THE ROLE OF CD44 AND SOX-2 CANCER STEM CELL MARKERS IN THE DEVELOPMENT AND PROGRESSION OF MUCOEPIDERMOID CARCINOMA AND PRIMARY SQUAMOUS CELL CARCINOMA OF SALIVARY GLANDS

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ABSTRACT

One of the greatest difficulties to reach a successful treatment of mucoepidermoid carcinoma (MEC) and primary squamous cell carcinoma (PSSC) of salivary glands is the resistance to different therapeutic procedures which advocate the existence of a subpopulation of cancer stem cell (CSC). In salivary gland tumors the hypothesis of cancer stem cell has not been fully understood.

Aim: To assess the expression of cancer stem cell markers CD44 and SOX2 in low and high-grades MEC and PSSC and to determine whether the expression of these markers can be used to predict tumor aggressiveness.

Materials and Methods: 4μm sections were prepared from paraffin-embedded blocks of 16 samples of MEC and 16 samples of PSSC of salivary glands (8 low-grade and 8 high- grade carcinomas for each of them). Staining of each specimen with hematoxylin and eosin, CD44 and SOX2 was done.

Results: All lesions of MEC and PSSC showed membranous CD44 and nuclear SOX2 expression. CD44 and SOX2 expression were significantly higher in high-grade tumors than in low-grade tumors. A strong positive correlation between CD44 and SOX2 expression was detected.

Conclusion: Cancer stem cells can be identified in MEC and PSSC using CD44 and SOX2 markers and may participate in tumor aggressiveness. The present study also suggests that patients with MEC and PSSC may benefit from therapies that comprise a combination of the two markers.

KEYWORDS: Mucoepidermoid carcinoma, primary squamous cell carcinoma, CD44, SOX2, immunohistochemistry

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INTRODUCTION

Salivary gland malignancies are relatively rare tumors.[1] Mucoepidermoid carcinoma (MEC) is considered one of the most prevalent malignant salivary gland neoplasms detected in the minor and major salivary glands. It affects children and adults.

MECs are classified histologically as low, intermediate and high grades.[2] Treatment of MEC consists of surgical resection with or without adjuvant radiotherapy, depending on the tumor grade. Meanwhile, there are no effective treatment options for patients with locally invasive, recurrent, or metastatic neoplasm.[3]

The prognosis of this tumor relies mostly on the histological grade, as high-grade tumors are very aggressive, while the low-grade ones are less aggressive and usually have a more benign nature. Studying the pathobiology of this malignancy and the mechanism implicated in therapy resistance is very important to enhance the quality of life and the survival of patients with MEC.[4]

Primary squamous cell carcinoma (PSCC) is a malignant epithelial neoplasm which has an aggressive behavior and accounts for less than 1% of all neoplasms of the salivary glands.[5,6,7] It is crucial to restrain the diagnosis of PSCC to major salivary glands because it is impossible to verify whether the neoplasm originates from minor salivary glands themselves or from the nearby mucosa.[8,9] PSSC may arise from a metaplastic salivary gland epithelium with a latency of 15-30 years. Ionizing radiation may lead to this metaplasia.[5,6,7,]

Treatment of PSSC consisted of surgical excision. Adjuvant radiotherapy or chemotherapy depends on the degree of tumor distribution.[10,11]

The most established theory for the etiopathogenesis of cancer is that the tumor begins as a solitary cell which has some features of that of the normal stem cells.[12,13] This cancer stem cells (CSCs) have an important role in tumor progression,

recurrence, therapeutic resistance and metastasis in many types of cancers as pancreatic cancer, breast cancer and head and neck cancer. So, identification and killing this CSCs could be an important goal of cancer therapy.[14,15,16]

Many studies characterized CSCs based on stem cell markers expression and correlated their expression with the clinical and histopathological features. Among the CSC markers are, CD133, CD24, CD44, SOX2, Oct4, Nanog and Bmi-1.[17,18,19]

Regarding salivary gland carcinomas, the role of stem cell markers is not fully investigated.[20]

CD44 is a transmembrane glycoprotein which regulates the expression of certain genes responsible for cell behaviors. It can transduce microenvironmental cellular signals to cell nucleus or cell membrane-associated proteins. Previous studies suggested that CD44 is a cancer stem cell marker and is an important regulator of cancer stemness, which includes the ability of self-renewal, tumor initiation, and tumor metastasis.[21,22,23]

SOX2 is one of the members of the SOX family gene. It is a transcription factor that shares in several embryonic processes as sex determination and plays a functional role in maintaining the embryonic stem cells state. Under certain conditions, SOX2 can reprogram differentiated cells into pluripotent stem cells.[24,25] Moreover, the overexpression of SOX2 is linked to poor differentiation of the tumor cells, lymph node metastasis, and bad prognosis.[26,27]

Studying of cancer stem cell markers in MEC can provide important information about the role of these markers in tumor development and progression. In addition, highlights the targets for future therapy.[28] No previous studies were done to evaluate the expression of stem cell markers in PSSC.

In the current work, the expression of CD44 and SOX2 in low and high- grades MEC and PSSC was investigated using immunohistochemical staining.

The aim is to assess the role of cancer stem cells in these neoplasms and to determine whether the expression of these markers can be used to predict tumor aggressiveness. In addition, the possible correlation between both markers was statistically analyzed.

MATERIALS AND METHODS

A total of sixteen archival paraffin blocks of mucoepidermoid carcinoma (8 low grade and 8 high- grade carcinomas) and sixteen archival paraffin blocks of primary squamous cell carcinoma of salivary glands (8 low grade and 8 high- grade carcinomas) were retrieved   from:

- The pathological files of General Pathology Department, Nasser Institute for Research and Treatment, Ministry of health and population.

- The pathological files of General Pathology Department, Al Hussein Hospital, Al Azhar University.

Section preparation:

Three sections from each formalin-fixed, paraffin-embedded tissue block were cut into 4 µm thickness and stained with the following:

a) First section stained with hematoxylin and eosin (H&E) to confirm the diagnosis of mucoepidermoid carcinoma and primary squamous cell carcinoma of salivary glands.

b) Second section stained with CD44 polyclonal antibody.

c) Third section stained with SOX2 monoclonal antibody.

Immunohistochemical (IHC) staining procedure

The sections were deparaffinized with xylene and rehydrated in graded ethanol for IHC staining with CD44 and SOX2 antibodies. Heat mediated antigen retrieval was done using citrate buffer PH (6.0), then the sections were immersed in hydrogen peroxide (H_2O_2) to block the endogenous peroxidase activity, washed in phosphate-buffered saline (PBS), and then protein blocking reagent was added and incubated for 20 minutes at 37°C within humid chamber to reduce the non-specific staining. The primary antibodies used in the present study were as follows:

- Concentrated polyclonal rabbit antibody for CD44 (Code No. PA5-21419 at dilution 1:100, Thermo Fisher Scientific USA).

- Concentrated monoclonal mouse antibody for SOX2 (Code No. MA1-014 at dilution 1:50, Thermo Fisher Scientific USA).

Sections were incubated with the primary antibody overnight.

The sections were then washed twice in PBS and treated with the labeled streptavidin- biotin complex (LSAB + System-HRP, Dako) at room temperature to bind the primary antibodies. Peroxidase activity was visualized by immersing the tissue sections in diaminobenzidine (Liquid DAB+ Substrate, Dako), which resulted in a brown reaction product. Finally, the sections were counterstained with Mayer's hematoxylin and cover-slipped.

Immunohistochemical Interpretation:

The immunoexpression of CD44 was evaluated by the presence of brown colored immunostaining reaction in the cell membrane of tumor cells.

Presence of brown colored immunostaining reaction in the nucleus of malignant cells was considered a positive reaction for SOX2.

In each slide, 5 microscopic fields showing the highest immunopositivity were selected and photomicrographed using image analyzer.

Immunoreactivity, for CD44 and SOX2 was assessed by estimating the area percentage of positive immunostained cells in relation to the area examined in each microscopic field using computerized image analyzer (Leica Qwin - Germany) at research unit (Faculty of Dentistry Cairo University).

The image analyzer consisted of a colored video camera, colored monitor, and hard disk of *hp* personal computer connected to the microscope and controlled by Leica Qwin 500 software. The image analyzer was calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. The area and area percentage reaction were measured using a magnification x200. Mean values were then obtained for each specimen.

Statistical analysis

Statistical analysis was then performed using a commercially available software program (SPSS 19; SPSS, Chicago, IL, USA).

As data was parametric, significance of the difference between groups was evaluated using oneway analysis of variance (ANOVA).

Pearson correlation test was used to study correlation between different parameters. The Pearson correlation coefficient is used to measure the strength of a linear association between two variables, where the value r = 1 means a perfect positive correlation and the value r = -1 means a perfect negative correlation.

The level of significance was set at P < 0.05.

RESULTS

1-Heamatoxylin and eosin stain findings

Histologically Low-grade MECs are characterized by increased cysts formation, minimal cytological atypia and large concentrations of mucous cells while high-grade MECs show a high proportion of squamous and intermediate cells and increased mitotic activity.

PSSC is characterized histologically by sheets and nests of malignant squamous cells which are well differentiated in low-grade tumors and poorly differentiated in high-grade tumors (figure1,A,B, C,D).

2-Immunohistochemical findings

CD44 immune-reactivity:

All lesions of MEC and PSSC showed immunopositivity for CD44 in the malignant cells especially in the cell membrane (figure 1, E,F,G, H).

SOX2 immune-reactivity:

All lesions of MEC and PSSC showed nuclear immunopositivity for SOX2 in the malignant cells (figure 1, I, J, K, L).

Results of the immunohistochemical staining:

Comparison between mucoepidermoid carcinoma and primary squamous cell carcinoma

Regarding CD44 area percent of immunoexpression, the highest mean value was recorded in MEC high grade, while the lowest value was recorded in MEC low grade, with a statistically significant difference (p<0.0001). Tukey's post hoc test revealed a statistically significant difference between every 2 groups (Table1, Graph 1).

Regarding SOX2 area percent of immunoexpression, a higher mean value was recorded in PSCC high grade, followed by MEC high grade, while the lowest value was recorded in PSCC low grade, with a statistically significant difference (p<0.0001). Tukey's post hoc test revealed no significant difference between MEC high grade and PSCC high grade. Moreover, there was no significant difference between MEC low grade and PSCC low grade (Table1, Graph 1).
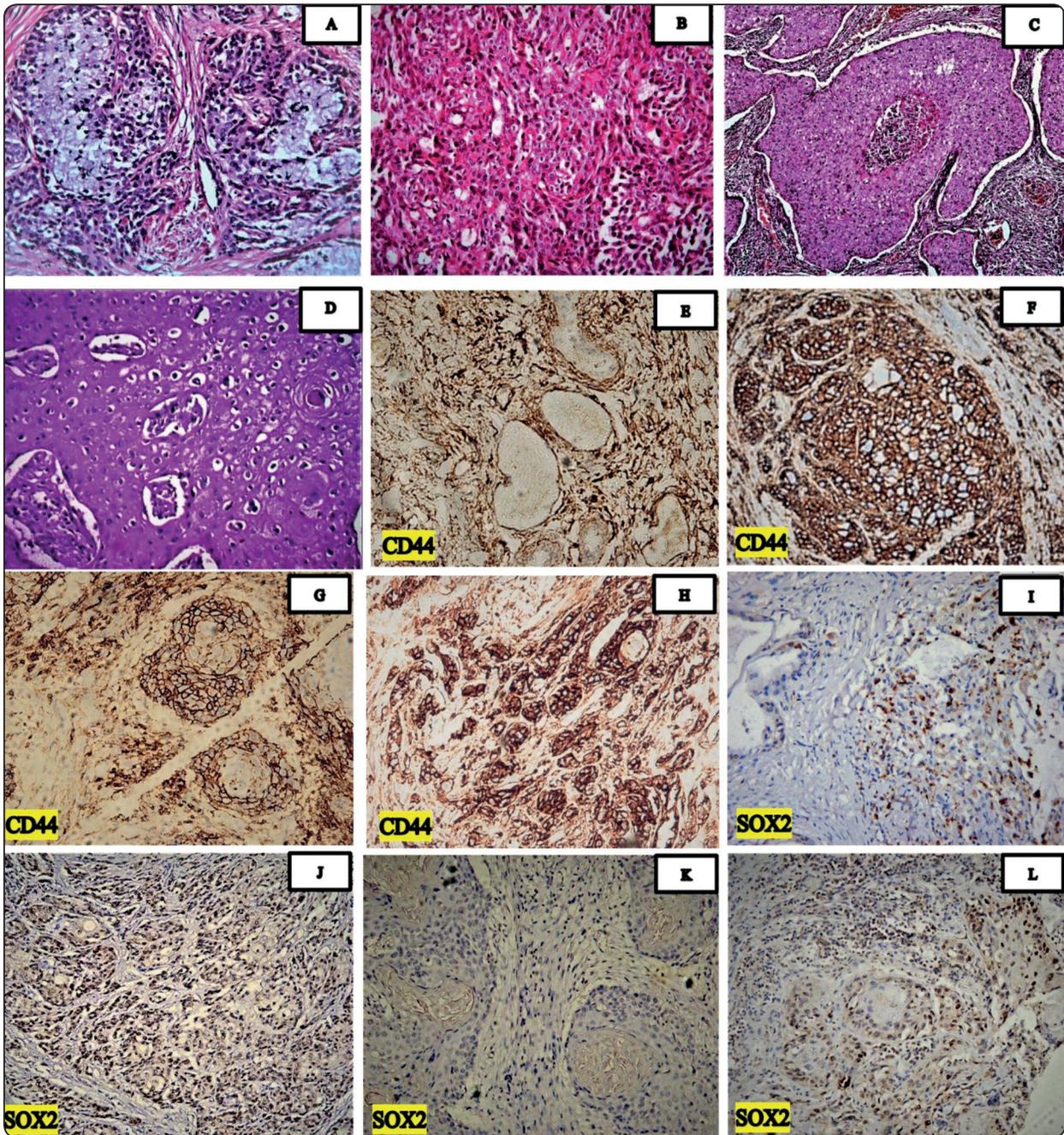
Fig. (1) (A): A photomicrograph of low- grade MEC showing cysts formation, minimal cytological atypia and large concentrations
        of mucous cells (H&E x200).

(B): A photomicrograph of high- grade MEC showing squamous and intermediate cells and increased mitotic activity (H&E x200).

(C): A photomicrograph of low- grade PSSC showing sheets of well differentiated malignant squamous cells (H&E x100).

(D): A photomicrograph of high- grade PSSC showing sheets of poorly differentiated malignant squamous cells (H&E x200).

(E): A photomicrograph of low- grade MEC showing immunopositivity for CD44 in the malignant cells (CD44 x100).

(F): A photomicrograph of high- grade MEC showing immunopositivity for CD44 in the malignant cells (CD44 x100).

(G): A photomicrograph of low- grade PSSC showing immunopositivity for CD44 in the malignant cells (CD44 x100).

(H): A photomicrograph of high- grade PSSC showing immunopositivity for CD44 in the malignant cells (CD44 x100).

(I): A photomicrograph of low- grade MEC showing immunopositivity for SOX2 in the malignant cells (SOX2 x200).

(J): A photomicrograph of high- grade MEC showing immunopositivity for SOX2 in the malignant cells (SOX2 x100).

(K): A photomicrograph of low- grade PSSC showing immunopositivity for SOX2 in the malignant cells (SOX2 x100).

(L): A photomicrograph of high grade PSSC showing immunopositivity for SOX2 in the malignant cells (SOX2 x100).

TABLE (1) Comparison between MEC and PSCC (ANOVA test)

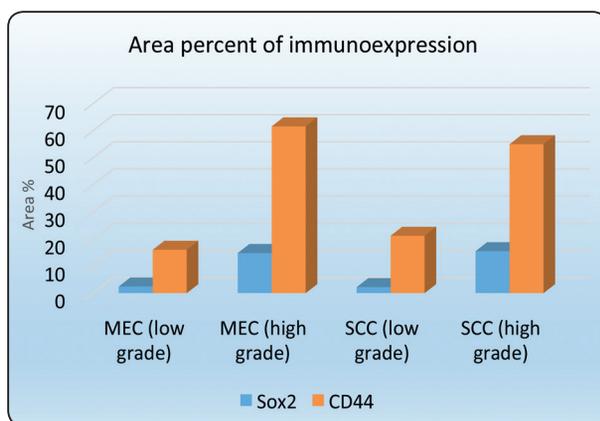|	SOX2 Area percent				CD44 Area percent			
	MEC (low grade)	MEC (high grade)	PSCC (low grade)	PSCC (high grade)	MEC (low grade)	MEC (high grade)	PSCC (low grade)	PSCC (high grade)
Mean	2.449 [b]	14.783[a]	2.205[b]	15.486[a]	15.99[d]	61.523[a]	21.159[c]	54.937[b]
Std Dev	0.749	4.255	0.828	5.344	4.569	5.764	3.228	3.663
Max	3.604	20.918	3.213	21.181	22.694	69.351	24.46	61.225
Min	1.556	8.902	1.319	9.469	11.739	54.658	17.592	52.18
F	45.769				237.08			
P value	$p < 0.0001$*				$p < 0.0001$*			

*Significance level $p < 0.05$, *significant*

Tukey's post hoc test: means sharing the same superscript letter are not significantly different
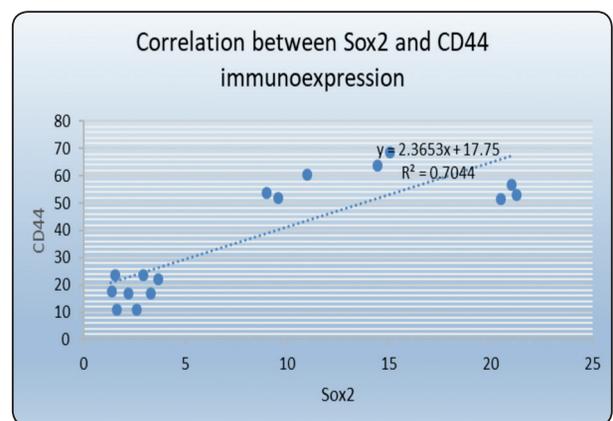
2- Correlation between expression of CD44 and SOX2

Pearson's correlation test revealed a strong positive correlation between SOX2 and CD44 area percent of immunoexpression (Table 2, Graph 2).

TABLE (2) Correlation between SOX2 and CD44 area percent of immunoexpression

R	R^2	Interpretation	P value
0.8393	0.7044	strong positive	0.0024* (significant)

Graph (1) Column chart showing mean SOX2 and CD44 area percent of immunoexpression in Mucoepidermoid carcinoma and Squamous cell carcinoma

Graph (2) Scatter plot showing correlation between SOX2 and CD44 area percent of immunoexpression

DISCUSSION

Salivary gland malignancies often show distant metastases several years after initial diagnosis, suggesting a subpopulation of cancer stem cells (CSCs) responsible for the late recurrences; however, the available models to characterize these rare cells are still limited.[29]

In general, in salivary gland tumors; the hypothesis of cancer stem cell has not been fully examined.[30]

Studies on other types of cancer revealed that cancer stem cells play a functional role in therapy resistance and tumor recurrence.[31,32,33,34]

Some studies indicated that cancer stem cells may play an important role in the pathobiology and prognosis of MEC of the salivary gland.[34,35] No previous studies had been done to investigate the role of CSCs in PSSC of SGs. However, the role of cancer stem cells in head and neck squamous cell carcinoma (HNSCC) was established and may yield new insights into the evolution of new therapeutic modalities for HNSCC.[16]

So, the present study aims to investigate the role of cancer stem cells in MEC and PSSC of SGs which may affect their progression. The expression of stem cell markers CD44 and SOX2 in low and high-grades MEC and PSSC were evaluated and correlated with histopathological parameters. In addition, the correlation between these two markers was detected to evaluate the possible impact of the combination of these two markers on the prognosis of MEC and PSSC.

The resistance of mucoepidermoid carcinomas to every therapeutic modality compounded with its relentless growth is a major clinical obstacle which may be related to cancer stem cells function.[35] A similar challenge is present for PSSC which is considered as an aggressive neoplasm with an unpredictable response to different therapeutic modalities. Despite adequate therapy, the 5-year survival rate of PSSC remains nearly 25–30%. However, further studies are needed to understand the pathobiology of PSSC.[36,37,38]

CD44 is an adhesion molecule which is expressed in normal and tumor tissues.[4] It is considered as a CSC marker which affords high malignant properties and therapeutic resistance.[39] An important function of CD44 in cancer is the protection of malignant cells from apoptosis through regulation of anti-apoptotic or per-apoptotic processes.[40,41]

Overexpressed CD44 was found to be significantly correlated with worse prognosis and malignant clinicopathological features in many types of cancers.[42]

In the current work, immunohistochemical analysis showed expression of CD44 in all cases of MEC and PSSC mainly in the cellular membrane.

Regarding CD44 area percent of immunoexpression, the highest mean value was recorded in MEC high grade followed by PSSC high grade, while the lowest value was recorded in MEC low grade, with a statistically significant difference.

Similarly, previous studies supported the results of the current work and demonstrated expression of CD44 in MEC with intense expression in solid histological type. This expression was higher in the invasive front of the tumor than in the central parts which indicates that CD44 may have a role in MEC progression.[4,28,35,43,44]

Previously, Adams et al.[35] and Warner et al.[45] reported that mucoepidermoid carcinoma comprised a small cancer stem cells population with increased carcinogenic potential. These cancer stem cells strongly expressed CD44. Their results indicated that therapies that destroy these carcinogenic cells could be of great benefit to MEC patients.

No available data for previous studies concerning the expression of CD44 in PSSC of SG but previous studies were done on HNSCC and are consistent with the present work.

A study by Joshua et al.[42] on HNSCC revealed expression of CD44 in all the tested samples. Furthermore, CD44 was found to play an essential role in resistance to chemotherapy and radiotherapy and may be involved in recurrence of head and neck squamous cell carcinomas.[46] In addition, its expression is positively correlated with metastasis, advanced T stage, perineural invasion, and shorter disease-free survival.[47,48]

SOX2 is a cancer stem cell marker that has an important role in embryonic development.[49] Furthermore, it has been linked to carcinogenesis as it can reprogram the differentiated somatic cells into a state similar to pluripotent stem cells.[35,50,51] This stemness may be responsible for the development and progression of tumors as it allows the ability to differentiation and self-renewal.[52]

So, previous studies suggested an association between SOX2 and tumorigenesis and correlate its expression with aggressive features in different types of malignancies.[53-55]

Regarding salivary gland cancer, the assessment of the expression of SOX2 and other stem cell markers needs further investigations.[20]

In the present study, nuclear expression of SOX2 was detected in all samples of MEC and PSSC. SOX2 area percent of immunoexpression, revealed a higher mean value in PSCC high grade, followed by MEC high grade, while the lowest value was recorded in PSCC low grade, with a statistically significant difference between high grade and low-grade samples of both tumors. On the other hand, no statistically significant difference between SOX2 expression in MEC and PSSC.

The study of Xu et al.[30] is the only available study concerning SOX2 expression in MEC. They concluded that a high prognostic achievement for palatal MEC could be established through a combination of CD44, CD133 and SOX2 markers.

Findings similar to the present work were observed in adenoid cystic carcinoma (ACC).

In the study of Dai et al.[56] SOX2 expression was associated with ACC development. They found a significant correlation between advanced T stage, distant metastasis and SOX2 expression advocating a clinical relevance of SOX2 expression to the progression of ACC. In addition, increased expression of SOX2 might be considered as one of the most essential prognostic factors and may represent a target for therapy in ACC.

In carcinoma ex- pleomorphic adenoma (CXPA) SOX2 seems to have a certain role in cancer development and maintenance of the infiltrative growth pattern most probably by adding CSC features to the neoplastic cells. Adding to this, increased SOX2 expression was correlated with increased mitotic activity, high histological grade and poor prognosis in this neoplasm.[20]

Previous studies on various types of tumors as lung cancer[57], hepatocellular carcinoma[58], gastric cancer[59], colorectal cancer[53], and laryngeal squamous cell carcinoma[26] demonstrated a correlation between the level of SOX2 expression and the progression and poor prognosis of tumors. Moreover, in breast cancer, SOX2 appears to be involved in chemotherapeutic resistance.[60,61]

In an experimental study, overexpression of SOX2 was reported to facilitate pancreatic cancer cells invasion and metastasis by promoting epithelial-mesenchymal transition.[62]

No available data for previous studies concerning the expression of SOX2 in PSSC of SG. Though overexpression of SOX2 protein may have an important role in initiation and progression in other types of squamous cell carcinomas.[63]

The above data was in accordance with the present results of SOX2 expression in PSSC of SG. So, the present results suggested that the level of SOX2 expression is correlated with MEC and PSSC progression and hence a poor prognosis.

Additionally, the current work detected a strong positive correlation between SOX2 and CD44 area

percent of immunoexpression. In line, the study of Xu et al.[30] revealed that the combination of CD44, CD133 and SOX2 is a powerful prognostic indicator for MEC patients.

Likewise, previous studies demonstrated a relation between SOX2 and CD44 expression. They identified a subpopulation of cancer stem cells that overexpress the two markers in HNSSC and concluded that SOX2 and CD44 are essential for the maintenance of the CSCs quiescence and self-renewal.[64,65]

Though, the results of the current work suggest that SOX2 and CD44 expression may be a useful indicator for the prognosis of MEC and PSCC of the SGs.

Collectively, in this work, the expression of SOX2 and CD44 was linked to the histopathological prognostic parameters in MEC and PSSC. Adding to this, the combination of these two markers could serve as an effective prognostic indicator.

CONCLUSION

Salivary gland MEC and PSSC exhibit a subpopulation of cancer stem cells. These cells can be detected by SOX2 and CD44 expression and may play an important role in tumor aggressiveness. These results also suggest that MEC and PSSC patients may get benefits from the therapeutic targeting of this cancer stem cells using a combination of the two markers. Nevertheless, the significance of these markers in the diagnosis and prognosis of MEC and PSSC requires further investigation.

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