Epithelial-to-mesenchymal transition: Event and core associates in bladder cancer

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1. ABSTRACT

Urothelial carcinoma of the bladder (UCB) shows different biological outcomes, diverse biological propensities for invading the muscularis as well as epithelial-to-mesenchymal transition (EMT), a dynamic key event during developmental processes, wound healing, and tissue repair. The EMT core molecules include EMT-activating transcription factors (EMT-ATFs), and a host of downstream effectors and target genes including extracellular inducers and growth factors. Here, we describe molecular regulatory determinants of mesenchymal-to-epithelial transition (MET) and more specifically EMT that allows a subset of urothelial cancer cells to gain mesenchymal traits with self-renewal potential. EMT accelerates tumor progression and poses a clinical challenge to anticancer therapies. Targeting the populations of tumor-initiating cells and those with a metastable phenotype provide the basis for the development of more reliable risk assessment of tumor progression and risk, and better treatment strategies of UCB.

2. INTRODUCTION

Bladder cancer is the second most common cancer of the genitourinary system and ranks ninth among the most frequently diagnosed malignancies worldwide (1). Majority (90%) of bladder cancers are histologically classified as urothelial carcinoma of the bladder (UCB), earlier known as transitional cell carcinoma. Schistosomiasis infections are identified as the root cause of non-urothelial histologies and are found to be more prevalent at least in part, in some areas of the world including Africa and the Middle East (1).

Higher rates of age-standardized incidences, multiple recurrences and heterogeneous nature of disease cause excessive burden on health care systems. The anatomical, genetical, hormonal and socio-economical status, environmental/industrial/occupational exposure to carcinogens/aromatic amines, use of tobacco, and prolonged use of arsenic-contaminated or chlorinated drinking water are the major risk factors for bladder cancer (2). These risk factors may lead to changes in urothelium, thus represent potential reasons for substantial molecular differences among clinically different types of bladder cancer.

Majority of UCB (80%) arise through the non-invasive papillary pathway, are diagnosed as papillary bladder tumors. These tumors are identified with the growth of hyperplastic urothelium towards the bladder lumen. Transurethral resection (TUR) of the bladder is the treatment of choice; however, still 50% to 90% of patients suffer frequent recurrences (3). Despite multiple recurrences, only 15%-20% of patients progress to muscle-invasive cancer. Muscle-invasive bladder cancer (MIBC) evolves from severe dysplasia or carcinoma *in situ* (CIS). Despite radical resection, tumors progress in 50% of such patients and the 5-year overall survival rate is reduced to 5%. Heterogeneous clinical outcomes, multifocal nature of tumor and higher rate of recurrences require attentive bladder cancer surveillance, thus makes the bladder cancer, an expensive disease to manage (4).

Detailed biological and molecular insights into the disease pathogenesis may help clinicians

Table 1. Molecular aberrations and their clinical implications in bladder cancer pathogenesis

Molecular alterations/ mutations during bladder cancer pathogenesis	Clinical implications in bladder cancer		References
At Genomic level			
-Chromosomes Y and 9 -Chromosome1q	Loss Gain	pTa: grade 1 and 2 tumors	7
-*p53* -DNA sequence copy number change of 2q, 5q, 6q, 8p and 11p -DNA sequence copy number change of 1q, 3p, 3q, 5p, 6p, 8q und 10p	Alterations Deletions Gain	pT1-4 tumors with invasive growth pattern	7
At Proteomic level			
-MAPK -Cyclin D1 expression -TERT, HRAS, FGFR, and PI3K -Phosphatase tensin homolog	Activated Increased Oncogenic mutations Oncogenic mutations	Papillary tumor	3, 4
- tumor protein 53 -cell cycle regulators -RB1 and CDKN2A /p16INK4A	Inactivation Loss of function Mutations	Muscle invasive disease	3
At Epigenetic level			
-*NPTX2, ZIC4, PAX5A, MGMT, IGSF4, GDF15, SOX11,HOXA9, MEIS1, VIM, STK11, MSH6, BRCA1, TBX2, TBX3, TERT, GATA2, DAPK1, CDH4, CCND2, GSTP1, CDKN2A, CDKN2B, WIF1, A2BP1* -Modification in Histone proteins:H2A, H2B, H3, and H4	DNA hypermethylation at promoter sites leads to bladder cancer progression		13
-Long non-coding RNA: TP73-AS1; LSI<u>NCT5</u>; H19; HOTAIR	Chromatin remodeling - Invasive bladder cancer		14
-Micro RNA:miR-200 family; miR-34; LIN28; let-7; miR-29b; miR-21; miR-429; miR-221; miR-301b; miR-23b; miR-148a-3p; miR-451; miR-433; miR-199a-5p; miR-30c	Chromatin regulation; Regulation at the post-transcriptional level		24, 51-56, 58-63, 80, 81, 89-92
At cellular level			
-CDH1, zona occludens 1, claudins, occludins, cytokeratins and components of basement membrane including collagen IV and laminin 1	Downregulation		10
-Fibroblast-specific protein 1, type 1 collagen, CDH2 and CDH3.	Upregulation		10

pT: Primary Tumor; MAPK: Mitogen-activated protein kinases; TERT: Telomerase reverse transcriptase; HRAS: Harvey rat sarcoma viral oncogene: FGFR: Fibroblast growth factor receptor; PI3K: Phosphoinositide 3-kinase; PTEN: Phosphatase tensin homolog; TP53: tumor protein 53; RB1: Retinoblastoma; CDKN2A: Cyclin dependent kinase inhibitor 2A/p16INK4A; NPTX2: Neuronal pentraxin 2; ZIC4: Zinc finger protein of the cerebellum 4; PAX5A: Paired box 5A transcription factor; MGMT: O(6)-Methylguanine-DNA methyltransferase; IGSF4: Novel immunoglobulin (Ig)-like intercellular adhesion molecule; GDF15: Growth/differentiation factor 15 ; SOX11: SRY-Box 11; HOXA9:Homeobox A9; MEIS1:Meis Homeobox1; VIM: Vimentin ; STK11: Serine/Threonine kinase 11; MSH6: MutS Homolog 6; BRCA1: Breast cancer type 1; TBX2: T-box transcriptional factor 2; TBX3: T-box transcriptional factor 3; TERT: Telomerase reverse transcriptase; GATA2: GATA binding protein 2; DAPK1:*Death-associated protein kinase 1*; CDH4: Cadherin 4; CCND2: Cyclin D2; GSTP1: Glutathione S-transferase pi 1; CDKN2A: Cyclin-dependent kinase Inhibitor 2A; CDKN2B: Cyclin-dependent kinase inhibitor 2B; WIF1: WNT Inhibitory Factor 1; A2BP1: Ataxin 2-Binding Protein 1; CDH1: E-cadherin; CDH2: N-cadherin; CDH3: P-cadherin; LSINCT5: long stress-induced noncoding transcript 5; HOTAIR: HOX transcript antisense RNA

for adequate treatment decisions at the time of its presentation; thereby minimize the risk of tumor recurrence and shorter survival probabilities of patients (5). Nevertheless discovering molecular markers for identifying patients with invasive tumors; high recurrence rates; and development of effective therapies for locally progressed and metastatic cancers, are some of the challenges in the clinical setting (6).

Findings of genome-wide approaches and candidate gene analysis validate the significance of mitogen-activated protein kinases (*MAPK*) activation; increased *cyclin D1* expression; oncogenic mutations in telomerase reverse transcriptase (*TERT*), Harvey rat sarcoma viral oncogene (*HRAS*), fibroblast growth factor receptor (*FGFR*), and phosphoinositide 3-kinase (*PI3K*), in the development of papillary cancer (4, 6). Increased chromosomal instability;

phosphatase tensin homolog (*PTEN*) inactivation; loss of tumor protein 53 (*TP53*) function; severe disturbances in cell cycle regulators, mutations in retinoblastoma (*RB1*) and cyclin-dependent kinase inhibitor 2A (*CDKN2A*)/*p16INK4A*; and altered DNA methylation and its effect on the expression of cell cycle regulators, promote muscle-invasive disease (6, 7). Mutations in *PI3K*, tumor suppressor genes, deleted in bladder cancer 1 (*DBC1*), tuberous sclerosis 1 (*TSC1*), *CDKN2A* and patched are noticed in both papillary and invasive tumors (8). Gene expression, whole genome array-CGH (comparative genomic hybridization) and mutation analyses validate *FGFR3, PIK3CA, KRAS, HRAS, NRAS, TP53, CDKN2A,* and *TSC1* genes as molecular signature tools in the classification of urothelial carcinoma into low-grade/high-grade as well as NMIBC/MIBC with high precisions and sensitivities (9) (Table 1). Gene expression profiling studies associate basal tumors

with better survival, while *p53*-like tumors with bone metastases and chemoresistant disease thereby identify subtypes of urothelial cancer that differ in their natural history and sensitivity to chemotherapy (32). Mutational status of DNA repair genes-Excision repair cross-complementing rodent repair deficiency, complementation group 2 (*ERCC2*), Fanconi anaemia complementation group C (*FANCC*), ataxia telangiectasia mutated (*ATM*) and *RB1*provide the basis of differential clinical responses to neoadjuvant platinum-based chemotherapies/targeted therapies thereby offer opportunities for personalization of bladder cancer therapy (33).

Genetic and epigenetic changes may lead to altered molecular pathways involved in tumor development, and subsequently, drive bladder tumor cells towards epithelial-to-mesenchymal transition (EMT) (10-12, 34). EMT endows sessile, non-motile urothelial cells to undergo complex reprogramming accompanied with the loss of apicobasal polarity and gain of migratory abilities. Molecular regulatory determinants regulate EMT, allow a subset of urothelial cancer cells to gain mesenchymal traits with self-renewal abilities, evade immune mechanisms and infiltrate the surrounding basement membrane. Tumor microenvironment influences tumor cells with mesenchymal phenotype to switch to epithelial phenotype via reverse process to EMT, known as mesenchymal-to-epithelial transition (MET) (35). Urothelial tumor cells then colonize to distant organ sites, establish pre-metastatic niches, and may survive against anticancer therapies (3). The present review extends our understanding of the regulatory functions of EMT/core associates and their clinical implications in urothelial tumorigenesis, which may facilitate the individualization of therapeutic decisions.

3. EMT/MET AND ITS CORE ASSOCIATES

EMT, an essential and naturally occurring physiological phenomenon, starts with alterations in cell morphology, cell-cell adhesion capabilities, apicobasal polarity and cell motility. Chain of events from the cells' inside to extracellular matrix (ECM) triggers loss of intracellular tight junctions, stress fiber redistribution, acquisition of mesenchymal-like traits with self-renewal abilities and increased invasive potential. Based on biological functions, the EMT program is classified as type 1, type 2 and type 3 EMT. Type 1 EMT is a natural gestational orchestration and vital for morphogenesis during embryonic development. Type 2 EMT functions in wound healing or fibrosis and is majorly identified in kidney, liver, lung, and intestine. Its association with the secretion of different inflammatory components that interact with ECM, causes epithelial layers to dissociate due to basement membrane degradation. Type 3 EMT, also known as oncogenic EMT, translates early-stage tumor into invasive malignancies. EMT

and MET promote cellular/epithelial plasticity by allowing tumor cells to switch between epithelial and mesenchymal phenotypes, thus induce metastatic dissemination (36).

Advancements in gene expression profiling, next-generation sequencing and the explosion of non-coding RNA provide host of information on essential roles of EMT in urothelial tumorigenesis and in its molecular subtyping. Downregulation of *CDH1* (E-cadherin), zona occludens 1, claudins, occludins, cytokeratins and components of basement membrane including collagen IV and laminin 1; and upregulation of fibroblast-specific protein 1, type 1 collagen, N-cadherin (*CDH2*) and P-cadherin (*CDH3*) are essential features of EMT at cellular level (Table 1). Reduced hemophilic interactions between E-cadherin on adjacent cells are responsible for the loss in cell-cell adhesion and increased cell motility. Its attenuated expression is reported to be associated with high progression rate of bladder cancer cells (32, 37-39). Focal loss in E-cadherin facilitates the release of β-catenin, thereby contributes to reduced anchorage to the actin cytoskeleton and poor mechanical stability. Nuclear translocation of β-catenin and activation of *Wnt* target genes result in EMT and increased metastasis (40). Interaction of N-cadherin with *FGFR1* dictates activation of MAPK/extracellular signal-regulated kinase (*ERK*), increases the expression of matrix metalloproteases 9 (MMP-9), and promotes tumor invasion. N-cadherin is considered as an independent marker for prognosis in low stage bladder tumors (41). Gain in N-cadherin regulates tumor invasiveness through the PI3K/protein kinase B (Akt) pathway. Recent studies document increased expression of P-cadherin as a marker of poor prognosis in bladder tumorigenesis. It is normally expressed in the basal cell layer of the urothelium. Forced expression of P-cadherin is shown to modulate catenin expression and enhance the migration of EJ and UM-UC-3/P-cadherin transfectants (42). Multivariate analysis identifies an independent association of expression levels of E-cadherin, MMP-9, and Twist; tumor size; and concomitant carcinoma *in situ* with intravesical recurrence-free survival of patients diagnosed with non-muscle invasive tumors (43).

Core associates of the event influence EMT/MET by the secretion of stroma-modulating ligands/ECM-degrading enzymes including VEGF, PDGF, basic FGF2, EGF receptor (EGFR), transforming growth factor β *(*TGFβ), tumor necrosis factor-α (TNF-α), Hedgehog, Notch and nuclear factor-kappa B (NF-kB) ligands; overexpression of EMT-activating transcription factors (EMT-ATFs); microRNAs; and oncoactivation of downstream signaling pathways; and overexpression of extracellular matrix (ECM)-degrading enzymes such as MMPs. These factors recruit mesenchymal progenitors, drive cellular
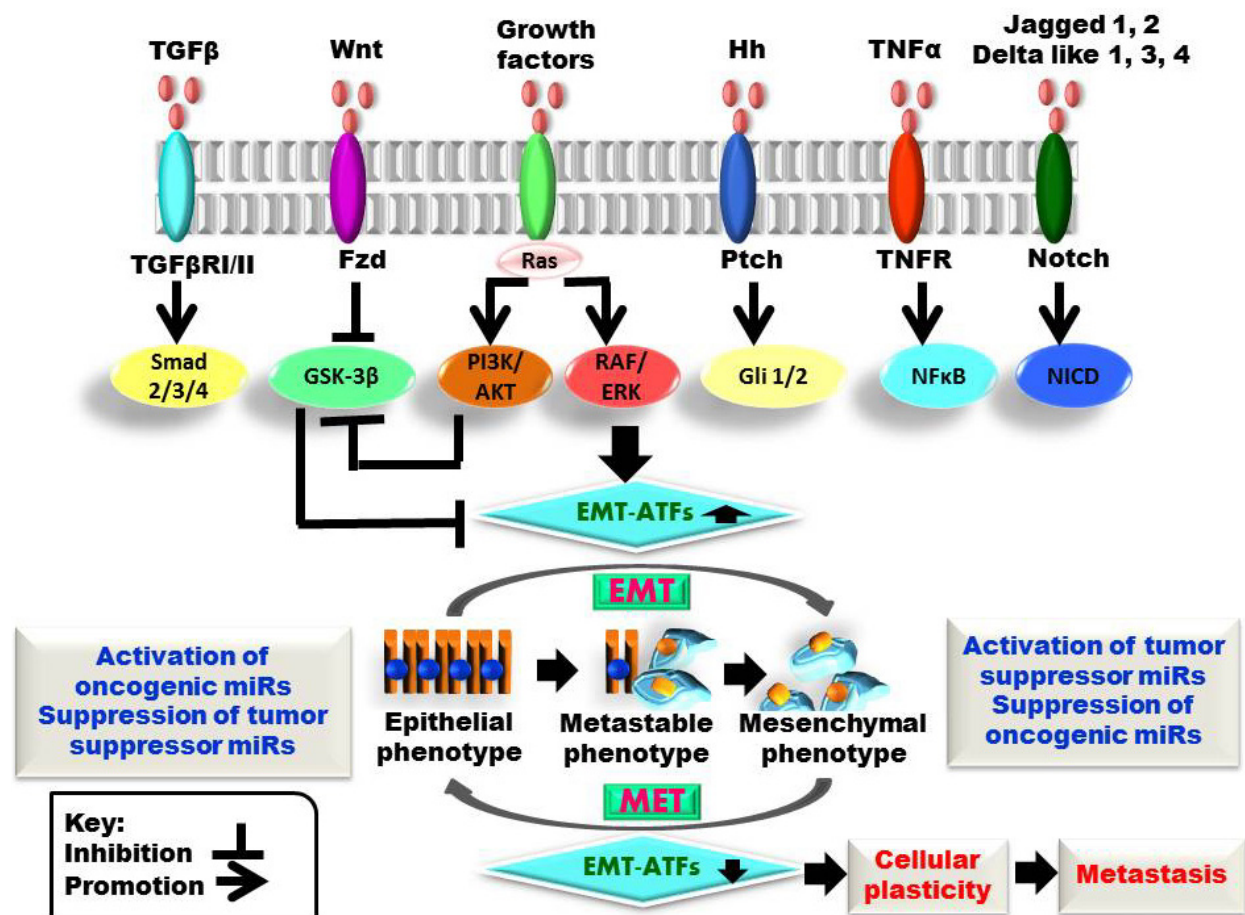
Figure 1. EMT and its core associates include (i) secretion of stroma-modulating growth factors; (ii) upregulation of EMT-ATFs; and (iii) activation of oncogenic microRNAs and suppression of tumor suppressor microRNAs. These factors activate oncosignaling pathways, elicit EMT/MET, recruit mesenchymal progenitors, drive cellular plasticity, stimulate angiogenesis and tumor invasion. EMT: Epithelial-to-mesenchymal transition; MET: Mesenchymal-to-epithelial transition; EMT-ATFs: EMT-activation transcription factors.

plasticity, stimulate angiogenesis and tumor invasion (Figure 1).

3.1. Stroma-modulating ligands and oncosignaling pathways

Stimulated secretion of growth factors and their binding to the receptors, activate multiple complex signaling cascades. Pathways interact through cross talk, mobilize transcription factors and elicit EMT response. Binding of growth factors results in concomitant activation of receptor tyrosine kinases (RTKs), ERK 1/2, p38 MAPK and tyrosine-protein kinases (Src). Regulatory functions of MAPK activation, ERK1/2 and p38 pathways in cigarette/tobacco smoke-triggered urocystic EMT are observed in human normal urothelial cells and BALB/c mice (44, 45). Targeting the smoke-induced MAPK activation and EMT may lead to chemoprevention of smoke-associated bladder cancer (44, 45). Underlying molecular mechanisms of enhanced MAPK signaling,

which inhibits apoptosis, induces morphological change, enhances migratory and invasive capacities and triggers EMT, have been examined (46). Integrin-linked kinase (ILK), a multifunctional adaptor protein, induces the expressions of key target proteins involved in PI3K/AKT/mechanistic target of rapamycin (mTOR) signaling pathway, thereby modulates cancer cell movement, cell cycle, EMT, metastasis and angiogenesis of xenograft bladder tumor (47). TNF-α stimulates MMP-9 expression by activating the transcription factor NF-κB, induces invasion and migration of 5637 human urinary bladder cancer cells (48). Suppression of Fas/Fasl and TNF-alpha/TNFR1 pathway in human bladder cancer cells *in vitro* and *in vivo,* mediates reduced apoptosis and enhanced tumor growth (49). NF-κB signaling pathway when activated, has been examined to be associated with upregulation of EMT markers, maintenance of stem cell marker, elevated expression of ABCB1, and cisplatin-induced bladder cancer chemoresistance (50). RNAi-mediated suppression

of *NF-κB* is associated with partial reversion of EMT and inhibition of the invasive ability of bladder cancer cell *in vitro* (51). Overexpression of HGF and EGF induces β-catenin signaling by stabilizing β-catenin. Its nuclear translocation along with lymphoid enhancer factor1/T-cell factor (LEF1/TCF) transcription factors drives the expression of target genes, promotes EMT, cell migration and significantly increases the ability to form tumors in a nude mouse xenograft model (52). Wnt7a depletion decreases levels of active β-catenin and its downstream target genes involved in EMT and ECM degradation and reduces cell invasion, in 5637 HMI and T24 bladder cancer cells (53). Secretion of Jagged1, 2 and Delta like1, 3, 4 ligands, when bind with Notch receptors, initiate Notch signaling. Cleavage of receptors by γ-secretase followed by the release of its intracellular domain and its translocation to the nucleus elicits expression of target genes. Recent studies report an association of Jagged2 expression status with tumor stage, grade, recurrence and metastasis of urothelial carcinomas (54). Jagged2 regulates cancer cell cycle from G1/S to G2/M, thus contributes to bladder cancer cells' proliferation and invasion. In another study, forced overexpression of the intracellular domain of Notch2 (N2ICD) has been shown to induce bladder cancer growth and invasion by cell-cycle progression, maintenance of stemness, and EMT activation. Its overexpression correlates with adverse disease parameters and worse prognosis in an orthotopic xenograft model (55). Complete inhibition of the Notch signaling has been identified to suppress EMT, inhibit cell proliferation and invasion, and reduce drug resistance in bladder cancer cells (56). Studies on mouse models report the effect of genetic inactivation of Notch signaling on the phosphorylation of ERK1 and ERK2 (ERK1/2) by downregulating dual-specificity phosphatases (DUSPs). Loss of Notch activity has been examined as a driving event in urothelial cancer (57). Sonic hedgehog (Shh) signaling activation is known to promote bladder cancer stem cell (CSC) phenotype, EMT and tumorigenicity. Overexpression of glioma-associated oncogene family zinc finger 1 (GLI1) transcription factor exerts migratory and invasive effects by stimulating EMT associated proteins in 5637 and T24 bladder cancer cells (58). Study by Pignot *et al.* explores the prognostic significance/constitutive expression of *Shh* ligand gene and *Gli*-inducible target genes [Forkhead Box M1 (*FOXM1*), insulin-like growth factor 2 (*IGF2*), osteoblast-specific factor 2 (*OSF2*), *H19*, and Secreted Phosphoprotein 1 (*SPP1*)] in most of the non-muscle invasive and 50% of muscle-invasive bladder cancers (59). Positive correlation of elevated expressions of Ki-67, Shh, Gli2, and N-cadherin with high stage and grade human bladder tumors is reported (60). Application of TGFβ induces bladder cancer cell migration and invasion via mTORC2 signaling by activating AKT, a selective target of mTORC2, in a SMAD2 (mothers against decapentaplegic homolog 2) and SMAD4 (mothers against decapentaplegic

homolog 4)-independent manner. Increased levels of TGFβ isoforms, receptors, and signaling components including phosphorylated SMAD2 associate with invasive bladder cancer, frequent recurrence and poor disease-specific survival (61). TGFβ1 treated HTB-9 xenograft mouse models exhibit strong evidence for a switch to a more stem cell-like phenotype, functional activation of Sox2, CD133, Nanog, octamer-binding transcription factor 4 (Oct4), bladder cancer specific stem cell markers CK5 and CK14, EMT activation and enhanced tumor growth (60). Small interfering RNA (siRNA) significantly suppresses the invasiveness and motility of T24 bladder cancer cells by inhibiting TGF-β1 signaling pathway and downregulating the expression of alpha3, beta1 and alpha2 integrin subunits and the activity of MMP-9 (62).

3.2. EMT-activating transcription factors

Snail1 (Snail), Snail2 (Slug) and Snail3 (Smuc) transcription factors, being the members of the Snail family share the SNAG domain at N terminus and zinc finger cluster at C terminus. Snail regulates target genes' expression by either binding with E-boxes at the promoter region of target genes or by histone modification (methylation/demethylation/deacetylation). Secretion of TGFβ, TNF-α, Notch, EGF, FGF, Shh, Wnt, SCF/c-kit, estrogens modulate transcriptional activities of Snail, thereby promote EMT (36). Association of Snail1 silencing with induced expression of E-cadherin; decreased vimentin, MMPs, and C-X-C chemokine receptor type 4 (CXCR4) levels, explain their crucial role in the progression and migration of UCB (63). Elevated levels of Slug and its involvement in EMT induction via control of cadherin switch, demonstrate that it is a potential marker for improving diagnosis and an important target for treating of muscle-invasive bladder cancers (64). Zinc-finger E-box binding homeobox (Zeb1) and Smad-interacting protein (SIP1)/Zeb2 transcription factors are conserved homologues belonging to Zeb family. Epigenetic modifications including SUMOylation by Pc2, acetylation by p300 and p300/CBP associated factor (pCAF), and phosphorylation, hypoxia-inducible factor-1α (HIF-1α), inflammatory cytokines and growth ligands (FGF, IGF, PDGF, TGFβ, Smad, Wnt and Notch), activate Zeb expression and promote EMT (65). Twist 1 and Twist 2, the basic helix-loop-helix (bHLH) factors, bind with target DNA, facilitate homo/hetero-dimerization, thus activate or repress transcriptional activities. The phosphorylation of specific amino acid residues of bHLH domain alters dimerization partner choice and binding affinity for DNA. Binding of Twist 1 with polycomb repressor complexes PRC1 and PRC2 and components of nucleosome remodeling deacetylase (NuRD) complex, at E-cadherin promoter regulate its repression. However, binding with H4K20 methyltransferase SET8 activates N-cadherin and suppresses E-cadherin. E12, another transcription

factor, interacts with Twist, facilitates hetero-dimerization and regulates transcription (66-68). Krüppel-like factor 4 (*KLF4*), an important prognostic marker, activates Twist 1, reduces E-cadherin and β-catenin expressions, induces vimentin and fibronectin expressions, regulates EMT/metastasis and predicts patients' survival (69).

3.3. MicroRNAs

MicroRNAs (miRs), ~ 21 - 23 nucleotide short double-stranded RNA molecules are frequently organized in clusters on all human chromosomes except Y chromosome, generally expressed as polycistronic transcripts and post-transcriptionally inhibit mRNA functions by binding to the 3'-untranslated region (3'-UTR) of their target mRNAs. Overexpression of miRs with oncogenic functions and downexpression of miRs with tumor suppressor functions play a significant role in tumor development and progression by regulating diverse cellular pathways (13).

Regulatory mechanism of *miR-323a-3p/MET/SMAD3/SNAIL* circuit in EMT induced progression of bladder cancer is well established (14). EMT core decision-making circuits, m*iR-200* family/Zeb and *miR-34* family/Snail, regulate *LIN28/let-7*and pull the stemness window away from the mesenchymal end of EMT axis. As a result, tumor cells with E/M phenotype gain stemness, and are associated with tumor aggressiveness/poor patient outcome. siRNA mediated silencing of Snail1 results in reduced levels of *miR-21* and *miR-29*b and triggers apoptosis in bladder cancer cells (15). *MiR-429* has been identified with the potential to reduce cell migratory abilities by reverting EMT via reducing Zeb1 and β-catenin, restoring the E-cadherin expression and inactivating MMP-2 (16). The expression of *miR-221* promotes *TGFβ1* induced EMT by reducing the expression of E-cadherin and inducing the expression of the mesenchymal markers, vimentin, fibronectin and N-cadherin. Its expression promotes loss of cell adhesion and is positively correlated with the malignant potential of bladder cancer cells (17). *MiR-301b* inhibits the expression of early growth response gene 1 (*EGR1*), regulates EMT, promotes proliferation and migration of human bladder cancer cells (T24) by (18).

Coordinated action between the Polycomb repression complex (PRC) members represses tumor suppressor functions of *miR-200*, post-transcriptionally regulates Zeb1 and Zeb2 and ultimately favors invasive bladder cancer development (70). Lower levels of *miR-200* family*, miR-205*, and *miR-192* in the urine sediment, significantly correlate with urinary expression of EMT markers (Zeb1, vimentin, TGFβ1, and Ras) (19). Overexpression of miR-23b, positively correlated with higher overall survival of bladder cancer patients, regulates Zeb1 levels, suppresses EMT, inhibits cell proliferation and impairs colony formation (20). Higher expression of miR-451 significantly upregulates E-cadherin; downregulates N-cadherin, vimentin and Snail; maintains bladder tumor cells in epithelial phenotype, inhibits EMT, and reduces the invasion and migration of tumor cells (21). Modification of tumor suppressor functions of *miR-148a-3p* by DNA methyltransferase 1 (DNMT1) affects the expression of its downstream target genes (*ERBB3, DNMT1 and AKT2*). In vitro experiments demonstrate the usefulness of overexpressed *miR-148a-3p* towards the development of effective therapies against bladder cancer. It regulates *ERBB3/AKT2/c-myc and ERBB3/AKT2/ Snail* signaling, and inhibits bladder cancer cell proliferation and EMT (22). Downregulated *miR-433* associates with proliferation, colony formation, migration, and invasion in bladder cancer cells. Lower expression levels of *miR-433* induce EMT response by upregulating *c-Met/Akt/GSK-3β/*Snail signaling pathway and thus promote bladder tumorigenesis (23). Recent studies reveal the protective functions of that *miR-199a-5p* against bladder tumorigenesis by targeting the 3' UTR of C-C chemokine receptor type 7 (*CCR7*) and regulating the expression of CCR7, MMP-9 and EMT-related proteins (24).

4. EMT/MET AND UROTHELIAL TUMORIGEN-ESIS: CLINICAL IMPLICATIONS

Epithelial lining of the bladder/urothelium is composed of highly differentiated, multinucleated umbrella cells towards bladder lumen; a variable number of multilayered intermediate cells; and unilayered basal cells with very high proliferative potential (Figure 2). Presence of slow cycling urothelial stem cells (USCs) in the basal cell layer contributes to high regenerative potential (73). Long life span; high colony-forming efficiency; high nuclear-cytoplasmic ratio; low granularity; and expressions of β1/β4 integrins, CD44, cytokeratins (CK-5/14, CK-17) and laminin receptor (LR) are the characteristic properties of basal USCs (72). Recent researches hypothesize the possibility of the existence of another independent pool of USCs, and support the formation of umbrella cells in p63-null mice that lack basal/intermediate cells (73). Regenerative/proliferative capacity diminishes with the differentiation of USCs into transit-amplifying cells of intermediate cell layer and then into umbrella cells. Studies on rat bladder validate the differential/maximal distribution of USCs in the caudal region of the bladder. This research suggests that maximal distribution of USCs may contribute to the relatively higher rate of incidence of carcinoma per surface area in this region (73). Mutagenic insults including genetic instability, epigenetic changes, mutations in oncogenes/tumor suppressor genes and aberrant molecular pathways
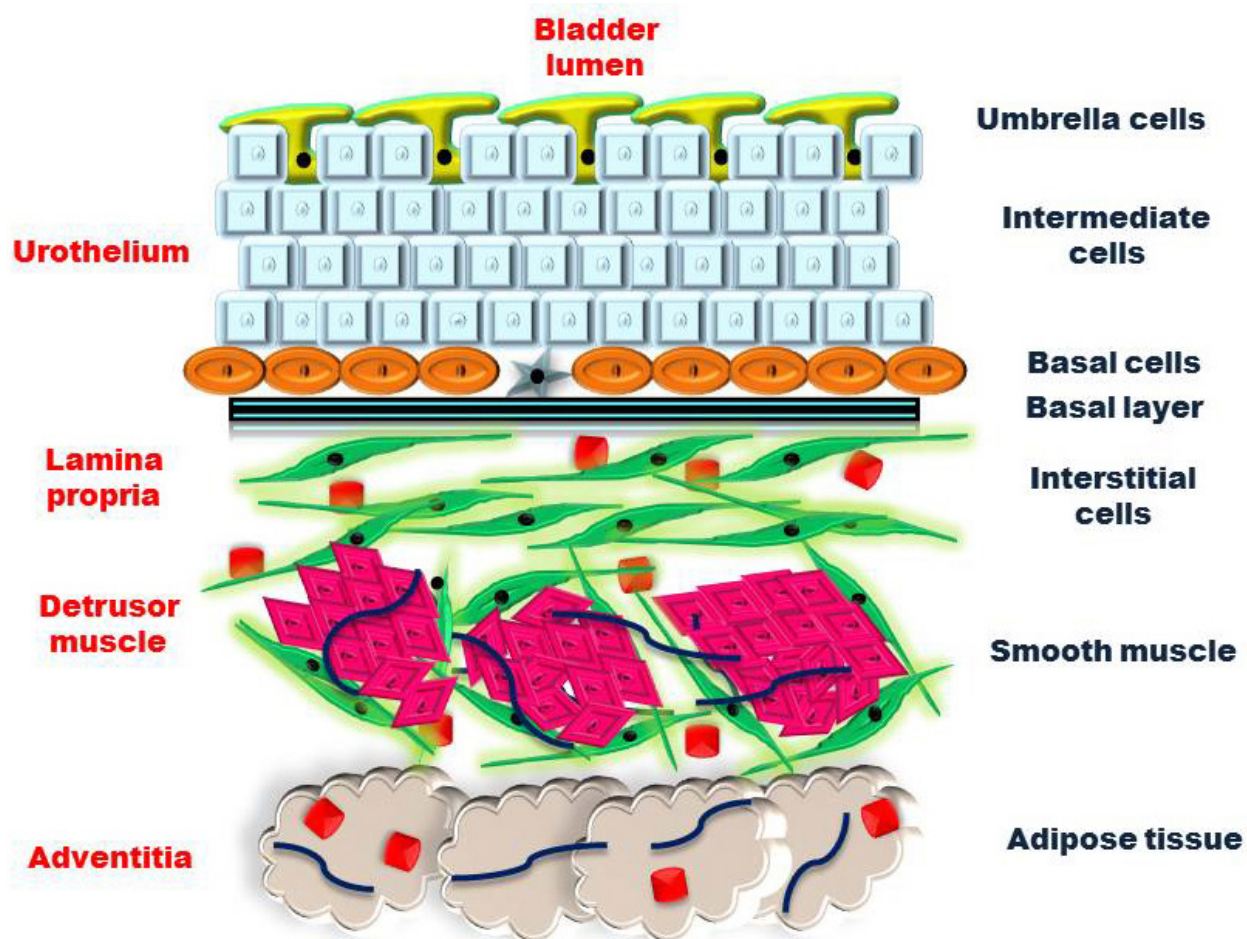
Figure 2. Schematic view of cross-section of the adult bladder wall showing the four major layers: (i) urothelium towards bladder lumen; (ii) lamina propria; (iii) detrusor smooth muscle layer; and (iv) adventitia. Smooth muscle bundles are aligned in lamina propria and detrusor muscle layer.

transform adult stem cells into a subset of unique urothelial cancer stem cells (UCSCs). UCSCs derived from differentiated progenies acquire tumorigenic properties. These cells are characterized by their enriched ability to conserve cellular heterogeneity by inducing xenograft tumors *in vivo* (74). Many cell surface markers are shared by adult stem cells and mature urothelial cells. Subsets of UCSCs, also known as side-population (SP), are examined by their enriched capacity to (i) express cell surface marker proteins including 67LR at the tumor-stromal interface, aldehyde dehydrogenase 1A1 (ALDH1A1^{hi}), CD44, CD44 splice variant6 (CD44v6), CD133, cytokeratins (CK5, CK7, CK17), early progenitor Nestin (NES), and low carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6^{low}); (ii) upregulate oncogenic genes including B lymphoma Moloney murine leukemia virus (*Mo-MLV*) insertion region 1 homolog (*BMI1*), β-catenin, *GLI1, NANOG, POU* domain class 5 transcription factor 1 (*POU5F1*)/*Oct4*, and signal transducer and activator of transcription 3 (*STAT3*); (iii) express high levels of ATP-binding

cassette (ABC) transporters and multidrug resistance pumps (MDRs); and (iv) efflux of vital dye Hoechst 33342 and DyeCycle violet (3).

Heterogenous populations of UroCSCs with high tumour-initiating potential, first identified in 2009, originate from normal stem cells/differentiated progenies with mutational defects, are associated with the acquisition of EMT phenotype (75). UroCSCs with hybrid epithelial/ mesenchymal (E/M) or metastable phenotype are associated with epithelial plasticity, tumor proliferation, quiescence, stemness at tumor bud level, successful colonization of distal target tissues, drug resistance, and poor survival probabilities of the patients. Powerful genome-wide screening techniques set challenges for diagnosing the two distinct types of bladder diseases, monitoring tumor recurrence/ metastasis and providing prognostic risk stratification by identifying predictive markers in the clinical setting.

High-grade NMIBC compared to low-grade NMIBC are observed with higher rates of amplification

of chromosome 6p22.3, *SOX4, E2F3*, Snail, Zeb, and Twist family genes . This supports their potential role as a regulator of bladder CSCs and validates them as predictive biomarkers for an early event in tumor progression (75). Sonic hedgehog promotes EMT induction and bladder carcinogenesis in initial tumor stages. Hh signaling contributes to drug resistance in the later clinical stages and ultimately leads to metastasis (76).

The induction of EMT, actin cytoskeleton remodeling and the overexpression of TGFβ signaling effector, cofilin, in a series of human bladder cancer specimens are significantly associated with bladder cancer progression (77). Whole genome mRNA expression profiling of xenograft models derived from human bladder cancer cell lines demonstrates the onset of reversible EMT via TGFβ/Snail pathway activation and its association with the accumulation of circulating tumor cells (CTCs) and regional/metastatic lesions (78). The findings of the experimental research performed by Liang *et al.* identify the mechanisms of TGFβ signaling in promoting EMT, cancer stemness and bladder cancer progression *in vivo* (79). Another study performed by Yu *et al.* demonstrates positive correlation between the loss of Nkx2.8, candidate tumor-suppressor gene, and lymph node metastasis. Overexpression of Twist1 reverses EMT inhibition by *Nkx2.8* and restores the invasive phenotype of urothelial carcinoma cells (80). PI3K-deregulated p27 binds Janus kinase 2 (JAK2), drives STAT3 recruitment/activation, upregulates Twist1, induces EMT, activates AKT and promotes cancer invasion and metastasis (81). SOX4, a potential regulator of the bladder cancer stemness, serves as a biomarker of tumor progression from NMIBC to MIBC and its expression correlates with advanced cancer stages and poor survival/prognosis (75). Addition of androgen receptor (AR) contributes to the development and progression of upper urinary tract urothelial cell carcinoma by significantly expanding the population of CSCs; increasing the clonogenicity/tumor sphere formation, upregulating the expression of Oct4, Bmi1 and Nanog, altering CSC-related miRNA profile, and promoting EMT (82). The positive relationship among higher levels of lactate dehydrogenase-A(LDH-A), CSC/EMT markers, cell proliferation, invasion and migration suggests an important function of *LDH-A* in malignant progression in invasive bladder cell line and muscle invasive patients (83). A study by Vantaku *et al.* examines higher expression of ganglioside GD2, cell proliferation, mesenchymal and cancer stem cell phenotypes in high grade, muscle-invasive bladder cancer tissues (84). Experimental research by Franzen *et al.* provides insights into the role of exosomes in the transition of bladder cancer into the invasive disease (85). Analysis of clinical specimens identifies a significant association of CD44 staining

with higher loco-regional failure rate, advanced clinical stage, and lower disease-specific survival rate for MIBC patients. The results of cellular experiments and orthotopic tumor models provide evidence on regulatory functions of activated IL-6 signaling in the induction of CD44 expression by providing a suitable microenvironment, activating STAT3, promoting EMT, and regulating programmed death ligand 1-mediated T-cell suppression (86). Exosomes isolated from T24/UMUC3 invasive bladder cancer cells and patient's urine and bladder barbotage samples potentially modulate signaling pathways in recipient urothelial cells. Bladder cancer-shed exosomes can induce EMT and show tumorigenic effects. These exosomes increase the expression of mesenchymal proteins, α-smooth muscle actin, S100A4 and Snail, reduce the expression of E-cadherin and β-catenin levels, thereby lead to increased migration and invasion of urothelial cells (85).

Upregulated expression of *circPRMT5*, a novel class of non-coding circular RNAs, in serum and urine exosomes positively associates with advanced clinical stage and worse survival of UCB patients. Clinical analysis from two independent cohorts of UCB reveals the regulatory effect of *circPRMT5* on cells' EMT via sponging *miR-30c* and its target genes (Snail1 and E-cadherin) in UCB progression (25). *MiR-200* and *miR-205* silencing, DNA hypermethylation and chromatin repression are proposed as possible prognostic markers in bladder cancer (26).

5. SUMMARY AND PERSPECTIVES: EMT/MET AND UROTHELIAL TUMORIGENESIS: THERAPEUTIC IMPLICATIONS

EMT, a trans-differentiation process, stimulates the expansion of cancer stem/progenitor cells and is thus responsible for increased invasiveness, drug resistance and angiogenesis. Tumor-associated stromal cells and ECM provide protective cancer stem cell niches, influence the tumor growth, and develop more aggressive tumor types. Understanding the regulatory functions of EMT and its core associates not only set challenges for their diagnostic and prognostic implications in risk stratification of pathologically similar urothelial tumors, but also provide the platform for the development of novel therapy. Combinational effective therapeutic strategies based on the potential use of drug/molecules may create an inhospitable microenvironment, suppress EMT and kill cancer stem/progenitor cells.

Subcutaneous bladder tumor regeneration system, developed by recombining benign human fetal bladder stromal cells with human bladder cancer cells, recapitulates malignant human bladder tumor architecture and mimics human disease. Tumor

cells in the presence of stroma exhibit EMT, cancer stemness, decreased differentiation, greater activation of oncogenic/ growth pathways, accelerated growth, and muscle invasion (87). Such efficient bladder tumor models may help elucidate the influences of stroma on tumor growth and development, and provide an accessible system for therapeutic testing.

Long-term treatment of urothelial cancer cells with drug/cisplatin does not result in enrichment of CD90 (+) cells with stemness and enhanced clonogenic characteristics. Nevertheless, these sublines display substantial phenotypic plasticity, express EMT associated markers, an altered pattern of CKs, Wnt-pathway target genes and contribute to the emergence of cisplatin resistance (88). Targeted therapies based on the use of inhibitors of urothelial plasticity by controlling EMT and its core regulators may suppress metastatic growth and tumor relapse. Epigenetic modifiers inhibit transcription factors involved in chromatin deregulation, suppress bladder cancer stem cell maintenance, and therefore, serve as the potential molecules of therapeutic significance (75). Myrtucommulone-A (MC-A), an anti-cancer novel agent, concomitantly inhibits multiple signaling pathways, ERK 1/2, p38 MAPK, and Src activity; downregulates EMT by affecting N-cadherin, β-catenin, Snail, and Slug expressions; and reduces cross-talk in bladder cancer cells (89). Enzalutamide, an AR inhibitor, has been shown to suppress its effects on tumorsphere formation. Targeting AR may lead to novel therapeutic approaches for genetically diversified urothelial carcinomas (84). Chemopreventive effects of curcumin reverse tobacco smoke-elicited activation of Wnt/β-catenin, urocystic EMT and acquisition of cancer stem cell properties (90). TGFβ receptor inhibitor treatment ablates TGFβ signaling; inhibits cancer cell proliferation, cancer stem cell population, and EMT; and suppresses invasive cancer progression (79). Silibinin, a nontoxic natural flavonoid, inhibits glycogen synthase kinase 3β (GSK3β) phosphorylation, promotes β-catenin nuclear translocation and transactivation and reverses EMT by suppressing Zeb1 levels and modulating the levels of cytokeratins, vimentin and MMP2. In addition, it suppresses the properties of UroCSCs, side population, spheroid colony formation, expression of stem cell factor CD44, thus represents a novel natural treatment in targeting bladder cancer metastasis (91). Owing to oncogenic functions of αv integrins in migration, EMT, and maintenance of ADH activity, treatment with an αv integrin antagonist or its knockdown in bladder cancer cell lines results in increased CDH1/CDH2 ratio; downregulation of Snal2, Nanog, Bmi1 and ADH; and decreased proliferative, migratory and clonogenic capacity (92).

Local administration of antagomiRNAs/antimiRNAs to suppress its oncogenic functions or miR replacement therapy to restore the loss of function, modifies stromal region, reverses EMT, and sensitizes CSCs to anti-cancer drugs. Differentiation therapies based on the stable expression of *miR-200* and treatment with anti-EGFR antibody cetuximab in mesenchymal human bladder cancer cell lines reduce cell migration, modulate EMT, induce differentiation, and restore the sensitivity of cancer cells to EGFR inhibitors (3). Overexpression of TP73-AS1, a long noncoding RNA (lncRNA) with tumor suppressor functions, diminishes EMT by upregulating E-cadherin and downregulating vimentin, snail, MMP-2, and MMP-9 (27). Increased expression of lncRNA, long stress-induced non-coding transcript 5 (LSINCT5), in human bladder cancer cell lines and its correlation with poor prognosis, enhanced Wnt/β-catenin signaling activation and EMT may validate it as a potential candidate for therapeutic intervention (28). Overexpression of *miR-323a-3p* significantly inhibits EMT and progression of bladder cancer by regulating *MET/SMAD3/SNAIL* circuit (14). Anti-metastatic effects/pro-apoptotic functions of *miR-34a* are determined to sufficiently reverse EMT by directly targeting CD44 gene and restoring the endogenous levels of E-cadherin in cancer cell lines (29).

EMT is reported to coordinately regulate maintenance of cancer stemness, drug resistance, angiogenesis and muscle invasion/metastasis, and is documented as a dominant feature in the biology of urothelial cancer. Successful elimination of subpopulation of UroCSCs and its differentiated progenies by targeting EMT and its core associates could be clinically beneficial in reversing EMT, preventing tumor relapse and prolonging survival of patients.

6. ACKNOWLEDGEMENTS

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Abbreviations: UCB: Urothelial carcinoma of bladder (UCB); TUR: Transurethral resection; NMIBC: Non-muscle invasive bladder cancer; MIBC: Muscle invasive bladder cancer; CIS: Carcinoma *in situ*; MAPK: Mitogen-activated protein kinases TERT: Telomerase reverse transcriptase;HRAS: Harvey rat sarcoma viral oncogene; FGFR: Fibroblast growth factor receptor; PI3K: Phosphoinositide 3-kinase; PTEN: Phosphatase tensin homolog; TP53: Tumor protein 53; RB1: Retinoblastoma 1; CDKN2A:Cyclin dependent kinase inhibitor 2A; DBC1: Deleted in bladder cancer 1; TSC1: Tuberous sclerosis; EMT: Epithelial-to-mesenchymal transition; MET: Mesenchymal-to-epithelial transition; CDH1: E-cadherin; CDH2: N-cadherin; CDH3: P-cadherin;ERK: Extracellular signal-regulated kinase; MMP9:Matrix metalloproteases 9; AKT: Protein kinase B; EGFR: EGF receptor; TGFβ: Transforming growth factorβ; TNF-α:Tumor necrosis factor-α: NF-kB: Nuclear factor-kappa B; ERCC2: Excision repair cross-complementing rodent repair deficiency, complementation group 2; FANCC: Fanconi anaemia complementation

group C; ATM:Ataxia telangiectasia mutated; EMT-ATFs: EMT-activating transcription factors; ECM: Extra cellular matrix; RTKs: Receptor tyrosine kinases (RTKs); ILK: Integrin-linked kinase; mTOR: Mechanistic target of rapamycin; LEF1/TCF: Lymphoid enhancer factor1/T-cell factor; Shh: Sonic hedgehog; CSC: Cancer stem cell; GLI: Glioma associated oncogene 1 (GLI); Gli-1: GLI family zinc finger 1; FOXM1: Forkhead Box M1; IGF2: Insulin-like growth factor 2 (IGF2), OSF2: Osteoblast-specific factor 2; SPP1: Secreted Phosphoprotein 1; SMAD2: Mothers against decapentaplegic homolog 2;SMAD4: Mothers against decapentaplegic homolog 4Oct4: Octamer-binding transcription factor 4 (Oct4), CXCR4: C-X-C chemokine receptor type 4; Zeb1: Zinc-finger E-box binding homeobox (Zeb) 1; SIP: Smad-interacting protein; CAF: CBP associating factor; HIF-1α:Hypoxia inducible factor-1α; PRC: Polycomb repressor complexes; NuRD: Nucleosome remodeling deacetylase; KLF4:Krüppel-like factor 4; miRs: MicroRNAs; 3'-UTR : 3'-untranslated region; EGR1: Early growth response gene 1; DNMT1: DNA methyltransferase 1; CCR7:C-C chemokine receptor type 7; USCs: Urothelial stem cells; CK: Cytokeratins; LR: Laminin receptor; UCSCs: Urothelial cancer stem cells; 67LR: 67 laminin receptor; ALDH1A1: Aldehyde dehydrogenase 1A1; CD44v6: CD44 splice variant; NES: Nestin; CEACAM6:Carcinoembryonic antigen-related cell adhesion molecule 6; Mo-MLV: Moloney murine leukemia virus; BMI1: B lymphoma Mo-MLV insertion region 1; POU5F1: Homolog POU domain class 5; STAT3:Signal transducer and activator of transcription 3; ABC: ATP-binding cassette; MDR1: Multidrug resistance pumps; CTCs: Circulating tumor cells; JAK 2: Janus kinase 2; LDH-A: Lactate dehydrogenase-A (LDH-A);MC-A: Myrtucommulone-A; lncRNA: Long noncoding RNA (lncRNA); AR: Androgen receptor; GSK3β: Glycogen synthase kinase 3 beta; Src: Tyrosine-protein kinases; N2ICD: Intracellular domain of Notch2; NPTX2: Neuronal pentraxin 2; ZIC4: Zinc finger protein of the cerebellum 4; PAX5A: Paired box 5A transcription factor; MGMT: O(6)-Methylguanine-DNA methyltransferase; IGSF4:Novel immunoglobulin (Ig)-like intercellular adhesion molecule; GDF15: Growth/differentiation factor 15; SOX11: SRY-Box 11; HOXA9:Homeobox A9; MEIS1: Meis Homeobox 1; VIM: Vimentin; STK11: Serine/Threonine kinase 11; MSH6: MutS Homolog 6; BRCA1: Breast cancer type 1; TBX2: T-box transcriptional factor 2; TBX3: T-box transcriptional factor 3; GATA2: GATA binding protein 2; DAPK1:Death-associated protein kinase 1; CDH4: Cadherin 4; CCND2: Cyclin D2; GSTP1:Glutathione S-transferase pi 1;CDKN2B:

Cyclin-dependent kinase Inhibitor 2B; WIF1: WNT Inhibitory Factor 1; A2BP1: Ataxin 2-Binding Protein 1; pT: Primary tumor; LSINCT5: Long stress-induced noncoding transcript 5; HOTAIR: HOX transcript antisense RNA

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