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CD44 POSITIVE CANCER STEM CELLS EXPRESS INVASION AND METASTASIS MARKERS: ANALYSIS OF THREE TONGUE SQUAMOUS CELL CARCINOMA CELL LINES

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ABSTRACT

Objectives: This study aimed to analyze the expression of epithelial-mesenchymal transition (EMT) markers, E-cadherin, and alpha-smooth muscle actin (α-SMA) in CD44+ cancer stem cells (CSCs) compared to parental cells to shed light on the possible role of CSCs in the invasion and metastasis of tongue squamous cell carcinoma (TSCC).

Materials and Methods: Three tongue squamous cell carcinoma cell lines were used in this study. Cells were cultured for cancer stem cell screening and isolation. Tumor cells underwent CD44-based fluorescence-activated cell sorting analysis. Subsequently the expression of E-cadherin and α-SMA in CSCs sub-population and parental cells was evaluated using quantitative real-time PCR.

Results: The three cell lines displayed stable CD44 expression levels. Moreover, E-cadherin recorded a significantly higher value in parental cells compared to CSCs sub-population, whereas α-SMA recorded a significantly lower value in parental cells compared to CSCs sub-population. A very strong negative correlation was noted between E-cadherin and each of α-SMA and CD44, whereas a very strong positive correlation was noted between α-SMA and CD44.

Conclusion: The findings of our study indicated that TSSC harbors a sub-population of CD44+ CSCs that exhibited enhanced EMT features compared to other cell populations. Our data provide new evidence in support of a potential link between α-SMA expression and CSC features which may have relevant implications for the development of cancer stem cell-based treatment options.

KEYWORDS: Tongue squamous cell carcinoma; Cancer stem cells; CD44; Epithelial-mesenchymal transition markers.

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INTRODUCTION

The most common malignancy affecting the oral and oropharyngeal mucosa is squamous cell carcinoma with tongue squamous cell carcinoma (TSCC) being the most aggressive form of oral cancer (Karatas et al., 2018; Shimura et al., 2019). Oral squamous cell carcinoma is typically treated with surgery, radiation therapy, and chemotherapeutic medications (Fukumoto et al., 2021). However, metastasis remains the prime cause of cancer-associated mortality (Jinesh et al., 2017).

Previous research reveals cellular heterogeneity in malignancies, in which populations of cancer cells consist of differentiated malignant cells and malignant cells with stem-like features, which are termed cancer stem cells (CSCs) (Liu et al., 2015). It is proposed that CSCs contribute significantly to tumor progression, recurrence, metastasis, and chemo-radio-resistance while the influence of other cancer cells appears to be less significant. CSCs are capable of self-renewal and differentiation within a tumor and may derive from the long-term or transitory amplification of normal stem cells (Fukumoto et al., 2022). Their formation may be linked to changes in intracellular signal transduction (Pozzi et al., 2015).

In terms of stem markers, CD44 has been listed as one of the crucial biomarkers for the isolation and identification of CSCs. It is a transmembrane receptor for hyaluronic acid. Extensive studies have revealed that CD44 plays an important role in the epithelial-mesenchymal transition (EMT). CD44 induces EMT in several types of cancer by downregulating epithelial markers and upregulating mesenchymal markers (Xu et al., 2015). And it has been found to be dysregulated in numerous malignancies including TSCC (Yanamoto et al., 2014; Wu et al., 2017).

In EMT cells lose their epithelial characteristics, such as cell adhesion, and acquire mesenchymal characteristics, such as cell motility (Tamura et al., 2018). However, EMT plays a role in the stemness acquisition of epithelial tumor cells, providing them with aggressive features and an invasive phenotype that leads to tumor metastasis and recurrence (Li et al., 2014).

Previous studies have revealed that CD44-targeted knockdown can attenuate cancer progression. This suggests that CD44 could be a promising target for cancer treatment (Xu et al., 2015). Furthermore, E-cadherin is an epithelial marker that plays a role in the downregulation of CD44 expression, its suppression increased CD44 protein level.

Meanwhile, EMT is linked to several transcription factors such as Snail and Slug, that suppress epithelial binding mediators including E-cadherin. E-cadherin is therefore regarded as the essential protein of EMT, and its reduced expression results in the disruption of cell-cell junctions (Wang & Zhou, 2013).

On the other hand, upregulation of the mesenchymal intermediate filament protein alpha-smooth muscle actin (α-SMA) is also a common occurrence during EMT, leading to the disruption of epithelial integrity (Liang et al., 2018). Cancer progression and EMT have both been associated with alterations in this gene expression (El-Kammar et al., 2019). There has been limited research on the expression of α-SMA in CSCs. Previous studies on ovarian and prostate cancer have indicated its possible role in CSC biology (Anggorowati et al., 2017; Masola et al., 2022).

In the present study, fluorescence-activated cell sorting was used to collect CD44-enriched cell populations. The unsorted total malignant cell lines were used as the parental cell population. To our knowledge, there is no prior data regarding α-SMA expression in oral CSCs. In addition, no prior research has compared E-cadherin and α-SMA expression in CSCs and parental cells. Thus, we aimed to analyze the expression of EMT markers, E-cadherin and α-SMA in CD44+ CSCs compared to parental cells to shed light on the possible role

of CSCs in invasion and metastasis of TSCC. We selected TSCC cell lines for analysis in this study as it is the most aggressive form of oral cancer (Karatas et al., 2018; Shimura et al., 2019) which requires novel diagnostic and prognostic markers for early detection.

MATERIALS AND METHODS

This work was conducted in 2023 at Ahram Canadian University, Egypt with the approval of our faculty's ethics committee (IRB00012891#58). It was performed in line with the principles of the Helsinki Declaration.

Oral Cancer Cell Lines Culture

We used in this study three human commonly utilized TSSC cell lines, (SCC-15, SCC-25, and HSC-3). They were received from the American Type Culture Collection (ATCC®CRL-1628, USA). The used cell lines were from the same origin to validate and confirm our findings. Cells were cultured in a monolayer in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (PS) at 37 °C with 5% CO2 atmosphere. The medium was refreshed every two days.

Fluorescence-activated cell sorting (FACS) for CD44 analysis

For CSCs isolation, CD44 pluriBead® anti-human suspension was used for the isolation of CD44+ CSCs from the cultured human OSCC cell lines according to the manual instructions provided. It was purchased from the cell separation Technology Company (PluriSelect, Germany). Briefly, pluriBead® uses non-magnetic monodispersed microparticles (beads). The cell surface was coated with monoclonal antibodies directed against specific antigens on the cell surface. During incubation, the target cells in suspension were bonded to the pluriBeads® and separated afterwards by a pluriStrainer® from the suspension. The cells were stained with a fluorescently labeled antibody specific to CD44 (CD44 PE antibody), and flow cytometry software was used to quantify the percentage of CD44+ cells in in each sample (FACS- sorted CD44+ cells). then CD44-enriched cell populations (CD44+) were collected. Further investigations were conducted to ensure that the CD44+ cells that were obtained had distinct stem-like features (CSCs sub-population). Unsorted total SCC-15, SCC-25, and HSC-3 cells were used as the parental cell population.

CD44+ cells culture

CD44+ cells were propagated in RPMI-1640 medium (R0883 Sigma-Aldrich) containing 1% PS and 10% bovine calf serum (Gibco BRL) at 37 °C with 5% CO2 atmosphere. The medium was changed every two days. Clone spheres of CD44+ cells were assessed by crystalline violet stains.

RNA quantification by Real time PCR

To elucidate the CSC-like features of CD44+ cells, PCR was performed to evaluate the expression of α-SMA and E-cadherin in CSCs sub-population and parental cells.

Total RNA was isolated utilizing RNA easy Mini Kit (Qiagen) and was analyzed for quality and quantity via a Beckman dual spectrophotometer (USA). To quantitatively measure the expression levels of mRNA α-SMA and E-cadherin, we utilized a High-capacity cDNA Reverse Transcriptase kit (Applied Biosystem, USA). The cDNA was subjected to amplification using the TaqMan PCR Master Kit (Fermentas) in a 48-well plate, utilizing the Step One apparatus (Applied Biosystem, USA), following the prescribed protocol. Normalization of target gene expression was performed by comparing it to the mean critical threshold (CT) values of the housekeeping gene β-actin.

The primer sequences of target genes were E-cadherin, forward primer: 5′-CGAGAGCTACACGTTCACGG-3′, reverse

primer: 5′ GGGTGTCGAGGGAAAAATAGG-3′, α-SMA, forward primer: 5′-ACTGAGCGTGGCTATTCCTCCGTT-3′, reverse primer: 5′ GCAGTGGCCATCTCATTTTCA-3′ and β-actin, forward primer: 5′-ACCGAGCGTGGCTACAGCTTCACC-3′,reverse primer: 5′AGCACCCGTGGCCATCTCTTTCTCG-3′

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 20 was employed for data management and statistical analysis. Mean and standard deviation were utilized to summarize numerical data. Normality was assessed by examining the data distribution and utilizing the Kolmogorov-Smirnov and Shapiro-Wilk tests. Using an independent t-test, we compared groups regarding normally distributed numeric variables.

The Pearson correlation test was used to evaluate the linear correlation between two variables. The value of the correlation strength ranges from -1 to 1, where -1 represents a total negative linear correlation, 0 represents no correlation, and +1 represents a total positive correlation. The strength of the correlation is interpreted as follows: The absolute value of r: .80-1.0 "very strong"; .60-.79 "strong"; .40-.59 "moderate"; .20-.39 "weak"; and .00-.19 "very weak". Significance was attributed to p-values that were less than or equal to 0.05.

RESULTS

FACS results

Using flow cytometry, the CD44+ cell population of the three cell lines was determined. The findings demonstrated that the percentage of CD44+ cells in SCC-15, SCC-25, and HSC-3 were 70.5±1.73%, 70±1.63%, and 71.25±1.5% respectively, suggesting that FACS enriched CD44+ cells effectively. (fig. 1a).

CD44+ cells culture

CD44+ subpopulations of the three cell lines showed mesenchymal morphology and clone spheres formation which could be an indicative sign of CSCs sub-population and was subjected for further analysis to confirm the presence of CSCs (fig. 1b and c).

3.3. PCR results

For the three cell lines, E-cadherin recorded a significantly higher value in parental cells compared to CSCs sub-population; whereas α-SMA recorded a significantly lower value in parental cells compared to CSCs sub-population (table 1 and fig. 2).

Correlation between markers

As regards CSCs in the three cell lines, a very strong negative correlation was noted between E-cadherin and each of α-SMA and CD44, whereas a very strong positive correlation was noted between α-SMA and CD44 (table 2 and fig. 3).

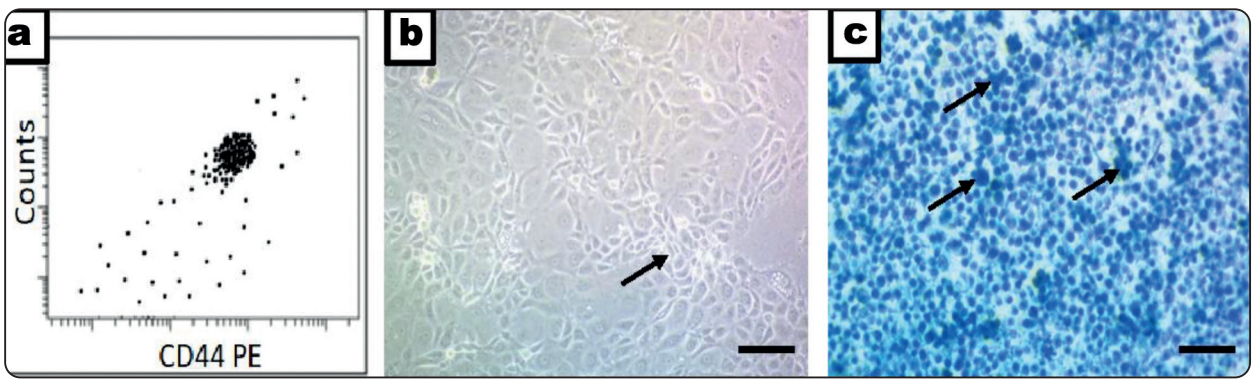

Fig. (1): FACS analysis for CD44 antigen (a). Cancer stem cells in culture. They were fusiform, the arrow indicates colony formation. Magnification at (40x) (b). Staining of clone spheres using crystal violet. Magnification at (40x) (c).

TABLE (1) Comparison of E-cadherin and α SMA in parental cells and CSCs sub-population

Marker	SCC-25			SCC-15			HSC-3		
	Parental cells	CSCs sub-population	P value	Parental cells	CSCs sub-population	P value	Parental cells	CSCs sub-population	P value
E-Cadherin	5.52±.62	1.46±.16	<0.001*	5.57±.52	1.53±.05	<0.001*	5.46±.52	1.48±.09	<0.001*
α-SMA	.97±.15	4.74±.13	<0.001*	.94±.12	4.89±.09	<0.001*	1±.08	4.84±.12	<0.001*

**** Statistically significant differences.***

TABLE (2) Correlation between E-cadherin, α-SMA, and CD44

Cell line			E-cadherin	α-SMA	CD44
SCC-25	E-cadherin	Pearson Correlation (R)		-.982-**	-.978-**
		P value		<0.001	<0.001
		Interpretation		VS (-ve)	VS (-ve)
	α-SMA	Pearson Correlation (R)	-.982-**		.998**
		P value	<0.001		<0.001
		Interpretation	VS (-ve)		VS (+ve)
	CD44	Pearson Correlation (R)	-.978-**	.998**	
		P value	<0.001	<0.001	
		Interpretation	VS (-ve)	VS (+ve)	
SCC-15	E-cadherin	Pearson Correlation (R)		-.987-**	-.989-**
		P value		<0.001	<0.001
		Interpretation		VS (-ve)	VS (-ve)
	α-SMA	Pearson Correlation (R)	-.987-**		.998**
		P value	<0.001		<0.001
		Interpretation	VS (-ve)		VS (+ve)
	CD44	Pearson Correlation (R)	-.989-**	.998**	
		P value	<0.001	<0.001	
		Interpretation	VS (-ve)	VS (+ve)	
HSC-3	E-cadherin	Pearson Correlation (R)		-.985-**	-.986-**
		P value		<0.001	<0.001
		Interpretation		VS (-ve)	VS (-ve)
	α-SMA	Pearson Correlation (R)	-.985-**		.998**
		P value	<0.001		<0.001
		Interpretation	VS (-ve)		VS (+ve)
	CD44	Pearson Correlation (R)	-.986-**	.998**	
		P value	<0.001	<0.001	
		Interpretation	VS (-ve)	VS (+ve)	

*****. Correlation is significant at the 0.01 level (2-tailed), VS= very strong***

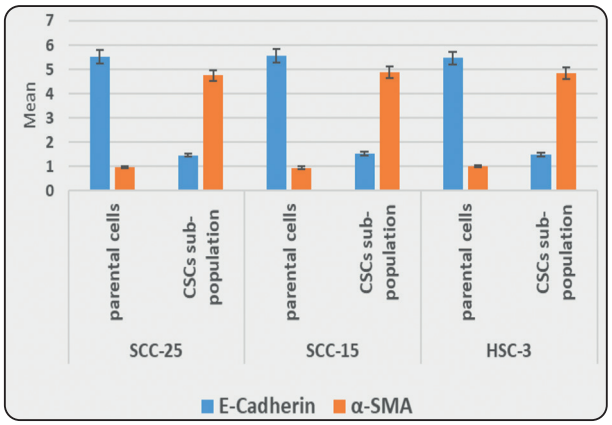

Fig. (2): Bar chart showing comparison of E-cadherin and α SMA expression in parental cells and CSCs sub-population.

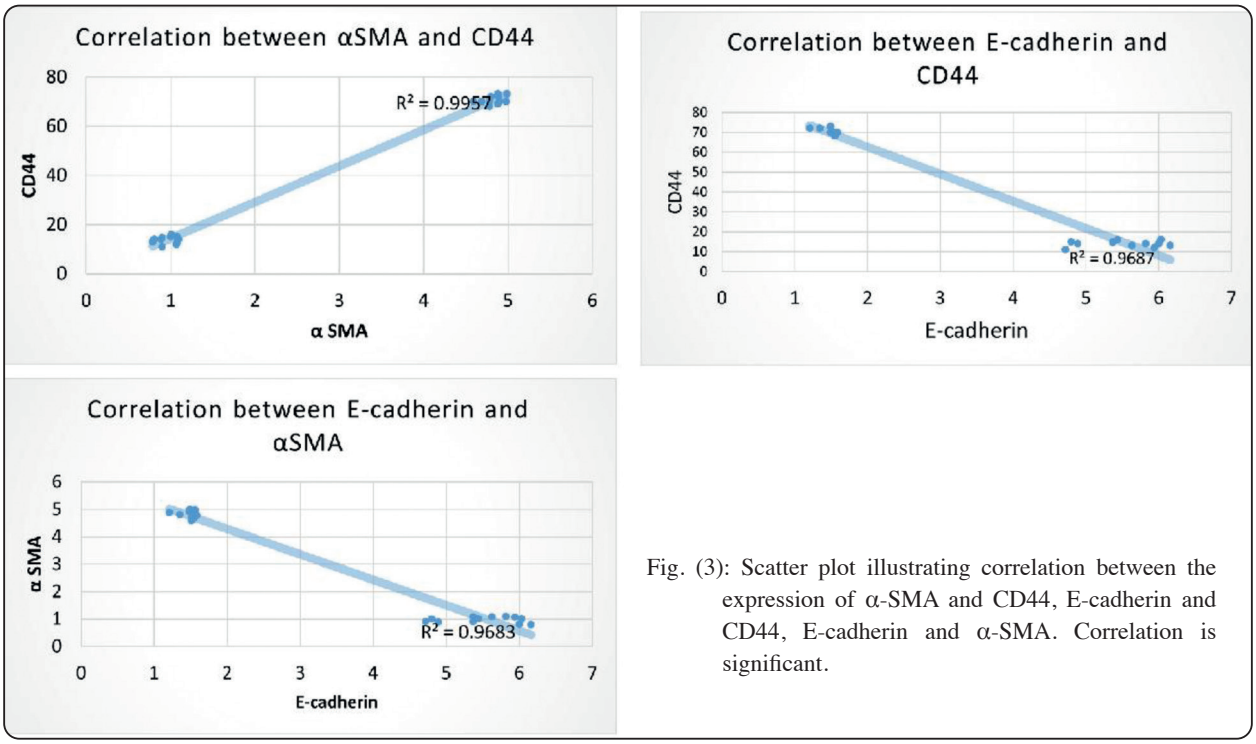

Fig. (3): Scatter plot illustrating correlation between the expression of α-SMA and CD44, E-cadherin and CD44, E-cadherin and α-SMA. Correlation is significant.

DISCUSSION

TSCC is the most prevalent type of oral cavity cancer. It is a very aggressive disease. New diagnostic and prognostic markers are required for TSCC as the majority of current chemoradiotherapy treatments fail to produce favorable clinical outcomes (Cui et al., 2017). The poor prognosis of oral squamous cell carcinoma is primarily attributable to the elevated rates of metastasis and recurrence. (Feller et al., 2013).

Understanding the molecular mechanism of metastasis is crucial for selecting an appropriate therapeutic target for cancer therapy. Nevertheless, substantial evidence indicates that cancer cell metastasis originates from CSCs suggesting a correlation between EMT stimuli and CSCs (Pan et al., 2016). Yet, the role of EMT in the progression of cancer and its impact on patient survival remains poorly understood (Masola et al., 2022).

So, the current study aimed to analyze the expression of EMT markers, E-cadherin and α-SMA in CD44+ CSCs compared to parental cells to shed light on the possible role of CSCs in invasion and metastasis of TSCC. Three TSCC cell lines were

used in this study. Utilizing malignant cell lines is advantageous as they are available in large amounts and are not contaminated with cancer-associated stroma or normal stem cells (Ghuwalewala et al., 2016).

CSCs have been identified by their ability to form spheres and their expression of stem-cell markers (Wang et al., 2014). It is well recognized that CD44+ cells could be separated from heterogeneous single-cell-prepared cancer cells using CD44-specific antibody labeling in conjunction with flow cytometry. These CD44+ cells have distinct stem cell characteristics, including self-renewal and tumorigenic abilities (Baniebrahimi et al., 2020).

Previous studies have pointed out CD44 cancer stem cell marker's significance in multistep TSCC carcinogenesis. In TSCC, the high staining intensity of CD44 was found to be a major predictor of poor prognosis. Furthermore, it was highly correlated with metastasis and recurrence (Lim et al., 2014;Yanamoto et al., 2014). In this respect, Pozzi et al. (2015) concluded that, a minimum of 200 CD44+ cancer cells were capable of constantly forming tumors while injections of thousands of cancer cells with different phenotypes failed to develop tumors.

In the present study, FACS analysis was used for CD44+ cell sorting, and enrichment; all the tested cell lines displayed stable CD44 expression levels. In addition, CD44+ subpopulations of the three cell lines showed mesenchymal morphology and clone spheres formation which could be an indicator of the presence of CSCs sub-population.

To further characterize the properties of CSC populations in TSCC, we investigated the expression of EMT markers (E-cadherin and α-SMA) in CSCs compared to the parental cells. It has been postulated that CSCs endure EMT to acquire invasive and migratory characteristics (Pan et al., 2016). During EMT, the expression of epithelial-specific proteins such as (cytokeratin and E-cadherin) would decrease, while the expression of mesenchymal-specific proteins such as (α-SMA and vimentin) rises (Lazarevic et al., 2020).

E-cadherin is a membrane protein involved in tumor invasion and metastasis. E-cadherin down regulation is considered a hallmark of EMT (Saltanatpour et al., 2019). As regards E-cadherin in the current study, a significantly higher value was recorded in the parental cells compared to CSCs sub-population across the three cell lines. Our findings are in line with a study by Farmakovskaya et al. (2016). In their study, the downregulation of E-cadherin led to a rise in CSC proportion. They hypothesized that the downregulation of E-cadherin could stimulate the upregulation of stem-maintaining transcription factors SOX2 and Oct3/4. This, in turn, could influence the acquisition of CSC characteristics.

On the other hand, α-SMA is a mesenchymal intermediate filament protein. In malignancy, it may also be observed in epithelial cells throughout the EMT process (Lazarevic et al., 2020). Stimulation of α-SMA expression has been linked to decreased disease-free survival in pancreatic, lung, and colorectal cancers (Tsujino et al., 2007; Lee et al., 2013; Sinn et al., 2014,). In breast cancer, patients with elevated α-SMA levels showed a worse prognosis than those with decreased α-SMA levels (Jeon et al., 2017). There is a lack of information regarding α-SMA expression in oral CSCs. To our knowledge, this research is the first investigation of α-SMA expression in oral cancer stem cells.

In this respect, our preliminary results revealed a significantly higher value of α-SMA in CSCs sub-population in comparison to parental cells. These results could be supported by previous studies on basal cell carcinoma, gastric, ovarian, and prostate cancer in which, the higher expression of α-SMA in malignant cells was significantly associated with increased tumorigenesis, migration, and invasion ability, indicating its possible role in CSC biology (Anggorowati et al., 2017; Jung et al., 2019; Jabour et al., 2022; Masola et al., 2022).

A recent study by Jabour et al. (2022) reported that the aggressive biological behavior of basal cell carcinoma can be predicted using α-SMA biomarker. They detected a significantly higher expression of α-SMA in the neoplastic cells of the infiltrative type compared to the other subtypes of basal cell carcinoma. Moreover, in a study on ovarian cancer, tumor cells expressing α-SMA are more invasive, more likely to metastasize, and have a worse prognosis (Anggorowati et al., 2017).

Furthermore, α-SMA promotes the migration and invasion of tumor cells via E-cadherin suppression (Jeon et al., 2017; Kim et al., 2019). This aligns with our findings as we observed a very strong negative correlation between E-cadherin and α-SMA. In addition, Farmakovskaya et al., (Farmakovskaya et al., 2016) discovered that downregulation of E-cadherin results in increased expression of genes that maintain the cell's stem state. They confirmed that E-cadherin-low cell lines may develop EMT characteristics as they changed their morphology, while E-cadherin-high cell lines and control cells maintained the epithelial features.

EMT can be influenced by various signaling pathways. These signaling pathways alter gene expression by modulating the major transcription factors including Snail, and Slug. Such pathways elevate the mesenchymal cell markers' expression, decrease the epithelial cell markers' expression, and ultimately transform the epithelial cells' phenotype into that of mesenchymal stem cells (Liang et al., 2018).

Snail possesses several binding sites located on E-cadherin; a crucial gene involved in EMT. Its reduced expression results in the failure of intercellular junctions and is strongly correlated with an increased EMT and metastatic potential (Saltanatpour et al., 2019).

As regards the correlation between E-cadherin and CD44, the present work revealed a very strong negative correlation between E-cadherin and CD44. A previous study by Ghuwalewala et al. (2016) supported our results in which CD44 high cells showed loss of E-cadherin. Shen et al. (2016) have also reported that the parental cells and CD44-low cells exhibited an epithelial phenotype with elevated E-cadherin expression.

A previous study on colon cancer cell lines revealed a decrease in E-cadherin expression in CD44-positive cells. The cells with downregulated E-cadherin resembled mesenchymal cells morphologically, indicating the role of E-cadherin-downregulation in the acquisition of cancer stem cell features (Saltanatpour et al., 2019).

Furthermore, the outcome of this study demonstrated a very strong positive correlation between α-SMA and CD44. Ghuwalewala et al. (2016) support our findings. They observed that CD44+ cells were mesenchymal in nature and were more invasive and migratory under in vitro circumstances. Recent research has demonstrated that CSCs' targeting can be an efficient cancer treatment strategy (Babaei et al., 2021). Indeed, researchers used hyaluronic acid (with its specific binding to CD44) as one of the vehicles to administer the targeted therapies (Pozzi et al., 2015).

Taken together, agents that primarily inhibit EMT not only repress EMT and metastasis but also repress the stem cell-like characteristics and drug resistance in various types of human cancers (Song et al., 2019). Therefore, our preliminary results regarding the expression of α-SMA by CSCs could be valuable in future studies focusing on α-SMA as a prognostic marker and targeted therapy in oral squamous cell carcinoma.

Ultimately, a limitation of this study is the lack of information regarding the expression of α-SMA in CSCs. More studies are required to confirm our findings and determine the precise function of this marker in CSCs and its potential as a therapeutic target. While this study has the potential to yield objective evidence, it presents a promising area for future investigation regarding the potential correlation between CSCs and EMT.

CONCLUSION

The findings of our study indicated that TSSC harbors a sub-population of CD44+ CSCs that exhibited enhanced EMT features compared to other cell populations. Our data could be considered as new evidence in support of the possible association between CSC properties and α-SMA expression. This may have relevant implications for the development of cancer stem cell-based treatment options.

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