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Analysis DNA Methylation Level of *STAT5a* and *E-cadherin* Genes in Formalin Fixed Paraffin Embedded Tissue Blocks of Iraqi Patients with Oral Lichen Planus

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Abstract: Oral lichen planus (OLP) is a persistent inflammatory condition. The exact cause is not fully comprehended. The study aimed to detect the methylation level of *STAT5a* and *E-Cadherin* genes in OLP and compare the findings with control. A total number of 60 samples of formalin-fixed paraffin-embedded tissue blocks were divided into 2 groups: 30 OLP and 30 normal oral mucosa collected from laboratory archives of the Oral Diagnosis Department at University of Baghdad/College of Dentistry. DNA was extracted, and bisulfite treatment was utilized, Then HRM-PCR was performed.The results found a significant decrease (P<0.05) in the methylation level of *STAT5a*, while there was a significant increase (P<0.05) in the methylation level of *E-Cadherin* in OLP compared to controls, In addition, there were significant molecular alterations in methylation level of studied genes accumulated incrementally from control to reticular to erosive form were present in higher-risk erosive variants of OLP. It was concluded that the aberrant methylation profile of studied genes may be associated with the pathogenesis and progression of OLP.

Keywords: *E-cadherin* gene, *STAT5a* gene, oral lichen planus, DNA methylation.

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Introduction

Oral lichen planus (OLP) is a chronic T cell-mediated inflammatory disease of the oral mucosa that creates a diagnostic and therapeutic challenge owing to its refractory course and recurring nature and its wide range of clinical presentations (1,2). OLP is categorised as a potentially malignant condition (PMD) affecting the lining of the mouth, with a transformation rate ranging from 0 to 6.25% (3). The precise aetiology of OLP remains obscure; nevertheless, genetic predisposition, systemic associations, and environmental factors such as dental metals, drugs, or viral infections have been linked in the development of OLP (4). Environmental variables are known to cause epigenetic changes, where the pattern of gene expression is altered without changing the DNA sequence. DNA methylation is an epigenetic mechanism that regulates gene expression by inhibiting genomic transcription. This is accomplished by adding a methyl group to the 5'-position of the cytosine ring (5, 6, 7). The Signal Transducer and Activator of Transcription 5A gene is a transcription

factor belonging to the STAT family. It plays a vital role in controlling gene expression in different types of cells and is essential for the cellular response to various cytokines and hormones. Consequently, it is crucial for regulating the functions of the immune system (8). Several studies have highlighted the role of STAT5a in many pathological conditions, such as inflammatory processes, autoimmune diseases, and malignant tumors (9, 10). Epithelial cadherin gene is a tumor suppressor gene that is considered an essential molecule for epithelial homeostasis by regulating epithelial architecture and tissue integrity (11). Epigenetic alterations may cause disruption of E-cadherin expression and cause the cells to lose their epithelial phenotype. However, the E-cadherin gene was suggested to play a role in the OLP pathogenesis scenario, but the evidence needs further building up (12). Temporal patterns of aberrant methylation can be used to forecast the likelihood and speed of malignant transformation, as well as the potential for disease regression. Due to these factors, abnormal DNA methylation is considered a highly suitable candidate for assessing its potential as a biomarker for early diagnosis in the progression of oral pre-cancer (13). The present study was conducted on DNA methylation patterns in OLP of the promoter regions of STAT5a and E-cadherin to comprehensively assess the extent of abnormal DNA methylation as a possible diagnostic indicator for the advancement of diseases, and to identify areas of limited understanding in existing literature in order to direct future research. Up to author Knowledge, there was no previous Iraqi study related to the investigation of the mechanism of epigenetic inheritance in OLP using FFPE tissue blocks of Iraqi patients that have recently been discussed to share significant contribution in pathophysiology of OLP, therefore, this study was performed.

Materials and methods

The present study included an observational retrospective case-control research done in Baghdad, Iraq, spanning from September 2022 to April 2023. Sixty Formalin-fixed paraffin-embedded (FFPE) tissue blocks OLP (n = 30) (reticular OLP (n = 15), erosive (n = 15) and normal mucosa (n=30) taken from the archive of the laboratory of oral and maxillofacial pathology, collage of dentistry, Baghdad university. The Institute of Personalized Medical Science, the College of Dentistry, and the University of Baghdad's ethical committee gave the study its stamp of approval (Ref:695/ date 2022, Dec./subject No.695722). Paraffin sections were prepared with a microtome and used for HRM PCR. The EasyPure® FFPE Tissue Genomic DNA Kit Reagent was utilised to isolate the whole DNA from FFPE Tissue, adhering to the instructions provided by the manufacturer. The genomic DNA was subsequently treated with sodium bisulfite using the EpiTect Quick DNA Bisulfite kit to modify the STAT5a and E-cadherin genes. This procedure transformed all cytosines that were not methylated into uracils, while maintaining the integrity of the methylated cytosines. The procedure was carried out in accordance with the manufacturer's guidelines.

Study primers

The primers were designed using the Primer 3plus V4 and double-checked by the University Code of

Student Conduct (UCSC) programs, furthermore, their reference sequences were stored in the National Center for Biotechnology Information (NCBI) database. They were synthesized and lyophilized by Alpha DNA Ltd. (Canada) (Table 1) displays all primer sequences utilized in this study's assays.

Table (1): The studies designed primers.

Primer	Sequence (5′→ 3′ direction)	Product size bp	Temp. °C
STAT5a (Gene methylation)			
Forward	AATTTAGGGGTTTAAAAGATGA	180	54
Reverse	ACTCTAAAAATCCCAAACCTAA		
E-cadherin (Gene methylation)			
Forward	TTTTTTGATTTTAGGTTTTAGTGA	210	54
Reverse	AATTCACCTACCRACCAC		

Quantitative methylation-specific HRM PCR assay. The Quantification Methylation-Specific HRM-PCR (qMSP) technique was used to examine the degree of DNA methylation in CpG islands. It was discovered that genomic DNA included methylation and unmethylated forms of E-cadherin/STAT5a, respectively. The cycling protocol was programmed for the following optimized cycles, as given in (Table 2) and the Quantitative HRM real-time PCR components mentioned in (Table 3).

Table (2): The thermal profile of HRM-PCR for detection methylation level (STAT5a/E-Cadherin).

Cycle step	Temperature	Time	Cycles
Initial activation	94 ºC	12 minute	1
Denaturation	94 ºC	15 seconds	40
Annealing	54 ºC	20 seconds	
Elongation	72 ºC	20 seconds	
Melting temp	60-90 ºC		1

Table (3): Quantitative HRM real-time PCR components.

Component	Volume
Eva Green HRM-PCR Mix	4µl
Primer Forward-(10 µM)	0.5 µl
Primer Reverse-(10 µM)	0.5 µl
DNA template	5 µl
H2O	10 µl
Total	20 µl

Statistical analysis

The Analysis of Statistics to find out how various factors affected the research parameters, the IBM SPSS Statistics 26 application was utilized. Results were compared using the T-Test, Pearson correlation, spearman correlation test. The data are reported as means ± standard deviation (SD), and statistical significance was determined at a threshold of $p < 0.05$ when comparing with the control group. Cut-off points, Sensitivity, specificity, area under the curve and confidence interval curve was also utilized (14).

Result and discussion

HRM-PCR analysis for studied genes presented in percentages graduated from 100% methylation to unmethylation explained by colors in HRM-PCR Figures (1,2).

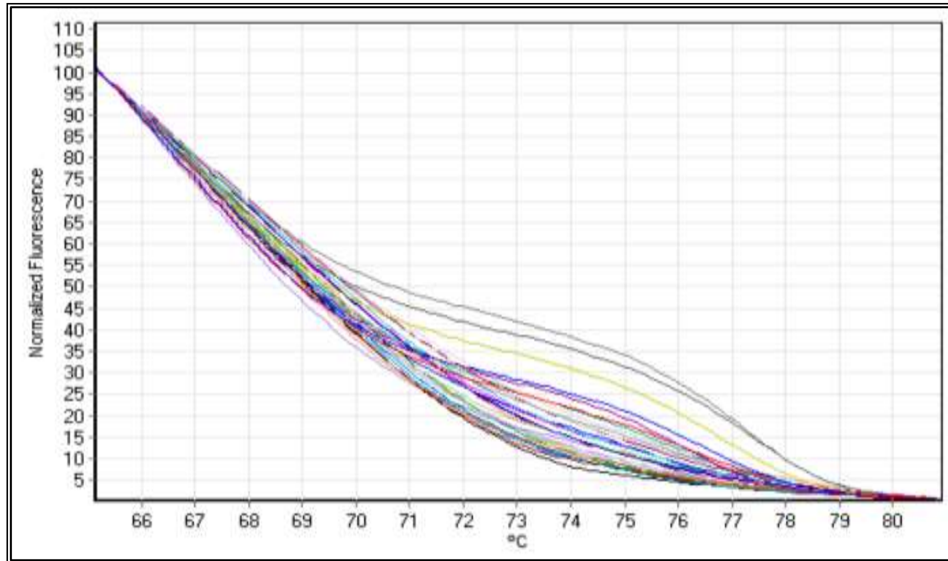

Figure (1): Graph of DNA Methylation by HRM-PCR Software Gene Using qPCR Technique for STAT5a Gene

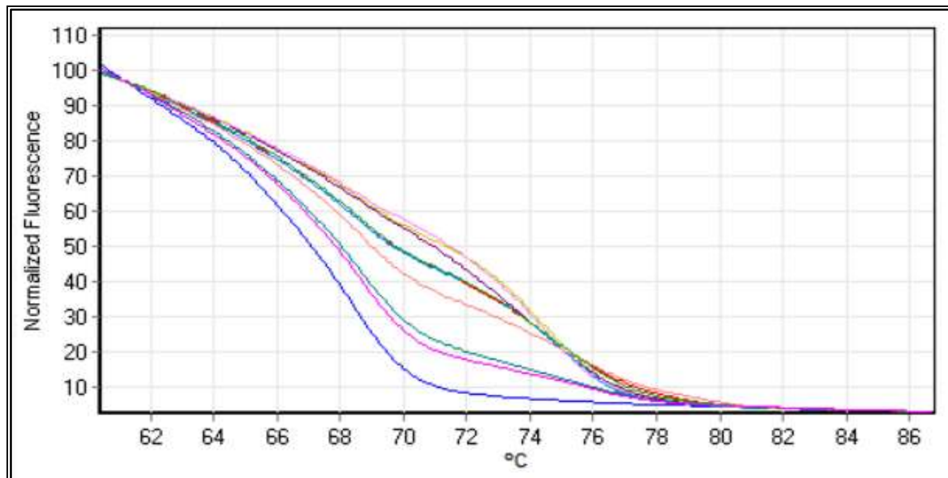

Figure (2): Graph of DNA Methylation by HRM-PCR Software Gene Using qPCR Technique for E-Cadherin Gene.

The DNA methylation levels of STAT5a were markedly reduced in OLP compared to the control group ($p < 0.05$). Conversely, the methylation level of E-Cadherin in OLP was dramatically elevated compared to OLP $p < 0.05$, (Table 4).

Table (4): Testing differences between methylation levels of studied gene and studied groups.

Studied Genes	Groups	No.	Mean	SD	Std. E.	t-test	
						t-value	Sig.
Stat5a	OLP	30 (50%)	25.50	14.38	2.62	-17.02	0.001
	Control	30 (50%)	82.03	11.12	2.03		
E-Cadherin	OLP	30 (50%)	75.78	13.60	2.48	12.86	0.001
	Control	30 (50%)	26.01	16.25	2.96		

No.: Number , SD: Standard deviation , SE: standard error , sig: significant , t-test : ratio of the difference between the mean of the two sample sets.

The methylation levels of studied genes were association with the clinicopathological findings of each patient. Regarding the age of OLP, The Person correlation for age of the study group with the methylation level of studied genes was described in (Table 5).

Table (5): person correlation between the age of OLP group with the methylation level of the studied group.

Studied gene	Pearson correlation	P value
STAT5a	-0.40	0.02
E-Cadherin	0.036	0.04

Pearson correlation: measures the strength and direction of the relationship between two variable.

The age of individuals showed a strong negative relationship with the methylation level of the STAT5a gene (r: -0.40, p: 0.02). Conversely, there was a significant positive association between the methylation level of the E-Cadherin gene and age (r: 0.03, p: 0.04).

The current findings indicate that there was no statistically significant difference ($p < 0.05$) observed in the methylation level of the examined gene between the gender groups of OLP. This is supported by the data presented in (Table 6).

Table (6): Analytic statistics of gender groups according to methylation level of studies genes.

Studied Gene	Classes	No.	Mean	SD	Std. E.	t-test	
						t-value	Sig.
STAT5a	Male	10 (33%)	19.98	10.89	3.44	-1.518	0.14
	Female	20 (66%)	28.26	15.35	3.43		
E-Cadherin	Male	10 (33%)	80.02	9.94	3.14	1.219	0.23
	Female	20 (66%)	73.66	14.87	3.32		

No.: Number, SD: Standard deviation, SE: standard error, sig: significant, t-test: ratio of the difference between the mean of the two sample sets.

In field of relation between clinical types of OLP and the methylation level of the studied gene as shown in (Table 7) which illustrates a significant difference between them.

Table (7): Analytic statistics of types groups according to methylation level of studies genes

Studied Gene	Type	No.	Mean	SD	Std. E.	t-test	
						t-value	Sig.
STAT5a	Erosive	15 (50%)	18.07	12.20	3.15	-3.26	0.03
	Reticular	15 (50%)	32.93	12.72	3.28		
E-Cadherin	Erosive	15 (50%)	81.39	12.47	3.22	2.44	0.02
	Reticular	15 (50%)	70.17	12.65	3.26		

No.: Number, SD: Standard deviation, SE: standard error, sig: significant, t-test: ratio of the difference between the mean of the two sample sets.

Semi quantitative scale of inflammation was used to assess the inflammation score of each OLP sample, the association between the Inflammation score of OLP samples and the methylation level of the studied genes revealed a significant association between them at a p-value (0.001) (Table 8).

Table (8): spearman correlation between the inflammatory score of OLP and methylation level of studied gene.

Studied gene	Spearman correlation	P value
STAT5a	-0.81	0.001
E-Cadherin	0.75	0.001

Spearman correlation: measures the strength and direction of association between two ranked variables.

Table (9) illustrate that the AUC for the studied gene was excellent in differentiation between groups with significant differences that AUC ranged from 0.95 to 0.98, the validity of the preceding markers in predicting a diagnosis of OLP was significantly different ($P< 0.05$).

Table (9): cut-off points, Sensitivity, specificity, area under the curve, and confidence interval of studied genes.

Studied genes	Cutoff Point	Sen.	Spec.	Area	Std. Error	Sig.	Asymp. 95% C.I.	
							L.b.	U.b.
STAT5a	47.65	0.93	0.97	0.95	0.03	0.00	0.88	1.02
E-cadherin	51.80	1.00	0.97	0.98	0.01	0.00	0.96	1.01

Sen.: sensitivity, Spec.: Specificity, Std error: standard error, Sig.: Significant, C.I: confident interval, L.b: Lower border U.b: upper border.

The interplay of genetic and environmental variables determines the likelihood of acquiring immune-mediated illnesses. Environmental changes are converted into long-lasting changes in gene expression by epigenetic processes of gene control (15). Despite the fact that one of the most researched and fully recognized epigenetic processes is DNA methylation (7,16), Few information is available on how methylation affects OLP's immune response regulation.

Epigenetic modification, namely mutational CpG methylation patterns, which silence tumour suppressor genes and/or activate oncogenes, is one of the initial molecular changes in the progression of oral illnesses (17).

The disease group samples in this study were FFEP tissue which has methylation profiles that may vary from those seen in blood or saliva because methylation patterns are tissue-specific (18, 19).

Frequent methylation of the *STAT5a* gene in OLP has been reported (20), wherein the STAT5a gene methylation level in OLP patients was found to be lower than that in healthy subjects, these findings are similar to those observed in the present study.

Previous research demonstrates that tumor growth is accelerated by E-cadherin hypermethylation, which is followed by a decrease in the protein's expression levels(21). Abnormal E-cadherin expression, which has been connected to malignancy in OLP, may be carried on by hypermethylation (22). Oral diseases, such as oral SCC and OLP, have been observed to feature E-cadherin that is hypermethylated (12). Hence, the possibility that abnormal E-cadherin expression contributes to the etiology of OLP should not be discounted.

To the authors' knowledge, the current study is the 1st molecular study interested in studying epigenetic change of OLP in Iraq and aimed to detect the methylation level of the STAT5a gene and E-Cadherin by using Formalin-fixed paraffin-embedded FFPE tissue of

OLP Iraqi patients and to compare their effect with that of the apparently healthy group.

In the analysis of risk factors linked to gene promoter methylation, our finding revealed a significant correlation was detected between the methylation level of the studied gene and the patient's age, Being older implies being exposed to environmental influences for a longer period of time and may lead to a different lifestyle, both of which might affect methylation patterns and raise the chance of developing age-related diseases in agreement with (23).

Despite several studies documented on the Arab population on the autosomes documented that more sites are highly methylated in females compared to males (24, 25), however, the present study has been demonstrated A non-significant difference regarding the link between methylation level of studied gene and sex groups.

OLP is recognized as an oral potential malignant condition, and the erosive type of OLP is thought to have a greater concretization rate (3). Our current study found that the methylation level of stat5a in erosive OLP was significantly lower than that in the reticular group, in contrast to the E-cadherin gene, which had significantly higher methylation than the reticular group in agreement with. This suggested that the degree of DNA methylation may be an indicator of how severe OLP is. The DNA methylation level may be used for early detection of OSCC in agreement with (12, 20).

Regarding the correlation between inflammation score of lesion and methylation level of studied genes the current study found there was a significant correlation between these two variables this in accordance with Shridhar (13), who identified DNA methylation as a potential biomarker of disease activity in OLP patients. And with another study connected by Sánchez-Siles (26), utilized methylation level of promotor region as prognosis marker to detect the severity of inflammation in erosive/atrophic OLP.

Conclusion

Aberrant DNA methylation patterns of *STAT5a* and *E-Cadherin* promoter regions resulted in the altered expression levels of these genes, which might be associated with the etiological mechanism of OLP. Patients with erosive OLP have more aberrant methylation levels than those with reticular form therefore we can consider the methylation level of studied genes as a potential biomarker to estimate the activity of OLP.

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