epithelial layer. Non-canonical Wnt ligand binding to FZD and co-localization of a co-receptor facilitate activation and localization of DVL to the inner membrane leaflet. This is followed by subsequent activation of actin-binding protein Profilin and small GTPases, RHO and RAC through parallel pathways. RHO and Profilin activation occurs through a DVL associated activator of morphogenesis 1 (DAAM1)-dependent manner while RAC activation is in DAAM1-independent manner. RHO-associated kinase (ROCK) and myosin downstream of RHO activation, participate in modification and rearrangement of the actin cytoskeleton (Figure 1). Functions of RacGTPase activated JNK in cytoskeletal modification are remain poorly characterized[25,26].

Activation of Wnt/Ca^{2+}pathway is mediated by binding of Wnt ligand to FZD, followed by activation of G-proteins and DVL. Activated DVL on binding with phosphodiesterase inhibits protein kinase G, increases intracellular calcium, as well as phospholipase C (PLC). Membrane-bound phospholipid phosphatidyl inositol 4,5-

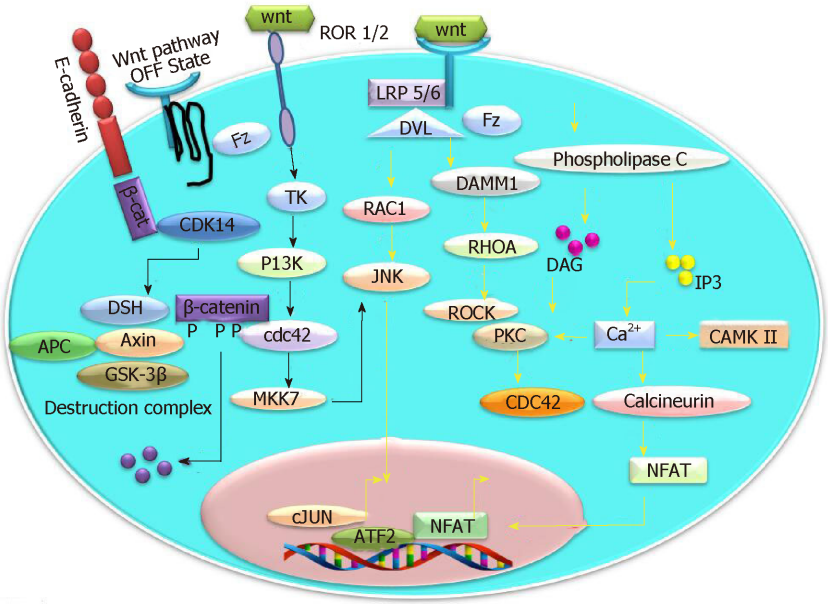

Figure 1 Wnt signaling with two intersecting networks: Canonical Wnt (or β-catenin-dependent) and non-canonical Wnt (or β-catenin-independent) pathways. Canonical Wnt pathway is shown in black color arrows whereas non-canonical pathway is shown in yellow color arrows.

bisphosphate produces inositol triphosphate (IP3) and 1,2-diacylglycerol (DAG) by activated PLC. IP3 triggers release of intracellular Ca^{2+}in the endoplasmic reticulum and activates downstream targets, including calcineurin, calmodulin-dependent protein kinase II (CamKII), and protein kinase C (PKC). Calcineurin and calmodulin activation result in dephosphorylation and stimulation of the nuclear factor associated with T cells transcription factors, thereby result in transcription of target genes which are critically involved in immune response and influence ventral cell fates in vertebrate embryos. PKC activation requires DAG, phosphorylates and activates GTPase [cell division control protein 42 (Cdc42)]. Activation of CamKII antagonizes β-catenin/TCF regulated transcription (Figure 1)[27,28].

Components of Wnt/β-catenin signaling pathway are by far the best characterized among Wnt pathways. Complexity of Wnt signals and their functional role in cell differentiation, polarization and migration are crucial during embryogenesis. Nevertheless, their aberrant activation results in the development of many human tumors and others diseases. One of the recent studies has shown the increased expression of LRP 5/6 in many tissues and its important role in the progression of many cancers including osteosarcoma, prostate cancer, lung squamous cell carcinoma, primary chronic lymphocytic leukemia, triple negative breast cancer, non small cell lung cancer and hepatocellular carcinoma[29]. Overexpression of WNT ligands, impaired cytosolic β-catenin degradation, modulated activities of the TCF/LEF transcription factors and epigenetic repression of WNT antagonists contribute to the pathogenesis of malignant neoplasms of the hematopoietic system[30]. Aberrant Wnt/β-catenin signaling due to mutations in APC and β-catenin genes is reported in colorectal cancer[31]. Study by Nagahata *et al*[32] reports the tumorigenic implication of upregulated DVL in 50% of ductal breast cancers. Deregulation of canonical Wnt signaling and its functions in promoting DNA damage repair and inhibiting apoptosis has been examined to be associated with radioresistance of multiple cancers[33].

Signaling proteins of canonical Wnt/β-catenin being the most prominent regulatory molecules of the urothelial stem cell signaling pathways may hold promise as an early diagnostic/ prediction markers. Genomic mutations and epigenetic changes are being examined to regulate the cancer stem cell phenotype by controlling the expression levels of Wnt/β-catenin components. Single nucleotide polymorphisms in about forty genes that participate in Wnt/β-catenin stem cell pathway are genotyped and the genomic variants have been reported to be associated with the etiology of the bladder cancer[34]. Aberrant Wnt signaling due to mutations in β-catenin in CSCs results in induced tumorigenic proliferation[35]. Missense/ inactivating mutations in APC tumor suppressor (in 13% of tumors) and frameshift deletions (in 3% of tumors) in regions adjacent to β-catenin binding sites result in β-catenin accumulation, dysregulation of Wnt/β-catenin in cancer cells or UCSCs, induce neoplastic proliferation and increase invasion potential[36]. Activated stromal

cells provide appropriate microenvironment to USCs, signalize urothelium with the canonical Wnt/ β-catenin pathway, thereby mediate proliferation in basal urothelial cells[37]. Differential expression of many Wnt signaling components between UCSCs and non-tumorigenic cancer cells including elevated protein levels of β-catenin, c-Myc, and Wnt10a ligand in UCSCs, while Wnt7 in papillary non-invasive carcinomas hypothesizes the involvement of stimulated Wnt signaling in urothelial differentiation, stem cell homeostasis and cancer stem cell maintenance[38-40]. Number of endogenous Wnt inhibitors and Wnt ligand antagonists are known which include: (1) WIF1: Binds to Wnt ligands and prevents them from triggering signaling; (2) Secreted frizzled-related proteins (SFRPs: Interact with Wnt proteins and prevent the latter from binding with FZD receptors); (3) Differentially expressed in ovarian carcinoma-2/disabled homolog 2 (DOC-2/DAB2: Associates with Axin and prevents its interaction with LRP5 co-receptor); and (4) Dickkopf-related proteins (DKKs: Isolate LRP6 co-receptor and antagonize Wnt/β-catenin pathway by reducing β-catenin levels). Epigenetic silencing of these antagonists due to DNA promoter methylation is well known mechanism in urothelial carcinogenesis. Methylation levels of six Wnt-antagonist genes including sFRP-1, sFRP-2, sFRP-4, sFRP-5, Wif-1, and Dkk-3 have been marked as M score and thus considered as novel epigenetic biomarker panel for UCC[19]. Study by Urakami *et al*[41] validates the methylation levels of sFRP-2 and Dkk-3 as significant independent predictors of UCC.

Multiple studies report the cooperativity between Wnt/β-catenin and other signaling networks in induction, maintenance of stem cell properties, cancer cell proliferation, invasion and metastasis. Aberrant Wnt/β-catenin mediated inactivation of GSK-3β, nuclear localization of β-catenin, and transcriptional activation of c-Myc and cyclinD1 drive human carcinogenesis through the induction of CSC features, bulk tumor proliferation and epithelial-to-mesenchymal transition (EMT)-like changes. EMT, a trans-differentiation process, is characterized by the loss of cell-cell adherence/loss of apico-basal polarity; transition of cells with epithelial (E) phenotype to mesenchymal (M) phenotype; and increased motility of cells. Mounting evidences suggest that lower levels of E-cadherin (epithelial marker protein) due to promoter hypermethylation may induce inappropriate responsiveness to Wnt factors. E-cadherin acts as an inhibitor of β-catenin/TCF-mediated transcription by sequestering β-catenin at the plasma membrane[42]. Recent studies report the association of specific genotype of E-cadherin with susceptibility to UCB and a worse clinical prognosis[43]. Prognostic molecular switching to an invasive phenotype is evidenced by the loss of E-cadherin and novel gain of N-cadherin during microarray analysis of 825 UCC samples from 572 patients[44]. Comparable expressions of E-cadherin and p63 vary with tumoral degree of differentiation and the tumoral depth of invasion in UCB. Transcriptional inactivation of p53 and TAp63 (an isoform of p63 with N-terminal transactivation domain) and loss of ΔNp63 (an isoform of p63 with truncated N-terminal) correlate with poor prognosis of invasive urothelial cancer. Loss of ΔNp63α followed by increased levels of N-cadherin and ERK (extracellular signal regulated kinase) signaling activation is proposed as one of the molecular mechanisms in the progression of invasive UCB[45]. Overexpression of GSK-3β was examined for attenuated cigarette smoke extract/tobacco smoke-triggered activation of Wnt/β-catenin, EMT induction, suppressed expression of CSCs markers including CD44, Nanog, Oct4 and ALDH1; reduced migration and invasion capacities of SV-HUC-1 cells[46].

Identification of key regulatory molecules in mechanistic regulation of Wnt/β-catenin signaling and current knowledge on Wnt antagonists would offer potential opportunities to develop new therapies by targeting this pathway primarily in UCSCs as well as in bulk tumor population.

THERAPEUTIC INTERVENTIONS BY TARGETING WNT/-CATENIN AND FUTURE PERSPECTIVES

Major mechanisms of chemoresistance are experienced by UCSCs and these include: (1) Enzymatic breakdown of drugs; (2) Efflux of drugs; (3) Inhibition of anti-tumor response; and (4) Inhibition of apoptosis. CSCs targeting treatment although is far from clinical use, but it is still widely believed to be promising approach for better curative effects. Hence, it is extremely important to comprehensively examine the genomic profile of CSCs and decipher the associated genes in order to establish intervention targets[47]. Global gene expression analysis provides detailed profiling of UCSCs and associates aggressive late stages of bladder tumor with the presence of activated gene signature. Given the important roles and pleiotropic effects of canonical Wnt signaling in cell self-renewal/maintenance of stemness, plasticity

regulation (differentiation/dedifferentiation) and tissue homeostasis, loss of Wnt/β-catenin pathway has been examined to be associated with the inhibition of stemness of UCSCs. Expression studies examine that dysregulated Wnt/β-catenin support dedifferentiation, EMT and hypoxia which might be responsible for the transformation of early bladder cancer cells into cancer stem cells. Role of Wnt/β-catenin pathway alone in regulating long-term tobacco smoke exposure triggered EMT and acquisition of CSCs properties (increased expression of CD44, ALDH1, Nanog and Oct4) has been well-demonstrated in SV-HUC-1 cells and the mice bladder[46].

Growing number of studies highlight the potential of Wnt/β-catenin as a druggable target for sensitizing the small subset of UCSCs as well as bulk tumor cells. Conserved small noncoding RNAs (about 22 nucleotides in length), known as microRNAs or miRs, negatively regulate the expression of target genes by binding to the 3′ untranslated region of target gene. Molecular functions of most of the miRs are still unclear, yet many miRs are established with tumor suppressive or oncogenic functions in bladder tumor proliferation, survival and resistance. Mass spectrum analyses revealed the overexpression of Wnt7a (Wnt family protein) in 5637 HMI cells with high invasion capabilities than in 5637 NMI cells with low invasion capabilities. Its higher levels are being associated with metastasis and worse clinical outcome in UBC patients. Re-introduction of tumor suppressive miR-370-3p, which is frequently lost in bladder cancer in 5637 HMI cells, lead to inhibition of Wnt7a and suppression of 5637 HMI cell invasion[48]. Significant association between higher levels of miR-374a (anti-tumor small non-coding RNA) and longer survival/ recurrence free survival of patients documents its inhibitory effect on tumor metastasis and aggressive biological behavior. Anti-tumor effects of miR-374a may be further explained by abrogating the invasive/metastatic potential, and promoting apoptosis after cisplatin treatment[49]. *In vitro* experiments in T24 and TCCSUP human bladder cancer cells determined miR-374a mediated inhibition of Wnt/β-catenin pathway by targeting Wnt5a. Antitumor effect of miR-532-5p overexpression is being reported by targeting high-mobility group protein B3 (HMGB3), downregulating Wnt/β-catenin signaling, and inhibiting the proliferation and invasion of bladder cancer cells[50]. Lower expression of miR-1826 in bladder cancer tissues as well as in J82, T24 and TCCSUP cell lines and its negative correlation with clinical tumor stage and tumor grade concludes its tumor suppressor role in bladder cancer. MiR-1826-transfected cancer cells exhibited increased apoptosis and G_1 cell cycle arrest *via* β-catenin/ MEK1/ VEGFC downregulation in bladder cancer[51].

In vitro studies validate the functional roles of oncomiRs, miR-23a and miR-27a in targeting and downregulating SFRP1 protein, a negative regulator of Wnt signaling pathway. Oncosuppression of miR-23a and miR-27a based therapies might be effective in reducing levels of β-catenin and suppressing proliferation, migration, invasion of cancer cells by sensitizing them to radiotherapies or chemotherapeutic drugs[52]. Target prediction and luciferase reporter assays were performed to determine the regulatory relationship between miR-940 and the components of Wnt/β-catenin pathway. Overexpression of miR-940 has been studied to be associated with increased levels of c-Myc, cyclin D1, and β-catenin and reduced expression levels of p27 and p-β-catenin. Experiments on miR-940 mimics/miR-940 inhibitor small interference RNA mediated knockdown of inositol polyphosphate-4-phosphatase type 1 A or GSK-3β resulted in regulation of malignant behavior of bladder cancer cells[53]. Down-expression of miR-940 *via* oncosuppressive approaches may affect the levels of direct targets and result in suppressed cell proliferation, migration, invasion and induced cell apoptosis. Oncogenic property of miR-135 in invasion and migration of bladder cancer has been determined by inducing EMT and suppressing GSK-3β *via* Wnt/β-catenin signaling. Therapeutic efficacy of miR-135a may be based on blocking its expression in cancer cells to control the levels of β-catenin, GSK-3β, c-Myc, cyclinD1, matrix metalloproteinase-7 (MMP-7), E-cadherin and Vimentin[54]. Wang *et al*[55] reported the cancer cell survival function of miR-92 *via* activated Wnt/c-Myc/MMP7 signaling by targeting GSK-3β. It would be interesting to examine its therapeutic potential by downregulating the miR-92 levels in bladder cancer cells.

Mediator complex subunit19 (Med19) is one of components of the mediator multiprotein complex which is known to increase DNA binding affinity of transcription factors to RNA polymerase II, thereby transcriptionally regulates target signaling molecules[56]. Wen and coworkers elucidated the critical role of Med19 in promoting invasive behavior and bone metastasis of bladder cancer cells by stimulating bone morphogenetic protein 2 (BMP-2)[57]. Short hairpin RNA mediated knockdown of Med19 was examined to remarkably reduce the expression of Wnt2, active β-catenin, cyclin D1 and MMP9, and increase the levels of GSK-3β and E-cadherin. Suppression of cell proliferation and migration in T24, UM-UC3 cells and 5637 *in vitro*, and inhibition of bladder tumor growth *in vivo* highlight the potential

role of Med19 as therapeutic target in the treatment of bladder cancer[58].

Curcumin with antioxidant, anti-inflammatory, anticancer and antifibrotic properties, is considered as one of the most promising chemopreventive agents. Interventional effects of curcumin in chronic tobacco smoke exposure-mediated urocystic EMT and acquisition of CSC like properties are validated *via* inhibition of activated Wnt/β-catenin. Suppressive effects of curcumin are further evidenced by increased GSK-3β, decreased β-catenin, weak expression of c-Myc, cyclin D1 both *in vitro* and *in vivo*[46]. In another study, a novel natural product analogue CYD 6-17 has been discovered to target β-catenin gene transcription by decreasing the binding of X-box binding protein 1 to the promoter region. CYD 6-17 with an IC50 at nM range, exhibits potent inhibitory effect on chemoresistant bladder cancer cells, thus offers rational therapeutic regimen to MIBC[59]. Novel mechanism for silibinin (a nontoxic natural flavonoid) in targeting or suppressing bladder cancer metastasis has been elucidated. Silibinin treatment to highly metastatic T24-L cell model suppressed cell migration/*in vitro* invasion, inhibited bladder cancer lung metastasis and prolonged animal survival *in vivo*. Dual block of EMT and stemness were identified as important therapeutic functions of silibinin mediated inactivation of β-catenin/zinc finger E-box binding homeobox 1 (ZEB1) signaling. Important findings of research study by Wu *et al*[60] on anticancer effects of silibinin include: (1) Blocking of GSK-3β phosphorylation and nuclear translocation/transactivation of β-catenin; (2) Downregulation of Zeb-1, cytokeratins, Vimentin and MMP2; and (3) Suppression of CSCs traits as evidenced by decreased side population, reduced spheroid colony formation, and lower expression of stem cell factor CD44 (Figure 2)[60]. Table 1 enlists the potential therapeutic agents/drugs/microRNA molecules which are being exploited in targeting the Wnt/β-catenin signaling components in UCSCs as well as bulk tumor progenies.

In vitro and *in vivo* studies provide detailed analysis on cross talk of Wnt signaling with other niche derived signals such as BMP, Hedgehog, TGF-β, Notch, FGF, VEGFC (vascular endothelial growth factor C). These integrated molecular pathways may act in a hierarchy, regulate each other and thus maintain organ specific tissue homeostasis by controlling stem cell signaling, proliferation or differentiation of progenitor cells. Nevertheless, stimulated Wnt/β-catenin and its regulatory circuitry have been deciphered to influence the cancer stem cell phenotype and invasive potential and are well characterized in many cancer types.

Understanding the coordinated activity of signaling pathways derived from bladder organ/tissue to elucidate the *in vivo* control of USC and UCSCs under various physiological and pathological conditions is urgently required. Unraveling the complexity of Wnt/β-catenin by investigating the hierarchical stimulation of signaling molecules and their effects on cancer phenotype may lead to discover potential therapeutic targets in the clinical management of Wnt-associated benign and malignant UCB.

Table 1 Therapeutic agents/ drugs/ microRNA molecules targeting Wnt/β-catenin signaling components in the treatment of urothelial carcinoma of bladder

Therapeutic molecules	Mechanism of action	Ref.
Therapeutic agents/ drugs		
Oridonin analogue CYD6-17	Targets β-catenin gene transcription by decreasing the binding affinity of X-box binding protein 1 to the promoter region	Chen *et al*[59]
Silibinin	Inhibits glycogen synthase kinase-3β (GSK-3β) phosphorylation, β-catenin nuclear translocation and transactivation, and zinc finger E-box binding homeobox 1 gene transcription	Wu *et al*[60]
Curcumin	Reverses tumor smoke-elicited activation of Wnt/β-catenin	Liang *et al*[46]
Mediator complex subunit 19 (Med19)	Med19 knockdown reduces the activity of Wnt/β-catenin pathway, and its target genes, including Wnt2, β-catenin, Cyclin-D1 and matrix metalloproteases 9	Yuan *et al*[58]
MicroRNAs		
Onco-microRNAs		
miR-135a	Downregulation of GSK-3β expression	Mao *et al*[54]
miR-940	Inhibits cell apoptosis *via* targeting inositol polyphosphate-4-phosphatase type 1 A/GSK-3β	Wang *et al*[53]
miR-27a	Targets and downregulates secreted frizzled-related proteins 1 protein, a negative regulator of Wnt signaling pathway	Meng *et al*[52]
miR-23a	Upregulation of β-catenin	Meng *et al*[52]
miR-92	Activates Wnt/cellular myelocytomatosis (c-Myc)/matrix metalloproteases 7 signaling by targeting GSK-3β	Wang *et al*[55]
Tumor supressor-microRNAs		
miR-532-5p	Targets high mobility protein B3 and downregulates Wnt/β-catenin signaling	Xie *et al*[50]
mir-139-5p	Blocks self-renewal of Urothelial cancer stem cells by inhibiting B lymphoma Moloney murine leukemia virus insertion region 1 homolog	Luo *et al*[61]
miR-200a	Directly interacts with 3'untranslated region of β-catenin and suppresses Wnt/β-catenin signaling	Su *et al*[62]
miR-374a	Downregulation of Wnt5a in T24 and TCCSUP bladder cancer cell lines	Chen *et al*[49]
miR-370-3p	Downregulation of Wnt7a in 5637 HM1 bladder cancer cell lines	Huang *et al*[48]
miR-1826	G1 cell cycle arrest *via* β-catenin/mitogen activated protein kinase 1/vascular endothelial growth factor C downregulation	Hirata *et al*[51]
miR-3619-5p	Downregulates β-catenin and cyclin-dependent kinase 2 and activates tumor protein 21	Zhang *et al*[63]

GSK-3β: Glycogen synthase kinase-3β.

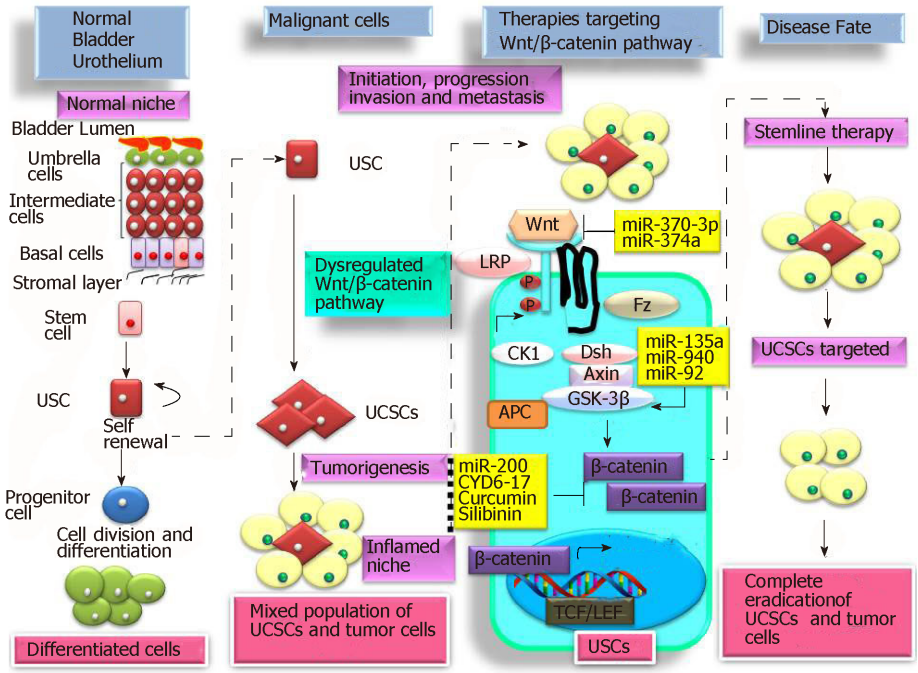

Figure 2 Potential therapies based on targeting the key regulatory molecules of canonical Wnt /β-catenin signaling in urothelial tumorigenesis.

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