Molecular and pathological signatures of epithelial–mesenchymal transitions at the cancer invasion front

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Accepted: 12 June 2008 / Published online: 22 July 2008 © Springer-Verlag 2008

Abstract  Reduction of epithelial cell–cell adhesion via the transcriptional repression of cadherins in combination with the acquisition of mesenchymal properties are key determinants of epithelial–mesenchymal transition (EMT). EMT is associated with early stages of carcinogenesis, cancer invasion and recurrence. Furthermore, the tumor stroma dictates EMT through intensive bidirectional communication. The pathological analysis of EMT signatures is critically, especially to determine the presence of cancer cells at the resection margins of a tumor. When diffusion barriers disappear, EMT markers may be detected in sera from cancer patients. The detection of EMT signatures is not only important for diagnosis but can also be exploited to enhance classical chemotherapy treatments. In conclusion, further detailed understanding of the contextual cues and molecular mediators that control EMT will be required in order to develop diagnostic tools and small molecule inhibitors with potential clinical implications.

Keywords  Stroma · Cadherin · EMT · Metastasis · Therapy

Introduction

Epithelial–mesenchymal transition (EMT) is a type of plasticity during which epithelial cells lose many of their epithelial characteristics and acquire properties that are typical of mesenchymal cells. Multiple terms, including epithelial–mesenchymal transformation and transdifferentiation have been used to refer to EMT. To understand this, it is important to review the terms epithelium and mesenchyme. An epithelium is a sheet of cells that adhere laterally to each other by cell-to-cell junctions. The epithelial layer is polarized in such a way that bottom and top can be defined as basal and apical (inside and outside). The filamentous actin cytoskeleton is apicobasally polarized and shows circumferential organization. Cytokeratins are the main intermediate filaments. The matrix-binding sites are on the basal face, mediating adhesion mainly to the basal lamina protein, laminin. In contrast, mesenchymal cells form a diffuse network with certain points on their surface adhering to their neighbors. Actin filaments form a dense cortical network of interacting fibers, and perhaps trans-cytoplasmic actin bundles. Vimentin is prominently expressed as an
intermediate filament. Contact sites to the extracellular matrix (ECM) are widely distributed all around the cells. The ECM forms a dense meshwork involving proteins like collagens and fibronectin. In contrast to epithelial cells, mesenchymal cells also have a more extended and elongated shape, and they have front-to-back leading edge polarity (Tarin et al. 2005; Lee et al. 2006; Thiery and Sleeman 2006; Hugo et al. 2007; Berx et al. 2007). In clinical practice pathologists use the terms “sarcomatous/sarcomatoid dedifferentiation” (i.e., “—resembling a sarcoma—”, a malignant mesenchymal tumor) or anaplasia (meaning “—to form backward—”) (Van Marck and Bracke 2005). EMT is not considered a requisite for invasion and metastasis, but compelling evidence shows that it can play an important role in determining the dissemination of tumors (Berx et al. 2007). In animal models, single non-dividing migratory cells delaminate from primary tumors as evidenced by powerful imaging techniques such as multiphoton microscopy (Condeelis and Segall 2003). The characterization of specific markers will undoubtedly help to identify the nature and origin of all mesenchymal-like cells found in the stroma and near the primary tumor.

Molecular and functional signatures of EMT

A key to EMT is the reduction of cell–cell adhesion by transcriptional repression of cadherins (adherens junctions), occludin and claudin (tight junctions), and desmoplakin (desmosomes) (Fig. 1). The Armadillo \(\beta\)-catenin protein is often lost from the cadherin-mediated cell–cell contacts and shuttles to the nucleus to potentiate EMT signaling events (Stemmer et al. 2008). Circumferential F-actin fibers of the cytoskeleton are replaced by a Rho-mediated network of stress fibers (occasionally positive for the myofibroblast marker \(\alpha\)-smooth muscle actin), at the tip of which ECM adhesion molecules localize, including integrins \(\alpha\)\(\beta\)\(_6\), fascin, and integrin linked kinase (ILK) (Bates et al. 2005; Vignjevic et al. 2007; Fuchs et al. 2008). These changes are sufficient for the cells to separate, lose apico-basal polarity and gain a more spindle shape, all facilitating cellular migration (Hugo et al. 2007; Lee et al. 2006). However, cells produced by EMT are not always motile and invasive. In fibrotic kidney, fibroblasts derived from tubular epithelia do not migrate (Kalluri and Neilson 2003). The expression of intermediate filaments is also changing during EMT, with vimentin being typical of mesenchymal cells and different types of cytokeratin being characteristic of epithelial cells. Matrix metalloproteases (MMP’s) such as MMP-1, -2, -3, -7 and -14 are frequently upregulated during EMT, potentially enabling cells to detach from each other (via cadherin ectodomain shedding) and to penetrate the basement membrane. ECM synthesis changes from basal lamina proteins to interstitial forms including collagen type I, fibronectin, secreted protein acidic and rich in cysteine (SPARC) and tenasin C (Nguyen et al. 2005). Furthermore, matricellular proteins, which bridge the functional and physical gap between ECM-associated proteins and cell surface proteins, were recently implicated in the EMT process. This is exemplified by induction of EMT in epithelial MCF-7 breast cancer cells by loss of the Wnt-induced signaling protein (WISP)-2 (Fritah et al. 2008). E-cadherin (epithelial cadherin) is essential for maintaining epithelial integrity of many embryonic and adult tissues. It has been known for a long time that the loss of epithelial differentiation in carcinomas is linked to abnormal, tumor-restricted reduction in the expression of E-cadherin (Semb and Christofori 1998; Strumane et al. 2004). Multiple mechanisms of E-cadherin loss have been described, including abnormal proteolytic cleavage, loss of heterozygosity and inactivating E-cadherin mutations, but lately the mechanism of transcriptional silencing is acquiring much attention. The loss of E-cadherin expression at the transcript level was first identified in several human cancer cell lines and later in different human cancers, including prostate, breast, colorectal and thyroid cancers (Reviewed in Van Aken et al. 2001; Strumane et al. 2004). This initiated the elaborate analysis of the mouse E-cadherin promoter, which has been of primary importance in unraveling the regulatory mechanisms of E-cadherin transcription. Analysis of the mouse proximal E-cadherin promoter identified E-box sequences that determine epithelium-specific expression (Behrens et al. 1991). Inactivation of these consensus E-box sequences resulted in transcriptional activity of the E-cadherin promoter in mesenchymal cells, which indicated the existence of repressors that silence E-cadherin expression in non-epithelial cells. A major breakthrough was the identification of a series of transcriptional repressors, such as Snail, Slug, SIP1/ZEB2 (Smad Interacting Protein)/(Zinc finger E-box Binding homeobox), deltaEF1/ZEB1 and the basic helix-loop-helix (HLH) transcription factor E47, which can directly bind the E-cadherin promoter and repress E-cadherin transcription (Cano et al. 2000; Batlle et al. 2000; Comijn et al. 2001; Eger et al. 2005). Importantly, these E-cadherin repressors have been linked to particular EMT processes during development (Nieto 2002; Vandewalle et al. 2005; Van de Putte et al. 2003). Indeed, overexpression of these transcription factors in epithelial cells not only results in loss of E-cadherin expression but also reprograms the cells toward a mesenchymal cell state. During EMT, transcription factors that reduce E-cadherin expression also downregulate other adhesion molecules and induce mesenchymal features in a coordinated manner (Cano et al. 2000; Vandewalle et al. 2005; De Craene et al. 2005; Aigner et al. 2007). Conditional expression of Snail in DLD-1 colorectal cancer cells
induces invasion of elongated, solitary cells into collagen type I gels (1 mg/ml) as evidenced by H&E stained paraffin sections from 14 day cultures (Fig. 2). In the absence of Snail expression DLD-1 cells form a monolayer on top of collagen type I gels. Interestingly, it was recently shown that knock-down of deltaEF1/ZEB1 in dedifferentiated human epithelial colon and breast cancer cell lines results in re-expression of E-cadherin and other epithelial differentiation markers (Eger et al. 2005; Spaderna et al. 2006, 2008). In addition, to the above-described transcription factors, several other factors that induce EMT with typical loss of E-cadherin expression have been identified, such as TWIST, homeobox (HOX)-B7, CArG box-binding factor-A (CBF-A), Mesenchyme Forkhead 1 (POXC2), and Krüppel-like factor (KLF)-8 (Venkov et al. 2007; Mani et al. 2007; Wu et al. 2006; Wang et al. 2007). Transrepression of E-cadherin transcription is accompanied by the induction of N-cadherin (neural cadherin) or cadherin-11 expression. This so-called cadherin switch (Vandewalle et al. 2005) is believed to contribute to malignant cancer progression (Wheelock et al. 2008). The expression of these transcription factors seems to be regulated by pathways known to promote tumor progression, including transforming growth factor (TGF)-β, vascular endothelial growth factor and scatter factor/hepatocyte growth factor (SF/HGF) (reviewed in Peinado et al. 2007). For instance, TGF-β mediates EMT by signaling via receptor similar to mothers against decapentaplegic (Smad)2/3 and through high-mobility group AT-hook (HMGa)-2 family of nonhistone chromatin proteins, the latter of which coordinates the expression of both Snail and Slug and the basic HLH-transcription factors Twist and Id2 (Thuault et al. 2006). Other recently described important modulators of EMT are small noncoding RNAs of 20- to 22-nucleotides (microRNAs, miRNAs) that inhibit gene expression at the post-transcriptional level: miR-141, miR-200b and miR-205 families control expression of ZEB1 and ZEB2 (Park et al. 2008; Gregory et al. 2008). Profiling miRNA expression of the NCI60 panel of human cell lines which are used for drug screening revealed that the miR-141 and miR-200b families of miRNAs were striking markers of the epithelial phenotype. Selective knockdown of miR-141, miR-200b and miR-205 family miRNAs was sufficient to reduce E-cadherin expression in a ZEB1/ZEB2-dependent way, leading to increased cell motility and thus EMT in epithelial MDCK3 and HCT116 cells. Conversely, ectopic expression of these miRNAs led to the re-expression of E-cadherin and epithelial phenotypes in MDCK cells that had undergone EMT and in mesenchymal MDA-MB-231 breast cancer cells (Park et al. 2008; Gregory et al. 2008). Each of the 400 miRNAs known to exist in mammalian cells has multiple targets, making them powerful regulators of complex processes such as differentiation and cancer progression. Indeed, a strong link between miRNA and human cancers has been established, as it has been demonstrated that miRNAs act either as oncogenes (e.g., miR-155, miR-17-5p and miR-21) (He et al. 2005; Voorhoeve et al. 2006) or tumor suppressors (e.g., miR-15a, miR-16-1 and let-7) (Calin et al. 2002; Takamizawa et al. 2004; Johnson et al. 2007; Yanaihara et al. 2006).

**Extracellular cues regulating EMT**

Bi-directional communication between cancer cells and their microenvironment, profoundly influences their behavior and fate. Primary tumors are heterogeneous and EMT is observed particularly at the cancer invasion front, suggesting the micro-environment as being essential in regulating EMT. For example, melanoma cells that give rise to invasive tumors when implanted into adult animals are reprogrammed into a non-aggressive phenotype when implanted into embryonic tissue (Hendrix et al. 2007). The stroma is essential for tissue integrity and in cancer it drives tissue invasion. Myofibroblasts (also called cancer-associated fibroblasts) are important components of the tumor stroma at the invasion front (De Wever and Mareel 2003; De Wever et al. 2008). In squamous cervix and colon carcinomas, Snail expression is restricted to cancer cells near stromal cells (Franci et al. 2006). Similarly, cancer cells accumulating nuclear β-catenin are distributed throughout the tumor mass but particularly along the invasion front (reviewed by Le et al. 2008). These results suggest that myofibroblasts docking at the invasion front regulate EMT of nearby cancer cells. This myofibroblast-induced EMT may be caused by transient heterotypic cell–cell contacts or by paracrine signals. In vitro, paracrine factors derived from breast tumor myofibroblasts induce EMT in PMC42-LA breast cancer cells, with upregulation of vimentin, loss of E-cadherin staining at cellular contacts and increased nuclear β-catenin signaling (Lebret et al. 2007). We previously demonstrated that tenasin C and SF/HGF produced by myofibroblasts provide convergent pro-invasive signaling to colon cancer cells (De Wever et al. 2004; Denys et al. 2008). ECM remodeling by myofibroblasts is implicated in progression of prostate cancer xenografts (Verona et al. 2007). Furthermore, increased collagen production and expression of α11/β1 integrin in combination with α-SMA mediated contractility has a profound effect on the tumor stiffness that is linked with malignancy (Zhu et al. 2007). Matrix stiffness perturbs epithelial morphogenesis by clustering integrins to enhance ILK activation and increase Rho-kinase (ROCK)-generated contractility and focal adhesions (Paszek et al. 2005; Hannigan et al. 2005). Furthermore, engagement of collagen-binding integrins promotes MMP-9-dependent shedding of the E-cadherin.
ectodomain in ovarian carcinoma cells (Symowicz et al. 2007). Another factor that is receiving considerable attention as an inducer of tumor metastasis is hypoxia (reduced oxygen). There is a strong correlation between tumor hypoxia on the one hand, and metastasis, poor prognosis and patient mortality on the other. This hypoxic response is mainly regulated by the hypoxia-inducible factor (HIF-1), a basic HLH transcription factor composed of two subunits, HIF-1α and HIF-1β. The HIF-1α subunit is regulated by oxygen tension, whereas HIF-1β is constitutively expressed. In breast MCF-7 and ovarian SKOV-3 carcinoma cell lines, Notch signaling is required to convert the hypoxia stimulus into a Snail dependent EMT program leading to increased motility and invasion (Sahlgren et al. 2008). Similarly, hypoxia or overexpression of HIF-1α promotes EMT and metastatic phenotypes in human carcinoma cells via direct induction of the E-cadherin repressor and EMT inducer Twist by direct binding of HIF-1α at the hypoxia response element in the TWIST proximal promoter (Yang et al. 2008). Accordingly, E-cadherin levels were down-regulated while vimentin and N-cadherin were induced in these hypoxic cancer cells. Importantly, these observations were extended in hypoxic human breast cancer cells showing cellular scattering, cell dissemination in vivo, high vimentin levels, translocation of E-cadherin in intracellular pools and nuclear translocation of the EMT inducer Snail (Lester et al. 2007). Furthermore, co-expression of HIF-1α and TWIST in primary tumors of head and neck cancer patients correlates with metastasis (Yang et al. 2008).

**EMT signatures at early stages of carcinogenesis and cancer recurrence in vivo**

Epidemiologic and clinical studies indicate that a strong association exists between estrogen exposure and increased breast cancer risk. Estrogen and/or estrogen metabolites may act as procarcinogens through ER-α—dependent or — independent mechanisms, and may induce genotoxic effects to initiate breast, prostate or other cancers (Yager and Davidson 2006; Huang et al. 2007). In support of these

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Fig. 1  Schematic representation of EMT markers. Upregulated factors implicated in cytoskeletal changes and the mesenchymal phenotype (green side) and repressed markers (red side) implicated in the maintenance of the epithelial phenotype. See text for more details.
observations, long-term treatment of spontaneously immortalized human breast epithelial cells by estradiol induced several phenotypic traits characteristic of EMT, including invasiveness, lower expression of the epithelial markers E-cadherin, keratins, and induction of Snail, vimentin, and the EMT inducer TGF-β (Huang et al. 2007).

To identify early events associated with myc-induced breast cancer, mouse mammary epithelial cells and stromal cells were distinguished using fibroblast specific protein (FSP) and epithelial-specific whey acidic protein (WAP) promoter driving transgenic expression of Cre recombinase constructs in transgenic animals, respectively (Trimboli et al. 2008). These two lineages were identified histologically by expression of the LacZ reporter gene from the Rosa26<sup>LoxP</sup> locus. Of note, early EMT events occurred in 25–50% of myc-initiated breast cancer in WAP-myc; FSP-cre; Rosa26<sup>LoxP</sup> mammary glands. The frequency of EMT in tumor mice with the FSP-cre transgene was slightly higher than in mice with the WAP-cre transgene, consistent with the concept that Fsp-1 expression is an early event in the EMT process. The MYC locus in the 131 patient samples was therefore analyzed for genome-wide LOH, using the adjacent polymorphic marker D8S1128. Trimboli et al. (2008) concluded that MYC amplification is associated with a predisposition of the epithelium to undergo EMT in human breast cancer.

Early appearance of several molecular alterations characteristic of EMT has been described in benign adenomas of the multiple intestinal neoplasia (<i>Min</i>) mouse model of familial adenomatous polyposis disease (Chen et al. 2008). Inactivation of the adenomatous polyposis coli tumor suppressor protein in this preclinical model recapitulates early events underlying the progression of human colorectal cancer. A strong vimentin signal was observed in the cytoplasm of <i>Min</i> colonic neoplastic cells with characteristic epithelial organization in 74 out of 79 tumors. Vimentin staining was absent from the adjacent normal epithelium. Microadenomas involving only three to five crypts in the colon and small intestine displayed elevated vimentin mRNA levels, suggesting that the classical EMT marker, vimentin can be detected at an early stage of tumorigenesis. Of note, <i>Min</i> mice on a tumor-resistant genetic background have reduced tumor multiplicities, longer life-span, and develop invasive intestinal tumors, that reached the submucosa and muscle layers. In this model, immunohistochemistry demonstrated strong vimentin expression in the epithelia of the primary tumor and in the neoplastic cells invading the submucosa and muscular layers (Chen et al. 2008). Vimentin expression was not systematically distributed in all neoplastic cells, but was present in patches in both central and peripheral tumor regions.

Breast cancer recurrence is an essential clinical manifestation of tumor progression and represents the principal cause of death from this disease. Using a conditional transgenic HER2/neu mouse model showing recurrence of HER2/neu-induced mammary tumors, Moody et al. (2005) demonstrated that the transcriptional repressor Snail is spontaneously upregulated in recurrent tumors in vivo and that recurrence is accompanied by EMT as evidenced by spindle shaped morphology with downregulation of
cytokeratin and E-cadherin and upregulation of FSP. Furthermore, Snail is sufficient to promote recurrence of mammary tumors in vivo, and high levels of Snail are predictive of shorter relapse-free survival in breast cancer patients.

Pathological signatures of EMT in tissue samples

Normal epithelial cells are incapable of invasion; they can move laterally in the plane of the epithelium while retaining adhesion to the underlying basement membrane. Active movement in other directions appears to be forbidden to them. Such departures from the plane of an epithelium depend on the acquisition of mesenchymal cell traits (and on the shedding of some of their native epithelial characteristics). As more mesenchymal traits are acquired, it becomes more difficult for the pathologist to distinguish these cells from the true mesenchymal cells that surround the neoplastic cells. This is especially important to determine the presence of cancer cells at the resection margins of a tumor. Figure 3 shows an H&E staining of a basal cell carcinoma with collective invading cancer cells and single migratory cancer cells released from the tumor mass. The analogy with mesenchymal fibroblasts or myofibroblasts present in the ECM is striking. Immunohistochemistry can help distinguish between cancer cells undergoing EMT and stromal fibroblasts, but it must be pointed out that most cancer cells undergoing EMT partially shut down epithelial markers while acquiring mesenchymal markers. Traditionally, most studies have been done by looking at the expression of the intermediate filaments, cytokeratin and vimentin. Routine immunohistochemistry employs a mixture of two different clones of monoclonal antibodies, AE1 and AE3 which cover most keratin subtypes (Goddard et al. 1991). The emergence of vimentin in epithelial cells of breast tumor correlates with a shorter post-operative survival of patients. Furthermore, the keratin/vimentin expression ratio is more predictive of a worse prognosis than vimentin expression alone (Thomas et al. 1999). In cervical cancer, vimentin expression is present in invasive carcinomas and in their lymph node metastases, but not in intra-epithelial neoplasia precursor lesions (Gilles et al. 1996). In villous human adenomas, which show high-grade dysplasia, the vimentin signals were negative in some samples, whereas others showed strong, patchy staining. Expression of E-cadherin varied inversely with vimentin expression independently of Snail and Twist. Conversely, vimentin expression correlated with Wnt and TGF-β signaling and with reduced levels of the Ki-67 proliferation marker (Chen et al. 2008). Cadherin switching may provide adhesion properties more suitable for a migrating cell. Reduced E-cadherin expression and gain of N-cadherin expression in cancer cells may be the result of EMT which is very difficult to detect in vivo. Double labeling of frozen sections with specific E- and N-cadherin antibodies show that invasive micropapillary carcinoma, which metastasizes easily to lymph nodes, contains cancer cells that are positive for N-cadherin, E-cadherin or both (Agiostratidou et al. 2007). In agreement with these findings, other researchers have observed N-cadherin expression in breast, colon, gastric, esophageal, pancreatic and prostate tumors (reviewed in Van Aken et al. 2001). The immunohistochemical staining reaction of β-catenin in normal epithelium and in most non-invasive carcinomas, is predominantly membranous, sometimes with faint cytoplasmic staining. In EMT, an aberrant expression can be seen, with sole cytoplasmic and/or nuclear staining. This supports the notion that competition between different cellular partners for the cytoplasmic pool of β-catenin influences its final incorporation either in cell adhesion or in signal transduction mechanisms. Nuclear accumulation of β-catenin is observed in colorectal cancer cells distributed along the invasion front but not in the tumor center (Hlubek et al. 2007). Furthermore, gene expression profiling revealed overexpression of EMT genes at the invasion front such as TWIST, FSP and tenasin C. Indeed, when β-catenin is released from the adherens junctions, it can enter the nucleus and lead to a transcription of putative invasion related genes. Another cadherin-binding protein, p120-catenin, shifts it localization from cell–cell junctions to the cytoplasm and this correlates with colon cancer progression and patient survival (Bellovin et al. 2005). Recent research has also documented the role of the Snail superfamily in mediating the loss of epithelial adhesion molecules. Immunohistochemical positivity of nuclear Snail correlates with increased vimentin and decreased E-cadherin expression in esophageal squamous cell carcinoma (Usami et al. 2008). The different repressors of E-cadherin transcription have already been associated with progression of different cancer types, e.g., breast (Cheng et al. 2001; Blanco et al. 2002; Moody et al. 2005; Yang et al. 2004; Martin et al. 2005) and gastric cancer (Rosivatz et al. 2002). Although the correlative expression data of the different E-cadherin repressors versus E-cadherin are overwhelming, care should be taken with their interpretation because many of these data are based on RT-PCR and on the use of antibodies with undefined specificity.

A large-scale tissue microarray-based immunohistochemical study of 479 invasive breast carcinomas revealed upregulation of mesenchymal markers (vimentin, α-SMA, N-cadherin and cadherin-11) and overexpression of proteins involved in ECM remodeling and invasion (SPARC, laminin and fascin), together with reduction of characteristic epithelial markers (E-cadherin and cytokeratin) particularly in breast tumors with the “basal-like phenotype” (Sarrió et al., 2008). Gene expression analysis using cDNA
Oncochip microarray of metastatic versus non-metastatic melanomas identified differentially expressed genes implicated in EMT. Validation of these expression data in an independent series of melanomas using tissue microarrays confirmed that the expression of a set of proteins included in the EMT group (N-cadherin, osteopontin and SPARC) was significantly associated with metastasis development (Alonso et al. 2007). A similar oligonucleotide microarray study on papillary thyroid carcinoma from central and invasive regions and of normal thyroid tissue was performed. The invasion fronts were consistently characterized by the presence of mesenchymal markers and absence of epithelial markers. Furthermore, immunohistochemical analysis revealed that overexpression of vimentin associated with papillary thyroid carcinoma cell invasion (Vasko et al. 2007). Molecular classification of fresh-frozen and formalin-fixed head and neck squamous cell carcinomas showed that genes involved in EMT are the most prominent molecular characteristics of high-risk tumors (Chung et al. 2006). In conclusion, we strongly insist that the occurrence of the EMT signature should be explored in large-scale studies of human clinical tumors in order to predict their metastatic potential.

Pathological signatures of EMT in biological fluids

A number of molecules related to EMT can be assessed in biological fluids from cancer patients. Particularly in serum they reflect either the pathogenesis or the consequences of the phenomenon. Table 1 summarizes their applications in different types of cancer.

Scatter factor/hepatocyte growth factor concentrations in serum are correlated with metastatic spread, they possess prognostic value, and they are useful for monitoring therapy. They can also be used as a urine parameter for diagnosis of transitional carcinoma of the bladder. Fibroblast growth factor-2 (FGF-2), another tyrosine kinase receptor ligand, is an endothelial-to-mesenchymal transformation marker, its serum concentrations correlate with tumor growth rate, volume, grading and staging. Its main clinical usefulness, however, resides in monitoring the effect of angiogenesis inhibitors. In prostatic cancer an inverse correlation between prostate-specific antigen (PSA) and FGF-2 has repeatedly been observed (Usami et al. 2008). While the EMT inducer TGF-β as a circulating marker is difficult to interpret, the serum concentrations of its functional activators, MMP-9 and CD44 (mainly its spliced isoform v6), have proven to possess prognostic information. Assessment of the urine concentrations of MMP-9 was claimed to contribute to the diagnosis of bladder cancer. Circulating tenascin-C, a matricellular protein acting in concert with SF/HGF, can discriminate between good and bad prognosis in numerous cancers, and its cut-off level was determined at 96 ng/ml. Tenascin-C serum concentrations also correlated with the vascularization of the tumors. A central player in cancer progression is p53, and this tumor suppressor’s level in circulation was found to be higher in cancer patients than in normal individuals. This is presumably the result of the increased biological half-life of many p53 mutants. Moreover, mutations in exons 5 and 6 are particularly immunogenic, and elicit detectable auto-immune anti-p53 antibodies in the circulation (IgM and IgG). Although p53 and its antibodies were shown to correlate with prognosis and therapeutic response of head- and neck cancer, their major applicability is in epidemiological studies, where mutagenic pressure from the environment has to be assessed.

The switch from E- to N-cadherin is considered typical for EMT, and enzymatically shed, soluble ectodomains from both cadherins have been detected at elevated levels in the serum of cancer patients reviewed in (De Wever et al. 2007). Conceptually the measurement of soluble E-cadherin (sE-cad) may be less straightforward, because
Table 1  Organ-related application of circulating EMT markers

| Organ or tissue/ marker | Organ-related application of circulating EMT markers |
|-------------------------|-----------------------------------------------------|
| Bile ducts              | SF/HGF                                               |
| Bladder                 | FGF-2                                               |
| CD44v6                  | Tenascin-C                                           |
| p53                     | Anti-p53                                             |
| sN-cad                  | sE-cad                                              |
| PSA                     | hK4                                                 |
| CEA                     | CA 15-3                                              |
| CA 19-9                 | CA125                                               |
| AFP                     |                                                     |

1 Assay on urine
2 Decreased concentrations indicate better prognosis
3 Also valid for CD44v5
4 Expressed by circulating cancer cells

E-cadherin is downregulated in carcinomas. Soluble N-cadherin (sN-cad), however, may be a very sensitive circulating marker, because it is upregulated in cancer cells and in stromal myofibroblasts. Two members of the kallikrein family, hK3 (or PSA) and hK4 (prostase) were shown to induce EMT in prostatic carcinoma cells in vitro (Lawrence et al. 2007), and both members are tumor markers for prostatic carcinoma. PSA is not only useful for the monitoring and follow up of therapy, it even contributes to the diagnosis and staging of prostatic carcinoma patients. Additional measurements have refined the interpretation of PSA values. These additional measurements include the rate of the PSA increase (more or less than 0.75 ng/ml or 20% a year), PSA density (serum PSA/prostatic volume), age-related reference values, determination of free/complexed PSA in serum and neural network assistance.

Loss of epithelial cell polarity and of junctional contacts in cancer, in combination with basement membrane breakdown, removes the barriers for apical markers to diffuse into the extracellular fluids. Some high molecular weight apical mucins, which normally circulate at extremely low concentrations, are sensitive markers for loss of cell–cell contact in cancer. Nowadays, they belong to the most frequently prescribed tumor markers in clinical chemistry, and are known as carcino-embryonic antigen (CEA), CA 15-3, CA 19-9 and CA 125. As a result of cell–cell contact loss in hepatocellular carcinoma, α-fetoprotein (AFP) is upregulated (Gleiberman et al. 1989), and has proven to be an excellent serum marker for this type of cancer.
Does EMT affect cancer treatment regimens?

Selective tyrosine kinase inhibitors are promising to treat cancers driven by activated tyrosine kinases such as Bcr-Abl in chronic myelogenous leukemia, c-Kit in gastrointestinal stromal tumors and EGFR receptor (EGFR) in non-small cell lung cancer (NSCLC). Gefitinib (Iressa) and erlotinib (Tarceva) are selective inhibitors of the EGFR receptor tyrosine kinase and is currently used to treat NSCLC patients (Guo et al. 2008). Although most NSCLC tumors express EGFR, only a fraction of patients with tumors dependent on EGFR for growth and survival respond clinically to EGFR inhibitors. These tumors appear to contain EGFR-activating mutations or have undergone amplification of EGFR gene copy number. Furthermore, a subset of non-small cell lung carcinomas that do not respond to erlotinib therapy have low levels of E-cadherin and higher levels of mesenchymal type transcripts such as vimentin and fibronectin (Thomson et al. 2005). The bulk of Thomson’s evidence is derived from NSCLC cell lines, but some confirmatory evidence comes from immunohistochemical staining of sections of human tumors (Yauch et al. 2005). Furthermore, ILK-mediated EMT predicts hepatocellular carcinoma sensitivity to EGFR-targeted therapies (Fuchs et al. 2008).

Alternatively, many routine cancer treatments may stimulate EMT and thus enhance invasion and metastasis. Chronic chemotherapy with oxaliplatin (a third generation platinum compound) induces EMT in colorectal cancer cell lines (Yang et al. 2006). In another example, ionizing radiation induces changes associated with EMT such as increased migration, F-actin stress fiber formation, and disturbed E-cadherin pattern in A549 lung epithelial cells in vitro (Jung et al. 2007). Human malignant gliomas are lethal neoplasms. Involved-field radiotherapy is the most important therapeutic measure. Most relapses originate from the close vicinity of the irradiated target field. Interestingly, sublethal doses of irradiation enhance the migration and invasiveness of human malignant glioma cell lines in vitro. IR-induced migration is p53-independent, and involves enhanced expression of αvβ3 integrin, altered MMP-2 and MMP-9 expression and activity profiles, altered expression of membrane type 1 MMP and tissue inhibitor of metalloproteinases-2, and altered BCL-2/BAX balance favoring resistance to apoptosis (Wild-Bode et al. 2001). Current approaches to cancer treatment involving more intensive radiotherapy regimens have been suggested to be associated with a higher incidence of local or distant metastasis (Jung et al. 2007). Therefore, a subset of patients may benefit from a combination of radiotherapy and EMT inhibitors. We did not find prospective randomized clinical trials providing level 1 evidence in favor of or against such side effects of treatment so our discussion is admittedly speculative.

Epithelial–mesenchymal transition signatures can be exploited to enhance classical chemotherapy treatments. Gain of N-cadherin expression is a typical example of the EMT signature and it has been linked with drug resistance (Zhang et al. 2007). Interestingly, ADH-1 (an N-cadherin cyclic pentapeptide antagonist) sensitizes N-cadherin-expressing melanoma cells to the anti-tumor activity of melphalan in melanoma models (Mariotti et al. 2007; Augustine et al. 2008). Recent work provides evidence to support the possibility of exploiting EMT as a potential therapeutic target in cancer (Sabbah et al. 2008).

Conclusion

Converging evidence using in vitro cell culture models, transgenic mouse models, gene signature microarray analysis and immunohistochemistry points out that EMT, amongst other mechanisms, is implicated in early steps of carcinogenesis, cancer cell invasion and metastasis and recurrence. The list of molecular signatures defining EMT is growing and will eventually lead to a clear definition useful for daily practice in pathology and clinical biology. Understanding the molecular basis of EMT is essential for designing small-molecule inhibitors targeting the EMT response. These can be used to enhance classical chemotherapy treatments.

Acknowledgments G. De Bruyne is gratefully acknowledged for reference management. This work was funded by Fonds Wetenschappelijk Onderzoek (FWO)-Vlaanderen (Brussels, Belgium). The Association for International Cancer Research (Scotland), the Scientific Exchange Program between the Flemish community and France (Grant I.2007.03) and FP7 (TUMIC) of the European Union. O. De Weyer and B. De Craene were supported by Bijzonder Onderzoeks Fonds (BOF, University Ghent, Belgium).

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