

Original Article

SIRT-1 and CD44 in The Saliva of Oral Squamous Cell Carcinoma, Dysplastic and Non-Dysplastic Oral Leukoplakia

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Abstract:

Objective: The objective of this study was to investigate the levels of salivary SIRT-1 and CD44 in patients suffering from oral squamous cell carcinoma (OSCC), dysplastic and non-dysplastic oral leukoplakia to assess their potential role as early diagnostic biomarkers for patients with oral leukoplakia and OSCC.

Methods: sixty patients were recruited from the outpatient clinic of the Department of Oral Medicine and Periodontology, Faculty of Dentistry, Cairo University. Participants were divided into four groups: 15 healthy individuals, 15 patients with non-dysplastic leukoplakia, 15 patients with dysplastic leukoplakia, and 15 patients with oral squamous cell carcinoma. Whole unstimulated saliva (WUS) was collected from all participants. Soluble CD44 (solCD44) and SIRT1 were measured using ELISA.

Results: CD44 was expressed in all the four study groups with different levels. OSCC showed a statistically significant highest CD44 level. While the control group showed the lowest CD44 level. There was no statistically significant difference between dysplastic leukoplakia and non-dysplastic leukoplakia groups. Similarly, there was no statistically significant difference between non-dysplastic leukoplakia and control groups. The means of solCD44 in ng/ml were 24.5 (non-dysplastic leukoplakia), 36.8 (dysplastic leukoplakia), 134.4 (OSCC), and only 11.3 (control). OSCC showed the statistically significantly highest SIRT-1 level. There was no statistically significant difference between dysplastic leukoplakia, non-dysplastic leukoplakia, and control groups; all showed statistically significantly lower SIRT-1 levels than the OSCC group. The means of SIRT-1 in pg/ml were 142.3 (non-dysplastic leukoplakia), 151.9 (dysplastic leukoplakia), 247.2 (OSCC), and 135.8 (control).

Conclusion: salivary SIRT-1 and CD44 could be used as a screening tool to improve early detection and diagnostic precision of oral leukoplakia and OSCC.

Keywords: SIRT-1, CD44, solCD44, OSCC, leukoplakia

Introduction:

Oral cancer is a serious public health problem as it represents the sixth most common human malignancy with a 50% five years survival rate (Kaur

et al., 2018) Most oral carcinomas develop from oral premalignant lesions such as oral leukoplakia and erythroplakia (Kaur et al., 2018). Unfortunately, oral cancer is usually detected at a later stage due to the lack of early detection (Lumerman et al., 1995)

Oral leukoplakia is the most common oral potentially malignant disorder (OPMD). Worldwide, the prevalence of leukoplakia is 0.2%–4.9% and the malignant transformation rate for dysplastic lesions is 3% - 6% depending on the lesion type and size and the length of follow-up. The presence of epithelial dysplasia histopathologically may be more relevant in predicting the malignant potential more than the clinical appearance (Smitha et al., 2011).

Many studies showed that the alterations in the extracellular matrix (ECM) are important in early tumor progression (Turley et al., 2002). Hyaluronan is an ECM protein with an important role in tumor growth, progression, and invasion (S. J. Wang et al., 2009). In many cell and tissue models, cells adhere to the hyaluronan via the Cluster of differentiation 44 (CD44) glycoprotein (Bourguignon et al., 2001). The interaction between hyaluronan and CD44 in cancer increases adhesion, migration, and invasion (Oran-Rousseau, 2010). Many studies showed that CD44 expression has been associated with aggressive behavior in different types of cancers. CD44 expression is also linked to cancer stem cells (CSCs), which have been known to play an important role in both tumor progression and poor prognosis in head and neck squamous cell carcinoma (HNSCC), and in other cancers (Kokko et al., 2011). That is why CD44 is identified as a candidate stem cell marker in the normal squamous epithelium and SCC and became a major focus of current cancer research (Saghravani et al., 2017). Although the increased expression of this biomarker in OSCC has been shown in many studies, other studies showed CD44 downregulation (Kokko et al., 2011).

Sirtuins (SIRT1-SIRT7) are a family of NAD⁺-dependent deacetylase enzymes which belong to class III histone deacetylases and have a role in many cellular processes including metabolism and cell cycle (Bartosch et al., 2016). The best-studied sirtuin member is SIRT1 which stands for (silent mating type information regulation 2 homolog1). SIRT1 either stimulates or inhibits various biological

processes, including gene expression regulation, cellular metabolism, stress response, and chemoresistance (Chen et al., 2012). However, its role in tumorigenesis is still mysterious. SIRT1 upregulation has been reported in various solid tumors (Brooks & Gu, 2009). SIRT1 has been demonstrated to silence different tumor suppressors for example p53, or activate tumor drivers for example the PTEN/PI3K/AKT pathway, so enhancing tumorigenesis (H. Wang et al., 2012). However, other studies have demonstrated the downregulation of SIRT1 in some tumors implying its role as a tumor suppressor instead (Pruitt et al., 2006).

Due to the contradiction in the current literature related to CD44 and SIRT-1, this study was conducted to measure the levels of salivary SIRT-1 and compare it to the results of CD44 in patients suffering from OSCC, dysplastic, and non-dysplastic oral leukoplakia to assess their potential role as salivary biomarkers for patients with oral leukoplakia and OSCC.

Materials and methods:

Sixty patients were recruited from the outpatient clinic of the Department of Oral Medicine and Periodontology, Faculty of Dentistry, Cairo University, age, and sex-matched. Participants were randomly recruited and divided into 4 groups: 15 systemically and orally healthy individuals as a control group, 15 patients diagnosed with non-dysplastic leukoplakia, 15 patients diagnosed with dysplastic leukoplakia, and 15 OSCC patients.

The research has been revised and approved by the Research Ethics Committee, Faculty of Dentistry, Cairo University. Research has been conducted in full accordance with the ethical principles, including the World Medical Association Declaration of Helsinki (version 2008). Informed written consents have been obtained from all individuals after explaining the nature and aim of the study.

All participants have undergone a detailed medical history according to the questionnaire of the modified Cornell Medical Index (Abramson, 1966). A thorough clinical examination has been performed on all participants. According to Warnakulasuriya et al 2007 (Warnakulasuriya et al., 2007), a provisional diagnosis of oral leukoplakia was made after excluding other conditions that clinically appear as white lesions. To differentiate between non-dysplastic and dysplastic oral leukoplakia and to confirm the diagnosis of clinically appearing OSCC, incisional biopsies were obtained and examined.

Saliva collection

Whole unstimulated saliva (WUS) was collected from all the participants. This collection was done using a standard technique according to Navazesh (NAVAZESH, 1993) All subjects were requested to refrain from eating, drinking, or chewing gum for at least an hour before the sampling. The participants were asked first to swallow, bend their heads forward, and spit out all saliva into 50-ml sterile centrifuge tubes for five minutes without swallowing. Saliva samples were centrifuged for 2 min at 10,000 xg and the clarified supernatants were filtered through a 0.45 μ m low protein binding membrane, separated into 0.5 ml aliquots, and frozen at -80°C to be analyzed later for SIRT-1 and CD44.

Quantitative detection of soluble human CD44:

The quantitative assays were performed at the Biochemistry Department, Faculty of Medicine, Cairo University. Soluble CD44 (solCD44) was measured using an ELISA kit provided by Thermo Fissure, Invitrogen, USA (Catalog # BMS209INST). An anti-human solCD44 coating antibody is adsorbed onto microwells. Human solCD44 present in the sample or standard binds to antibodies adsorbed to the microwells. An HRP-conjugated anti-human solCD44 antibody is then added and bound to human solCD44 captured by the first antibody. Following incubation, the unbound HRP conjugated anti-human solCD44 was removed

during the wash step, and substrate solution reactive with HRP is added to the wells. A colored product was formed in proportion to the number of human solCD44 present in the sample or standard. The reaction was terminated by the addition of acid and absorbance was measured at 450 nm. A standard curve is prepared from six human solCD44 standard dilutions and the human solCD44 concentration determined.

Determination of SIRT-1 level

The used kit was a sandwich enzyme immunoassay for quantitative measurement of SIRT1 in humans and was provided by MyBioSource, USA (Catalog No. MBS2601311). The microtiter plate provided in this kit has been pre-coated with an antibody specific to SIRT1. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody which was specific to SIRT1. Then, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. After TMB substrate solution was added, only those wells that contained SIRT1, biotin-conjugated antibody, and enzyme-conjugated Avidin exhibit a color change. The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of $450\text{nm} \pm 10\text{nm}$. The concentration of SIRT1 in the samples was then determined by comparing the O.D. of all the samples to the standard curve.

Statistical analysis:

Numerical data were tested for normality by tests of normality (Kolmogorov-Smirnov and Shapiro-Wilk tests). The age data showed normal distribution while the salivary markers levels showed non-normal distribution. Data were presented as mean (M), standard deviation (SD) for normally distributed data and median, and Inter-Quartile Range (IQR) values for non-normally distributed data.

For parametric data; a one-way ANOVA test was used to compare between the four test groups.

Bonferroni's posthoc test was used for pair-wise comparisons when the ANOVA test was found significant. For non-parametric data; the Kruskal-Wallis test was used to compare between the four test groups. Dunn's test was used for pair-wise comparisons. Spearman's correlation coefficient was used to determine significant correlations between different variables. Qualitative data were presented as frequencies and percentages. A Chi-square test was used for the comparisons.

ROC (Receiver Operating Characteristic) curve was constructed to determine the cut-off values of different markers for differentiation between the test groups. Areas under the ROC curve (AUCs) were compared using a z-statistic test.

The significance level was set at $P \leq 0.05$. Statistical analysis was performed using IBM® SPSS® Statistics Version 30 for Windows. ROC curve analysis was performed using MedCalc Version 11.3 for Windows (MedCalc Software bvba).

Results:

1. Demographic data:

The current study included 60 individuals: 15 patients with non-dysplastic leukoplakia, and 15 patients with dysplastic leukoplakia, 15 patients with OSCC, and 15 healthy control. The mean ages of the four groups were: 41.1, 47.3, 46.5, 28 years respectively. There was no statistically significant difference between gender distributions in the four groups as shown in table 1.

2. Salivary Biomarkers:

CD44 and SIRT-1 levels were determined in salivary samples utilizing ELISA. CD44 was expressed in all four study groups with different levels. OSCC showed a statistically significant highest solCD44 level. While the control group showed the lowest solCD44 level. There was no statistically significant difference between dysplastic leukoplakia and non-dysplastic leukoplakia groups; both showed

statistically significantly lower solCD44 levels than the OSCC group. There was also no statistically significant difference between non-dysplastic leukoplakia and control groups with a non-statistically significant difference from the non-dysplastic leukoplakia group and a statistically significantly lower level than OSCC and dysplastic oral leukoplakia groups. The means of solCD44 in ng/ml were 24.5 (non-dysplastic oral leukoplakia), 36.8 (dysplastic oral leukoplakia), 134.4 (OSCC), and only 11.3 (control). as shown in table 2.

SIRT-1: OSCC showed the statistically significantly highest SIRT-1 level. There was no statistically significant difference between dysplastic leukoplakia, non-dysplastic leukoplakia, and control groups; all showed statistically significantly lower SIRT-1 levels than the OSCC group. The means of SIRT-1 in pg/ml were 142.3 (non-dysplastic oral leukoplakia), 151.9 (dysplastic oral leukoplakia), 247.2 (OSCC), and 135.8 (control) as shown in table 2.

3. Correlation between different variables

There was a statistically significant direct (positive) correlation between the age, solCD44, and SIRT-1 levels i.e. older age is associated with higher solCD44 and SIRT-1 levels and vice versa. There was a statistically significant direct (positive) correlation between solCD44 and SIRT-1 levels i.e. an increase in solCD44 level is associated with an increase in SIRT-1 level and vice versa as seen in table 3.

4. Diagnostic accuracy of the two markers (ROC curve analysis)

Differentiation between non-dysplastic oral leukoplakia and control groups:

ROC curve analysis of the two markers for differentiation between non-dysplastic oral leukoplakia and control groups is presented in table (4) and figure (1).

Table (1): Descriptive statistics and results of comparisons between base line characteristics in the four groups

	Non-dysplastic leukoplakia (n = 15)	Dysplastic leukoplakia (n = 15)	OSCC (n = 15)	Control (n = 15)	P-value
Age (Years)					
Mean (SD)	41.1 (6.2) ^A	47.3 (12.2) ^A	46.5 (12.9) ^A	28 (6.2) ^B	<0.001*
Gender [n (%)]					
Male	9/15 (60%)	9/15 (60%)	6/15 (40%)	7/15 (46.7%)	0.691
Female	6/15 (40%)	6/15 (40%)	9/15 (60%)	8/15 (53.3%)	

*: Significant at $P \leq 0.05$, Different superscripts in the same row are statistically significantly different

Table (2): Descriptive statistics and results of Kruskal-Wallis test for comparison between markers levels in the four study groups

Marker	Non-dysplastic oral leukoplakia (n = 15)	Dysplastic oral leukoplakia (n = 15)	OSCC (n = 15)	Control (n = 15)	P-value
SCD44 (ng/ml)					
Median (IQR)	24.2 (22.8 – 25.9) ^{BC}	38.8 (32.1 – 41.3) ^B	124.7 (76.4 – 195.7) ^A	10.6 (8.8 – 14.3) ^C	<0.001*
Mean (SD)	24.5 (3.5)	36.8 (4.1)	134.4 (63.8)	11.3 (2.9)	
SIRT-1 (pg/ml)**					
Median (IQR)	140.2 (139.3 – 146.3) ^B	155.8 (142.3 – 160.2) ^B	255.7 (211.4 – 285.3) ^A	133.5 (122.3 – 155.6) ^B	<0.001*
Mean (SD)	142.3 (5.9)	151.9 (7.9)	247.2 (52.3)	135.8 (18.2)	

*: Significant at $P \leq 0.05$, Different superscripts in the same row are statistically significantly different

** : picograms per milliliter. A picogram is one-trillionth of a gram

Table (3): Results of Spearman's correlation coefficient for the correlation between different variables

Variables	Age		SCD44		SIRT-1	
	Correlation coefficient	P-value	Correlation coefficient	P-value	Correlation coefficient	P-value
Age	-	-	0.529	<0.001*	0.363	0.004*
SCD44	-	-	-	-	0.777	<0.001*

*: Significant at $P \leq 0.05$

Table (4): Cut-off values for different markers and the corresponding sensitivity, specificity, predictive values, diagnostic accuracy, Area Under the ROC curve (AUC) and 95% confidence interval (95% CI) of the (AUC) for differentiation between non-dysplastic oral leukoplakia and control groups

Marker	Cut-off value	Sensitivity %	Specificity %	+PV %	-PV %	Diagnostic accuracy %	AUC	95% CI
SCD44	16.8	100	100	100	100	100	1.000	0.884 – 1.000
SIRT-1	134.2	100	60	71.4	100	80	0.671	0.467 – 0.831

+PV: Positive Predictive Value, -PV: Negative Predictive Value

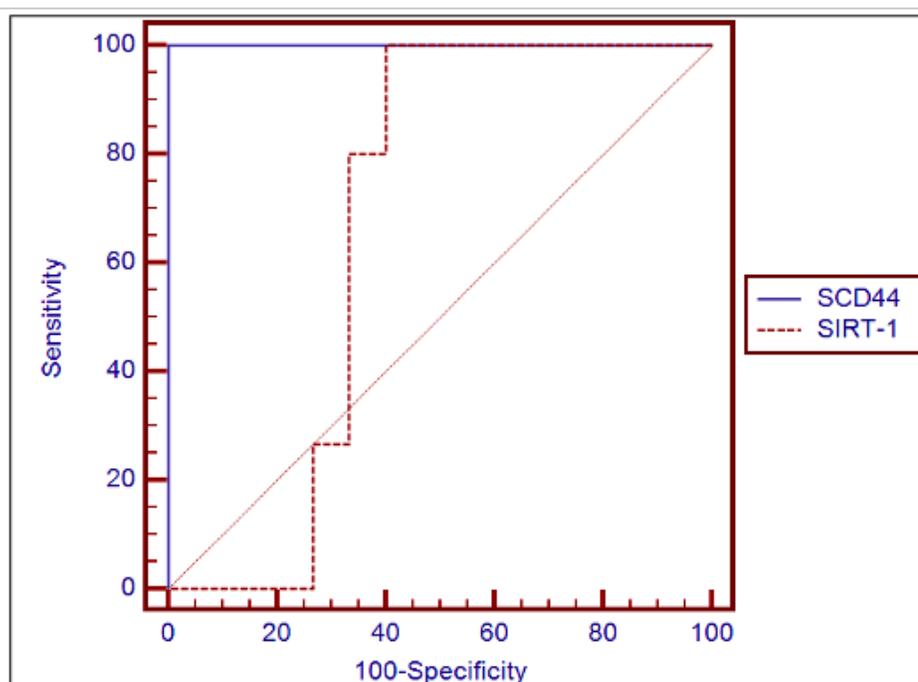


Figure (1). ROC curves of the two markers for differentiation between Non-dysplastic Leukoplakia and control groups

Table (5): Cut-off values for different markers and the corresponding sensitivity, specificity, predictive values, diagnostic accuracy, Area Under the ROC curve (AUC) and 95% confidence interval (95% CI) of the (AUC) for differentiation between Dysplastic leukoplakia and control groups

Marker	Cut-off value	Sensitivity %	Specificity %	+PV %	-PV %	Diagnostic accuracy %	AUC	95% CI
SCD44	16.8	100	100	100	100	100	1.000	0.884 – 1.000
SIRT-1	136.3	100	66.7	75	100	83.4	0.764	0.575 – 0.899

+PV: Positive Predictive Value, -PV: Negative Predictive Value

A cut-off value of 16.8 ng/ml; solCD44 showed 100% diagnostic accuracy while at a cut-off value of 134.2 pg/ml; SIRT-1 showed 80% diagnostic accuracy for differentiation between Non-dysplastic oral leukoplakia and control groups. Pair-wise comparison between areas under the ROC curve (AUC) of the two markers showed that solCD44 showed statistically significantly higher AUC than SIRT-1 for differentiation between Non-dysplastic Leukoplakia and control groups.

Differentiation between dysplastic oral leukoplakia and control groups:

ROC curve analysis of the two markers for differentiation between dysplastic oral leukoplakia and control groups is presented in table (5) and figure (2). A cut-off value of 16.8 ng/ml; solCD44 showed 100% diagnostic accuracy while at a cut-off value of 136.3 pg/ml; SIRT-1 showed 83.4% diagnostic accuracy for differentiation between Dysplastic Leukoplakia and control groups. Pair-wise comparison between areas under the ROC curve (AUC) of the two markers showed that solCD44 showed statistically significantly higher AUC than SIRT-1 for differentiation between Dysplastic Leukoplakia and control groups.

Differentiation between OSCC and control groups

ROC curve analysis of the two markers for differentiation between OSCC and control groups is presented in table (6) and figure (3). At a cut-off value of 16.8 ng/ml; solCD44 showed 100% diagnostic accuracy while at a cut-off value of 165.7 pg/ml; SIRT-1 showed 93.4% diagnostic accuracy for differentiation between OSCC and control groups. Pair-wise comparison between areas under the ROC curve (AUC) of the two markers showed that there was no statistically significant difference between the two markers for differentiation between OSCC and control groups.

Differentiation between non-dysplastic oral leukoplakia and dysplastic oral leukoplakia groups:

ROC curve analysis of the two markers for differentiation between non-dysplastic oral leukoplakia and dysplastic oral leukoplakia groups is presented in table (7) and figure (4). At a cut-off value of 25.9 ng/ml; solCD44 showed 93.4% diagnostic accuracy while at a cut-off value of 140.2 pg/ml; SIRT-1 showed 76.7% diagnostic accuracy for differentiation between Non-dysplastic oral leukoplakia and dysplastic oral leukoplakia groups. Pair-wise comparison between areas under the ROC curve (AUC) of the two markers showed that SsolCD44 showed statistically significantly higher AUC than SIRT-1 for differentiation between Non-dysplastic Leukoplakia and Dysplastic Leukoplakia groups.

Differentiation between dysplastic oral leukoplakia and OSCC groups

ROC curve analysis of the two markers for differentiation between dysplastic oral leukoplakia and OSCC groups is presented in table (8) and figure (5). At a cut-off value of 41.7 ng/ml; solCD44 showed 100% diagnostic accuracy while at a cut-off value of 160.2 pg/ml; SIRT-1 showed 93.4% diagnostic accuracy for differentiation between dysplastic oral leukoplakia and OSCC groups. Pair-wise comparison between areas under the ROC curve (AUC) of the two markers showed that there was no statistically significant difference between the two markers for differentiation between dysplastic oral leukoplakia and OSCC groups.

Discussion

More than 50% of oral carcinomas seen in the dental specialist clinics are in advanced stages already at the time of presentation due to the lack of awareness of the patients and early detection skills of the general practitioners.

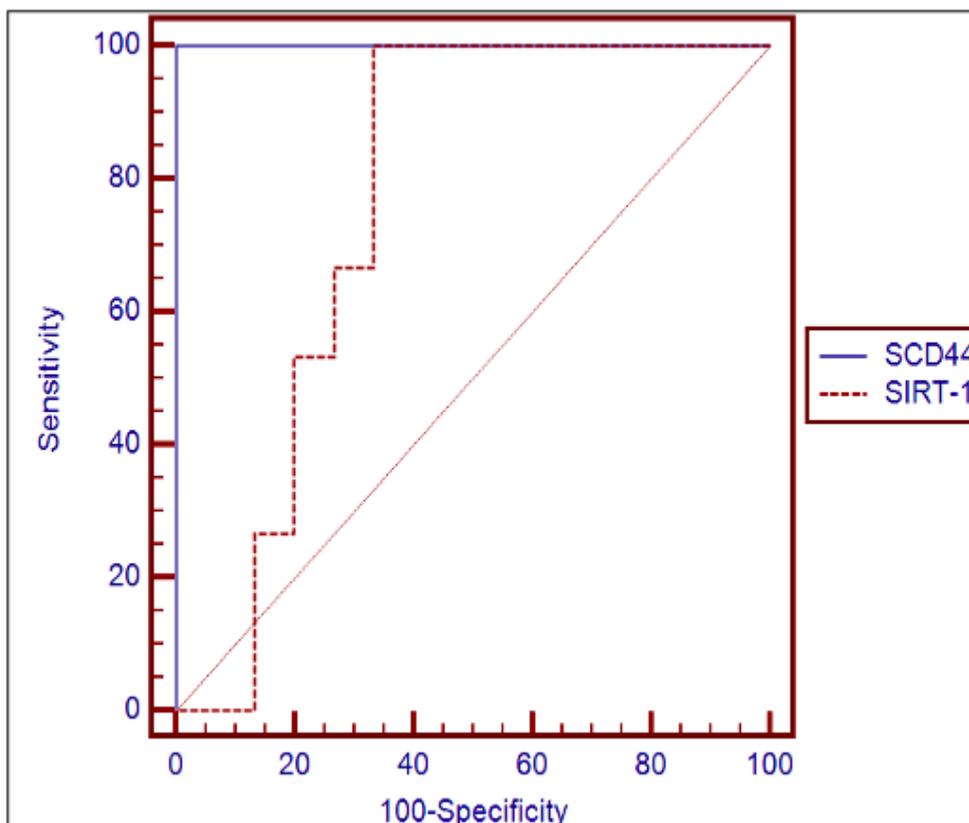


Figure (2). ROC curves of the two markers for differentiation between dysplastic oral leukoplakia and control groups

Table (6): Cut-off values for different markers and the corresponding sensitivity, specificity, predictive values, diagnostic accuracy, Area Under the ROC curve (AUC) and 95% confidence interval (95% CI) of the (AUC) for differentiation between dysplastic leukoplakia and control groups

Marker	Cut-off value	Sensitivity %	Specificity %	+PV %	-PV %	Diagnostic accuracy %	AUC	95% CI
SCD44	16.8	100	100	100	100	100	1.000	0.884 – 1.000
SIRT-1	165.7	86.7	100	100	88.2	93.4	0.978	0.845 – 1.000

+PV: Positive Predictive Value, -PV: Negative Predictive Value

Unfortunately, oral cancers which treated in advanced stages have a poor prognosis and 5-year survival of only 50%. Even for those who survive, the quality of life is badly affected with many

disabilities particularly in eating, swallowing, and speech. Early detection of oral cancer significantly improves survival and contributes to less morbidity following treatment.

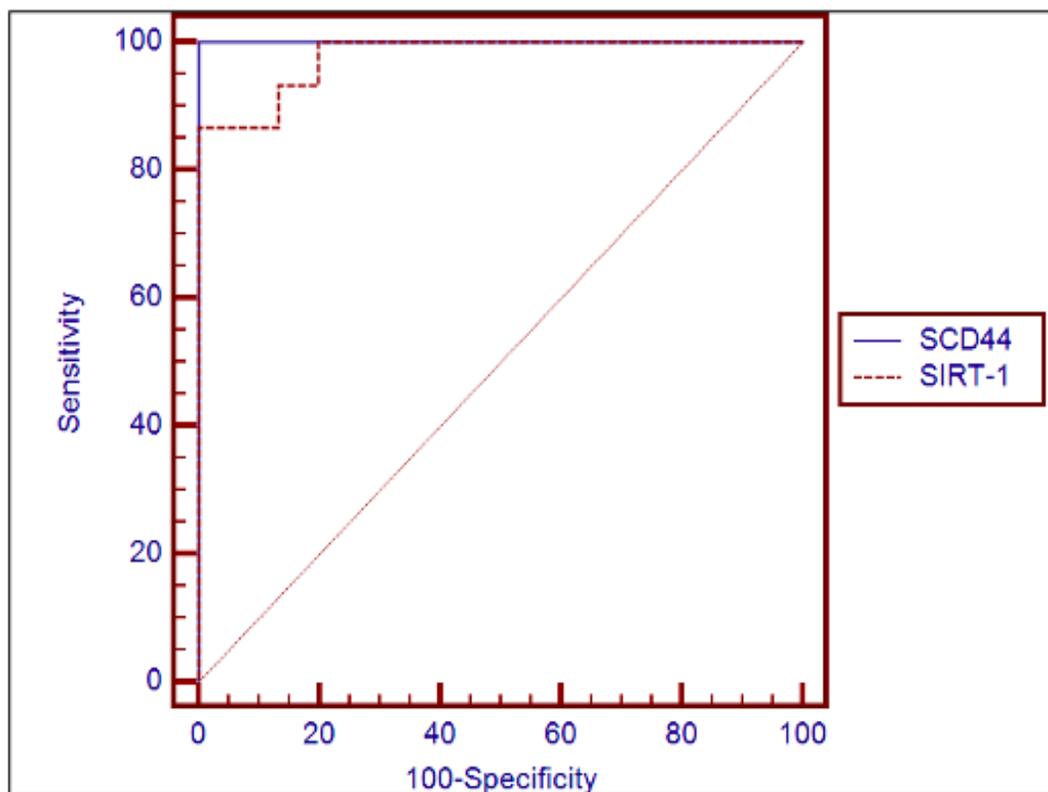


Figure (3). ROC curves of the two markers for differentiation between OSCC and control groups

Table (7): Cut-off values for different markers and the corresponding sensitivity, specificity, predictive values, diagnostic accuracy, Area Under the ROC curve (AUC) and 95% confidence interval (95% CI) of the (AUC) for differentiation between Non-Dysplastic leukoplakia and Dysplastic Leukoplakia groups

Marker	Cut-off value	Sensitivity %	Specificity %	+PV %	-PV %	Diagnostic accuracy %	AUC	95% CI
SCD44	25.9	86.7	100	100	88.2	93.4	0.982	0.853 – 1.000
SIRT-1	140.2	53.3	100	100	68.2	76.7	0.840	0.661 – 0.948

Table (8): Cut-off values for different markers and the corresponding sensitivity, specificity, predictive values, diagnostic accuracy, Area Under the ROC curve (AUC) and 95% confidence interval (95% CI) of the (AUC) for differentiation between Dysplastic leukoplakia and OSCC groups

Marker	Cut-off value	Sensitivity %	Specificity %	+PV %	-PV %	Diagnostic accuracy %	AUC	95% CI
SCD44	41.7	100	100	100	100	100	1.000	0.884 – 1.000
SIRT-1	160.2	100	86.7	88.2	100	93.4	0.960	0.818 – 0.998

+PV: Positive Predictive Value, -PV: Negative Predictive Value

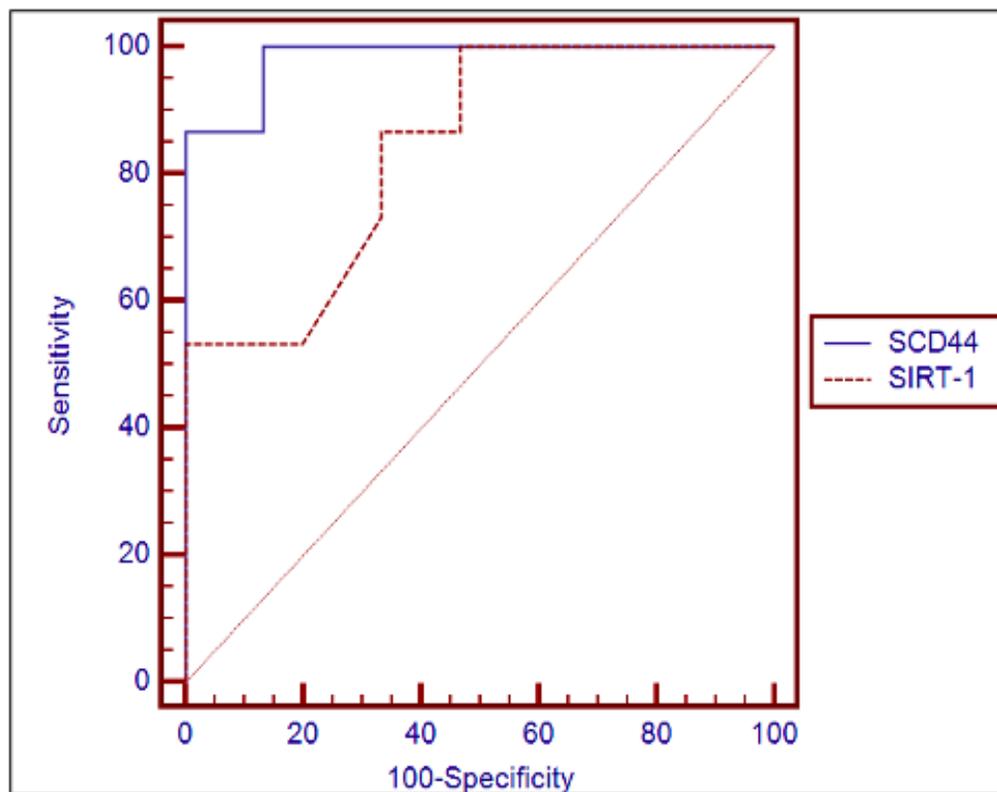


Figure (4). ROC curves of the two markers for differentiation between non-dysplastic oral leukoplakia and dysplastic oral leukoplakia groups

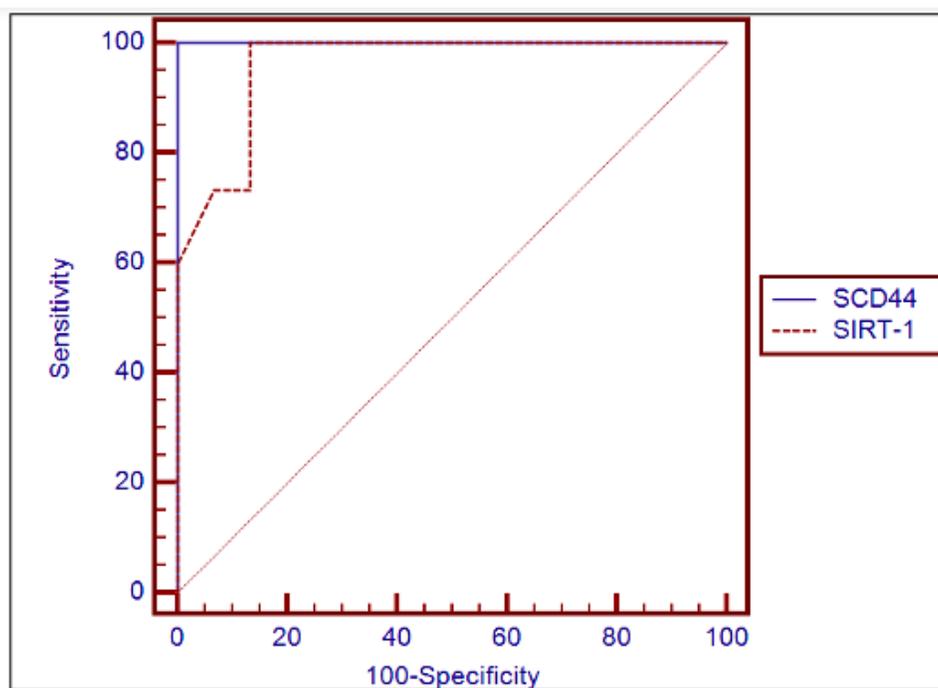


Figure (5). ROC curves of the two markers for differentiation between Dysplastic Leukoplakia and OSCC groups

Fortunately, some oral cancers are preceded by premalignant changes (recently called the oral potentially malignant disorders or OPMD) and if the dysplasia is detected before the development of a tumor, there is a possibility to intervene and reduce the risk of cancer development. However, the transformation rates of these OPMDs vary, their risk assessment is complicated and cannot be easily performed in primary care units by clinicians with limited experience in the management of these disorders (Warnakulasuriya, 2017).

The latest technology in oral cancer research has directed at the development of possible diagnostic tools at the clinical and molecular levels for the early detection and diagnosis of oral cancer. The detection of oral cancer or OPMD is based on clinical visual examination and palpation of the affected site and the examination of neck lymph nodes. Most oral cancers presented as either new swellings or persistent non-healing ulcers, or red or a white lesion on the oral mucosa. These signs are not unique to cancer and many benign conditions may have similar clinical representations (Warnakulasuriya, 2017). For this reason, primary care practitioners have difficulty detecting these early changes. Studies show that diagnosis based on signs and symptoms only lack specificity and the predictive value of referring a patient as suspected of oral cancer was low and estimated at 7.9% (Warnakulasuriya, 2017). Despite its limitations, tissue biopsy is still the gold standard for oral cancer diagnosis, but this technique requires training and is invasive, painful, time-consuming, and expensive (Kaur et al., 2018). Other clinical diagnostic technologies are toluidine chloride or toluidine blue dyes, salivary diagnostic biomarkers, and lastly optical imaging systems (Warnakulasuriya, 2017)

Cancer-initiating cells (CICs), or cancer stem cells (CSCs) constitute only a minor population of cells within the tumor, but they are crucial for tumor maintenance and progression. Current evidence

indicates that the great majority of the cancer cells within a tumor cannot sustain the lesion and only a small group of cells, which is the (CSCs), with their self-renewal properties are capable of spreading the tumor.

CICs can be enriched by CIC markers, the most common of which is CD44. CD44 has been used as a CIC marker in several hematopoietic and epithelial malignancies because of its widespread expression. Also, CD44 is a cell adhesion molecule known as the 'homing receptor'. It was found that the expression of the variant isoform of CD44 (CD44v) caused a metastatic phenotype in locally growing tumor cells. There is strong evidence for the significance of CD44 expression in the progression of many tumor types (Shimada et al., 2009; Zöller, 2011)

On the other hand, SIRT1, a nicotinamide adenine dinucleotide (NAD⁺)-dependent histone deacetylase enzyme which is found to be significantly elevated in human prostate, primary colon, and skin cancers (Zhang & Wang, 2013) Recent evidence indicates that epigenetic changes may 'addict' cancer cells to altered signaling during the early stages of tumor development (Ostojić et al., 2009). SIRT1 plays an important role in epigenetic modifications.

In the present study, we compared the levels of the CD44 salivary samples of healthy control with oral squamous cell carcinoma and patients with oral leukoplakia with or without dysplasia. We evaluated the correlation between the age and the level of expression of CD44 and SIRT-1. There was a statistically significant positive correlation between the age, CD44, and SIRT-1 levels i.e. older age is associated with higher CD44 and SIRT-1 levels and vice versa. This is consistent with most oral cancer studies which correlate age with the risk of oral cancer development. It has been accepted for a long time that carcinomas are associated with older age. This may be due to the longer time of exposure to the environmental carcinogens, in addition to the

cumulative effect of these carcinogens (Ahmad et al., 2015).

This is in contrast to Saghravani et al. who observed no relationship between CD44 expression and patients age or gender (Saghravani et al., 2017) We demonstrated increased salivary solCD44 level in all the four study groups with the highest expression presented in OSCC with a statistically significant difference with the other groups while the control groups showed the lowest expression of CD44. Those findings were similar to the results of Khamis et al., who concluded that the salivary levels of CD44 in patients with OSCC were significantly higher than the levels in patients with benign epithelial tumors, and healthy subjects. They also concluded that the intense, moderate, and weak expressions were associated with poorly, moderately, or well-differentiated histological grading respectively. So, CD44 expression can be used in OSCC staging as well. (Khamis et al., 2017). Our results also supported the results of Saghravani et al. who concluded that the immune-expression of CD44 was found in all OSCC samples (Saghravani et al., 2017). According to Ohkoshi et al, the up-regulation of CD44 functions as a survival mechanism for the cells to escape apoptosis in response to DNA damage repair in head and neck squamous cell carcinoma (Ohkoshi & Umemura, 2017).

Our results were in contrast to Seyedmajidi, et al. who found no statistically significant difference in the serum and salivary solCD44 level between the patient group and control group (Seyedmajidi et al., 2018). Also, it is opposite to the results by Naga et al. who found the expression of CD44 higher in control healthy oral tissues. They proposed that CD44 mediates the adhesive properties and signaling for the epithelial cells to migrate upward. It regulates the interaction of growth factors and their receptors. Because increased cellular proliferation is a result of

enhanced binding of the growth factors with their receptors, which is correlated to loss of CD44. Thus, CD44 could help in cell adhesion and cell-cell interactions (Naga et al., 2019).

All these conflicting results may have many explanations. That might be due to the lower numbers of patients in the conducted studies, different sampling methods either tissue, salivary, or serum sampling with the use of different antibodies, different oral cancer staging, and different localization of CD44 expression within the tumor tissue. In addition to the presence of different variants of CD44.

Similar results were found regarding SIRT-1 expression levels as OSCC showed the statistically significant and highest level. These results were in contrast to Kang et al. (Kang et al., 2018) who found that SIRT1 levels were significantly lower in patients with OSCC than in the control group. Devaraj et al. reported that both the tumor suppressor role and carcinogenic role of SIRT1 are reported in oral cancer, so its role is still ambiguous. In oral cancer, SIRT2 is not studied enough and so its role remains obscure (Ezhilarasan et al., 2021).

There was no statistically significant difference between dysplastic oral leukoplakia, non-dysplastic oral leukoplakia, and control groups; all showed statistically significantly lower SIRT-1 levels than the OSCC group. This finding may be in contradiction with the findings by Farah and Fox (2019) who found changes in the molecular profile of oral leukoplakia with dysplasia including downregulated ECM (Farah & Fox, 2019).

Also, there was a statistically significant positive correlation between solCD44 and SIRT-1 levels i.e. an increase in solCD44 level is associated with an increase in SIRT-1 level and vice versa. Up to our knowledge, there was no study combining the use of those two markers to diagnose OSCC.

At a cut-off value of 16.8 ng/ml; solCD44 showed 100% diagnostic accuracy while at a cut-off value of

134.2 pg/ml; SIRT-1 showed 80% diagnostic accuracy for differentiation between non-dysplastic oral leukoplakia and control groups. These findings are approaching the results by Franzmann et al. (2007) who studied CD44 in the saliva of HNSCC patients using ELISA and found sensitivity range from 62% to 70%, and specificity ranged from 75% to 88% depending on both the cutoff point and the site of the lesion (Franzmann et al., 2007).

Pair-wise comparison between areas under the ROC curve (AUC) of the two markers showed that solCD44 showed statistically significantly higher AUC than SIRT-1 for differentiation between Non-dysplastic Leukoplakia and control groups. Up to our knowledge, there was no study comparing those two markers in OSCC.

Conclusion

Salivary SIRT-1 and CD44 could be used as a screening tool to improve early detection and diagnostic precision of oral leukoplakia and OSCC.

Recommendations:

1. Further studies with larger participants numbers needed to study SIRT-1 level on OSCC due to the controversial results in the literature.
2. Further studies are needed to study those biomarkers in different stages of OSCC.
3. Further studies are needed to advance our understanding of the mechanisms of SIRT-1 and CD-44 in cancer development, and also in the development of novel therapeutic targets in OSCC treatment.

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