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CD44 expression as a potential favorable marker for prognosis in mucoepidermoid carcinoma of salivary gland

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ABSTRACT

Background: Cluster of differentiation 44 (CD44) is one of the markers of cancer stem cells belonging to the cell adhesion molecule family, which may play a role in tumorigenesis of mucoepidermoid carcinoma (MEC) of the salivary gland. The histological grade is one of the most important factors for prognosis. This study was aimed to investigate the association between CD44 expressions with histological grade in MEC of the salivary gland.

Methods: In A cross-sectional study, 34 cases diagnosed with MEC in the Department of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia, Dr. Cipto Mangunkusumo Hospital, Jakarta, Indonesia from 2012 to 2017 were included. Histological grade was classified into low-, moderate-, and high-grade. CD44 expression was done by immunohistochemistry staining and classified into weak and strong expressions.

Results: Eighteen cases (52.9%) were high-grade, 3 cases (8.8%) were moderate, and 13 cases (38.2%) were low-grade. Perineural invasion was found in 7 cases (20.6%) and mostly in high-grade tumors. CD44 expression was significantly associated with histological grade ($p = 0.002$, OR=0.09, 95%CI=0.02–0.48). Strong CD44 expression was found more in low-grade MEC.

Conclusions: This study suggests that strong CD44 expression may act as a potential favorable predictive factor for prognosis in the MEC of the salivary gland.

Keywords: cancer stem cell, CD44 antigen, mucoepidermoid carcinoma.
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INTRODUCTION

Mucoepidermoid carcinoma (MEC) is the most common salivary gland malignancy which can be found in children and adults and is estimated to be about 34% of salivary gland malignancies. Tumorigenesis of MEC still remains unclear. Histologically, mucoepidermoid carcinoma is comprised of epidermoid cells, mucus cells and intermediate cells. Retrospective studies consistently showed a correlation between recurrence and metastatic behavior with histological grade, which confirmed that the histological grade of MEC is a strong predictor for prognosis and the main factor for management decision.[1-6] Cancer stem cell (CSC) was thought to have a pivotal role in various pathogenesis of malignancy. CSC are multipotent cells that can differentiate and self-renewal in the process of tumorigenesis, metastasis, and resistance in therapy or recurrence of the tumor.[7-9] CD44 is the most common stem cell (SC) marker in the salivary gland.[10] CD44 expression increased the potential of initiation and progression of the tumor.[11] CD44 binds with HA and extracellular matrix (ECM) molecule, which regulates CSC ability to self-renewal, maintenance, colonization in metastasis, and therefore resistance to chemotherapy. CD44 expressions in MEC showed increment compared to the normal salivary gland.[5] In MEC, the mean area fraction of CD44-positive cells was higher in high-grade than low-grade.[12] Association histological grade may indicate a role in pathogenesis with CSC.[12] Despite most research showing that CD44 supports tumor progression, there were also several studies that revealed the opposite result. Several studies showed CD44 expressions as a better prognostic indicator, such as in epithelial ovarian cancer and breast cancer.[13,14] In breast tumors, CD44 showed more positivity in breast papilloma than papillary carcinoma.[15] Until now, the role of CD44 in tumorigenesis is still inconclusive. CD44 perhaps could act as a biomarker and promising therapeutic target in the future. This study was aimed to investigate the CD44 expression and analyze it with histological grade in MEC of the salivary gland.

METHODS

This cross-sectional study was conducted on MEC of salivary gland histopathologically samples. The samples were obtained with consecutive sampling methods, from the year 2012 to 2017 archives of the Department of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia/Dr. Cipto Mangunkusumo Hospital, Jakarta, Indonesia has adequate block paraffin included in this study. Cases with double primer tumors were

excluded. Histopathological findings were determined by three independent-blinded reviewers to classify as a low-, moderate-, and high–grade tumor based on the Armed Forces Institute of Pathology (AFIP) grading system.[16] Subject's characteristics include age, gender, and tumor location were documented. The Ethics Committee approved this study of the Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia, with number 0429/UN2.F1/ETIK/2018.

Immunohistochemistry CD44

Paraffin blocks were sectioned 4µm thick, placed on poly-L-lysine layered object-glass, and heated on 55–58^{0}C hotplate. Slides were deparaffinized with xylol and rehydrated with alcohol. Antigen retrieval using 0.1M NaOH citrate buffer (pH 7.0) and heat-induced by 121^{0}C autoclave. The slides were then washed with phosphate buffer saline (PBS) at pH 7.4. Block endogenous peroxidase using hydrogen peroxide in 3% methanol at room temperature and washed in running water. Non-specific protein blocking using Novolink Protein Block (Novocastra™ RE7102, USA) in this study. The slides were incubated with primary antibody CD44 (ThermoFisher, clone 156-3C11, dilution 1:200), washed with PBS, and incubated with biotinylated secondary antibody (Novolink, Novocastra RE7111, USA). The slides were washed with PBS, then incubated with 3,3'-diaminobenzidine, washed with deionized water and running water. Counterstaining with Hematoxylin Lillie's Mayer then washed. The slides were soaked in lithium carbonate then washed. Dehydrated with alcohol and clearing with xylol, then mounted. Positive control was using tonsil tissue, and negative control was obtained by skipping primary antibody incubation.

Immunohistochemistry CD44 analysis

CD44 expression is positive in the cell membrane. The expression of CD44 was analyzed using a histoscore (H-score) system. For H-score assessment, representative fields were captured at random at x 400 magnification using Leica DM500 integrated with Leica Microsystem ICC50 W Microscope Camera. The staining intensity in the malignant cell membrane was scored as 0, +1, +2, or +3 corresponding to the presence of negative, weak, moderate, and strong brown staining, respectively, based on Wolff et al.[17] The total number of cells stained at each intensity were counted until it reached 500 cells in total. ImageJ counted the quantity and quality of CD44 expression. The average positive rate was calculated, and the following formula was applied:

H-score: [1x(% cells +1) + 2x(% cells +2) + 3x(% cells +3)]

H-score between 0 and 300 were obtained and calculated into percentage. The receiver operating characteristic (ROC) curve was used to classify H-score results into weak expression and strong expression.

Statistical analysis

Statistical analyses were using SPSS version 24.0 (IBM Corp., USA). The association of categorical variables was evaluated with the Chi-Square test and Fisher's exact test. The mean difference was evaluated by a T-test. A p-value of <0.05 was considered significant.

RESULTS

There were 34 cases that meet inclusion criteria as shown in Table 1.

Histopathologic characteristic and CD44 expression

Eighteen cases (52.9%) were classified as high-grade, 3 cases (8.8%) were moderate, and 13 cases (38.2%) were low-grade. Perineural invasion was found in 7 cases (206%). Most cases with perineural invasion were found in a high-grade tumor. Necrosis was found in 7 cases (20.6%). Anaplasia was found in 24 cases (70.6%). Lymphovascular invasion was found in 2 cases (5.8%) and both cases in high-grade tumors.

CD44 expression in the normal salivary gland was strong in serous cells, negative in mucus cells, and weak-to-moderate in several ductal cells (Figure 1). CD44 expression in MEC showed positivity with variable intensity (Figure 2).

CD44 expression and histological grade of MEC

Based on histological grade, CD44 expression in low-grade showed 79.32 (±25.97), moderate-grade was 79.96

Table 1. Demographic and clinicopathological findings.

Variable	Category	Frequency, n(%)
Age, mean (SD)		42.76 (15.52)
Gender	Male	18 (52.9)
	Female	16 (47.1)
Tumor location	Parotid	20 (58.8)
	Submandibular	2 (5.9)
	Sublingual	1 (2.9)
	Minor salivary gland	11 (32.4)
Histological grade	Low	13 (38.2)
	Moderate	3 (8.8)
	High	18 (52.9)
Cystic component (%)	≥20	13 (38.2)
	<20	21 (61.8)
Perineural invasion	Yes	7 (20.6)
	No	27 (79.4)
Necrosis	Yes	7 (20.6)
	No	27 (79.4)
Mitosis	<4/10 HPF	19 (55.9)
	≥4/10 HPF	15 (44.1)
Anaplasia	Yes	24 (70.6)
	No	10 (29.4)
Lymphovascular invasion	Yes	2 (5.8)
	No	32 (94.2)

SD=standard deviation; HPF=high-power fields

(±25.58) and high-grade was 58.56 (±24.01) (Table 2). In comparison analysis, showed lower expression in moderate/high-grade tumors than low-grade tumors even though no significant mean differences (Table 3 and Figure 3).

ROC curve was performed to make a cut-off of CD44 expression. From the analysis, AUC was 0.744 and cut-off was 76.7% with a sensitivity of 76.9% and specificity of 76.2% (Figure 4). Fifteen cases (41.1%) showed strong CD44 expression and 19 cases (55.9%) showed weak CD44 expression. Low-grade tumors showed stronger CD44 expression than high-grade (66.7% versus 23.80%). CD44 expression was found to be associated with histological grade significantly (p=0.002, OR=0.094, 95%CI=0.018–0.481) (Table 4). Necrosis and mitosis were associated with CD44 expression. There were only 2 cases of lymphovascular invasion.

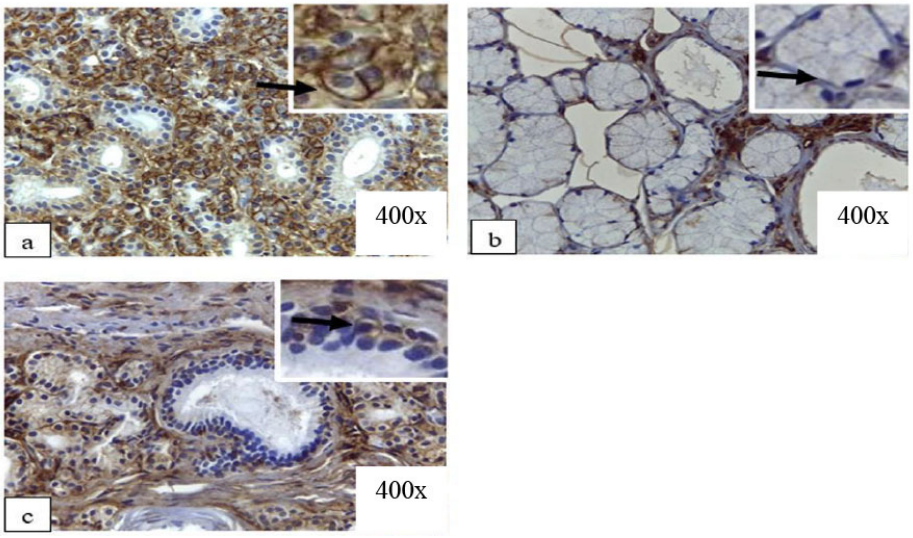

Figure 1. CD44 expression in normal salivary gland (arrow, 400X). (a) The strong intensity in serous cells; (b) negative in mucus cells; (c) weak to moderate intensity in several ductal cells. CD44 = cluster of differentiation 44.

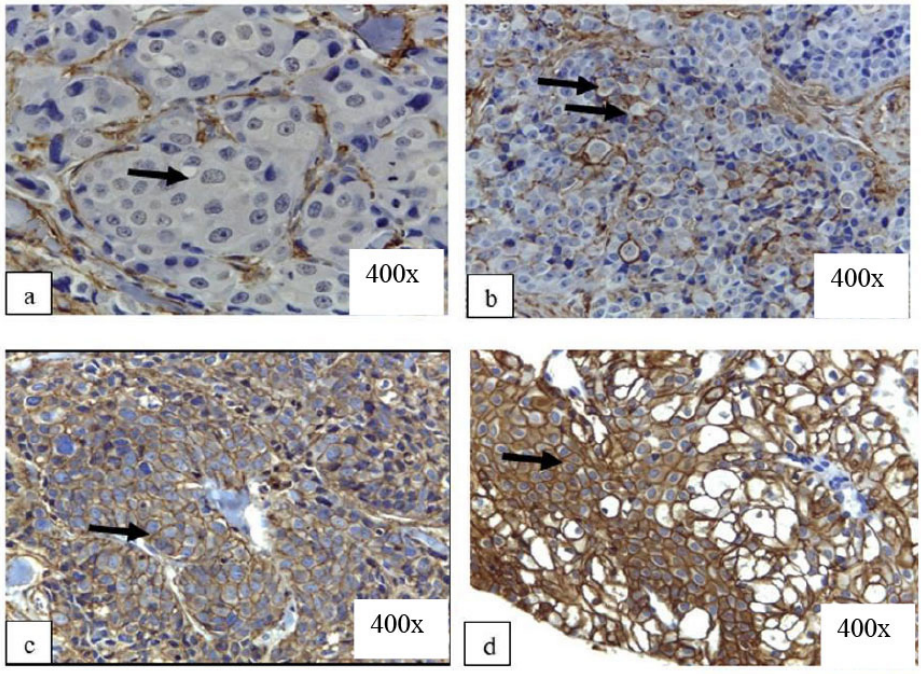

Figure 2. CD44 expression in MEC (arrow, 400X). (a) Negative; (b) weak intensity; (c) moderate intensity; (d) strong intensity. CD44=cluster of differentiation 44; MEC=mucoepidermoid carcinoma

DISCUSSION

High-grade MEC have more aggressive behavior compared to low-grade.[18] Our study showed perineural and lymphovascular invasion mostly found in high-grade MEC which correspond to Ali et al. and McHugh et al.[2,4] Perineural and lymphovascular invasion are associated significantly with high-grade tumors and have poor survival rate due to increased recurrence rate locally, regionally, and distant metastasis, thus the presence should always be documented and for which the treatment should be more aggressive. Nevertheless, the pathogenesis of perineural spread remains poorly understood.

Even though various treatment modality has been developed, the quality of life and survival in salivary gland malignancy remain low, especially in an advanced stage. It shows that CSC may play a pivotal role in pathogenesis and tumor progression. However, the role of CSC in the salivary gland is not widely known, particularly MEC.[19] MEC has been believed to arise from pluripotent reserve cells in the excretory duct of the salivary gland. Those cells have the ability to differentiate into the squamous cells, columnar cells, and mucus cells, which was concord with MEC that consist of epidermoid cells, intermediate cells, and mucus cells. Several studies showed that CSC are resistant to chemotherapy and radiation.[7-9] It was believed that the CSC will be in the phase of G0 or quiescent as a response to stimulus, so the cells resistant to chemotherapy agents that work depend on the cell cycle or if the therapeutic target is proliferative cells.[16]

CD44 is one of the CSC markers and is an adhesion molecule. Yet, the prognostic role of CD44 still remains controversial. Recent research showed that CD44 has participated in oncogenesis signals that promote tumor progression. In breast cancer, CD44 expression is associated with distant metastasis and reduced survival.[20,21] In head and neck malignancy, increased CD44 expression correlates with aggressiveness and poor prognosis.[22] CD44 expression changes when the

Table 2. **CD44 expression characteristic based on histological grade.**

	Category	CD44 Expression (H-score)	
		Mean ± SD	Min / Max
Histological grade	Low	79.32 ± 25.97	3.00 / 99.53
	Moderate	79.96 ± 25.58	50.47 / 96.20
	High	58.56 ± 24.01	0 / 98.93

Table 3. **CD44 expression mean comparison.**

	Category	Mean ± SD	Min / Max	*p*
Histological grade	Low	79.32 ± 25.97	3.00 / 99.53	0.05*
	Moderate/High	61.61 ± 24.79	0 / 98.93	

*T-test

tumor develops which caused alteration in cell adhesion with ECM. The function of CD44 molecular structure is as the receptor for HA and other components of ECM, thus CSC can detect environment changes and mediate signal transduction to regulate stem behavior. CD44 binds with HA and ECM, such as osteopontin which is a component of metastatic niche, then activated Nanog-signal transducer and activator of transcription 3, OCT4-Sox2-Nanog, or c-Src kinase signaling tract to upregulate microRNA (miR)-21 or downregulate miR-203. That mechanism regulates CSC's ability to self-renewal, maintenance and therefore resistance to chemotherapy. These mechanisms show that CD44 is really important for cancer cells to adapt to the new environment and is needed for colonization in metastasis.[11] However, other studies showed that decreased CD44 expression is associated with poor prognosis in melanoma, prostate cancer, and colorectal cancer.[23-25]

Our study showed that there was a difference in CD44 expression in ductal cells of normal salivary gland compared to tumor cells, though in this study it cannot be analyzed statistically. In tumor cells, CD44 expression showed stronger expression more in low-grade MEC. On the contrary, high-grade MEC showed mostly weak expression. Statistically, this CD44 expression is associated significantly with histological grading. These differences in expression compared to other studies may support the hypothesis that there is a dual function in CD44 which leads to inconsistent results in many studies. CD44 can activate and inhibit oncogenic signals.[25] Pro- or anti-tumorigenesis signal depends on the signal from stromal cells.

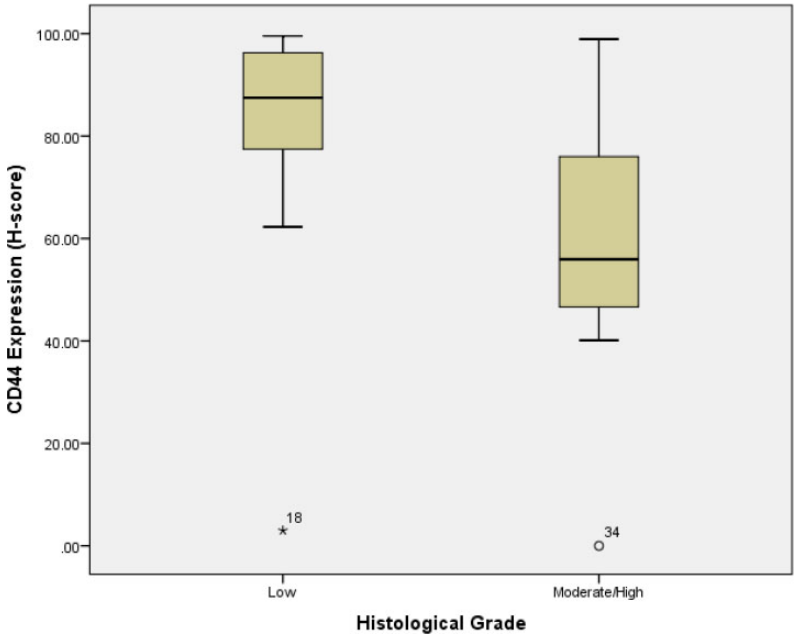

Figure 3. Box plot showed the difference of CD44 expression between low and moderate/high-grade groups.

However, the extra- and intra-cellular mechanism still needs further study.

Various studies showed CD44 can respond differently when bound with HA.[27] HA is divided into high molecular weight (HMW)-HA and low molecular weight (LMW)-HA. In normal tissue, HA presents as HMW-HA and functions as an anti-angiogenic and anti-inflammatory.[28] When CD44 binds with HMW-HA, growth and invasion of tumor cells are inhibited. CD44 binds with Merlin to inhibit Ras activation which later inhibits invasion, growth, and motility of cancer cells. In addition, this binding suppresses the activation of EGFR, while LMW-HA has the function to enhance the proliferation and migration of endothelial cells by pro-angiogenic capabilities. CD44 binds with LMW-HA will have an opposite response by activating signaling pathways to promote cell migration and invasion.[26] The mechanism of this binding in MEC is still not clearly understood.

The limitation of this study is prognostic analysis could not be done due to lack of clinical data and number of the cases. To evaluate further the role of CSC in MEC requires a combination with other CSC markers, which is not evaluated in this study.

CONCLUSION

Our study showed CD44 expression is associated with histological grade and high-grade tumors tend to have lower

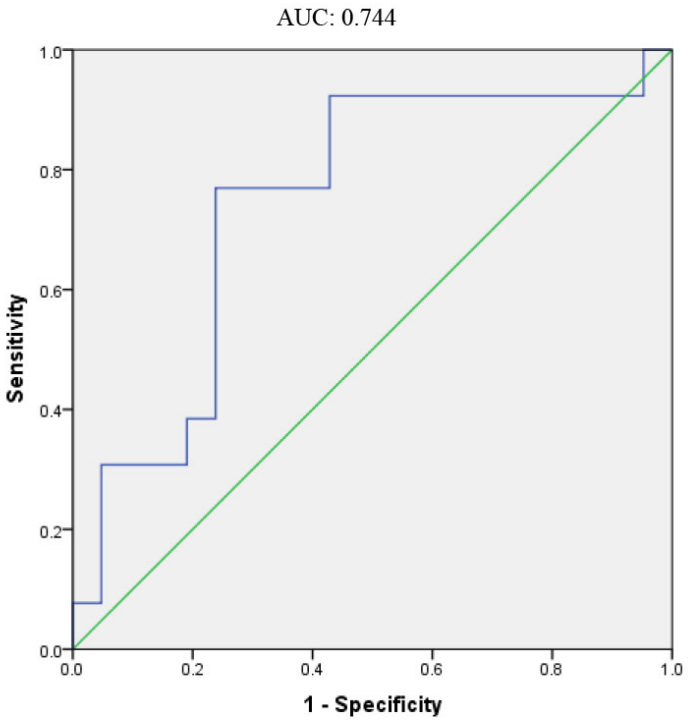

Figure 4. Receiver operating characteristics (ROC) curve for CD44 expression. AUC=area under the curve.

CD44 expression. Overexpression of CD44 presumably does not always a bad prognostic factor in the MEC of the salivary gland. Nevertheless, the prognostic value of CD44 expression and environment cues that affect CD44 responses is still an interesting matter for further investigation.

CONFLICT OF INTEREST

The authors declare that there is no competing interest regarding the manuscript.

ETHICAL CLEARANCE

This study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia with number 0429/UN2.F1/ETIK/2018.

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Table 4. CD44 expression and clinicopathological findings.

Variable	CD44 expression		*p*	OR	95%CI
	-/+	++			
Gender					
Male	12	6	0.18*	2.57	0.64 – 10.34
Female	7	9			
Tumor location					
Parotis	12	8	0.99†	N/A	N/A
Submandibula	1	1			
Sublingual	1	0			
Minor	5	6			
Histological grade					
Low	3	10	0.002*	0.094	0.018 – 0.48
Moderate/High	16	5			
Cystic component (%)					
≥20	5	8	0.11*	0.31	0.074 – 1.32
<20	14	7			
Perineural invasion					
Yes	5	2	0.43‡	0.43	0.071 – 2.62
No	14	13			
Necrosis					
Yes	7	0	0.01‡	0.44	0.29 – 0.68
No	12	15			
Mitosis					
<4/10 HPF	6	13	0.00*	0.07	0.01 – 0.42
≥4/10 HPF	13	2			
Anaplasia					
Yes	15	9	0.28‡	0.40	0.09 – 1.81
No	4	6			

*Chi-Square test; ‡Fisher exact test

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AUTHORS CONTRIBUTION

S.M, L.R, D.R.H conceived and performed experiments, wrote the manuscript. KK participated in the design and analysis of the study. All authors read and approved the final manuscript.

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