Review Article

EPITHELIAL MESENCHYMAL TRANSISION AND CANCER ASSOCIATED FIBROBLASTS IN THE ADENOID CYSTIC ADENOCARCINOMA: A LITERATURE REVIEW

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Abstract

Human adenoid cystic carcinoma (AdCC) is characterized by diffused invasion of the tumor into adjacent organs and early distant metastasis. Epithelial mesenchymal transition is considered prerequisites for cancer cells to metastasize. Exploring the relationship between these processes and their underlying mechanism of action is a promising way to better understand AdCC tumors. Genetic and cell biology studies indicate that tumor progression is not just determined by malignant cancer cells themselves, but also by tumor stroma. Evidence is increasing that the fibroblasts in tumor stroma remain permanently activated and serve as important promoters of tumor growth, invasion and metastasis. These activated fibroblasts are often termed as cancer-associated fibroblasts (CAFs), tumor-associated fibroblasts, or myofibroblasts due to their expression of α-smooth muscle actin. However, α-SMA expression alone will not identify all CAFs. AdCC in the oral cavity is rare, scarce immunohistochemical studies of AdCC in the oral cavity have been performed. So, the aim of the current study was to determine epithelial mesenchymal transition and cancer associated fibroblasts in the stroma of adenoid cystic adenocarcinoma using PubMed and Medline database English literature by the terms "Mesenchymal transition", "Fibroblasts", "Cancer" and "Adenoid cystic adenocarcinoma". In conclusion, it is reported CAF exhibited the most important defining feature of CAF by promoting cancer progression. Vimentin and α-SMA plays a key role in and progression of the cancer. It appears that the immunohistochemical profile of AdCC in the oral cavity is identical to major salivary glands. More series involving greater numbers of cases are needed to confirm these findings.

Key words: *Epithelial mesenchymal transition, Carcinoma associated fibroblast, α-SMA, Vimentin, E cadherin.*

Introduction

Mucosal melanoma (MM) and AdCC of the salivary glands belong to rare cases of head and neck cancer. The MM originates from melanocytes of mucosal epithelia and makes up to 1% of all melanomas.[1] However, more than half of all cases can be found in the head and neck region (MMHN). There is compelling evidence highlighting molecular and clinical differences between cutaneous and mucosal melanoma in terms of tumor growth, metastasis, and pathogenesis. MMHN is characterized by an infiltrative and local destructive growth pattern, and overall survival in these patients is more often limited by local recurrences rather than by distant metastases.[2] AdCC originates from epithelial cells of the major or minor salivary glands of the head and neck, and is the second most common cancer of the salivary glands. AdCC is a neurotropic tumor with an infiltrative growth pattern preferentially along nerve fibers and a high tendency to local spread and early distant metastases, limiting therapeutic options with a curative intent of treatment.[3] Epithelial-to-mesenchymal transition (EMT) is a complex process in which epithelial cells lose their characteristic features and acquire a mesenchymal-like phenotype. Phenotypic hallmarks of EMT are the loss of cell-cell junctions, loss of apical-basal polarity, and acquisition of migratory and invasive properties. It resembles a fundamental process in embryonic development and during wound healing. In a pathophysiologic context, cancer cells are able to reactivate the EMT program to gain new properties such as accelerated motility and treatment resistance. A large range of developmental and growth factor signals can drive EMT by triggering genetic and epigenetic programs, which are under the control of or regulate a core set of transcription factors.[4] Carcinoma-associated fibroblasts (CAFs) are critical in determining tumor invasion and metastasis. However, the role of CAFs in the invasion of salivary gland AdCC is poorly understood. In this study, we isolated primary CAFs from two AdCC patients. AdCC-derived CAFs expressed typical CAF biomarkers and showed increased migration and invasion activity. Conditioned medium collected from CAFs significantly promoted AdCC cell migration and invasion. Co-culture of CAFs with AdCC cells in a microfluidic device further revealed that CAFs localized at the invasion front and AdCC cells followed the track behind the CAFs. Genetic and cell biology studies indicate that tumor progression is not just determined by malignant cancer cells themselves, but also by tumor stroma. Evidence is increasing that the fibroblasts in tumor stroma remain permanently activated and serve as important promoters of tumor growth, invasion and metastasis. These activated fibroblasts are often termed as CAFs, tumor-associated fibroblasts, or myofibroblasts due to their expression of α-smooth muscle actin. However, α-SMA expression alone will not identify all CAFs. So, this paper is part of Ph.D. thesis and the aim of the current literature review was to determine EMT and cancer associated fibroblasts in the adenoid cystic adenocarcinoma.

Material and Methods

The keywords used for the literature search for this review was peer-reviewed articles following keywords: Mesenchymal transition × Fibroblasts × Cancer × Adenoid cystic adeno carcinoma × Immuno histochemical markers × E-cadherin × α-SMA × Vimentin. Related articles were also scrutinized. Hand search was also driven. The search was

carried out using Biological Abstracts, Chemical Abstracts, and the data bank of the PubMed and Medline database updated to 2018. The references found in the search were then studied in detail.

Adenoid cystic carcinoma (AdCC)

The AdCC is one of the most frequent malignant tumors arising in the salivary glands, representing about 2–6.5% of tumors of the head and neck region. It shows a strong capacity for local invasion and a high incidence of distant metastasis. AdCC cells can be found at a considerable distance beyond the clinical boundaries of the tumor. In addition, tumor cells can extensively invade bone before there is radiographic evidence of osseous destruction. Elucidating the mechanisms of AdCC invasion might thus provide new strategy for AdCC therapy.[5] Typically, AdCC grows slowly, but spreads relentlessly into adjacent tissues. The frequencies of recurrence and distant metastasis of AdCC are markedly high, with 40-60% of AdCC patients developing distant metastases in the lungs, bone and soft tissues.[6] Unlike common malignant tumors, AdCC cells produce large amounts of extracellular matrix, consisting of collagen, elastin, basal lamina components and mucopolysaccharides.[7] These ECM components can accumulate in intercellular spaces, resulting in the formation of a pseudocyst, which is the characteristic architecture of AdCC. Consequently, as AdCC cells can become surrounded by their own ECM, in addition to normal host connective tissue, the mechanisms of invasion and metastasis of AdCC cells are unique from those of other malignant tumors of the oral cavity, particularly in the interaction with the ECM.[7]

Polymorphous low-grade adenocarcinoma (PLGA)

Polymorphous low-grade adenocarcinoma (PLGA) occurs more frequently in minor salivary glands and rarely as a primary neoplasm in major salivary glands. It tends to be a low-grade malignancy with recurrences recorded up to 10 years after treatment. The local recurrence rates vary from 10.3 to 17% and the distant metastases are very rare). The overlapping histological features of AdCC and PLGA occasionally may result in a diagnostic pitfall and especially when small biopsies do not contribute to distinguish between these tumors. In these cases, immunohistochemistry may be necessary or desirable to suggest or confirm a diagnosis.[8] It is studied immunohistochemically four cases of PLGA and based on their results as well as on the results of a similar study of AdCC suggested that the detection of epithelial membrane antigen (EMA) and carcinoembryonic antigen (CEA) may assist to distinguish AdCC from PLGA. Because the number of examined cases of PLGA was small further studies were suggested to confirm this possibility.[9]

Cancer associated fibroblast (CAF)

CAF is a general term that describes activated fibroblasts of different origin which express differing markers depending on their location. CAFs are the most abundant stromal cells in the tumor microenvironment.[10] They are derived from various origins, such as normal fibroblasts, smooth muscle cells, endothelial cells, myoepithelial cells, or mesenchymal stem cells. CAFs support tumor growth by both secreting many growth factors and stimulating angiogenesis. Based on immunohistochemical data, several CAF markers were identified including vimentin, fibroblast-specific protein-1(FSP-1), fibroblast-activation protein (FAP), α-SMA Among the potential sources of CAFs are local activated fibroblasts, mesenchymal stem cells and cells which have undergone epithelial to EMT. Fibroblasts are the primary cell type of connective tissue and form the extracellular matrix. Local non activated fibroblasts can undergo activation by tumor cells which secrete TGF-beta and SDF-1.[11] CAFs influence tumor development and progression by secreting many growth factors, interleukins and matrix metalloproteases (MMPs). Once transformed to their activated phenotype, CAFs promote tumor growth and metastasis. This is in contrast to disease free fibroblasts which exert an anti-tumorigenic effect. The tumor promotion occurs through the release of factors that promote cell growth and recruit new cells into the tumor microenvironment. SDF1 (CXCL12) promotes survival of cancer cells and is a chemo attractant for other stromal cells. IL-6 is a pro-inflammatory cytokine which is produced in substantial amounts by CAFs. IL-6 produced by CAFs also promoted tumor development in a cell model system. In addition to promoting tumor development by enhancing proliferation and cell recruitment, CAFs also release pro-angiogenic factors. Release of the VEGF and PDGF cause recruitment and proliferation of endothelial cells to form new blood vessel development.[12] CAFs also help the tumor cells to metastasize to secondary sites. One method of achieving this is through their release of TGF-beta. TGF-beta signalling is capable of transforming stationary endothelial cells into mobile mesenchymal cells by EMT. Similarly, hepatocyte growth factor (HGF) can also induce EMT enabling cells to migrate more efficiently. In addition to creating a more mobile cell, CAFs can also edit the ECM to further facilitate invasion and metastasis. Release and activation of MMPs (MMP_9 and MMP_{14}) degrade type IV and V collagens and other extracellular matrix proteins enabling cancer cell migration. Recent evidence has shown that CAFs are capable of shielding tumors from the effect of various treatments. Type I collagen secreted by CAFs creates a physical barrier between the tumor cells and the drugs diffusing from the blood stream. CAFs also mediated treatment resistance in response to gefitinib through activation of Met and EGFR/Met pathway crosstalk. Two further studies have shown that CAFs mediated resistance to anti-angiogenic therapy and Cetuximab.[13]

Epithelial-mesenchymal transition (EMT)

The EMT plays an important role in cancer progression as well as in embryonic development. During this process, the epithelial cells undergo a morphologic change and acquire a mesenchymal phenotype, which often leads to tumor invasion and metastasis. EMT is characterized by down-regulation of cell adhesion molecule E-cadherin, a

transmembrane protein needed for stable adherent junctions between adjacent cells, and is regulated by certain E-cadherin transcriptional repressors including basic helix-loop-helix transcription factors such as zinc finger E-box binding homeobox 2 (ZEB2).[14] These EMT markers are considered to be important contributors to cancer progression. Loss of E-cadherin is a hallmark of the EMT, resulting in cells acquiring invasive and metastatic properties (14). EMT has been extensively studied in various malignancies such as breast, ovarian, pancreatic, colorectal and gastric cancers. E-cadherin loss and its promoter hypermethylation status have been earlier reported in eyelid SGC; however, its role in EMT remains to be explored.[15]

Immunohistochemical markers

The AdCC in the oral cavity is rare, scarce immunohistochemical studies of AdCC in the oral cavity have been performed. Immunohistochemically, AdCC was consistently positive for cytokeratin (CK) AE1/3, CK 34βE12, CK5/6, CK7, CK14, CK18, p63, CA19-9, c-KIT (CD117), PDGFRA, MUC1, and Ki-67. AdCC was consistently negative for CK8, CK20, desmin, S100 protein, CD34, chromogranin, MUC2, MUC5AC and MUC6. Some AdCCs were positive for CK CAM5.2, CK19, EMA, CEA, vimentin, α-smooth muscle actin, p53, CD10 and synaptophysin. These results may provide basic knowledge of AdCC of the oral cavity. However, in the current paper we listed some of these markers. Also, Ashraf *et al*.[16] on value of immunohistochemical markers in pleomorphic adenoma and AdCC of the salivary gland there was no difference between luminal and stromal expression of six cytokeratin markers (CK7, CK 8, CK 13, CK 14, CK 17, and CK 18) in both typical and atypical areas except SMA that was not expressed in the atypical area. No statistically significant differences were found between the expression of CK7, CK 8, CK 14 and CK 18 in both typical and atypical areas of AdCC group, with no expression of SMA in atypical area.

E-cadherin

Cadherins constitute a large family of cell surface proteins, including E (epithelial)-, N (neural)-, VE (vascular-endothelial)-, P (placental)-, R (retinal)-, and K (kidney)-cadherins. Classical cadherins are single-pass transmembrane proteins which participate in Ca^{2+}-dependent cell adhesion that is necessary to form solid tissues. E-cadherin is functionally linked to the generation of a polarized epithelial phenotype. E-cadherin/β-catenin protein complexes are involved actively in epithelial to mesenchymal (EMT) and mesenchymal to epithelial (MET) transitions, which play a particularly important role in embryo development, tissue fibrosis, and cancer progression. The process of EMT is characterized by differentiated epithelial cells that undergo a phenotypic conversion that gives rise to the matrix-producing fibroblasts and myofibroblasts. Epithelial cells lose their marker proteins such as E-cadherin, zonula occludens-1 (ZO-1), cytokeratin, gain of a mesenchymal phenotype with expression of mesenchymal proteins including vimentin, α-smooth muscle actin (α-SMA), fibroblast-specific protein-1 (FSP1) and production of interstitial matrix components type I collagen and fibronectin.[17]

Fibrosis is an active extracellular matrix (ECM) biosynthetic process and represents the final pathway of chronic failure of many organs. Evidence of EMT has been reported in kidney, lung, liver, eye, and serosal membranes suggesting that EMT can be closely associated with the pathogenesis of fibrotic disorders in those organs.[17] E-cadherin downregulation responsible for the loss of cell-cell adhesion and β-catenin up regulation for the subsequent transcriptome program of EMT are two important changes in the process of fibrosis. It is reported that 36% of renal fibroblasts, the main effector cells in kidney fibrosis responsible for ECM production, originate from renal tubular epithelial cells via EMT despite conflicting evidence about the relative importance of various sources of myofibroblasts was reported. Decreased expression of E-cadherin and early expression of EMT-related markers as well as β-catenin cytoplasmic translocation have been detected in kidney specimens from patients with glomerulonephritis and diabetic and chronic allograft nephropathies. Similar changes appear in lung epithelial cells of patients with idiopathic pulmonary fibrosis and usual interstitial pneumonia and in biliary epithelial cells of patients with primary biliary cirrhosis, primary sclerosing cholangitis and alcoholic liver disease. The transition of retinal pigment epithelial cells into myofibroblasts is observed in patients with proliferative vitreoretinopathy, and peritoneal mesothelial cells from fluid effluents of dialyzed patients show a mesenchymal phenotype with reduced E-cadherin expression.[18] Nuclear *β*-catenin immunoreactivity suggests aberrant activation of Wnt/*β*-catenin signalling and subsequent EMT in the pathogenesis of a number of fibrotic disorders. In addition to the nuclear translocation of *β*-catenin, we previously reported that matrix metalloproteinase (MMP)-mediated E-cadherin disruption led directly to tubular epithelial cell EMT is an important mechanism for the cancer development and initial step of metastasis.[19] Disruption of E-cadherin /*β*-catenin might contribute to tumor aberrant morphogenetic effects. Loss of E-cadherin expression or loss of its normal localization at cell-cell contacts is consistently observed at sites of EMT during tumor progression. E-cadherin expression level is often inversely correlated with the tumor malignancy. Cases of invasive lobular carcinoma have been associated commonly with the loss of E-cadherin expression as a result of E-cadherin gene mutation and promoter hypermethylation. Evidence from animal models has shown that conditional deletion of E-cadherin in p53-deficient mouse mammary epithelium promoted tumor initiation and progression to invasion and metastasis. *β*-catenin is a critical element in the canonical Wnt signalling of tumorigenesis. Activating mutations of *β*-catenin or inactivating mutations of APC or Axin have been found to be associated with a wide variety of human malignancies, such as colorectal, ovarian endometrial, heptocellular,

desmoid and pancreatic tumor.[20] Cancer studies suggest that deregulated β-catenin signalling promotes tumorigenesis by inducing expression of oncogenes such as c-myc and cyclin D1. Stabilizing mutations in the β-catenin N-terminal sequence have been found in 25% of metaplastic breast cancers. Increased cytoplasmic and nuclear β-catenin levels have been observed in 40% of primary breast cancers and correlated with poor prognosis and worse patient survival.[20]

Alpha- smooth muscle actin (α-SMA)

Alpha-smooth muscle actin (α-SMA) is the actin isoform that predominates within vascular smooth-muscle cells and plays an important role in fibrogenesis. Myofibroblasts are metabolically and morphologically distinctive fibroblasts expressing alpha-SMA, and their activation plays a key role in development of the fibrotic response. In an activated state, myofibroblasts cease to proliferate and start to synthesize large amounts of extracellular component proteins. The expression of α-SMA is correlated with the activation of myofibroblasts. In contrast, the expression of alpha-SMA in cells made quiescent by cell-cell contact was lower than that in cells made quiescent by serum starvation.[20]

Vimentin

Intermediate filament (IF) proteins are best known for their cell type specificity and their static structural role as components of the vertebrate cell cytoskeleton. There is growing evidence for IF involvement in a variety of dynamic cellular functions, including intercellular and cell-to-extracellular matrix signal transduction, cellular motility, and tumor cell invasiveness.[21] Vimentin, a 57 kDa protein, is one of the most widely expressed and highly conserved proteins of the type III IF protein family. During murine development, vimentin expression commences on embryonic day 8.5 and its expression is predominant in the primitive streak stage, while in adult vimentin expression is limited to connective tissue mesenchymal cells, in CNS and in muscle.[21] Infidelity of IF expression, expression of the mesenchymal marker vimentin by an epithelial cell, or expression of more than one type of IF (coexpression) has been observed *in vitro* in certain tumor cell lines and in cellular and tissue samples of certain human malignancies. *In vitro* model of keratin and vimentin coexpression in breast cancer cells (developed as stable transfectants) has revealed that these keratin/vimentin IF-positive cells display increased proliferation rates, invasive potential, clonogenicity, and tumorigenicity when compared with keratin-positive/vimentin-negative controls displaying low invasive potential. The keratin/vimentin-positive, transfected breast cancer cells demonstrated networks of well-formed IFs, which appear as interwoven keratin and vimentin filamentous networks.[21] The *in-situ* appearance of vimentin immunoreactivity in human breast cancer cells, which normally express only the epithelial IF marker keratin, has been reported in up to 61% of human breast cancers. The immunohistochemical detection of vimentin expression in invasive breast cancer has also been associated with biomarkers of poor prognosis and adverse clinical outcomes.[20]

Reports

The metastatic spread of tumor cells is a complex multi-step process. In order to metastasize, tumor cells first need to invade through a basement membrane, detach from the primary tumor mass, enter the circulation, and travel to a distant secondary site where they expand rapidly.[22] Each of these steps is essential and requires interactions between tumor cells and their microenvironment. Unlike common malignant tumors, AdCC cells produce large amounts of extracellular matrix, consisting of collagen, elastin, basal lamina components and mucopolysaccharides. These ECM components can accumulate in intercellular spaces, resulting in the formation of a pseudocyst, which is the characteristic architecture of the AdCC. Consequently, as AdCC cells can become surrounded by their own ECM, in addition to normal host connective tissue, the mechanisms of invasion and metastasis of the AdCC cells are unique from those of other malignant tumors of the oral cavity, particularly in the interaction with the ECM. In this regard, Ishii *et al*.[23] significant down-regulation of cell adhesion molecules, such as cadherins and integrin subunits was observed. The loss of E-cadherin and integrins and the gain of vimentin in AdCCS-M GFP cells were confirmed by immunoblotting. These results suggest that EMT is a putative event in AdCC metastasis that induces tumor cell dissemination from the primary tumor site. Also, Jia *et al*.[24] reported the EGFR/PI3K/Akt pathway acts as the common regulator for EMT-like transformation, as confirmed by their specific inhibitors. Gefitinib and LY294003 restored the sensibilities of anoikis-resistant cells to anoikis and simultaneously impaired their metastatic potential. In breast cancer, CAFs promote cancer cell growth, angiogenesis, and invasion by C-X-C motif chemokine 12 (CXCL12), MMP9, and MMP14. In prostate cancer, CAFs have been shown to affect the proliferation and facilitate the invasiveness of cancer cells by CXCL12, CXCL14, MMP2 and MMP3. In lung cancer, CAFs promote the proliferation and invasiveness of cancer cells by high expression of Forkhead box F1 and CXCL12.[25] AdCC-derived CAFs might promote cancer invasion by expressing MMP2 and CXCL12. This cell-type is mostly defined based on morphological characteristics and the expression of markers such as VIM, α-SMA, FAP, and FSP-1.VIM is a common marker for mesenchymal cells expressed in both normal fibroblasts and CAFs. Although α-SMA is a biomarker which is widely used to detect CAFs, the functional significance of α-SMA-expressing fibroblasts in tumor stroma is uncertain. Some researchers reported that these α-SMA-expressing fibroblasts promoted tumor growth and angiogenesis.[25] By contrast, transgenic mice with the ability to delete α-SMA-expressing fibroblasts in pancreatic cancer developed invasive, undifferentiated tumors with enhanced hypoxia, epithelial-to-mesenchymal transition, and cancer stem cells, with diminished animal survival. Recently Kong *et al*.[26] on established and characterized of a carcinoma-associated fibroblast cell line derived from a human

salivary gland adenoid cystic carcinoma and revealed CAF-SA CM significantly promoted the migration rates of both SACC-83 and SACCLM cells. CAF-SA CM enhanced the invasions abilities of both SACC-83 and SACC-LM. Fibroblasts extracted from two AdCC cases were not only positive for common fibroblastic markers such as VIM, but also positive for activated fibroblastic markers such as α-SMA, FAP and FSP-1. In addition, we found that almost all AdCC-derived CAFs expressed FAP, while only some CAFs expressed α-SMA. These findings suggest the cellular heterogeneity of AdCC-derived CAFs.[26] On comparison of immunohistochemical expression of e-cadherin in malignant salivary gland tumors. Prabhu *et al*.[27] reported negative expression was evident in canalicular adenoma, AdCC, mucoepidermoid carcinoma and adenocarcinoma. Statistically significant reduction in reactivity was evident in mucoepidermoid carcinoma and adenocarcinoma, when compared to pleomorphic adenoma.

Conclusions

CAFs are one of the most abundant and important stromal cells in tumor microenvironment. The most important defining feature of CAFs compared to normal fibroblasts is their capacity to stimulate cancer invasion and metastasis. They regulate the growth, invasion and metastasis of tumor cells and stimulate angiogenesis, recruiting bone marrow-derived cells and immunocytes.[28] CAFs support tumor growth by both secreting many growth factors and stimulating angiogenesis. Based on immunohistochemical data, several CAF markers were identified including vimentin, FSP-1, FAP, α-SMA.[29] Vimentin and α-SMA plays a key role in and progression of the cancer. It appears that the immunohistochemical profile of AdCC in the oral cavity is identical in major salivary glands.

This paper is part of Ph.D. thesis. More series involving greater numbers of cases are needed to confirm these findings.

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