Abstract

Background: Cancer stem cells (CSCs) may participate in angiogenesis by lining the wall of tumor vessels. Aim: The current study aimed to present the role of vimentin and CD44 in inducing vasculogenic mimicry (VM) and epithelial–mesenchymal transition (EMT) in different grades of mucoepidermoid carcinoma (MEC). Materials and Methods: A total of 63 MEC samples were collected from the archive of Department of Pathology of Taleghani Educational Hospital, Tehran, Iran. Vimentin and CD44/periodic acid–Schiff double staining was performed. Statistical Analysis: Chi-square test was used to examine the differences with categorical variables. Significance level was set at 0.05. Pearson’s correlation coefficient was used to assess the colocalization of the markers. Results: There were statistically significant differences between tumor grade and the expression levels of vimentin and CD44 (P = 0.000). Conclusion: Our results may disclose a definite relationship between microvessel density (MVD), VM, EMT, and CSCs in MEC samples. Thus, it is reasonable to suggest that CSCs are related to angiogenesis and VM.

Keywords: Angiogenesis, CD44, mucoepidermoid carcinoma, vimentin

Introduction

Malignant salivary gland tumors constitute <5% of all head-and-neck malignancies. Mucoepidermoid carcinoma (MEC) accounts for 35% of all malignant salivary gland tumors[1] and is mostly found in the parotid gland.[2] According to Brandwein et al., category, MECs are classified as low, intermediate, and high grade depending on criteria such as the intracystic component, tumor invasion front, vascular, lymphatic, and bony invasion, perineural spread, nuclear atypia, number of mitoses, and the presence of necrosis.[3‑5] Histologically, MEC comprises variable proportions of muco-secretory, intermediately, and epidermoid cells. Due to heterogeneity of the morphological and histological features of MEC, it is useful to examine the relevance of molecular markers with clinical and pathologic features.[1,6]

Folkman first coined a theory regarding tumor angiogenesis in 1971. He proposed that a tumor produces its own new vasculature from the existing blood vessels. Therefore, the inhibition of angiogenesis has become a new strategy for anticancer therapy. Recently, antitumor angiogenic therapies have been challenged. Thus, new drugs are needed. Many studies have hypothesized that tumor blood vessels develop by endothelial cells, but a growing body of evidence suggests that some cancer blood vessels are not lined by endothelial cells.[7] Maniotis et al. found that the aggressive melanoma cells themselves make vascular-like channels directly and function as tumor blood vessels to facilitate tumor blood perfusion through a process called “vasculogenic mimicry” (VM).[8] Vasculogenic tumor cells promote tumor growth and cancer metastasis.[9,10] Previous studies found that VM exists in many cancers and is a prognostic factor of poor prognosis.[10] VM can be identified by the detection of periodic acid–Schiff (PAS)-positive loops surrounded by tumor cells (not endothelial cells), with or without red blood cells in it.[11] VM and epithelial–mesenchymal transition (EMT) are phenomena to acquire the capability of invasion and metastasis.[11] Highly aggressive epithelial tumor cells may overexpress the mesenchymal phenotype through EMT during VM formation.[12] Similar to VM, EMT is well correlated with invasion and lymph node metastasis.[13] A

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A growing body of research indicates that vascular channels are lined by both tumor cells and endothelial cells in some cancers called “mosaicism.”[14]

In recent decades, another hypothesis has become more significant in cancer research. This is about cancer stem cells (CSCs) describing a small subset of tumor cells which can replicate. CSCs are not only capable of self-renewal but also can reproduce the whole phenotype of the original tumor. They are responsible for cancer relapse, chemo (radio) therapy resistance, invasion, and metastasis.[15] Cancer metastasis starts from invasion of cancer cells through the wall of small blood vessels or lymph vessels.[9,16] Then, cancer cells settle into a niche to promote proliferation, vasculogenesis, and metastasis.[9,17] Recently, it has been shown that CSCs may participate in angiogenesis by lining the wall of tumor vessels.[18] In addition, CSCs exist in advanced tumors with lymph node metastasis.[10] On the other hand, previous studies have shown that CSCs can induce EMT, a phenomenon which promotes tumor cell invasion and metastasis.[19] A growing body of evidence has shown that cells with EMT phenotype are important sources for CSCs, suggesting their biological similarities.[20] In fact, cancer cells undergoing EMT may resemble CSCs.[21]

Vimentin is a marker of EMT.[22] Elevated vimentin expression has been detected in several cancers such as melanoma and prostate cancer.[23] In some cancers such as esophageal squamous cell carcinoma, the increased expression level of vimentin is associated with a higher incidence of lymph node metastasis.[19,24] CD44 is a cell surface glycoprotein involved in cell–cell interactions, cell migration, and adhesion; therefore, it plays a major role in tumorigenesis and metastatic progression for a variety of tumors.[25] Increased CD44 expression level has been detected in cancers such as head-and-neck cancer, breast cancer, and prostate cancer. Furthermore, CD44 has been described as a CSC marker in head-and-neck squamous cell carcinoma (HNSCC), and CD44+ cells have been proposed as the tumor-initiating CSCs in HNSCC which can re-establish the original tumor heterogeneity.[26,27] In addition, CD44 is correlated with tumor grade, recurrence, and poor prognosis in oral squamous cell carcinoma (OSCC).[15] Although EMT promotes the CSC signature, the regulatory mechanism of CSC and EMT is still unclear. The identification of biomarkers related to EMT and CSCs may provide a chance to develop drugs targeting EMT and CSCs.[28,29]

The current study aimed to present the role of vimentin and CD44 in inducing VM, EMT, and to identify the CSC niche in different grades of MEC.

Materials and Methods

Patients and tissue samples

A total of 63 MEC samples (21 samples each grade) were collected from the archive of Department of Pathology of Taleghani Educational Hospital, Tehran, Iran. There were 30 cases from parotid gland, 20 cases from submandibular gland, and 13 cases from minor salivary glands. Adjacent normal salivary gland tissue (from parotid, submandibular gland, and minor salivary glands) served as control group. Hematoxylin and eosin staining was performed to confirm the previous diagnosis. Histologically, MECs were classified as low, intermediate, or high grade on the basis of the presence of cystic spaces, proportion of mucous cells, growth pattern, type of invasion, and cytological atypia.[4,5,30]

Detection and scoring

Vimentin expression was detected in the cytoplasm of cancer cells. CD44 expression was detected in the membrane of the tumor cells. Microvessel density (MVD) was determined by light microscopy examination of stained sections at the “hot spot.” Fields of the greatest neovascularization were identified by light microscope at low power (×100). The average vessel count of the five fields (×400) was regarded as the MVD. The MVD was classified as either high (≥15) or low (<15); 15 was considered as the median value of MVD in our study. VM was also assessed. Vimentin and CD44/PAS double staining was used to validate VM. VM was identified by the detection of PAS-positive loops surrounded by tumor cells (not endothelial cells), with or without red blood cells in it. The abundance of positive cells for both biomarkers was graded as follows: 1 (weak) for <20% positive cells, 2 (moderate) for 20%–50% positive cells, and 3 (strong) for >50% positive cells.[31]

Statistical analysis

Analyses were conducted through SPSS software version 22.0 (SPSS, Inc., Chicago, IL, USA). Chi-square
test was used to examine the differences with categorical variables. Significant level was set at 0.05. Pearson’s correlation was used to assess the colocalization of the markers.

**Results**

A total of 63 samples (35 men, 55.6% and 28 women, 44.4%) were used for IHC study. Age ranged from 20 to 70 years with a mean age of 50.3 years. There were statistically significant differences between tumor grade and MVD ($P = 0.000$), between tumor grade and VM ($P = 0.000$), and also between tumor grade and the expression levels of vimentin and CD44 ($P = 0.000$). In addition, there was a strong positive correlation between tumor grade and vimentin expression level (Pearson’s $r = 0.857$, $P < 0.000$) and between tumor grade and CD44 expression level (Pearson’s $r = 0.611$, $P < 0.000$). There was also a positive correlation between VM and vimentin expression level (Pearson’s $r = 0.600$, $P < 0.000$) and between VM and CD44 expression level (Pearson’s $r = 0.388$, $P < 0.002$).

The details are summarized in Tables 1 and 2.

**Table 1: The relationships between Vimentin, CD44 expression and histopathological variables in different grades of MEC**

| Histopathological variables | Low grade | Intermediate grade | High grade | $P$ |
|----------------------------|-----------|--------------------|------------|-----|
| MVD >15        | 16 (76.2%) | 0                  | 0          | 0.000 |
| MVD ≤15        | 5 (23.8%)  | 21 (100%)          | 21 (100%)  |     |
| VM Positive    | 0         | 9 (42.9%)          | 14 (66.7%) | 0.000 |
| VM Negative    | 21 (100%) | 12 (57.1%)         | 7 (33.3%)  |     |
| Vimentin Weak  | 20 (95.2%)| 1 (4.8%)           | 1 (4.8%)   | 0.000 |
| Vimentin Moderate | 1 (4.8%)  | 18 (85.7%)         | 4 (19%)    |     |
| Vimentin Strong | 0         | 2 (9.5%)           | 16 (76.2%) |     |
| CD44 Weak and Moderate | 19 (90.5%) | 7 (33.3%) | 5 (23.8%) | 0.000 |
| CD44 Strong    | 2 (9.5%)  | 14 (66.7%)         | 16 (76.2%) |     |

**Discussion**

In this study, the expression levels of vimentin and CD44 were examined in the normal salivary gland tissue and in the MEC tissue of different histological grades.

Angiogenesis is a hallmark of cancer, and VM is another way to supply oxygen and nutrition to the cancer cells. VM vessels are lined by tumor cells and do not require endothelial cells. Previous studies have shown that VM has a crucial role in tumor progression and metastasis. For instance, aggressive melanoma contains a lot of tumor cell-lined vasculatures. In our series, VM was present in 9/21 (42.9%) of intermediate- and 14/21 (66.7%) of high-grade samples (altogether 23/63; 36.5%) and was significantly associated with tumor grade [Figures 1 and 2]. In a previous study, VM was found in 40% of adenoid cystic carcinoma (AdCC) samples. In this study, a positive correlation between VM rate and tumor grade was indicated. In another study, tumor cell-lined vessel was found in 18/33 (54.5%) cases of OSCC. VM has been shown to be present in 21/84 (25%) of gastrointestinal stromal tumors, which was significantly associated with tumor grade and liver metastasis. On performing IHC staining for 99 tissue samples of hepatocellular carcinoma, VM was observed in 12 cases (12%). Microvessel density (MVD) may give useful information about tumor behavior. In the present study, MVD count was higher in intermediate- and high-grade samples compared to that of low-grade cases. In a previous investigation on MEC, MVD was associated with clinical stage, histologic grade, and tumor recurrence. Another study on MEC demonstrated a positive association between histologic grade of MEC tumor samples and the expression level of caveolin-1. In addition, MVD was significantly correlated with clinical stage, vascular invasion, and metastasis in patients with ACC. Similar to our study, a previous study on prostate cancer found a significant association between microvessel count and tumor grade. Previous reports found intense angiogenesis at the periphery of malignant salivary gland tumors. Besides, a moderate vascular endothelial growth factor (VEGF)-positive staining was found in low-grade MECs, while intensity was increased in high-grade MECs. In this study, the VEGF expression...
was mainly observed in epidermoid and intermediate cells and was mild or absent in mucous cells.\textsuperscript{[37]} In our study, the MVD was higher at the periphery of the tumor and at the invasive front in intermediate- and high-grade tumors. In addition, intratumoral MVD was mostly high in intermediate- and high-grade tumors. Another study on MEC also found a positive correlation between intratumoral MVD and histological grade.\textsuperscript{[18]} A previous investigation on MEC showed a higher CD34 positivity in intratumoral vessels compared to those of peritumoral regions.\textsuperscript{[20,39]} Moreover, another study on CD133 expression level in ACC showed that CD133\textsuperscript{+} cells were able to organize VM which contributes to cancer migration and invasion.\textsuperscript{[19]} MVD count, VM, and expression of CD133, another CSC marker, are also correlated with tumor grade in renal cell carcinoma.\textsuperscript{[18]} These findings explain the role of angiogenesis in tumor progression, invasion, and metastasis.

According to the results obtained in the current investigation, overexpression of vimentin was mainly found in epithelial cells, especially at the periphery of epithelioid cellular islands in intermediate- and high-grade samples indicating that the reserve cells gained EMT properties [Figures 3 and 4]. Vimentin is a marker of EMT. Besides, vimentin is a useful marker for the identification of neoplastic myoepithelial cells,\textsuperscript{[40]} which is expressed in salivary myoepithelial cells.\textsuperscript{[40]} Therefore, some vimentin-positive cells inside the epithelioid cellular islands may indicate the presence of myoepithelial cells. Moreover, vimentin positivity was found in some detached tumor cells, especially around the blood vessels, and in the stroma of invasive front [Figure 5]. It means that both reserve cells and/or myoepithelial cells may contribute in EMT. In a previous study on HNSCC, strong positivity of vimentin was found in the microenvironment. The authors proposed that tumor cell mobility can facilitate cancer progression and metastasis.\textsuperscript{[41]} Previous studies have reported the
similar findings\cite{42,43} which may also indicate the EMT phenomenon. Recent studies have shown that increased vimentin expression level is correlated with EMT process of cancers such as breast cancer\cite{44} and is indicative of aggressive tumor behavior and poor prognosis in OSCC.\cite{45} Furthermore, vimentin expression is associated with lymph node metastasis of esophageal squamous cell carcinoma.\cite{19} In melanoma samples, overexpression of vimentin acts as a predictive factor of hematogenous metastasis.\cite{46} Moreover, EMT is associated with the presence of VM; therefore, there may be an alternative mechanism of VM in epithelial neoplasm.\cite{47}

Regarding CD44 expression in this study, weak expression was observed in 12.7%, moderate expression in 36.5%, and strong expression in 50.8% of all cases. There was a significant association between CD44 expression level and tumor grade [Figures 6-8]. These findings are consistent with other data showing that low expression of CD44 is associated with less aggressive tumors.\cite{30} These results may indicate an increased number of CSCs by tumor progression. Another study on MEC cases showed weak CD44 positivity in 13.3%, moderate CD44 positivity in 20%, and strong CD44 positivity in 53.3% of cases. This study also found moderate-to-strong CD44 expression in 87.5% of high-grade tumors.\cite{30} A previous study on MEC and the expression pattern of CSC markers demonstrated CD44 positivity in both cystic and solid tumors. The authors proposed that CD44 is necessary to get the aggressive CSC phenotype.\cite{22} Another study on MEC detected weak-to-strong patchy membranous epidermoid cell staining.\cite{48} A previous report indicated that CD44-positive tumor cells present CSC properties in HNSCC.\cite{26} Among them, intensity of CD44 expression was strong in 55.8% of cases, moderate in 32.6% of samples, and weak in 11.6% of cases. Besides, CD44+ tumor cell nests were mainly located at the periphery of the tumor close to the stroma.\cite{49} In another study on HNSCC, CD44

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**Figure 5:** Immunohistochemical analysis of vimentin in mucoepidermoid carcinoma lesions. Close section shows detached vimentin-positive tumor cells around the vessels and in the stroma (×250)

**Figure 6:** Identifying CD44 expression in low-grade tumor. The cystic structure is lined by epithelial cells which are stained by CD44 (black arrow). The mucous cells are positive for periodic acid–Schiff staining (×250)

**Figure 7:** Double-positive staining of CD44 and periodic acid–Schiff show a moderate positive staining of CD44 in epithelial cell islands of intermediate-grade mucoepidermoid carcinoma (×250)

**Figure 8:** Immunohistochemical analysis of CD44 indicates significantly increased expression of epidermoid cells in high-grade mucoepidermoid carcinoma (×250)
expression was detected in 89.6% of cases. The authors suggested a direct correlation among CD44 expression, CSCs, and the aggressiveness of tumors. Similar to a study on head-and-neck cancer, our study showed CD44+ positivity in the stroma and at the invasive front of the tumor which may indicate the ability of CD44+ cells undergoing EMT as well as having the characteristics of CSCs to promote metastasis [Figure 9]. CD44 expression is related to other tumor characteristics such as EMT and CSCs. In the present report, CD44 expression was observed at the periphery of ductal structures and cystic areas, where myoepithelial cells are normally located. A previous report on ACC showed the expression of CD44 in myoepithelial component, not in ductal structure. As CD44 is a stem cell marker, these findings may be another proof to suggest the role of myoepithelial cells in controlling salivary gland tumor growth. We also found that the cancer cells at the invasive front were positive for vimentin and CD44. Growing evidence has indicated that the EMT is closely correlated with CSCs, and tumor cells which have the ability of undergoing EMT also have the characteristics of CSCs. A growing body of evidence found the presence of CSCs predominantly at the tumor–host interface which have acquired acquisition EMT phenotype as well as stemness. Furthermore, other reports have demonstrated that EMT is sufficient to provoke a cell population with stem cell characteristics. CSCs can be found in specialized areas known as the “niche.” In some tissues such as brain, CSCs aggregate in the perivascular areas which are called “perivascular niches.” CSCs may also take part in angiogenesis by forming the wall of tumor vessels. These findings may explain that the anti-angiogenic treatment fails in some types of cancers. According to the findings of our study, CD44 and vimentin positivity was found in the detached cells, especially around the vessels [Figures 5 and 10]. MEC is a tumor with lymph node metastasis. Distant metastasis in cases of MEC has not been reported. On the other hand, CSC existence has been reported in advanced tumors with lymph node metastasis. Our findings may explain the ability of MECs in lymph node metastasis, not in distant metastasis.

**Conclusion**

This study found a positive correlation between VM rate and tumor grade. In addition, the present study indicated a higher MVD in advanced tumors. Therefore, our results may disclose a relationship between MVD, VM, EMT, and CSCs in MEC samples. Thus, it is reasonable to suggest that CSCs are related to angiogenesis and VM. Taken together, it could be demonstrated that MECs contain the CSCs since they share the same markers in the tumor cells, in the stroma, and at the invasive front of the tumor. The combined detection of vimentin and CD44, to some extent, has a significantly increased value for determining MEC prognosis. While vimentin and CD44 play an important role in the control of angiogenesis, they are not the only genes that are involved in the control of angiogenesis. Although we have identified a link between vimentin and CD44 expression and MEC aggression, more research is needed to determine whether the expression levels of vimentin and CD44 in MEC could serve as prognostic markers or an indicator of response to therapy. Besides, our results profoundly indicated that a targeting strategy against VM is most urgently needed, and vimentin and CD44 would be ideal targets for therapy, as they would target multiple aspects of tumor biology. The ability of cancer cells to find alternative growth signaling pathways also needs to be considered.

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Conflicts of interest

There are no conflicts of interest.

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