Epithelial-to-Mesenchymal Transition

Subjects: Otorhinolaryngology | Oncology

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Epithelial-to-mesenchymal transition is a well-studied phenomenon in embryology and occurs during the morphogenesis of organs. It is described as epithelial-to-mesenchymal transition (EMT) type I. The molecular procedure of EMT is also reprogrammed in the healing of wounds and the pathological fibrosis of organs, known as EMT type II. EMT III is the type that is implicated in tumor metastasis. While initially focusing on the abolishment of epithelial and acquisition of mesenchymal characteristics by the tumor cells, the idea behind EMT currently incorporates all the phenotypic and molecular characteristics that enable tumor cells to migrate, survive, and proliferate in distant tissues. In other words, it is a complete model of molecular processes signaled by specific factors called inducers. This model progresses via cross-linked molecular pathways, concluding with functional and structural modifications that make the carcinoma cells metastatic. These modifications are mediated by molecules known as the effectors of EMT.

epithelial to mesenchymal transition (EMT) laryngeal carcinoma metastasis

1. The Role of EMT Effectors in EMT Characteristics

1.1. Loss of Adhesion

One of the fundamental characteristics of epithelial cells on the mucosal surfaces is their polarity. An additional characteristic is the existence of adhesion structures that attach every epithelial cell with its adjacent cell and the basal membrane. Such adhesion structures, including tight junctions, zona occludens, desmosomes, and hemidesmosomes, are constructed by adhesion molecules, such as E-cadherins [1]. E-cadherins are transmembrane proteins that create a homophilic bond with E-cadherins of the adjacent cell. At the same time, E-cadherins are anchored to the actin filaments of the cytoskeleton via a molecular complex consisting of p-120 and β-catenins. A hallmark of EMT is the abolishment of E-cadherins. The loss of E-cadherin can happen mainly when its promoter is downregulated by transcription factors, especially Snail, Slug, and ZEB2 [2]. Secondarily, E-cadherins can be abolished due to their cleavage by metalloproteinases (MMPs) or their phosphorylation and subsequent degradation by the ubiquitin–proteasome system [3][4]. E-cadherin abolishment results in the epithelial cells' detachment from each other and the basement membrane. Due to the dismantling of the molecular complex, p-120 and β-catenins are released into the cytoplasm. When these catenins reach the nucleus, they function as transcription factors that enhance the expression of crucial molecules, such as MMPs and N-cadherin that drive EMT progression. The "cadherin switch" is a critical event that enhances tumor cells' migratory capacity [5].

In laryngeal carcinoma clinical samples studied with immunohistochemistry, advanced staging and decreased differentiation correlate with E-cadherin downregulation, β-catenin nuclear translocation, and expression of the transcription factors Snail, Slug, and ZEB2 [6][7]. Co-expression of E-cadherin/β-catenin in laryngeal tissue samples also correlates with clinicopathological parameters such as lymph node metastasis and overall patient survival [7][8]. Additionally, a cadherin switch, with N-cadherin replacing E-cadherin, is evident in laryngeal carcinoma cell lines [7][9]. The Twist transcription factor regulates N-cadherin expression in laryngeal carcinoma cell lines [9]. Another study on Hep-2 cell lines has shown that the knockdown of Snail can inhibit EMT [10].

1.2. Remodeling of the Cytoskeleton

During the course of EMT, epithelial cells undergo alterations of their cytoskeleton. Activation of Rho GTPases by free p-120 catenins leads to another hallmark trait of EMT, the substitution of normal epithelial cytokeratins by vimentin [11][12][13][14]. Modifications of the cytoskeleton, additionally, contribute to the loss of polarity, the acquisition of spindle cell morphology, and the creation of structures that facilitate migration, such as lamellipodia [15][16].

When EMT develops, typical modifications in the cytoskeleton occur in laryngeal carcinoma tissue samples, with the replacement of cytokeratins by vimentin in higher-grade neoplasms [17]. Spindle-cell squamous laryngeal carcinoma is a rare entity whose nature is not clarified. However, it is shown in other carcinomas that spindle-cell morphology is acquired during EMT [18]. Interestingly, one study supports that laryngeal carcinoma cells that metastasize through lymphatic vessels are exposed to fluid shear stress that can induce EMT, cytoskeleton modifications, and lamellipodia formation [19]. Lamellipodia enhance local invasiveness in different head and neck carcinomas [19][20].

1.3. Evasion of Apoptosis

Apoptosis is a programmed cell death met both in pathological and physiological situations in tissues. Apoptosis is mediated by molecules known as caspases, which can be activated by various molecular pathways [21]. An apoptotic cell is removed without leaving behind an inflammatory reaction. One typical condition that induces a type of apoptosis in epithelial cells, known as anoikis, is their detachment from the basal membrane [22][23]. The basal membrane sends survival signals to the cells through the integrins of the epithelial cells' focal adhesions [24]. Normally, the epithelial tumor cells that detach from the basal membrane while becoming metastatic should die by anoikis. However, through EMT, molecular pathways such as ILK-Akt and MAPKs preserve the tumor cell's survival signals [25][26]. Notably, apoptosis evasion is related to resistance to various chemotherapeutic drugs [27].

On the other hand, specific molecular pathways activated during EMT lead tumor cells to a condition called autophagy, a catabolic process that normally aids cellular homeostasis by eliminating damaged organelles and molecules. In cancer-associated autophagy, the tumor cells intentionally destroy some of their organelles to reduce their energy needs. This way, tumor cells can remain dormant until they find favorable conditions to proliferate again [28]. The interplay between EMT and autophagy is complex: autophagy may initiate or suppress EMT but is also activated by EMT-related signaling pathways, i.e., hypoxia or TGF-b [28]. One of the most important regulators

of autophagy, mTOR, is also a downstream effector of the PI3K-Akt pathway of EMT [29]. However, the final effect of EMT on autophagy may be related to the cell type and the stage of tumor progression: in early phases of tumorigenesis, autophagy may impede the EMT process; on the other hand, once cells have undergone EMT, they may promote and utilize autophagy as a means of apoptosis and immune surveillance evasion, increasing their metastatic potential [30].

In several head and neck neoplasias, including laryngeal carcinoma, activation of focal adhesion kinase (FAK) has been shown to protect cancer cells from caspase-mediated anoikis [31]. Caspases have been suggested as promising targets for laryngeal carcinoma therapy [32]. Additionally, studies with cell cultures have shown retinoids to suppress FAK, leading to apoptosis and G2/M cell cycle blockage [33].

Novel molecules involved in apoptosis, such as the tumor suppressor programmed cell death 4 protein (PCDP4), have shown emerging roles in EMT of the laryngeal carcinoma contributing to the cadherin switch process. In vivo experiments in mice have shown that PCD4 silencing is associated with dysregulation of the Wnt-β-catenin and the STAT3-miR-21 signaling pathways, suggesting crosstalk of PDCD4 with important regulators of EMT [34].

1.4. Evasion of Immune Surveillance

Transcription factors activated during EMT lead to the expression of cytokines, such as TGF-β, which also functions as a master immune regulator [35][36]. As evident in several neoplasias, enhanced expression of TGF-β may prevent the recognition of tumor cells by T-helper lymphocytes [37]. Additionally, EMT leads cancer cells to reduce the expression of HLA proteins of the histocompatibility system and eventually to evasion of immune surveillance [38].

In laryngeal carcinoma, TGF-β regulates cells of the innate and adaptive immune system [39]. Upon exposure to TGF-β, the primary antigen-presenting cells, the dendritic cells, facilitate immune tolerance and become inactive and immobile [40]. Moreover, TGF-β skews T-naïve lymphocytes' differentiation towards T-regulatory lymphocytes (T-regs) instead of the immune-potent Th1 lymphocytes [39]. This condition renders the immune surveillance of malignant cells in laryngeal carcinoma less effective.

1.5. Upregulation of Metalloproteinases (MMPs)

Transcription factors such as AP-1 and β-catenin, which accumulate in the nucleus as a consequence of the activation of the molecular pathways of EMT, upregulate the expression of the metalloproteinases [41]. MMPs enhance tumor cells' migratory capacity in several ways. They can activate signaling molecules for EMT, such asFGF, and cleave E-cadherin, which leads to dismantling adhesion structures [42][43]. They also participate in cytoskeleton remodeling and the evasion of apoptosis [44][45]. Additionally, MMPs can activate VEGF, a crucial factor for neovascularization [46]. Moreover, MMPs on the lamellipodia can pave the way through stroma by degradation of collagen fibers [47]. Finally, substances that emerge from stroma after cleavage by MMPs, such as Elastin, can act as chemotactic factors that contribute to the migratory capacity of tumor cells [48].

Less differentiated laryngeal carcinomas appear with enhanced expression of metalloproteinases such as MMP-2 and MMP-9 and suppressed expression of the tissue inhibitors of metalloproteinases TIMP -1 and -2 [49]. Thus, metalloproteinase expression has been suggested as a prognostic factor for laryngeal carcinoma [50]. Other immunohistochemical studies have additionally shown that the primary source of MMP-9 in laryngeal carcinoma that promotes EMT is the inflammatory cells in the tumor stroma [51].

1.6. Neovascularization

The formation of new vessels (neovascularization or neoangiogenesis) is a crucial procedure for developing metastasis since it facilitates the supply of oxygen and nutrients to solid tumor cells for a tumor to grow beyond a certain size (1–2 mm^3 in diameter) [52]. These newly formed vessels are usually small in diameter; hence, the measurement of microvessel density (MVD) is a long-established method to quantify associated neoangiogenesis in several tumors [53]. Two of the molecular pathways that function as master regulators of EMT, RTKs—Ras—MAPK and Src—PI3K—Akt, contribute to neovascularization [54]. Additionally, the upregulation of MMPs is related to the activation of VEGF-A, a growth factor implicated in creating new blood vessels [55]. MMPs also contribute to VEGF-D activation, which is implicated in forming new lymph vessels [56]. Interestingly, enhanced expression of the EMT-related transcription factor Twist is involved in the phenomenon of vascular mimicry [57]. This phenomenon consists of channels in the cancer mass that imitate vessels, which are not typically formed by endothelial cells. Nevertheless, this type of pseudovessels can also pave the way for metastasis of the epithelial tumor cells [58].

Available data suggest that in laryngeal carcinoma tissue samples, molecular pathways that intersect and end up with the activation of the transcription factor ZEB2 mediate the mesenchymal transition of the epithelial cells and upregulate the expression of genes that mediate neovascular formation by activated endothelial cells [59]. Moreover, vascular mimicry has also been studied in laryngeal carcinomas, showing enhancement of lymph node metastasis [60]. Vascular mimicry has been correlated with clinicopathological parameters in laryngeal carcinomas, such as histopathology grade and disease-specific and metastasis-free survival [60]. Finally, lymphangiogenesis in the early stages also contributes to the dissemination of laryngeal carcinoma and is related to local and locoregional recurrence [61].

1.7. Acquisition of Stem-Cell Traits

A proportion of cells in every tissue appear with stem cell properties, the most characteristic being self-renewal and potency [62]. Self-renewal is the ability of the cells to go through numerous cycles of division while maintaining their undifferentiated state. Potency is the capacity to differentiate into specialized cell types. These properties are crucial for metastasis but also for resistance to chemotherapeutics. Cells acquire characteristic surface antigens of stemness, typically CD44+, under the regulation of molecular pathways of EMT, such as the pathways of TGF-β, Wnt/β-catenin, Notch/Hedgehog, and miRNAs [63]. A possible model that has been suggested is that among the tumor cells, those which will undergo EMT acquire stem-cell properties and promote metastasis.

In the laryngeal carcinoma cell line, Hep-2, the downregulation of miRNA-145 contributed to the acquisition of stem-cell properties, such as potency and self-renewal, by carcinoma cells, such as the expression of CD-133 [64]. This condition occurs mainly in hypoxic environments and is related to higher proliferation and colony formation ability of the Hep-2 cell lines [65]. Additional markers associated with stemness in laryngeal carcinoma include the expression of aldehyde dehydrogenase (ALDH) and the cell-surface glycoprotein CD44+ [65]. More specifically, CD44+ functions as a receptor for hyaluronic acid involved in cell adhesion and migration. Carcinoma cell lines that acquired stem-cell properties exhibit self-regeneration and resistance to radiotherapy [66]. Eventually, these conditions are related to poorer clinicopathological parameters, such as advanced stage and recurrence in laryngeal carcinoma [67].

1.8. Altered Interaction of EMT Cells with Tumor Stroma

Tumor cells develop a particular interaction with the underlying stroma that promotes migration and metastasis. Tumor cells exert functional modifications to the tissue stroma leading to its activation. Subsequently, the activated stroma cells mediate the EMT of the tumor cells by contributing to the signaling of this molecular phenomenon [68] [69]. A new cell, the cancer-associated fibroblast (CAF), appears in the activated stroma, characterized by the presence of alpha-smooth muscle actin (α-SMA) [70]. Several theories speculate on the origin of CAFs. The most prevalent theories support their origin from preexisting fibroblasts transformed under the effect of TGF-β and IL-6 produced by the tumor and inflammation cells [71]. In a vicious circle, tumor cells create CAFs, and, subsequently, CAFs induce EMT of tumor cells through Wnt, FGF, and TGF-β production, endowing them with a migratory capacity [69][72]. Additionally, CAFs produce MMPs, angiogenetic, and chemotactic factors for inflammatory cells, mediating multiple stages of the molecular process of metastasis. It is noteworthy that interleukins, miRNAs, and growth factors from the tumor site can travel in exosomes and activate the stroma of distant tissues [70]. Exosomes are nanometer-scale membrane vesicles deriving from the tumor and tumor-microenvironment cells and are released into the circulation. Exosomes can prepare the microenvironment of the future metastatic niche, demonstrating a refined manifestation of the “seed and soil” theory [73].

In the larynx, α-SMA-expressing CAFs in the stroma are detected only in invasive carcinoma and not in the stroma of the adjacent normal tissue [74][75]. Moreover, several studies have shown that miRNAs that have selectively enriched exosomes, such as miR 1246, miR 1290, miR 335 5p, miR 127 3p, and miR 122 5p, are distinct compared to miRNAs in the parental carcinoma cells [76]. Crucial exosomalmiRNAs derived from the CAFs of laryngeal carcinoma stroma promote migration and invasion, as shown in cultures from cancer specimens [77][78].

2. Inducers and Pathways of EMT

2.1. The Pathway of TGF-β

TGF-β is secreted by epithelial tumor cells and CAFs of the activated stroma [79]. It actuates its molecular pathway through transmembrane receptors that activate a family of cytoplasmic molecules called Smads [79]. Smads form complexes that function as transcription factors in the nucleus to express or repress crucial molecules for EMT.

Interestingly, the TGF-β pathway may present both tumor-promoting and tumor-suppressive roles [80]. This dual role is related to the co-activators or co-repressors of the Smad complexes in the nucleus, the type of promoter of the various genes, and the different molecular pathways that are activated in the specific cell at a given time [80]. Moreover, the TGF-β signaling, apart from canonical, is also triggering non-canonical pathways. Typical molecular pathways activated during EMT, such as Wnt, Ras, and PI3K-Ras, which are cross-linked with TGF-β canonical pathway, can affect the final result of TGF-β signaling [81].

There are several indications of the dual role of TGF-β in laryngeal carcinoma. TGF-β has an enhanced expression in laryngeal carcinoma compared to the adjacent normal tissues, supporting its tumor-promoting role [82]. Additionally, TGF-β pathway downstream genes act as an immune suppressor in head and neck carcinomas, negatively affecting the survival indexes [83]. In another study, the downregulation of the TGF-β receptor II is shown to be an early event in carcinogenesis, indicating the TGF-β pathway tumor-suppressive role at the initial stages of carcinoma progression [84].

2.2. Molecular Pathway of Wnt

The typical hallmark of EMT is the downregulation of the adhesion molecule E-cadherin. A consequence of this condition is the release in the cytoplasm of β-catenins. B-catenins form a complex with E-cadherins, and when they are set free in the cytoplasm, they normally bind on a molecular complex formed by APC, Gsk-3b, and Axin. This complex leads β-catenin to degradation by the ubiquitin-proteasome system [85]. Cancer-associated fibroblasts in the activated stroma of tumor cells secrete Wnt molecules [86]. Wnt induces EMT, signaling via a Frizzled (Fz) family transmembrane receptor. When the receptor is activated, a signal is transmitted to the phosphoprotein Dishevelled (Dsh), which leads to translocation of the negative Wnt regulator, Axin. The stereotactic alteration of the complex reduces the affinity of β-catenins to it, thus, leading to the accumulation of β-catenin in the cytoplasm and subsequently translocation in the nucleus, where β-catenin functions as a transcription co-factor of TCF/LEF. This complex of transcription factors is crucial for the molecular modifications of EMT on the tumor cells [87]. Recently, non-canonical Wnt pathways have been described to cross-link other EMT molecular pathways [88].

In laryngeal carcinoma, several long coding RNAs have been shown to signal via the Wnt/β-catenin pathway [89] [90]. Other long coding RNAs, such as NEF, exhibit a tumor-suppressive role targeting the Wnt pathway [91]. Additionally, in laryngeal carcinoma tissue samples and cell lines, the FOXP4 transcription factor regulates EMT as it directly bounds to the LEF promoter, a downstream effector of the Wnt pathway [92]. Moreover, even the main effector of the Hippo signaling pathway, Yes-associated protein, is implicated in the Wnt/β-catenin pathway in laryngeal carcinoma cell lines [93]. Finally, it has been shown that aberrant Wnt signaling has a prognostic value in early laryngeal carcinomas [94].

2.3. Molecular Pathways of Growth Factors

Growth factors reach tumor cells via paracrine and autocrine loops. Their signaling is mediated through the cell-surface receptors of tyrosine kinase (RTKs) [95]. The molecular pathways that RTKs activate include Ras [96], Rho

[97], Src [98], and Notch-Hedgehog [99], which participate in the majority of the molecular phenomena that define EMT.

The Src molecule plays a crucial role in laryngeal carcinoma pathogenesis since siRNA silences inhibit carcinoma growth and regulates apoptosis through the Src/PI3K/Akt pathway in vitro and in vivo [100][101]. Notch 1 and 2 are other molecules implicated in cell growth, aberrant angiogenesis, acquisition of stem cell traits, anti-apoptosis, and the metastasis of laryngeal carcinoma [102][103]. Knockdown of Notch 1 in Hep-2 cells inhibited molecules with nodal roles in the development of EMT, such as p-Akt, cyclin D1, and Bcl-2 [104]. Moreover, K-Ras over-expression has been correlated with dedifferentiation in laryngeal carcinoma cells [105]. Ras has also been found to be regulated by miR-21 in the larynx. More specifically, inhibition of miR-21 by antisense oligonucleotides led to decreased levels of Ras and significant suppression of laryngeal tumor cells' migratory capacity [106].

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