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A Diverse Range of Biomarkers that Narrate Epithelial Mesenchymal Transition in Oncological Events. A Review

Surekha L. Chavan[1]*, Revati S. Deshmukh[1], Harsh Parekh[2] and Pratap N. Mukhopadhyaya[2]

[1]Department of Oral Pathology and Microbiology, Bharti Vidyapeeth University Dental College and Hospital, Pune, India.
[2]Department of Biotechnology, JJT University, Rajasthan, India.

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***For Correspondence**

Dr. Pratap N Mukhopadhyaya, B-604, Kapil Aasmant, Pashan-Sus Road, Pashan, Pune-411021, Maharashtra, India. Tel: +91 9881153425; E-mail: pnm.istra@yahoo.com .

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ABSTRACT

Stromal tissues has regions of mesenchymal stem cells which provide the primary cell support for growth and repair. While the origin of mesenchymal stem cells remains an intense subject of discussion, broadly it is believed to be perivascular cells form mesenchymal stem cells to be the original source in most tissues. The role of mesenchymal cells in invasion of tumor and metastasis as well as tissue repair however is still not well understood.

A large number of genes and proteins expressed from them have now been shown to reflect intricate molecular events and behaviors during transition of cells from epithelial to mesenchymal identity. This review discusses the various types of epithelial mesenchymal transition and a vast number and diverse nature of genes that collectively and/or sequentially function to bring about this important molecular event that is by far the most significant one also in the domain of oncology.

INTRODUCTION

Epithelial mesenchymal transition was first identified by Elizabeth Hay in 1967. Epithelial and mesenchymal cells are often characterized by unique morphology of the multicellular structures and various other phenotypes (Shook and Keller, 2003). Mostly epithelial cells are attached to their neighboring cells by specific junctional complexes which are majorly formed by adheren junction, tight junction, gap junction and desmosomes. Epithelial cells tightly associate with their neighbors inhibiting the movement and dissociation from the epithelial layer. Typically, epithelial sheets are polarized between apical and basal surfaces. The major determinants of these surfaces include, (i) organization of specific junction like structures, (ii) presence of basement membrane, (iii) the localization of adhesion molecules and (iv) polarization of actin cytoskeleton. Epithelial cells undergo dramatic phenotypic changes that reflect their transformation to mesenchymal cells. It is a highly conserved cellular program that allows polarized immotile epithelial cells to convert to motile mesenchymal cells.

Whereas mesenchymal cells are completely opposite in nature, they do not form layers of cells nor do they contain intercellular adhesion complexes. The fact that mesenchymal cells can freely migrate is due to its end-to-end polarity and focal adhesion, unlike epithelial cells. Despite of their interaction with neighboring cells, they lack the apico-basal polarity as exhibited by epithelial cells. Owing to their

migratory property mesenchymal cells play a very important role in organ development, as they can cover a huge distance across the embryo. The properties of a messenchymal cell include enhanced migratory capacity, increased production of extracellular matrix (ECM) components, invasiveness and high resistance to apoptosis.

Epithelial to mesenchymal transition (EMT) is a phenomenon in which epithelial cells which are normally attached to a basement membrane undergoes multiple biochemical changes that enable it to assume a mesenchymal cell phenotype. The completion of EMT is characterized by complete degradation of basement membrane and the formation of a well-defined mesenchymal cell which can migrate from the epithelial layer to a distinct place. However initiation of EMT is not as simple as it seems, and number of molecular processes are involved in this process. Firstly the transcription factors are activated followed by expression of cell surface proteins, and later the cytoskeletal proteins are reorganized and new proteins are formed. Upon completion of protein formation, the enzymes that help in degradation of ECM components are formed which result in changes in expression of specific microRNAs. Eventually it was learned that, during specific steps of embryogenesis and organ development the epithelial cells appear to be plastic and thus able to move back and forth between epithelial and mesenchymal states via processes of EMT and MET (Mesenchymal to Epithelial Transition) (Lee et al., 2006). For e.g. EMT is required for gastrulation (Thiery and Sleeman, 2006), while MET occurs during somitogenesis (Christ and Ordahl, 1995) and kidney development (Funayama et al., 1999).

Different terms to describe cellular plasticity are; EM Transformation, Interaction, Transition and Transdifferentiation. EMT in normal physiologic process is seen during embryogenesis and tissue morphogenesis, mesoderm, neural crest, cardiac valve, secondary palate.

Types of EMT: As discussed earlier EMT is an example of cell plasticity that generates new mesenchymal cell types from epithelial cells. EMT can be divided into three distinct sub-types: Type I EMT is involved in embryogenesis and organ development. In this type, the mesenchymal cells formed undergo MET to form secondary epithelia. Moreover, this type of EMT results in systemic spread across circulation as it does not involve an invasive phenotype nor does it cause fibrosis. The EMTs associated with wound healing, tissue regeneration and organ fibrosis comprise of type II EMT. Unlike type I, type II EMTs generates fibroblasts and other related cells in response to an injury caused by trauma or inflammation. Once inflammation is repaired EMT is also ceased as seen in wound healing and tissue regeneration. During type III EMT, some cells retain epithelial traits while acquiring mesenchymal features while others shed most of their epithelial features and fully turn into messenchymal cells. This type of EMT is seen in carcinoma cells that have formed solid tumors at one site, and cells from these solid tumors have the potential to migrate elsewhere through blood stream and form secondary tumors at other sites through MET. Although they are three distinct type of EMTs, but all the three EMTs may be induced and regulated by common sets of stimuli, signal transduction pathways, transcription factors and posttranslational

regulations (Thiery and Sleeman, 2006; Wynn, 2008; Kalluri and Weinberg, 2009; Lo'pez-Novoa and Nieto, 2009; Zeisberg and Neilson, 2009).

Hence to be able to identify these significant transitions, biochemical markers are used which are discussed as follows:

Epithelial to Mesenchymal Transition markers:-

The process of EMT involves the following steps or components:

1. Activation of transcription factors
2. Expression of cell-surface markers
3. Cytoskeletal proteins expression
4. ECM proteins
5. Micro RNAs

The EMT markers can also be classified in the similar pattern. Table 1 shows a glimpse of markers that will be discussed in this review.

Table 1: Up and down regulated expression elements during epithelial mesenchymal transition.

Up regulated markers during EMT			Down regulated markers during EMT		
Class	**Name of marker**	**Reference**	**Class**	**Name of marker**	**Reference**
Cell-surface proteins	N-Cadherin	46-73	Cell-surface proteins	E-cadherin	14-45
	Integrins -α5β1 integrin -αVβ6 integrin	89-93 94-114 115-125		Zonula Occludens-1 (ZO-1)	74-88
Cyto-skeletal markers	B-catenin	126-135	Extracellular Matrix Proteins	Laminin-1	153-160
	Vimentin	136-146			
Extracellular Matrix Proteins	Fibronectin	147-152			
	Laminin-5	153-160			
Transcription Factors	Snail1 Snail2 (Slug)	161-173			

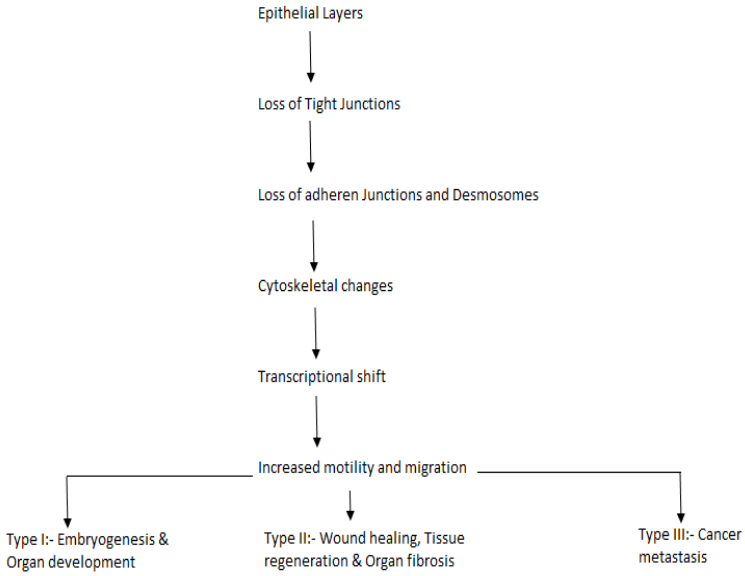

Figure 1: progressive stages of epithelial mesenchymal transition in cancer.

1 Cell-surface protein markers:-

i. Cadherins:-

Cadherins are a large family of membrane associated glycoprotein, marked by the presence of multiple repeats of 100 amino-acids, extracellular domain (ECD) (Humphries and Newham, 1998). Cadherins are further divided into various sub families. The basic cadherin family consists of N, P, OB and E-cadherin (Angst *et al.,* 2001). These cadherins localize in specific sites of cell-cell adhesion which are known as adheren junctions, where cadherin molecules form a stable linkage with actin cytoskeleton. Another family of cadherin group is desmosomes-associated cadherins which form intracellular linkage to intermediate filaments rather than actin filaments (Hynes, 1999). The final family of cadherin group comprises of poto-cadherins, which contains complex gene sub-family that helps in development of nervous system (Wu and Maniatis, 1999). The important markers are discussed in detail below.

a. E-Cadherin:-

The E-cadherin gene CDH1 is located on chromosome 8 in mice and on chromosome 16 in humans (Berx *et al.,* 1995). The role of e-cadherin in embryonic development is very well proven, wherein the e-cadherin knock-out mice model did not survive as a consequence of lack of trophectoderm formation (Larue *et al.,* 1994; Larue *et al.,* 1996). This function of e-cadherin can be attributed to the presence of promoter region containing various activating and silencing sequences, few such activating sequences being the CCAAT and GC boxes (Peinado *et al.,* 2004). While the negative binding regulators are Snail, Slug, E12/E47 and the zinc finger factors ZEB1 and ZEB2 (van Roy and Berx, 2008).

E-cadherin is a glycoprotein containing 5 different extracellular domain, four of which are typical cadherin domains, while the 5th is known as the membrane proximal extracellular domain (MPED). It also contains

four cadherin cysteines which are essential for e-cadherin function (van Roy and Berx, 2008). E-cadherin generally binds to another e-cadherin molecule in a hemophilic manner as well as binding to the same cell type (homotypic fashion) (Koch *et al.,* 2004). E-cadherin is expressed in most adult epithelial tissue and also acts as potent tumor suppressor (Gumbiner, 1996; Gumbiner, 2005; Tunggal *et al.,* 2005; Halbleib and Nelson, 2006). Thus loss of e-cadherin results in EMT, a crucial process in tissue repair and tumor progression (Lee *et al.,* 2006).

Loss of function, down-regulation, or complete shut-down of e-cadherin, mutation of e-cadherin gene or any other factor that interfere with the integrity of this gene leads to increase in carcinoma cells. Loss of e-cadherin function is directly correlated to loss of epithelial morphology and rise in metastatic carcinoma cells (Reitmacher *et al.,* 1995). Mutation in CDH1 gene has been reported in different forms of cancer (Berx *et al.,* 1998). Migration and invasion of bladder cancer cells showed reduced expression of e-cadherin (Du *et al.,* 2014). A membrane bound zinc dependent metalloprotease, CD10 expression is found to be increased, where as E-cadherin expression was found to be reduced in both squamous and transitional cell carcinoma (Omran, 2012). In case of urothelial cell carcinoma also reduced expression of E-cadherin was noted suggesting progression, invasion and metastasis of cancer cells (Omran, 2012). A meta-analysis study clearly indicates that hypermethylation of E-cadherin is associated with bladder cancer and was more prevalent in Asian populations as compared to Caucasian populations (Li *et al.,* 2014). Loss of E-cadherin is mostly associated with tumor cells, but soluble E-cadherin could be detected in urine of bladder cancer patients, and the amount of E-cadherin is related to tumor size and lymph node metastasis (Salama *et al.,* 2013).

Talking about brain cancers, glioblastoma is the most common form it. Loss of E-cadherin is associated with most glioblastoma tissues and is found in the differentiation status of glioblastoma (Yang *et al.,* 2012; Wu *et al.,* 2013). In a comparison study of tumor tissues and gliobalstoma cell lines, E-cadherin was found to be rare in the later (Perego *et al.,* 2010; Lewis-Tuffin *et al.,* 2010). Loss of E-cadherin is characterized in aggressive breast cancer such as aggressive lobular carcinoma (Sarri'o *et al.,* 2004). The surface epithelium and the intestinal epithelium have expression of E-cadherin (Do'gan *et al.,* 1995). Loss of E-cadherin will reduce cell-cell adhesiveness in turn affecting the phenotypic characteristic and physiological state of colon cancer (Wheelock, 1990). Similar to bladder cancer patients, urine samples of colon cancer patients shows presence of soluble E-cadherin fragments (Katayama *et al.,* 1994).

Immunohistochemical staining clearly indicates an association between E-cadherin loss and invasive endometrial cancer (Mell *et al.,* 2004). As the tumor stage increases loss in E-cadherin expression and hypermethylation also increases (Park *et al.,* 2008). In head and neck cancer cell lines, reduced E-cadherin expression leads to loss of epithelioid cell morphology (Tomson *et al.,* 1996). In mouse models of liver cancer, loss of E-cadherin resulted in metastasis (Ding *et al.,* 2010). Loss of E-cadherin function/expression could induce migration and promote EMT in liver cancer (Grosse-Steffen *et al.,* 2012; Liu *et al.,* 2013). Genetic mutation in E-cadherin is the primary reason of E-cadherin inactivation in

lung cancer (Fei *et al.,* 2002). Absence of E-cadherin in non-small cell lung cancer resulted in activation of MEK/ERK or EGFR pathway (Bae *et al.,* 2013).

b. N-Cadherin:

The N-cadherin gene is located on chromosome 18 in mice (Miyatani *et al.,* 1989), which is in complete homology with humans. Also N-cadherin was found to be homologous with other cadherins in the family (Wallis *et al.,* 1994). N-cadherin was first identified in 1982 by Grunwald as a 130kD molecule in chick neural retina, which was protected from proteolysis by calcium (Grunwald *et al.,* 1982). The entire gene was mapped to 250kb region on chromosome 18q11.2, comprised of 16 exons. The protein contains five different extracellular cadhrein repeats namely EC1 to EC5 decoded by exon 4 to 13, a trans membrane protein encoded by exon 13 and 14, and also a cytoplasmic part encoded by exons 14 to 16 (Harada *et al.,* 2002). N-cadherin mostly forms homotypic hemophilic interactions, but in some cases heterotypic hemophilic and heterophilic interactions have also been formed (Shan *et al.,* 2000).

The up-regulation of N-cadherin at the transcription level requires certain proteins for e.g. during early mesoderm formation transcription factor twist initiates the N-cadherin expression. The subsequent increase in the level of N-cadherin is monitored by other transcription factor snail (Oda *et al.,* 1998). The turnover of N-cadherin is under the supervision of an important regulator $P120^{ctn}$. Knockdown of $p120^{ctn}$ with siRNA (small interfering RNA) rapidly degrades cadherins (Davis *et al.,* 2003). It has also been reported that Caspase-3, metalloproteases (MMP), and presenilin cleaves N-cadherin giving rise to different fragments (Paradies and Grunwald, 1993; Hunter *et al.,* 2001; Marambaud *et al.,* 2003). The role of these newly formed fragments is yet to be known, except for the intracellular fragment of N-cadherin formed by PS1 cleavage. This fragment forms a complex with CREB binding protein in cytoplasm and promotes the degradation of CBP (Paradies and Grunwald, 1993).

N-cadherin plays an important role in embryo formation. In, N-cadherin knockout mice model, the mice die after 10 days of gestation. The embryo of such model showed major heart defects underdeveloped neural tubes and somites (Larue *et al.,* 1996). N-cadherin is also responsible for complete cardiac development including formation of pericardiac mesoderm, cardiac looping morphogenesis, sorting out precardiac mesoderm and trabeculation of myocardial wall (Hatta *et al.,* 1987; Takeichi, 1988). Puch et al. (2001) showed the involvement of N-cadherin in early hematopoietic cells in the bone marrow (Puch *et al.,* 2001). N-cadherin is also expressed in osteoblast differentiation (Ferrari *et al.,* 2000). BMP-2, phorbal ester and FGF-2 increase the level of N-cadherin, whereas TNF-α and IL-1 are responsible for downregulation of expression (Marie, 2002). N-cadherin plays an important role in skeletal muscle differentiation. Slug plays an important role in regulating the expression of N-cadherin throughout skeletal muscle differential. Slug downregulates cadherins leading to loss of cell-cell interaction and allowing cells to migrate. When the neural crests of two cells are still attached N-cadherins are expressed, but once they start migrating N-cadherin is donregulated (Nieto, 2001; Pla *et al.,* 2001).

N-cadherin is been reported to be over expressed in various types of cancers. In esophageal squamous cell carcinoma (ESCC) over expression of N-cadherin was in correlation to invasion, differentiation, and lymph node metastasis (Li *et al.,* 2009). In another study, ESCC cell lines (EC9706) N-cadherin was knocked out. The results suggested that in absence of Ncadherin the cell cycle could be arrested at G0/G1 phase, induce cell apoptosis and inhibit tumour formation (Li *et al.,* 2010). Takahito Kamikihara et al. (2002), suggested that neoexpression of only N-cadherin can be used as a prognostic marker in gastric cancer irrespective of E-cadherin expression (Kamikihara *et al.,* 2002). The role of N-cadherin in colon cancer was identified for the first time in 2001 by Erika Rosivatz. They tried to relate the events of EMT in colon cancer patients with N-cadherin and E-cadherin expression with respect to regulators Snail1, SIP1 and TWIST (Rosivatz *et al.,* 2004). Similar findings were observed in pancreatic carcinoma patients (Nakajima *et al.,* 2004). The role of N-cadherin antagonist ADH-1 has also been studied well. ADH-1 has a significant anti-tumor activity against N-cadherin expressing pancreatic cancer cells in mouse model of pancreatic cancer (Shintani *et al.,* 2008).

Over-expresion of N-cadherin was found to be in complete correlation with positive node status, poorly differentiated carcinoma and poor Nottingham Prognostic Index in 132 patients suffering from ductal breast carcinoma (ElMoniem and Zaghloul, 2011). The presence of N-cadherin in breast cancer patients prevents the re-expression of E-cadherin in mesenchymal mammary carcinoma cells (Rezaei *et al.,* 2012). The 5 year survival rate of patients suffering from NSCLC was lower in N-cadherin positive tumours as compared to that of N-cadherin negative tumours (Nakashima *et al.,* 2003). The presence of N-cadherin has also been reported at the cell-cell borders of human bladder carcinoma cell lines (Mialhe *et al.,* 2000).

ii. Zonula-Occludens 1 (ZO-1):

Tight junctions (TJ) are cell-cell adhesion structures mostly present in epithelial cells, between the apical plasma membrane. TJ mostly functions as a gate which regulates the flow of ions, water and other molecules through a specific pathway. It also restricts the movement of lipids and proteins within the membrane. They are the most apical components of the junctional complex found between the cells (Harhaj and Antonetti, 2004). TJ is composed of multiple signaling proteins comprising of occludin, caludin, adhesion molecules, other linker proteins such as zonula occludens family members (ZO-1/2/3) (Furuse *et al.,* 1993; Gardner *et al.,* 1996; Hirase *et al.,* 1997).

The first ever protein to be identified as component of TJ was ZO-1 in 1988, followed by ZO-2 and ZO-3 that co-associates with ZO-1 (Anderson *et al.,* 1988). ZO-1 was found to be a 210-225 kDa fragment while ZO-2 and ZO-3 were comparatively smaller measuring 180 kDa and 13kDa respectively. All the three iso-forms of *zonula occluden* are found within the tight junctions of both epithelial as well as endothelial cells (Harhaj and Antonetti, 2004). As an exception ZO-1 and ZO-2 are also found in adherine junction apart from tight junction, such as fibroblasts and cardiac myocytes (Itoh *et al.,* 1999). All the three ZO proteins are identical in structure containing 3 PDZ domains, a single SH3 domain and a non-

catalytic guanylate kinase (GuK) domain (Woods and Bryant, 1993). Apart from the above mentioned three distinct domains these proteins contains additional 4 domains namely, a basic domain, an acidic domain, a proline rich C-terminus and a leucine zipper (Beatch *et al.,* 1996; Haskins *et al.,* 1998).

As discussed earlier ZO has many binding sites, and various other cellular proteins bind to these sites via multiple protein binding domains. One of the most important function of ZO-1 is to bind specifically to occludin, this association occurs at the N-terminal half of ZO-1. The claudins-1 to -8 is also been reported to bind with ZO-1, and this binding takes place between C-terminus regions of Claudins and PDZ-1 domain of ZO-1 (Harhaj and Antonetti, 2004). In addition to TJ proteins, ZO-1 also binds to adherens junction protein, gap junction protein and β-catenin (Harhaj and Antonetti, 2004). ZO-1 has also been reported to interact with actin cytoskeleton, the proline rich C-terminus of ZO-1 that binds with F-actin making it important link between occludin and actin cytoskeleton (Fanning *et al.,* 1998).

ZO-1 has been reported to be under expressed in many types of cancers. In a study of breast cancer patients, the paraffin-embedded breast cancer samples were screened for both ZO-1 as well as E-cadherin expression. The results were as expected, normal tissue showed high expression of ZO-1 but the expression was lost in around 69% of the breast cancer samples, E-cadherin also showed similar kind of expression (Hoover *et al.,* 1998). One of the studies also shows that ZO-1 is an important factor which regulates IL-8 in breast cancer patients independent of the β-catenin pathway (Brysse *et al.,* 2012). Similar findings were observed in patients suffering from NSCLC, where around 101 patients suffering from NSCLC and 61 benign tissue samples were screened for expression of ZO-1 against control group. The difference in the ZO-1 mRNA expression in carcinoma group and control group was found to be statistically significant (Wang *et al.,* 2011). In prostate cancer cell lines also the expression of ZO-1 and other tight junction proteins were reduced. This reduction was mediated by hepatocyte growth factor (HGF). HGF directly affected the redistribution of ZO-1, ZO-2 and ZO-3 away from the tight junction leading to increase in metastasis (Martin *et al.,* 2014).

iii. Integrins:

Integrins play an important role in regulating intracellular responses including proliferation, migration and differentiation. Although they were first identified as only cell adhesion molecules and receptors for extracellular matrix (ECM) (Aoudjit and Vuori, 2012), Integrins are combination of αβ proteins that form heterodimers, and these associations defines the specificity of adhesion to ECM proteins. In all there are 18 α and 8 β subunits, forming a large family of 24 αβ integrins. They are composed of transmembrane domain, an extracellular domain and a short cytoplasmic tail. Mostly the α subunit is responsible for the high specificity of the integrins (Aota *et al.,* 1999). Integrins are either activated by binding to its ligand or by binding of an activator protein such as talin, these binding resulting in conformational changes in its structure from a low affinity state to a high affinity state (Shattil *et al.,* 2010). Among the large integrins family only few of them are important for e.g. few integrins involved in angiogenesis and vascular development are α5, α4, αv, β1 subunits (Yang *et al.,* 1995; Carlson *et al.,* 2008).

a. α5β1 integrin:

Increased rate of apoptosis were observed in three different colon cancer cell lines KM20, KML4A and KM12C, when these cell lines were treated with anti-α5 integrin inhibitory antibody (Murillo *et al.,* 2004). The upregulation of α5 subunit in colon cancer is under the direct control of PTHrP and ZEB2, leading to upregulation of cell invasion during EMT (Anderson *et al.,* 2007; Nam *et al.,* 2012). Lunaisn, a peptide isolated from soyabean suppresses the activity of is α5β1 activity, this inturn potentiates the effect of oxaliplatin preventing colon cancer metastasis (Dia and Mejia, 2011). In ovarian cancer cells, α5β1 integrin is activated by fibronectin secreted by peritoneal tissue, thereby increasing its invasive capacity via increase in MMP-9 activity (Shibata *et al.,* 1997). Different ovarian cancer cell lines express α5β1 integrin, but their peritoneal wall preparation capacity in mice is impaired by anti α5β1 antibodies (Yokoyama *et al.,* 2007). In one of the study it is clearly mentioned that, α5β1 expression is inversely proportional to E-cadherin expression, and it was also shown to increase adhesion of tumor cells and metastasis (Sawada *et al.,* 2008).

Many studies imply the proinvasive role of α5β1 integrin in breast cancer (Ignatoski *et al.,* 2000; Jia *et al.,* 2004; Maschler *et al.,* 2005). The upregulation of α5β1 integrin is driven by oncogene ERBB2 which is strongly associated to poor prognosis and high metastasis in mammary adenocarcinoma tumor cells (Spangenberg *et al.,* 2006). Similar kind of relation was found between α5β1 integrin expression and E-cadherin expression, as found in ovarian cancer cell lines (Wu *et al.,* 2006). Upregulation of α5β1 integrin in some breast cancer cells is associated with over expression of steroid receptor co-activator (SRC-1), leading to increased cell adhesion and migration (Qin *et al.,* 2011). A negative correlation was established between α5β1 integrin overexpression and survival rate in NSCLC patients (Adachi *et al.,* 2000). α5β1 integrin was expressed more frequently in tumors with lymph nodes than in patients without metastasis (Han *et al.,* 2003). The role of α5β1 intgerin has also been shown in progression of glioblastomas, following perinecrotic or perivascular pattern of expression (Gingras *et al.,* 1995; Riemenschneider *et al.,* 2005). The role of α5β1 integrin has been well established in proliferation, invasion, migration and resistance to chemotherapy in different glioma cell lines (Martinkova *et al.,* 2010). Similar kind of expression of α5β1 was observed in melanomas (Beliveau *et al.,* 2000; Beliveau *et al.,* 2001; Landreville *et al.,* 2011).

b. αVβ6 integrin:

αVβ6 integrin is exclusively expressed in epithelial cells only, while during embryogenesis high level of αVβ6 integrin is found in skin, lung and kidney epithelia. But as growth progresses its expression is downregulated (Breuss *et al.,* 1993; Breuss *et al.,* 1995). High expression of αVβ6 integrin is also observed throughout the wound healing process (Haapasalmi *et al.,* 1996). αVβ6 integrins are also expressed in a wide variety of cancers. Basal Cell Carcinoma (BCC's), are the most common type of skin cancers, which can be either nodular or morphoeic. In one of the study it has been shown that expression of αVβ6 integrin was lower in nodular BCC, but its expression markedly increased in

morphoeic variants (Marsh *et al.,* 2008). Higher expression of αVβ6 integrin in colon cancer patients was associated with more aggressive disease outcome (Bates, 2005). Increased expression of αVβ6 integrin in turn activates MMP3 which is responsible for enhanced cell migration and metastases in oral squamous cell carcinoma patients (Li *et al.,* 2003; Ramos *et al.,* 2009). Ovarian cancer cell lines also showed the expression of αVβ6 integrin, where grade III had high expression while grade I had low expression. The expression of αVβ6 in Oovarian cancer ranges from high to moderate and sometimes low (Ahmed *et al.,* 2002). High levels of αVβ6 ecpression is also been observen in pancreatic cancer (Sipos *et al.,* 2004) as well as endometrial cancer (Hecht *et al.,* 2008; Zhang *et al.,* 2008).

1. Cyto-skeletal markers:

There are several markers that come into this category namely β- catenin, Vimentin, α-SMA, Fibroblast-specific proteins 1 (FSP-1). Catenins also known as cadherins-associated proteins or β-catenin, is a 88kDa protein having dual function of cell-cell adhesion and gene transcription. β-catenin was first identified in 1990's as a component cell adhesion complex, but later its role in regulation of Wnt signaling pathway was described (McCrea *et al.,* 1991; Kemler, 1993). β-catenin structure mainly comprises of 40 amino acids long repeats termed as armadillo repeats, and these repeats fold into a single rigid protein domain known as armadillo domain (Gottardi and Peifer, 2008). The N-terminal domain contains a conserved short linear motif which is responsible for binding β-TrCP, whereas the C-terminal domain acts as a strong transactivator when recruited on DNA (Xing *et al.,* 2008). β-catenin is also a part of the protein complex that forms the adherens junctions, which are important for the creation and maintenance of epithelial layers and barriers (Brembeck *et al.,* 2006). β-catenin is also essential for early embryonic development, wherein knock out model of β-catenin failed to develop mesoderm and initiate gastrulation (Haegel *et al.,* 1995). High levels of β-catenin in certain cell types are required to maintain its pluripotency.

Is has been reported that mutations in β-catenin gene leads to inactivation of tumor suppressor gene APC (Adenomatous Polyposis Coli), in most of the colon cancer patients (Morin *et al.,* 1997). A study of 92 colorectal cancers and 57 cancer cell lines revealed that mutations in β-catenin gene mostly occurred in exon 3 and also exhibited widespread microsatellite instability (Kitaeva *et al.,* 1997). Aberrant expression of β-catenin serves as a surrogate marker for Wnt signaling activation. These findings were confirmed in a cohort study of 245 patients suffering from invasive breast cancer. Out of 245 patients screen 221 patients were found to contain β-catenin mutation; however the expression varied in between the grades of breast cancer: low grade showing less β-catenin expression (Geyer *et al.,* 2011). β-catenin once activated, translocates to the nucleus and binds to T cell factor family members and activates its target genes. In breast cancer cells, it has been reported that β-catenin activates cyclin D1 thus causing poor prognosis for breast cancer patients (Lin *et al.,* 2000).

Vimentin is a protein belonging type III intermediate filament (IF) set of proteins which is mostly expressed in messenchymal cells (Eriksson *et al.,* 2009). Typical structure of vimentin consists of 3

domains namely central α-helix domain, non-helical domain and carboxyl tail domains. The stability of the entire coiled-coil dimers is imparted by periodic distribution of basic and acidic amino acids (Fuchs and Weber, 1994). Vimentins main function is to maintain the integrity of the cytosol by supporting the organelles of cytosol. It is essential for maintaining the integrity of cytoplasm, cell shape, stabilizing cytoskeletal interaction (Goldman *et al.,* 1996). Vimentin is commonly used as marker for EMT during embryogenesis. Expression of vimentin in mice is found right from the beginning of endoderm formation and is continuously expressed until mesoderm is formed (Colucci-Guyon *et al.,* 1994). Vimentin was found to be overexpressed in prostate cancer cell line CL1, but after experimental removal of its expression decrease in invasiveness of the tumor was observed (Singh *et al.,* 2003). Similar kind of expression was observed in lung cancers where vimentin was detected in moderately and well differentiated adenocarcinomas and giant cell carcinoma (Upton *et al.,* 1986). Vimentin overexpression was also found in various different types of cancers such as cendometrial cancer (Coppola *et al.,* 1998), certain types of lymphomas (Gustmann *et al.,* 1991), cervical cancer (Gilles *et al.,* 1996), thyroid carcinoma (Yamamoto *et al.,* 1992), and renal cell carcinoma (Williams *et al.,* 2009).

2. Extracellular matrix proteins:

a. Fibronectin:

Fibronectin is a high molecular weight glycoprotein (440kDa) indigenous to extracellular matrix protein, binding to integrins. Fibronectin always occurs as a dimer, wherein two monomer units are linked by disulfide bonds (Pankov and Yamada, 2002). Two types of fibronectin are found in human body, namely soluble plasma fibronectin mainly found in blood plasma and insoluble cellular fibronectin found in the extracellular matrix. Major functions of fibronectin include growth, cell adhesion, migration and differentiation, in wound healing and embryonic development (Williams *et al.,* 2008). Fibronectin is composed of three different types fo repeating units namely, type I, type II and type III. In all fibronectin contains 12 type I repeats, 2 type II repeats and 16-17 types III repeats, this comprising of almost 90% of its sequence.

In a study of 110 breast cancer patients, the results indicated that fibronectin expression was in significant correlation with histological grade, tumor size and MMP-9 expression (Fernandez-Garcia *et al.,* 2014). Serum and urine examination of 113 colorectal cancer patients (CRC) determined the presence of high levels of fibronectin. This level continued to rise along with the cancer progression, indicating increase in levels of fibronectin along with progression of colorectal cancer (Saito *et al.,* 2008). The effect of radiation therapy on patient's suffering from head and neck cancers was studied and was correlated with fibronectin expression. No significant changes were observed in fibronectin expression before and after radiation therapy, however the expression varied based on the progression of disease (Nishioka *et al.,* 1993). However one of the study shows completely different results, showing that a fragment of fibronectin plays an important role in inhibiting tumor growth, metastasis and angiogenesis. This fragment of fibronectic is obtained by treating soluble fibronectin with a III-1C, a 76 amino acid long

polypeptide derived from type III fibronectin (Yi and Ruoslahti, 2001).This arise a doubt on the credibility of fibronectin as marker of EMT.

b. Laminins:

Laminin is an integral and important part of of the basal lamina which is in turn a part of basement membrane. Various β1 and β4 integrins, a receptor tyrosine phsphatase, heparan sulfates and other cell surface proteins acts as receptor for laminin. In all twelve different types of laminins have been identified till now in mammals (Burgeson *et al.,* 1994; Miner *et al.,* 1995; Hutter *et al.,* 2000). Laminins are trimeric in nature containing α-chain, β-chain and γ-chain.

As discussed above laminin plays a very vital role in maintaining the integrity of basement membrane, thus its expression is either disrupted or attenuated in many forms of cancers. The γ2 chain of the laminin-5 plays a very vital role in epithelial cancer cells. This γ2 chain is majorly expressed in the cytoplasm of the epithelial cancer cells, thereby indicating the role of laminin-5 in epithelial cancers (Giannelli and Antonaci, 2001). It has also been reported that various laminin isoforms that are major component of basement membrane are found to be over expressed in human breast tumors. For instance Fujita et al. (2005) identified three different isoforms of laminin namely laminin-2, -8 and -10 which were overexpressed in breast cancer cells (Fujita *et al.,* 2005). Laminin-5 was found to be overexpressed in 6 patients suffering from adenocarcinoma when compared to the expression in normal mucosa. This study indicates that laminin-5 can be used a biomarker in evaluation of invasive cervical adenocarcinoma (Imura *et al.,* 2012). It has also been reported that laminin-5 directly regulates the expression of α6β1 integrin and thereby promotes cell motility in pancreatic cancer (Li *et al.,* 2004). The role of laminin-5 in hepatocellular carcinoma has also been established; in this type of cancer laminin-5 promotes cell proliferation by directly interacting with α6β4 and α3β1 integrins (Bergamini *et al.,* 2007).

3. Transcription Factors:

a. Snail superfamily:

Snail superfamily genes are very well known due to their important role in the process of EMT. In 1987 Boulay et al, showed the importance of snail in the formation of mesoderm (Boulay *et al.,* 1987). Few years later while carrying out snail loss of function experiments in chick embryo it was shown that how snail genes play role in triggering EMT (Nieto *et al.,* 1994). Many different pathways play a role in activation of the snail genes, for instance TGFβ1 induces snail1 in hepatocytes (Spagnoli *et al.,* 2000) as well as in epithelia (Peinado *et al.,* 2003) whereas TGFβ2 induces snail2 during heart development (Romano and Runyan, 2000). EGFR pathway also plays a vital role in activation of various snail genes (Yamashita *et al.,* 2004). Apart from EMT snail genes also play an important role protecting cell from cell death.

Overexpression of snail1 gene has been reported in tumors induced mouse skin; here snail genes act as an E-cadherin repressor thereby promoting malignancy (Perl *et al.,* 1998; Cano *et al.,* 2000). There is an inverse relation in expression of snail1 and E-cadheirn and this hypothesis is established in breast

tumors (Blanco *et al.,* 2002), gastric cancers (Rosivatz *et al.,* 2002), hepatocellular carcinoma (Miyoshi *et al.,* 2005) and colon cancers (Palmer *et al.,* 2004). Moreover in colon cancer snail1 not only down regulates the expression of E-cadherin but also represses the expression of vitamin D receptor gene (Palmer *et al.,* 2004). In breast cancer patients it was found that snail1 expression was related with differentiation and metastasis whereas snail2 expression was reported to suppress tumor suppressor gene BRCA2 (Tripathi *et al.,* 2005).

Identification of new markers is of utmost significance and will be a useful knowledge to observe Epithelial Mesenchymal Transition , as it may have contribution during neoplasia as well. The research in this domain is expected to reach a pinnacle in the coming years with newer animal models and biomarkers destined to throw light on a vast number of unanswered questions. The identity of EMT-inducing micro-environmental signals, the type of cellular events that make them accept such signals and the complex signaling cascade that maintain the EMT phenomenon across all cells are only few of them.

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