Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 3, 2014

Original Article

ROLE OF CADHERIN SWITCHING IN EMT AND PROSTATE CANCER METASTASIS - A TOPIC REVISITED

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Received: 21 Jan 2014, Revised and Accepted: 21 Apr 2014

ABSTRACT

The conversion of the sessile epithelial cells to the motile mesenchymal phenotype (EMT transition) involves the characteristic switching of E-cadherin to N-cadherin and is a "signature-like event", involving the TGFβ1-mediated pathway, in the process of invasion and metastasis of prostate cancer cells –a process commonly observed in other cancer cells. The transcriptional epigenetic repression of E-cadherin is associated with and regulated by the expression of ZEB1 – zinc finger homeo-domain transcription repressor, which in turn, is regulated by specific microRNAs. The role of IGF-1, correlatable with ZEB1, through equivocal, may have an important staging-dependent differential role in prostate cancer. Other transcription factors (Snail, Slug & E-47), when expressed, induce, among other signaling molecules, the expression of IGF-1 and Wnt-5. This, in turn, causes E-cadherin repression. One of the major, common downstream pro-survival effector protein is PI3K/Akt and is regulated both by the Ras as well as the TGF-β1 pathways. This pivotal protein is known to protect the cells against TGFβ1-mediated apoptosis and plays an important role in EMT and metastasis. Repression of E-cadherin, is accompanied by the Twist1-dependent expression of N-cadherin. Corroborative evidence supports the abnormal activation of the Wnt/β catenin pathway and this pathway has been strongly implicated in prostate cancer invasion and metastasis pathways while catenin-independent pathways have also been reported apart from important epigenetic mechanisms regulating the inhibitors of the pathway like (Wnt inhibitory factor-1 -WIF-1). This review provides the reader with an update on the role of important signaling molecules and a better molecular understanding of cadherin switching – lessons that can be applied in cancer biology and chemoprevention by ethno-pharmacological and bio-pharmaceutical approaches.

Keywords: E-cadherin, N-cadherin, EMT, Transcription factors, Metastasis, Prostate cancer.

INTRODUCTION

Prostate Cancer, more precisely prostate adeno-carcinoma, is one of the most commonly diagnosed cancers in men (globally), and one of the leading causes of cancer-related deaths in western countries with the incidence being lower in Asia. However, the changing lifestyle and the westernization in terms of increased consumption of fatty food and obesity, apart from improvements in diagnosis and life expectancy, has contributed to the rising incidence of prostate cancer in developing countries. Further, an unfavorable stage distribution has also been reported[1],[2][3] Since this disease is the outcome of a complex interplay between environmental factors as well as the underlying genotype, race and ethnicity are important considerations in the evaluation of gene-gene and gene-environment interactions[4]. This changing risk profile is also mirrored in the decrease in the risk between Asian immigrants and natives in the developed countries [3]. Deaths due to prostate cancer occur mostly due to metastases formed by the tumor cells at secondary sites, particularly bones. Prostate carcinoma forms metastases at secondary sites in a well recognised pattern which involve the axial skeleton and local lymph nodes[5][6]. Metastases are seen in other sites as well such as lungs, brain and liver, but to a lesser extent [6][7]. Skeletal metastases formed by prostate cancer are more frequently osteoblastic in nature and is known to follow an order in terms of frequency with differences in the stage-dependent distribution in the different regions of the spine. This prelidiction for the spinal localization, early in the metastatic process, is consistent with the reported backward spread via the veins in addition to the involvement of the vena cava-based process[8][9].

The propensity of prostate cancer cells to metastasize to secondary organs has been explained by a number of hypotheses. Batson proposed that retention of prostate cancer cells to bones might be due to the retrograde flow of prostate cancer cells in veins[10][11]. The famous "Seed and Soil" hypothesis for metastasis of cancer proposed by Paget suggests that the micro-environment of the secondary site (soil) determines the selectivity of the cancer cells (seed). This theory still holds forth today, as the potential of a tumor cell to metastasize to a secondary site is dependent on its interactions with the micro-environment of secondary site as well as on factors, which promote tumor cell survival, angiogenesis, invasion and metastasis[12][13]. Also, there is evidence of prostate cancer cells in their journey towards acquiring the metastatic phenotype, become osteoblastic, through inductive influences with bone stromal cells. This leads to an alteration in critical transcription factors (Cbfa and MSX) which, in turn, can favor the expression of genes like osteopontin (OPN), osteocalcin (OC) and bone sialoprotein (BSP)[14][15]. There are certain common steps in the metastatic cascade that must occur in all forms of cancer. These sequential and selective steps, with certain stochastic components, involve the loss of cell adhesion at a primary site, invasion, migration, and survival and growth of tumor cells at a secondary site with heterogeneity, both within a single cell and between metastases, as one of its hallmark features[16][17]. The very first requirement for a cell to metastasize is that it loses its adhesion with surrounding cells. Loss of cell adhesion at a primary site is believed to be mediated by Epithelial-Mesenchymal Transition, where the epithelial cells loses their cell-cell junctions and characteristic features and become more motile [18]. In this regard, they re-active an embryonic program, wherein the sessile epithelial cells acquire mesenchymal features and become motile. Further, conversion renders the transformed cells with stem cell-like properties (resistance to therapy and apoptosis, apart from a decrease in senescence as well as the ability to evade the immune system) with the acquisition of the invasive phenotype being important for metastasis to occur[19],[20]. Cadherin switching is one of the aspect of epithelial-mesenchymal transition, where cells switch expression of their characteristic cadherins and express unusual cadherins at **adherans** junctions which affect the phenotype and behaviour of the cells due to a change in the isoform of the cell adhesion protein[21][22][23]. Loss of E-cadherin with increase in expression of N-cadherin is the most remarkable event occurring when a tumor cell acquires metastatic properties[24][25].

Cadherins

Cadherins are the major cell adhesion molecules. They are calcium-dependent adhesion molecules and play a crucial role in the spatial

segregation of cell types and organisation of different tissues during embryonic development[26][27][28]. Cadherins interact with other cadherins on adjacent cells by a complex of proteins called catenins. The catenins bind to the actin cytoskeleton of the cell. The cadherin-catenin complex forms the classic adherans junctions which integrate the epithelial cells in a mechanical unit. Cadherins join cells together by homophillic binding, binds to the same type of cadherin on another cell. Cell adhesion by cadherins is mediated by both the homophillic binding of extra cellular domains and binding of cytoplasmic domain of cadherin with actin cytoskeleton[29][30]. Homophillic binding between the extracellular domains of cadherins is initiated and stabilized by binding of Ca^{2+}[31][32]. E-cadherin, also known as uvomorulin, is expressed on all the early embryonic cells of mammals. Later its expression is restricted to epithelial cells. Mesenchymal cells, which are less polarized and more motile than epithelial cells, express N-cadherin (neural cadherin) and various other cadherins such as R-cadherin and cadherin-11[33][34][35]. VE-cadherin is expressed specifically by endothelial cells at the junctional complex. Endothelial cells also express N-cadherin whose function is unknown as they are not expressed at the junctions. E-cadherin is expressed by epithelial cells where it provides the mechanical strength to the tissue, however many epithelium-derived cancer cells loose the expression of E-cadherin[36][37][38]. Enzymatic activity is not found in classical cadherins and catenins but in adherans junctions, they can associate with kinase and phophatase enzymes such as Fer and PTP1B[39][40]. Adhesion of E-cadherin activates phospatidylinositol 3-kinase (PI3-K) and Akt/protein kinase B[41]. Akt is a serine/threonine kinase which is activated by growth factors and integrin adhesion. Akt plays a regulating role in various metabolic pathways and apoptotic pathways. Phosphorylation of threonine 308 (Thr-308) and serine 453 (Ser-473) by 3-phosphoinositide dependent kinase 1 or phosphoinositide dependent kinase 2 results in activation of Akt[42]. Upon activation, Akt phosphorylates various substrates that suppress apoptosis. When a cell receives apoptotic signals, cell fate is determined by the balance between pro-apoptotic and anti-apoptotic proteins of the Bcl 2 family genes. The pro-apoptotic proteins of Bcl-2 family includes Bad, Bik and Bid and the anti-apoptotic proteins include Bcl-2[43] and Bcl-xL [44]. Formation of homodimers of Bcl-2 in mitochondrial membrane prevents the activation of caspase-9 while formation of heterodimers of Bcl-2 and Bad activates caspase-9[45]. Regulation of apoptotic pathway by Akt involves the phosphorylation of Bad on Serine 136 thereby preventing the formation of heterodimers in mitochondrial membrane[46].

N-cadherin is typically expressed by mesenchymal cells which are more motile in nature than epithelial cells. Studies have reported unusual expression of N-cadherin in epithelium derived tumors and this upregulation of expression of N-cadherin promotes cell motility and invasiveness[47][48]. This shift in the expression of cadherins from E-cadherin to N-cadherin occur during gastrulation where it affects the phenotype of participating cells and helps in the separation of different types of cells, for example, a shift in expression from E-cadherin to N-cadherin helps the segregation of neural tube from the epithelium [49][50].

Cadherin switching and its role in prostate cancer metastasis

Cadherin switching usually refers to shifting of E-cadherin expression to N-cadherin expression but also involves conditions where N-cadherin expression is upregulated without a significant change in expression of E-cadherin and also situations where other cadherins like R-cadherin, P-cadherin, T-cadherin and cadherin-11 etc, are co expressed with E-cadherin[51][52][53]. Cadherin switching has been reported to be an important event occurring during metastatic progression of a tumor by enhancing the invasiveness of the tumor cells[54][55][56][57]. Decrease in expression of E-cadherin and increase in N-cadherin expression has been observed in various metastatic tumors. Studies on prostate cancer cell lines have also reported upregulation of N-cadherin expression that might mediate a homotypic adhesion between prostate cancer cells and stromal fibroblasts and facilitate metastasis[58]. Invasion of prostate cancer proceed through the surrounding stroma, migration to the perineural space and finally penetrate the capsule to escape from the primary location[59][60]. In addition to facilitating the escape from prostate gland N-cadherin expression might also aid the invasion of local blood vessels by the tumor cells. As endothelial cells also express N-cadherin in extra-junctional spaces, with an unclear role[61], a homotypic interaction between prostate cancer cells and endothelial cells promote metastasis by allowing access to the blood vascular system, possibly involving the IL-6-TGF-β-MMP-9 pathway, as demonstrated by *ex vivo* cell culture experiments[62]. Cadherin switching, decreased expression of E-cadherin and increased expression of N-cadherin, was observed in LNCaP-19 tumor cells, as the tumor progressed towards a stage of androgen independency suggestive of a correlation between cadherin switching, invasiveness and androgen independency in prostate cancer[63]. Studies have shown that apoptosis is induced in both normal and cancer cells, when cadherin adhesion is disrupted[64]. Also, increased Akt expression has been observed in androgen-independent metastatic prostate cancer cells[65].

In most of the epithelial malignancies, a key step in metastasis of carcinomas of breast and prostate is the transcriptional repression of E-cadherin gene[66]. Although the mechanisms which regulate the abnormal expression of N-cadherin in carcinoma progression are yet unknown, it has been shown that N-cadherin expression during epithelial-mesenchymal transition is induced by TGFβ1 through GTPase RhoA signalling[67] while at the later stages, prostate cancer cells are resistant to this growth factor[68]. A basic helix-loop-helix transcription factor Twist-1 which regulates the expression of E-cadherin and increased expression of mesenchymal genes during morphogenesis has been shown to be up-regulated in breast and prostate carcinomas[69].

A study on the role of Twist-1 in regulating N-cadherin expression has shown that increased accumulation of Twist-1 in nucleus results in β1 integrin mediated cell adhesion. Twist-1 directly binds to an E-box cis-element located in the first intron of the human N-cadherin gene and initiates the transcription of N-cadherin[70]. N-cadherin also plays dual functional roles in homophillic cell-cell adhesion and regulation of apoptosis. Studies involving PC3 cell lines have shown that homophillic adhesion of N-cadherin is linked to Akt signalling and inhibition of mitochondrial apoptotic pathway. Homophillic adhesion between extracellular domains of N-cadherin provides specific signals that regulate the levels of Bcl-2 by recruitment and activation of PI3-kinase and phosphorylation of Akt which leads to phosphorylation of Bad at Ser-136 and stabilizes Bcl-2[71].

Role of ZEB1 in promoting EMT in prostate cancer cells

Zinc finger enhancer binding protein (ZEB1) is a zinc finger homeo-domain transcription repressor and is known to regulate developmental processes like muscle, lymphoid differentiation and skeletal patterning[72]. ZEB1 expression has also been shown to be elevated in various malignancies like breast, lung and colorectal cancer [73][74][75]. It repress the expression of E-cadherin by interacting with CANNTG sequence in the promoter region and recruiting histone deacetylase, thereby resulting in chromatin condensation and gene silencing[72][73]. *In-vitro* studies to investigate the relationship between expression of ZEB1 and prostate cancer, using metastatic prostate cancer cell lines DU-145, PC-3, $ARCaP_E$ and $ARCaP_M$ and poorly tumorigenic cell line LNCaP as well as its bone- derived sub line C4-2B, has shown that ZEB1 mRNA and protein expression is undetectable in normal prostate cells, moderately expressed in low Gleason score tumors and highly expressed in tumors with high Gleason score, suggesting its relation with aggressiveness and grade of tumor.

Expression of ZEB1 is dependent on MEK signaling as the inhibition of MEK/ERK reduces the expression of ZEB1 in $ARCaP_E$ cell lines. Inhibition of MEK/ERK also suppresses the expression of β-catenin (also reported to regulate cadherin-11), however, it does not have any effect on the expression of endogenous mesenchymal markers like fibronectin and vimentin. Inhibition of ZEB1 by using ZEB1-siRNA revealed decreased migration rate in otherwise aggressive $ARCaP_M$ cell lines suggesting that that ZEB1 also plays a role in suppressing the expression of E-cadherin and promoting the expression of N-cadherin[76]. Specifically, it has been observed that

nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase (SIRT1) deacetylates histone H3, following recruitment to the E-cadherin proximal promoter by ZEB1. This, in turn, reduces the binding of RNA polymerase II to the transcriptional start site, ultimately suppressing E-cadherin transcription[77]. However, other experiments have demonstrated that exit from EMT involves an up-regulation of E-cadherin, despite the persistent expression of ZEB1 providing evidence for the need to use an appropriate model system for attempts to replicate E-cadherin expression in human cancers[78].

Expression of IGF is also correlated with the expression of ZEB1 in the serum of patients with high Gleason Score Prostate cancer, suggesting a role of IGF1 signaling in the over-expression of ZEB1. Highly aggressive cell lines like $ARCaP_M$ has a 2 fold increase in the expression of phosphorylated IGF-IRβ[79] Further, the expression of ZEB1 in MEK/ERK suppressed cell lines is restored on treatment with IGF-1 (a factor that also induces the expression of Twist and is known to promote EMT)[80]. While corroborative data indicate the involvement of IGF-1, via ZEB1, in prostate cancer initiation, a large study (ProtecT trial) which evaluated the relationship between circulating insulin-like growth factors (IGFs) and prostate cancer has found no role for circulating serum IGF-1 with reference to this cancer[81]. However, more recent evidence seems to indicate that a better evaluation of the nature of the relationship may be done by measuring the levels of distinct isoforms during the progression of prostate cancer[82] with suitable and acceptable histo-pathological correlates.

Role of snail, slug and E-47 factors in EMT and metastasis

Zinc finger factors Snail, Slug and basic Helix-Loop-Helix factor E-47, like ZEB1, also plays an important role in EMT. These factors, when expressed, induce a similar kind of phenotype which exhibit complete EMT[83][84][85]. Gene profiling studies involving MDCK-Snail, MDCK-Slug and MDCK-E-47 cell lines have shown an up-regulation of transcription factor IGF-1, cell proliferation and signaling factor Wnt-5 and various other genes related to EMT, angiogenesis, metabolism, transport and basic cellular functions. About 36% of the EMT-related genes were coordinately regulated by all the 3 genes, while the remaining was regulated by one or more of the 3 afore-said transcription factors. Regulation of over-expressed EMT-related genes in these cell lines is in a similar fashion by either of these factors provides evidence for their important role in EMT and imparting metastatic & invasive potential to tumorous cells[86].

Apart from the insulin-like growth factors, hepatocyte growth factor[87], epidermal growth factor[88], fibroblast growth factor[89], the transforming growth factor[90] plays an important role in EMT processes [91]. miR-200 and miR-205 have been recently shown to modulate the function of ZEB1 and ZEB2 (transcriptional repressors of E-cadherin gene expression), thereby playing an important role in TGFβ-induced EMT[92].

Role of TGFβ and oncogenic ras in EMT and metastasis

Oncogenic Ras (normally mitogenic) and Transforming Growth Factor-β (TGFβ) (normally growth inhibitory) and TGFβ Receptor are known to play important roles in EMT and metastasis. TGFβR signaling is required for EMT, invasion and metastasis in cancer cell through a Rho-dependent mechanism as mentioned earlier[93][94][95]. However, controversial to this is the known, paradoxical role of TGFβ in tumor suppression by growth inhibition and it functions as a tumor suppressor gene[96]. Downstream signaling pathways of oncogenic Ras are complex and involve many feedback loops as well as cross-talk with other pathways. It is mediated through Raf/MEK/ERK signaling and is required for TGFβ-induced EMT and metastasis. In addition, the activation of PI3K (phosphatidylinositol 3 kinase) – another TGFβ-regulated pathway, by downstream signaling of Ras oncogene, protects the cell from TGFβ induced apoptosis, thereby suggesting that tumor metastasis and EMT depends on mutual harmony between expression of TGFβ and PI3 Kinase[97][98][99].

Role of Wnt in EMT and metastasis

Gene profiling studies of various cancer cell lines have revealed an up-regulation of cell proliferation and signaling factor WNT-5. Wnt (wingless type) pathway plays a central role in the development of tissues during embryonic stages. It has also been shown that abnormal activation of Wnt pathway is involved in rendering metastatic potential and invasiveness in Prostate cancer cells[100][101]. Wnt pathway participates in cell invasion, proliferation, metastasis and angiogenesis by the regulation of target Wnt genes. Earlier, it was believed that activating mutations in β-catenin were the dominant mechanism in activation of Wnt in cancerous cells[102] but studies have shown that despite presence of these downstream activating mutations, presence of secreted Wnt antagonists like secreted Frizzled-related protein (sFRP)family, Dickkopf (Dkk) family and Wnt inhibitory factor-1[103][104][105] can suppress Wnt signaling suggestive of an autocrine Wnt signaling involved in tumor progression[106][107]. Corroborative evidence has been provided for the role of β-catenin (mRNA & protein levels), at the level of the cadherin-11 3'UTR, even though β-catenin-independent regulation of cadherin-11 has also been observed[108].

WIF-1 has been shown to inhibit the growth of various tumors and its expression was observed to be downregulated in 64% of primary prostate cancer specimens[109][110]. Studies using PC3 cancer cell lines revealed that inhibition of WIF-1 in most prostate cancer cells is due to hyper-methylation of its promoter[111]. Ectopic expression of WIF-1 in prostate cancer cell lines results in the upregulation of epithelial markers and increase in the protein levels of E-cadherin and keratin-18 as well as downregulates mesenchymal markers N-cadherin, fibronectin and vimentin, thereby resulting in the reversal of EMT.

Thus modulation of EMT markers is associated with the inhibition of Wnt signaling by WIF-1[112]. Inhibition of Wnt signaling down regulates the expression of Slug/Twist transcription factors, which are known to promote EMT. Restoration of WIF-1 in PC3 cell lines, thus resulted in the complete reversal of EMT, by inducing the expression of epithelial markers E-cadherin and keratin-18 and suppression of mesenchymal markers N-cadherin and vimentin, suppression of cell motility by down regulation of matrix metalloproteinases-2 and 9 and down regulation of transcription factors Slug/Twist[113]. Such mechanistic insights, in addition to those provided by E-cadherin conditional knock out and cadherin-11 knock-out animals, provide opportunities for development of molecules (like dietary polyphenols), that can potentially be used for the reversal of EMT[106] and cause the induction of programmed cell death or apoptosis[114].

ACKNOWLEDGEMENT

The authors would like to profusely thank the Management of VIT University for their infrastructural support and their constant source of encouraging and inspiring words which made this manuscript possible.

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