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Relation between epithelial-mesenchymal transition and colorectal cancer prognosis: Impact of SMAD4 and β -catenin markers

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Abstract

Colorectal cancer (CRC) is one of the most prevalent and deadly neoplasms. It develops through several molecular pathways and varies in clinical presentation and patient prognosis. The prognosis of CRC is influenced by variable pathologic and molecular variables. Understanding the molecular basis that promote CRC progression, invasive and metastatic features, is important for establishing effective CRC therapeutic strategies. Epithelial mesenchymal transition (EMT) is regarded to be key hallmark of CRC carcinogenesis, providing metastatic capacities to cancer cells by promoting motility and invasion. During this process, epithelial markers such as E-cadherin are down-regulated, whereas mesenchymal markers such as Vimentin are up-regulated. Several signaling pathways crosstalk to each other to initiate and maintain the process of EMT. Both the transforming growth factor- β (TGF- β)/SMAD4 and wingless-type mouse mammary tumor virus integration site family (Wnt)/ β -catenin pathways, often cooperatively, induce EMT during cancer metastasis. Assessment of EMT-related markers, is important to predict the aggressive behavior of tumors, and is associated with higher risk for local or distant recurrence and poor survival outcomes in patients with CRC.

INTRODUCTION

Colorectal cancer (CRC) is one of the world's most frequent cancers. It is the 3rd most prevalent cancer (10.2%) and the 2nd leading cause of cancer specific death (9.2%) globally (1). CRC accounts for 6.1 percent of all cancers in Egypt, including 5.8 percent of male cancers and 6.2 percent of female cancers, as well as 3.4 percent of overall cancer specific mortality (2).

As being a heterogeneous disease, CRC develops through several molecular pathways, is impacted by numerous environmental and genetic risk factors, and varies in clinical presentation and patient prognosis (3). The prognosis of CRC is mostly influenced by tumor stage. Advanced staging (including depth of tumor invasion in the intestinal wall, lymph node involvement, serosal invasion, peritoneal carcinomatosis, and distant metastasis) has been linked to a greater risk of cancer recurrence and a shorter life expectancy (4).

However, within the same tumor stage, many pathologic and molecular variables influence prognosis and responsiveness to therapy. The presence of tumor residue at the resection margins of a CRC specimen either gross or microscopically, poor histological differentiation, mucinous and signet ring carcinoma histologic subtypes, high grade tumor budding at the invasive tumor margin, lymphovascular and perineural invasion, and low levels of lymphocytic infiltration are all poor prognostic pathological factors. Tumor protein p53 (p53) tumor suppressor gene mutation, human epidermal growth factor receptor 2 (HER2) amplification, and Kirsten rat sarcoma viral oncogene homolog (KRAS)/ v-raf murine sarcoma

viral oncogene homolog B (BRAF)-mutations and microsatellite stability are all poor prognostic molecular factors (5).

Most CRC cases are discovered at a late stage. Understanding the molecular mechanisms underlying the genetic and epigenetic alterations that promote CRC progression, uncontrolled proliferation and cell survival, followed by invasive and metastatic features, is thus crucial for establishing effective CRC therapeutic strategies and combating drug resistance (6).

The complicated biologic process of epithelial mesenchymal transition (EMT) has been cited as a significant hallmark of carcinogenesis, providing metastatic capacities to cancer cells by promoting motility, invasion, acquiring stem cell properties, and displaying marked treatment resistance (7).

I) Epithelial mesenchymal transition

Epithelial mesenchymal transition (EMT) can be defined as a process in which epithelial cells lose their apical-basal polarity and intercellular connections and transform into mesenchymal cells with motile activity and high invasiveness (8).

Cells exhibit reduced activation of epithelial genes such as epithelial-cadherin (E-cadherin) associated with downregulation of epithelial markers as well as increased activation of mesenchymal genes such as Vimentin along with elevation of mesenchymal markers throughout the EMT process (9).

Molecular alterations during EMT results in a variety of phenotypic changes, including converting epithelial cobblestone appearance to dispersed spindle-like cells with migratory pseudopodium. Furthermore, intermediate filaments in epithelial cells are typically switched from cytokeratins to Vimentin (10). EMT can be represented histologically by the presence of tumor budding, which is described as the presence of single tumor cells or tiny clusters of up to 4 dedifferentiated cells at the invasive front of tumor (11).

E-cadherin is a calcium-dependent glycoprotein found on the cell surface. It is responsible for the integrity of intercellular adhesion, the polarity and integrity of epithelial cells, and the maintenance of proper function (12). The E-cadherin molecule consists of an external domain that interacts with neighboring cells and an intracellular region that is connected with multi-protein complexes such as α , β and p120 catenin. β -catenin binds to the actin microfilaments of the cytoskeleton, which are important in cell shape and migratory control (Figure 1) (13).

A) Molecular and immunohistochemical markers in EMT

1. E-cadherin/ β -catenin complex

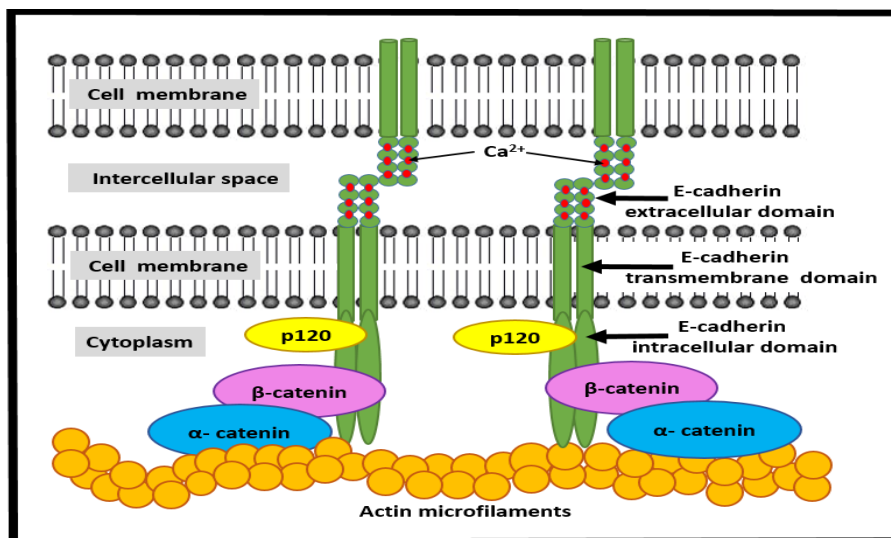


Figure 1. E-cadherin/catenin multi-protein complex at adherens junctions. E-cadherin can dimerise and form clusters between epithelial cells. E-cadherin dimers interact with adjacent ones in the neighboring cell via E-cadherin extracellular domains forming cell-cell adhesion. The stability of E-cadherin extracellular domains and the homophilic intercellular interactions require the presence of Ca^{+2} ions. E-cadherin molecules bind to p120 and β -catenin via their intracellular domains. These cadherin/ catenin complexes allow α -catenin to link the adherens junction to actin cytoskeletons.

The promoter regions of E-cadherin gene (*CDH1*) contain DNA sequences that allows direct binding of certain transcription regulators and control gene expression (14). Reduced or lost E-cadherin expression in tumors is thought to be a

characteristic of EMT induction (15). Furthermore, decreased E-cadherin expression is related with poor survival and may represent a substantial independent poor prognostic indicator. (16).

At the same time of decreased E-cadherin expression, cytoplasmic accumulation of β -catenin is supposed to occur (17). β -catenin is a protein that is involved in the Wnt signalling pathway. This pathway's dysfunction is critical in CRC carcinogenesis because it leads to nuclear translocation of β -catenin and activation of EMT-target genes (18). Low membranous and high nuclear expression levels of β -catenin may be linked to aggressive morphological features and poor prognosis (19).

2. *Vimentin*

Vimentin is an intermediate filament protein found in mesenchymal cells. Vimentin expression in epithelial cells is associated with cytoskeletal protein reconstruction and the acquisition of spindle like fibroblast migratory features. Vimentin expression as a mesenchymal marker changes inversely with E-cadherin expression as a structural adhesion protein as cancer progresses. Vimentin aberrant expression is strongly connected with invasion and metastasis in CRC and is significantly related to poor overall survival (20, 21).

3. *Transcription factors*

There are three main groups of transcriptional factors that are thought to be master regulators of different EMT events and are implicated in induction of EMT by suppression of E-cadherin expression. These are the SNAIL zinc-finger family, the basic helix-loop-helix (bHLH) family and the zinc-finger E-box-binding homeobox family of proteins (ZEB) (17).

Other transcription factors are implicated in EMT and the progression of CRC, including T-cell factor/lymphoid enhancer-binding factor (TCF/LEF-1). LEF-1 overexpression can induce EMT by activating nuclear β -catenin. β -catenin interacts to LEF-1 transcription complexes, which inhibit the expression of the E-cadherin gene. (22).

B) Signaling pathways and mechanisms involved in EMT.

Epithelial mesenchymal transition can be triggered by variable complex interacting and synergistic signaling pathways. The TGF- β pathway, a powerful EMT promoter, collaborates with other signaling pathways, such as Wnt, to induce complete EMT (9).

1. Transforming growth factor- β /Bone morphogenetic protein signaling pathway

Transforming growth factor- β (TGF- β) has a complex role in carcinogenesis. TGF- β works as a tumor suppressor in the early stages of tumor growth, inducing growth arrest and death, and as a tumor enhancer in the later stages of tumor growth, initiating cancer growth (23).

Transforming growth factor- β regulates gene transcription through signal transduction by Small Mother Against Decapentaplegic (SMAD) proteins or other SMAD independent pathways as the phosphatidylinositol 3-kinase (PI3K) and its downstream molecule protein kinase B (PKB; also known as AKT), Mitogen-activated protein kinase (MEK)/extracellular-signal-regulated kinase (ERK) pathway, JNK/p38 pathways and also through short and long term actin reorganization. These pathways can both work independently and through regulation SMAD complexes (24).

Small Mother Against Decapentaplegic (SMADs) are proteins presented in the cytoplasm downstream of TGF- β receptor complexes. They transport signals from cell membrane to nucleus in order to control gene transcription. SMADs are classified into three subfamilies based on their function. The first group is receptor-activated SMADS (R-SMADS) as SMAD1, SMAD2, SMAD3, SMAD5, SMAD8 are direct substrates of TGF- β family receptor kinases. The second group of SMADs associates with R-Smads and are referred to as common mediator SMADs (Co-SMADs) as SMAD4. The last group comprises the inhibitory SMADS (I-SMADS) as SMAD6 and SMAD7 that inhibit the signaling function of the other mentioned two groups (25).

The typical TGF- β signaling occurs when TGF- β ligands binds to the TGF- β receptors, allowing phosphorylation of the intracellular signal transducers SMAD2 and SMAD3. Activated SMAD2/3 proteins, hetero-oligomerize with SMAD4, then translocate to the nucleus to regulate TGF- β target gene transcription. The bone morphogenetic protein (BMP) signaling is similar to the basic TGF- β pathway; however, BMP ligands bind to BMP receptors and use phosphorylated SMAD1/5/8 instead of SMAD2/3 complexes (26). SMAD4 is considered as a central intracellular mediator of the TGF- β signaling pathway. However, to acquire target specificity, SMADs must interact with cofactors such as EMT-promoting transcription factors (Figure 2) (27).

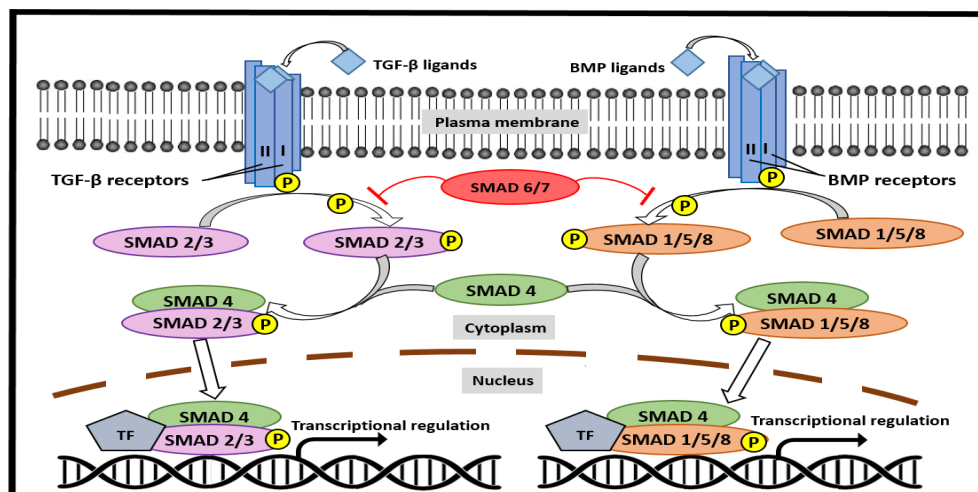


Figure 2. SMAD dependent TGF- β /BMP pathway. After TGF- β /BMP ligand binding, TGF- β receptors propagate the signal by recruiting and phosphorylating Smad2/3, while the corresponding BMP receptors recruit and phosphorylate SMAD 1, 5, and 8. These receptor-activated SMADs (R-SMADs), subsequently, can form heteromeric complexes with the common mediator (Co-SMAD: SMAD4) which are subsequently transported into the nucleus and interact with cofactors such as EMT-promoting transcription factors to regulate the transcription of target genes. Inhibitory SMADs (I-SMADS: SMAD6 and 7) can counteract the signals transduced by TGF- β /BMP-receptors as part of feedback loops by competing with R-SMADs for receptor binding, thereby inhibiting R-SMAD phosphorylation.

Nearly 80% of CRC is associated with mutations in either the TGF- β receptor, or in the signal transducers SMAD gene family (28). The loss of essential tumor suppressor genes on chromosome 18q is one of the most frequent

cytogenetic abnormalities in colorectal cancers. DCC (deleted in colorectal carcinoma) and SMAD2 and are among the genes targeted by these deletions (29). Interestingly, SMAD4, commonly known as the dpc4 (deleted in

pancreatic cancer 4) gene since it was shown to be mutated in around 55% of pancreatic tumors, is also located on chromosome 18q21 and mutated in 20-30% of CRC cases (30).

Loss of SMAD4 gene expression has been proposed as a late phase when cancers develop invasive potential and is associated with metastasis, and it has been shown to be strongly associated with poor survival regardless of stage (31). Furthermore, decreased Smad4 expression may indicate early recurrence after curative therapy as well as poor response to 5-fluorouracil based chemotherapy (32).

2. *Wnt/β-catenin/LEF-1 signaling pathway*

In the classic wnt signaling pathway, on absence of signaling, β-catenin as a central mediator is typically sequestered at the adherens junctions (E-cadherin, p120, β-catenin, α-catenin). A destruction protein complex that contains adenomatous polyposis coli (APC), Axin, and Glycogen synthase kinase 3 beta (GSK3 β), binds

and phosphorylates free cytoplasmic β-catenin, therefore targeting it for ubiquitination and proteolytic degradation (Figure 3-A) (33).

Misregulation of Wnt signaling is implicated in many tumors including CRC. Owing to aberrant activation, the Wnt ligands binds to a seven-pass transmembrane Frizzled (Fz) receptor and its co-receptors; low-density lipoprotein receptor related protein 5/6 (LRP 5/6). Disheveled (Dvl), a cytoplasmic protein, is then recruited, phosphorylated, and activated. Dvl activation recruits Axin to cell membrane and inhibit GSK3 β (34). These events result in deactivation of the "destruction complex" and the suppression of β-catenin phosphorylation degradation, resulting in the stability of β-catenin. As a result, great amounts of β-catenin accumulate in the cytoplasm prior to travelling to the nucleus to bind with TCF/LEF-1 transcription factors, transforming them from repressors to transcriptional activators of Wnt target genes transiently (Figure 3-B) (35).

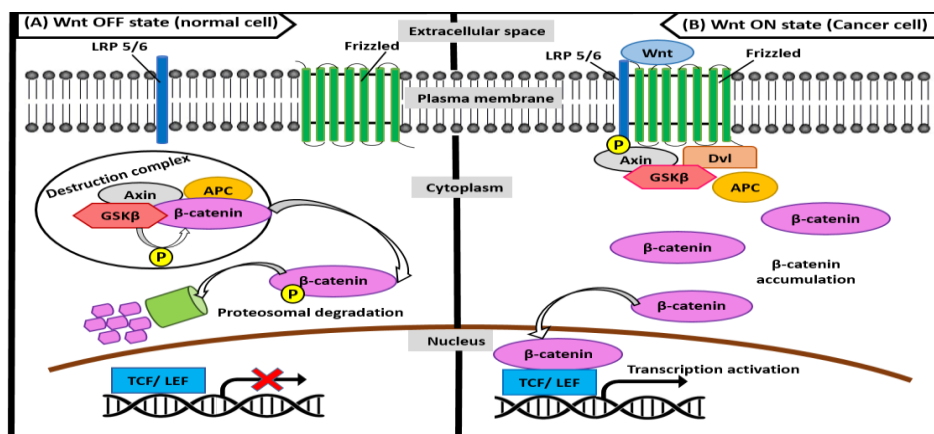


Figure 3. Classic Wnt/ β-catenin pathway. A) **Wnt OFF signaling state:** In the absence of Wnt ligand, cytoplasmic β-catenin associates with APC and Axin and is continuously phosphorylated by GSK 3β within a destruction multi-protein complex (marked by circle). The phosphorylated β-catenin, subsequently undergoes ubiquitination and proteasomal degradation. B) **Wnt ON signaling state:** Binding Wnt proteins to frizzled receptors and the LRP 5/6 co-receptor, induces phosphorylation of the co-receptors LRP5/6, which forms a docking site for Axin. The Dvl protein recruits the Axin to cell membrane. This disassembles the destruction complex and inhibits phosphorylation and destruction of β-catenin molecules by GSK3β, allowing their accumulation in the cytoplasm, then translocation to the nucleus, association with TCF/LEF and subsequent transcriptional activation of WNT target genes.

Mutations in pathway components observed in metastatic CRC emphasize the importance of the Wnt/ β -catenin signaling system in EMT during cancer progression (26). In most cases of CRC, inactivating mutations in the APC gene lead to loss of APC activity, as well as intracellular β -catenin stabilization and constitutive signaling to the nucleus. This induces the expression of downstream genes, including those required for invasive growth (33).

Inactivation of APC only may not be enough to completely activate Wnt signaling. Other mutational events may be required to facilitate invasion and metastasis (28). Loss of APC function is often followed by activation of the rat sarcoma viral oncogene homolog (RAS) and mutations in additional RAS pathway genes, such as v-raf murine sarcoma viral oncogene homolog B (BRAF), along the adenoma-to-carcinoma sequence. These events lead to phosphorylation of β -catenin, its release from E-cadherin at the adherens junction, and increased Wnt signaling to the nucleus (33).

Activating mutations of β -catenin that make it resistant to proteolytic degradation have been found in colorectal tumors lacking APC mutations. Wnt target gene transcriptional deregulation occurs in both cases. Among these target genes are prominent EMT regulators such as SNAIL, which suppresses E-cadherin transcription (36).

In addition to Wnt pathway activation, CRCs exhibit upregulation of the expression of Wnt pathway feedback inhibitors, especially Axin2. As a result of this, GSK3 is redirected to the cytoplasm, leaving SNAIL unphosphorylated and transcriptionally active. This SNAIL

stabilization is followed by E-cadherin suppression and EMT induction (37).

C) Crosstalk of signaling pathways in EMT

Signaling coordination occurs during EMT, and different pathways converge on common EMT targets (38). Both the Wnt and TGF- β signalling pathways appear to cooperate together to influence gene expression changes during EMT. TGF- β causes adherens junction breakdown, enabling β -catenin to concentrate in the nucleus and feed into Wnt signalling via a complex with LEF-1 (39). LEF-1 may also form a complex with SMAD proteins, bind E-cadherin gene sequences, limiting E-cadherin production and inducing EMT (40).

III. Summary and Conclusion

Epithelial mesenchymal transition has been proposed as a critical mechanism during cancer progression, endowing cancer cells with greater motility and invasiveness, allowing them to seed metastasis. As a result, EMT pathways are gaining attention as a potential therapeutic approach in cancer treatment that might be targeted to limit tumor cell spread in early stage patients. The identification of altered immunohistochemical expression of EMT-related proteins and their inducer pathways, primarily TGF- β and Wnt signaling cascades, is important for predicting tumor aggressiveness and associated with an increased risk of local or distant recurrence and poor survival outcomes in patients with CRC.

Abbreviations:

APC: adenomatous polyposis coli, **CRC:** colorectal cancer, **EMT:** epithelial mesenchymal transition, **SMAD:** Small Mother Against Decapentaplegic, **TCF/ LEF-1:** T-cell factor/

lymphoid enhancer-binding factor, **TGF- β** : transforming growth factor- β , **Wnt**: wingless-type mouse mammary tumor virus integration site family.

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