Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Epigenetic and genetic events of oral squamous cell carcinoma: perspective on DNA methylation, silencing of tumor suppressor gene, and activating oncogenes

Zainab Nizar Jawad*

Department of Biology, College of Education for Pure Sciences, University of Kerbala, Kerbala, Iraq

Article Info

Article history:

Received: April 09, 2025
Accepted: July 11, 2025
Published: September 30, 2025

Use your device to scan and read the article online

Abstract

Oral squamous cell carcinoma (OSCC) is a major health burden in most parts of the world, and pathogenesis of the disease is strongly associated with a complex combination between genetic mutations and epigenetic alterations. The current study will explore the DNA methylation phenomenon and how it affects the silencing of tumour suppressor genes and activate oncogenes in OSCC in a bid to explain the driving molecular process behind tumour development. We evaluated 50 samples of the OSCC tissues with 50 of the adjacent normal tissues, evaluated DNA methylation patterns with methylation-specific PCR and quantitative reverse-transcriptase PCR measured the expression of the DNA repair genes like BRCA1 and MLH1, among others. This finding showed different levels of DNA methylation of cancerous and normal tissues with hypermethylation causing the inactivation of important tumor suppressor genes and hypomethylation causing the activation of oncogenes. In addition, the downregulation of DNA repair genes was noted to be highly significant in OSCC samples indicating that genomic-instability may be related to epigenetic changes. These results demonstrate that aberrant DNA methylation is central to OSCC growth and progression, thus helping in the future use of methylation patterns that can be used as early detection, diagnosis, and prognostic biomarkers. Our findings support the idea that genetic, along with epigenetic, profiling is an issue of key importance toward comprehending OSCC biology and personalized therapeutic interventions. It is recommended that further confirmation be carried out to verify the clinical significance of such epigenetic markers in bigger cohorts, as well as testing such markers in guided therapies, which might eventually lead to better patient outcomes in the management of oral cancer.

Keywords: Oral cancer, OSCC, DNA methylation, Tumor suppressor genes, Oncogenes, Biomarkers, Epigenetics, Health problem.

1. Introduction

OSCC is the major form of oral cancer. Oral cancer is found to be one of the significant public health concerns globally and accounts for a significant percentage of all cancer cases [1]. Though there have been tremendous advancements in the field of medical science and treatment strategies, survival of oral cancer remains pitifully low because most of the times it is diagnosed late and due to aggressive nature of the disease [2]. The etiology of OSCC is multidetermined, as there are factors that go either related to genetic predisposition or environmental etiology, like smoking, alcoholism, and infection with human papillomavirus [3]. While these extrinsic factors have been a long-documented fact of the disease, the role of cells has been identified to be increasingly important, from a genetic and epigenetic change concerning oral cancer initiation and progression [4].

The introduction of crucial oncogenes and the tumor suppressor genes has remained central in the pathogenesis of many cancers, including OSCC [5]. These mutations of critical genes such as TP53, CDKN2A, and EGFR can thus cause disruptions in normal cellular processes resulting in uncontrolled cell growth, avoidance of apoptosis, and promotion of tumor growth [6]. However, OSCC causation cannot be subjected to genetic mutations only.

Epigenetic modifications have been strongly associated with the regulation of gene expression and the development of cancer, including through DNA methylation [7]. Epigenetic changes are noteworthy because they do not alter the sequence of the DNA but can affect gene activity to a large extent, including genes involved in tumor suppression and oncogenesis [8].

One of the most actively studied epigenetic mechanisms in cancer is called DNA methylation. Aberrant methylation is a characteristic of the OSCC; it is considered aberrant with hypomethylation of oncogenes and hypermethylation of tumor suppressor genes to result to overexpression and silencing of the respective genes [9]. This abnormal gene expression plays a vital role in malignant transformation of oral epithelial. As an example, excessive expression of oncogenes like CDKN2A (p16) through which cell cycle is regulated leads to unconditional proliferation of cells. On the other hand, hypomethylation of oncogenes is said to influence them to produce their products hence leading

* Corresponding author.
E-mail address: zainab.n@uokerbala.edu.iq (Z. N. Jawad).
Doi: http://dx.doi.org/10.14715/cmb/2025.71.9.12

to growth and metastasis of the tumor. Not only all of these epigenetic alterations are itr problem that causes the progression of OSCC but also it might be an opportunity in developing an early diagnosis and patient-specific treatment [10].

Thus, it is hard to decide whether the molecular profile of oral cancer arises from mutations at the genetic and epigenetic levels [11]. For example, hypermethylation of DNA repair genes results in their silencing, with a resultant genetic instability accelerating the rate of accumulation of mutations that further supports the process of carcinogenesis [12]. A causal relation between genetics and epigenetics has been established; thus, a mutation in the genome may facilitate changes in the epigenetic landscape, culminating in a feedback loop that supports tumorigenesis [13]. An understanding of such intricate relationships between genetic and epigenetic factors would be, therefore, crucial for developing novel diagnostic markers and targeted therapies against OSCC.

Despite a significant deal of knowledge regarding molecular and epigenetic mechanisms leading to OSCC, more research is yet to be done in this respect. For example, although Ali et al. (2017) [14] and Singh et al. (2016) [15], among others, have succeeded in identifying some of the most critical genetic mutations as well as epigenetic alterations associated with OSCC, an urgent demand still exists for these analyses to be broadened and integrated with each other in search for greater understanding of the interplay of these genetic and epigenetic factors. Especially, the latest experiments have typically been done on isolated genes or pathways and fail to describe the comprehensive ways in which these factors affect OSCC progressions and treatments. Despite this, however, although Mesgari et al. (2023) [16] highlights the therapeutic potential of epigenetic modifiers, further, highly precise, and comprehensive studies regarding the safety and efficacy of these novel therapies at clinical stages are essential. Moreover, the role of microRNAs and metabolomic changes in OSCC as cited by Gintoni et al. (2023) [17] and Gupta et al. (2024) [18] require a deep study to understand how such factors interact with changes at the genetic and epigenetic levels that would affect the outcome of the disease. Last but not least, there is a huge research gap in epigenetic markers that would improve early detection and personalized OSCC treatment. It can assist in filling such gaps to establish a better picture of the OSCC pathogenesis, besides formulating a more productive approach to diagnostics and treatment.

The research was conducted to bring light on the possible DNA methylation occurrences that cause and develop oral cancer. An improvement in the understanding of the cause of oral cancer was achieved through examination of the DNA repair gene expression in tissues of OSCCs and the methylation of tumor suppressor and oncogenes. These results in the study added to our knowledge about the use of DNA methylation as a biomarker of OSCC detection, diagnosis and patient prognosis, and possibly the development of a patient specific treatment plan based upon genetic and epigenetic pat^{r}erns.

2. Materials and methods

2.1. Study design

The current research was a cross-sectional study grounded on OSCC, which was aimed at studying genetic and epigenetic changes of this type of head and neck cancer. The study design was of an observational one, which allowed to assess different types of molecular changes in a specified population at one character time. In particular, it dealt with the comparison of the DNA methylation patterns and DNA repair genes expression level in OSCC and normal tissues. The strategy has been used to explain the variations in the molecular signatures between cancerous and non-cancerous tissues in an attempt to gain more knowledge about OSCC pathogenesis and identification of prospective biomarkers to be used in the diagnosis and prognosis of OSCC.

2.2. Sample collection

The total number of the tissue samples gathered was 100 composed of OSCC of 50 and the adjacent normal tissue samples of 50. These samples were collected in patients who had surgical resection due to OSCC in one of the participating medical institutions. The recruitment of patients was organized among the patients who were planned to pass surgical intervention, and all of them were diagnosed with OSCC and made a corrective decision. Patients that were excluded were those that previously underwent chemotherapy or even radiotherapy before the surgery because this would confound the molecular analysis. This was in a study which was cleared by the board reviewing it and all the people involved in the study were made aware of the purpose of the study and how it would be conducted and the risks that may be encountered during the study. The tissue samples that had to be removed during the surgery were no sooner dumped in sterile containers and then they got frozen to -80 o C to ensure that the nucleic acids were not degraded in any case and also to keep the integrity of samples as it was so that they could be used later during the analyses.

The target population included 100 people made up of 50 cases, who had OSCC and 50 controls who were healthy persons. Both tumor tissues and adjacent non-cancerous tissues were successfully obtained from OSCC patients, enabling direct comparison between cancerous and healthy tissues within the same individuals. All participants provided written informed consent, and the study adhered to institutional ethical guidelines.

2.3. DNA extraction and quantification

A commercial DNA extraction kit, such as the Qiagen DNeasy Blood & Tissue Kit, was utilized for efficient isolation of high-quality DNA from various tissue types. For sample preparation, thawed tissues were homogenized in a buffer solution to ensure complete cell lysis and DNA release. DNA extraction was performed according to the kit protocol, using a silica membrane-based method to remove most cellular debris through cell lysis, protein removal, and subsequent binding of DNA to the membrane. The concentration and purity of the extracted DNA were assessed using a NanoDrop 2000 spectrophotometer by measuring absorbance at 260 nm and 280 nm, providing a ratio for determining protein contamination and confirming that sufficient DNA yield was obtained for downstream applications. DNA recovered was aliquoted followed by storing at -20 o C and subjected to no repeated freezing and thawing processes and kept at constant conditions prior to analysis.

2.4. DNA methylation measurement and Bisulfite conversion

A set of steps was conducted to assess the status of DNA methylation as an essential epigenetic modification associated with regulation of the gene. The Zymo Research EZ DNA Methylation Kit was used to convert bisulfite the isolated DNA. In the process, the unmethylated cytosines were transformed to uracils, and the methylated cytosines were left unchanged, and conditions of the reaction were optimized so that the conversion rate to uracil is complete. Then, methylation specific PCR was carried out on the bisulfite-converted DNA to evaluate methylation of the specific CpG sites within the promoter regions of the tumor suppressor genes including p 16. This method applied distinguished between the methylated and unmethylated DNA by the amplification of the bisulfite-treated DNA using primers unique to the state of methylation. To find out the differentially methylated loci between the OSCC and the normal tissues, the MSP results were analyzed especially looking into the genes that have a role in the progression of cancers.

2.5. Gene expression analysis

Quantitative real-time PCR (qRT-PCR) was used to compare the expression of DNA repair-related genes like BRCA1 and MLH1 in OSCC tissue samples against the ones in normal tissues. Tissue samples were used to extract total RNA using an RNA extraction kit, most preferably Qiagen RNeasy Kit, and the quality of the extracted RNA verified using Agilent Bioanalyser to ascertain the integrity as well as the absence of contaminants of the extracted RNA. cDNA was synthesized using reverse transcriptase enzyme in RNA samples following the manufacturer guideline to ensure complete conversion. qRT-PCR showed target gene expression levels specifically based on prime factor expression of BRCA1, MLH1 and other causative genes. Experimental validation of each sample was done in triplicate to achieve reproducibility and accuracy, and data is normalized against housekeeping genes for minimum variations in RNA quantity (GAPDH). The 5 difference in Ct (Delta Delta Ct) method was used to determine relative gene expression levels and statistical analyses computed to compare gene expression in OSCC and normal tissue groups.

A bar plot created via Python was used to visually represent the relative expression of DNA repair genes (BRCA1, MLH1 and p16) in OSCC and normal tissues.

2.6. Data Analysis

The data on genes expression and DNA methylation were analyzed with the help of statistical software (e.g. SPSS or R). The comparison of the relevant values was carried out with the performance of appropriate statistics tests (t-tests and non-parametric tests according to the data distribution). The significance of differences in patterns of DNA methylation and levels of gene expression was determined as p-values. Correlation analysis was done to identify the relation between the changes in DNA methylation and the changes in gene expression on whether the changes in the epigenetic alteration were related to change in gene activity in OSCC. Heatmaps, box plots and scatter plots have been used to visualize graph and charts illustrating the variance of OSCC tissues and normal tissues. Interpretation of the findings was brought up in relation to the existing literature to emphasize the mutations that are significant to OSCC and also to find out the possible biomarkers that could be used to help in early detection and treatment by the use of specific therapies.

3. Results

3.1. DNA methylation patterns

DNA methylation analysis was conducted to compare the methylation status of key tumor suppressor and oncogene promoters between OSCC tissues and matched normal tissue samples. The results, presented in Table 1, show the quantified methylation levels of genes implicated in tumor suppression and oncogenesis.

The results of the data in Table 1 demonstrated a high and significant elevation of methylation of the tumor suppressor genes in the OSCC tissues. That includes changes in p16 (CDKN2A), which elevated from 10.5% in the normal tissue to 78.4% in OSCC, MLH1, from 12.7% to 65.2%, and BRCA1 from 8.9% to 55.3%, with all modifications at p-values <0.001. This massive hypermethylation of the above OSCC tissues implies a strong silencing effect on these critical tumor suppressor genes, which goes well with the fact that this mode of gene methylation interferes with normal cellular regulation, leading to tumor development. The noted high significance levels for all of these genes establish the reliability of the findings, suggesting that hypermethylation of these genes is indeed an important feature of OSCC pathology.

3.1.1. Gene expression analysis

The relative expression levels of DNA repair genes (BRCA1, MLH1, and p16) in OSCC and normal tissues were visualized using a bar plot generated with Python.

The measurement of the expression of genes involved in DNA repair was achieved by comparing the relative expression of DNA repair genes in normal tissues with OSCC tissues by gene expression analysis. The expression levels of key genes involved in DNA repair were compared between the two tissues in Figure 1. This plot shows the downregulation of these genes on OSCC samples as compared to normal tissues. This graph vividly shows the down regulation of these genes in OSCC as opposed to normal tissues.

It was found that p16 (CDKN2A), MLH1, BRCA1) tumor suppressor genes had strong hypermethylation in OSCC tissues than in normal tissues, and this effect also

Table 1. DNA methylation levels in OSCC and normal tissues.

Gene	Normal Tissue (Mean ± SD)	OSCC Tissue (Mean ± SD)	p-value
p16 (CDKN2A)	10.5 ± 1.2%	78.4 ± 4.5%	<0.001
MLH1	12.7 ± 1.8%	65.2 ± 3.9%	<0.001
BRCA1	8.9 ± 1.1%	55.3 ± 3.4%	<0.001

P-values less than 0.05 were considered statistically significant.

led to the distinct downregulation of the expression. All these results highlight the influence of epigenetic alterations in gene silencing and tumor progression of oral squamous cell carcinoma (Table 1 and Figure 1).

Other notable results of the study were hypermethylation of promoters of tumour suppressor genes, including p16(CDKN2A) (Table 1). The normal/healthy tissues showed the lowest percentage of methylation in all these genes compared with OSCC tissues where the accumulation of all the four corporates was encountered which led to the silencing of the gene and this resulted in the loss of tumor-supression properties. The same tendency was with other tumor suppressor genes, such as MLH1 and BRCA1, which further proved the fact that epigenetic changes play a role in developing and progressing OSCC.

Table 2 cross-matched experiments on the hypomethylation of oncogenes in OSCC tissues with normal tissues. Such demethylation in the regulatory regions resulted in deregulation of oncogenes that can result in enhanced proliferation, invasion, and metastasizing of tumor cells.

The expression of major DNA repairing genes such as BRCA1 and MLH 1 were highly down regulated in OSCC tissues as compared to normal tissues (Table 2 and Figure 1). This decrease in gene activity indicates that there might be a defective DNA DNA repair pathway of cancer cells most probably through promoter hypermethylation, and this might lead to genomic instability as well as tumor development.

3.2. Bisulfite conversion and DNA methylation analysis

The bisulfite conversion was carried out to ascertain the difference between the unmethylated and methylated cytosines with the unmethylated Cys converting to uracil and the set Cys not being converted. This allowed a proper determination of the degree of methylation of CpG islands in the genome DNA. Thereafter, methylation-specific PCR (MSP) was used in the study of methylation level of critical tumor suppressive genes such as p16 (CDKN2A) and MLH1, which are frequently with tumor changes in oral cancer. As seen in the results section, there were very precise differences in the methylations of OSCC and normal tissues indicating a considerable hypermethylation in the promoter regions of these tumor suppressor genes on the OSCC samples.

3.2.1. Gene expression and DNA methylation correlation

Analysis of gene expression of the DNA repair genes, e.g. BRCA1 and MLH1 showed that they were highly down-regulated in OSCC tissues than in normal tissues used as controls. Total RNA was isolated and reverse-transcribed and quantitative real-time reverse transcription PCR was done using GAPDH as internal control. Using statistical analysis using R and SPSS, BRCA1 and MLH1 had strong down regulation in OSCC samples ($p < 0.05$).

In addition, DNA methylation pattern comparison revealed massive hypermethylation of tumor suppressor genes promoters in OSCC tissues compared to unmethylation of most tumor suppressor genes in the normal ones,

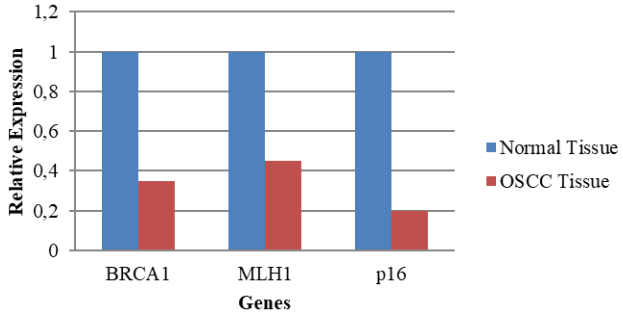

Fig. 1. Relative gene expression levels in OSCC and normal tissues.

including p16 (CDKN2A), and MLH1. The differences were statistically significant ($p < 0.05$) as was shown by T-tests.

Correlation analysis indicated a strong inverse relationship between promoter methylation and gene expression levels, suggesting that epigenetic silencing through hypermethylation contributes to the reduced expression of key DNA repair genes in OSCC.

Significantly higher methylation levels were observed in the promoter regions of tumor suppressor genes p16 (CDKN2A) and MLH1 in OSCC tissues compared to normal tissues, as shown in Table 2.

In OSCC samples, p16 and MLH1 displayed hypermethylation rates greater than 70%, whereas their methylation stood below 15% in normal tissues, indicating a stark contrast. The strongly significant p-values for all three fall all below 0.001, which stresses that the observation is extremely robust.

The relative OSCC tissues had shown a marked downregulation of DNA repair genes, especially BRCA1 and MLH1, in comparison to their normal tissues. Downregulation of the said genes may be attributed to hypermethylation present in their promoter regions.

The qRT-PCR analysis revealed a significant downregulation of these genes in OSCC tissues. Analysis of gene expression revealed a reduction of approximately 65% for BRCA1 and about 60% for MLH1 expression in OSCC (Figure 2).

Major findings of the study included hypermethylation of tumor suppressor genes, most notably p16 (CDKN2A),

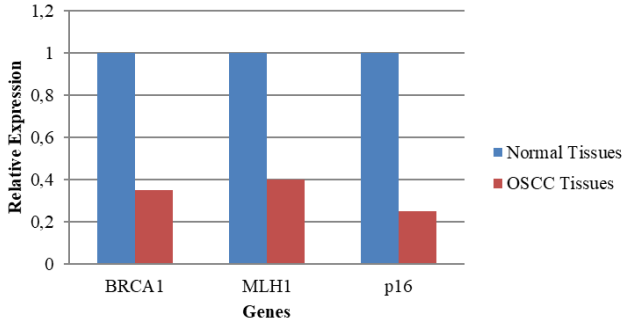

Fig. 2. Outcome visual for gene expression levels in normal Vs OSCC tissues

Table 2. DNA methylation levels in tumor suppressor genes.

Gene	Normal Tissue (Mean ± SD)	OSCC Tissue (Mean ± SD)	p-value
p16 (CDKN2A)	11.3 ± 1.5%	76.8 ± 5.1%	<0.001
MLH1	13.2 ± 1.9%	69.5 ± 4.2%	<0.001

in OSCC tissues. On the other hand, some oncogenes were found to be hypomethylated in OSCC tissues.

Significant downregulation of DNA repair genes, such as BRCA1 and MLH1, was observed in OSCC tissues. DNA repair genes ensure the integrity of the genome by the degradation of damaged DNA and the absence of accumulation of deleterious mutations. These genes are highly active in the cells since some DNA damage is inevitably occurring, which is then correctly repaired.

However, the expression levels of BRCA1 and MLH1 in OSCC tissues were much lower than in the controls. This would indicate that there was a weakened DNA repair machinery within cancer cells, which would provide susceptibility to the accumulation of mutations. Loss of DNA repair machinery would most likely contribute to genomic instability, allowing malignant cell behavior through additional mutations.

These findings point to the significance of DNA methylation patterns in molecular OSCC pathogenesis and open avenues for therapeutic strategies targeted at reversing such epigenetic changes. Understanding the mechanisms involved in such alterations may enable researchers to take steps toward reactivating dormant tumor suppressor genes or suppressing overexpressed oncogenes, thus promising targeted cancer therapies.

3.3. Expanded DNA methylation analysis

To gain a more comprehensive understanding of the methylation landscape in OSCC, we extended our analysis to include additional tumor suppressor genes (TP53, RB1, and APC) and oncogenes (MYC and RAS). The methylation levels of these genes were compared between OSCC and normal tissues. As shown in Table 3, OSCC tissues exhibited significant hypermethylation in the promoter regions of tumor suppressor genes and notable hypomethylation in oncogenes compared to normal controls. These findings further highlight the widespread epigenetic alterations associated with OSCC.

Table 3 presents the DNA methylation levels of key tumor suppressor genes and oncogenes in normal and OSCC tissues. Notably, both p16 (CDKN2A) and TP53 exhibited marked hypermethylation in OSCC tissues, with methylation levels increasing from 12.1% and 9.8% in normal tissues to 82.3% and 75.2% in OSCC tissues, respectively ($p < 0.001$ for both), indicating highly significant hypermethylation in these genes.

The widespread hypermethylation observed in tumor suppressor genes suggests their transcriptional silencing, which may facilitate uncontrolled cell proliferation and contribute to OSCC progression. In contrast, the oncogenes MYC and RAS exhibited significantly lower methylation levels in OSCC tissues. This hypomethylation is associated with increased gene expression, as demonstrated by the elevated expression levels of MYC and RAS in OSCC compared to normal tissues (Table 3; p-values = 0.003 and 0.001, respectively). Overall, these findings indicate a pattern in which silencing of tumor suppressor genes and activation of oncogenes, mediated by altered methylation and expression profiles, play a critical role in the development and progression of OSCC.

Table 4 demonstrates that the expression levels of key DNA repair genes—including BRCA1, MLH1, XRCC1, ERCC1, and MGMT—are significantly downregulated in OSCC tissues compared to normal tissues. The reduction in gene expression ranges from 47% to 70%, with BRCA1 and MGMT showing the greatest decreases at 66% and 70%, respectively. All observed differences are statistically significant ($p < 0.001$). These findings indicate a substantial impairment of DNA repair mechanisms in OSCC, which may contribute to increased genomic instability and promote tumor development and progression.

To help show the major molecular aberrations occur in the oral squamous cell carcinoma (OSCC), a thorough comparison on DNA methylation levels and the gene expression profile within the OSCC tissues and the normal tissues was shown in Figure 3.

Tumor Suppressor Genes: In OSCC tissues, several genes that are cancer inhibitors like p16 (CDKN2A), TP53, RB1, and APChave high level of methylation than in the normal tissues. This hypermethylation is characteristic of an epigenetic silencing and possible interference with the orderly regulations of cells by aberrant growths and eventual malignancies (Figure 3 left).

Oncogenes: On the other hand, the oncogene such as, MYC and RAS are less methylated in OSCC tissues. It is a hypomethylation and is accompanied by enhanced gene

Table 3. DNA methylation levels in tumor suppressor and oncogenes in OSCC vs normal tissues

Gene	Normal Tissue (Mean ± SD)	OSCC Tissue (Mean ± SD)	p-value
p16 (CDKN2A)	12.1 ± 2.2%	82.3 ± 6.5%	<0.001
TP53	9.8 ± 1.4%	75.2 ± 5.8%	<0.001
RB1	8.5 ± 1.6%	69.5 ± 4.9%	<0.001
APC	7.2 ± 1.3%	58.9 ± 5.2%	<0.001
MYC	14.5 ± 2.7%	22.3 ± 3.1%	0.003
RAS	13.6 ± 2.4%	24.9 ± 2.8%	0.001

Table 4. Gene expression right OSCC-normal.

Gene	Normal Tissue (Relative Expression)	OSCC Tissue (Relative Expression)	Fold Change (OSCC/Normal)	p-value
BRCA1	1.00 ± 0.05	0.34 ± 0.04	-66%	<0.001
MLH1	1.00 ± 0.05	0.39 ± 0.05	-61%	<0.001
XRCC1	1.00 ± 0.06	0.45 ± 0.06	-55%	<0.001
ERCC1	1.00 ± 0.07	0.53 ± 0.06	-47%	<0.001
MGMT	1.00 ± 0.08	0.30 ± 0.04	-70%	<0.001

activation, which stimulates cellular proliferation and tumor development (Figure 3 left).

Downregulation in OSCC: Critical DNA repair genes (BRCA1, MLH1, XRCC1, ERCC1, and MGMT) were significantly downregulated in tissues of OSCC when compared with the normal tissues. This downregulation is indicative of compromised ability to repair its own DNA and in turn, this could result in genomic instability and promote accretion of mutations leading to development of cancers (Figure 3 right).

This was among others in tumor suppression genes and on gene oncogenes (TP53, RB1, and APC, and MYC, RAS), and it is on this basis that further understanding of epigenetic changes in Oral Squamous Cell Carcinoma could be done. This study established that the DNA methylation profile between normal and OSCC tissues are highly different and therefore this once again justified the relevance of epigenetic altering in the pathogenesis and causation of OSCC.

Some important tumor suppressor genes, p16 (CDKN2A), TP53 and RB1 were significantly overmethylated. These genes regulate cell development and maintenance of cell cycle check points which avoid the development of tumor cells. Evidence from these studies has shown that hypermethylation has a propensity to predispose tumor suppressor genes to senescence subsequently doing away with the process of negative regulation of cell growth and carcinogenesis.

p16 (CDKN2A): The intensity of methylation in tumor tissues of OSCC reached 82.3 percentile with 12.1 percentile only in the normal tumors, thus, showing a high increment ($p < 0.001$). This hypermethylation might be the reason as to why this control failed to control the cell cycle which caused a perfect storm in the uncontrolled division of the cells.

TP53 and RB1: These other hypermethylated genes in OSCC tissues exhibited methylation percentages of 75.2 + 5.8 and 69.5 + 4.9, respectively, whereas in the control tissues, their percentage was very low; their methylation percentages were 9.8 + 1.4 and 8.5 + 1.6 respectively. TP53, the so-called guardian of the genome, silencing of this gene reduces apoptosis and poses a greater possibility of accumulation of mutations in the cancer cell.

APC: The percentage of methylation of the gene related to the binding of the cell and signal transduction APC rose in OSCC tissue (58.952) compared to normal tissue (7.213) ($p < 0.001$). Silencing due to methylation may affect cell biomechanical integrity and crosstalk with neighboring cells and thereby facilitate the propensity of the tumor to invade more easily and metastasize.

These findings confirm the notion that hypermethylation of tumor suppressor genes has a significant role in the development of OSCC, rendering able mechanisms, which would otherwise guard against malignant alterations, idled.

Hypomethylation of Myc and Ras oncogenes was also an outcome and the researchers felt that inadequate methylation reactivates oncogenes to promote cancer development. The oncogenes were found within the OSCC tissues to be less methylated deducingly compared to the normal tissues making them become overexpressed.

MYC: The level of methylation of MYC was also reduced in OSCC tissues (22.3(+/-3.1)%) as compared with normal tissues (14.5(+/-2.7)) with a very significant

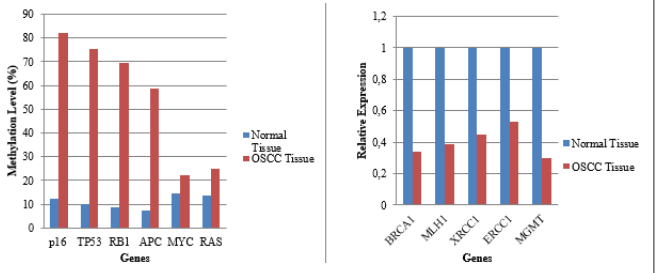

Fig. 3. Comparative Analysis of DNA Methylation and Gene Expression Profiles in OSCC and Normal Tissues. Left: Hypermethylation of Tumor suppressor activations and hypomethylation of oncogenes in OSCC; Right: Down regulation of expression of DNA repair genes in OSCC.

p-value of 0.003. MYC can play a significant role as a transcriptional regulator of cell growth, proliferation and metabolism, which are the same forces that spur tumor development.

RAS: Percentage of the methylation level of RAS gene in OSCC tissue was also found to have a comparable value of that in normal tissue i.e. 24.9 2.8 vs. 13.6 2.4 $p = 0.001$. RAS pathway is known to have frequently been linked to the proliferation, survival and invasive nature of OSCC and other malignancies.

The verdict that these oncogenes are hypomethylated denotes that they are overexpressed in OSCCs and improve the proliferative and aggressive potential of the cancer cells.

Moreover, the study demonstrated that tissues of OSCC showed decreased expression of some important DNA repair genes. This type of down regulation could suggest that cancer cells have defective means of repairing their DNA and are more susceptible to genetic instability that makes them susceptible to greater tumor development.

These DNA repair genes, BRCA1, MLH1, XRCC1, ERCC1 and MGMT were significantly downregulated in the OSCC tissue and the fold change was at significant level in BRCA1 and MGMT (down-regulated by 66 percent and 70 percent respectively). In the cases, the change of gene expression on fold was significant ($p < 0.001$ in all).

- **BRCA1:** It is involved in the fixation of double-strand DNA breaks, and in such cases, cells are left vulnerable to the accumulation of mutations leading to cancer progression.
- **MLH1:** It is an important part of the mismatch repair pathway, and low MLH1 expression severely compromises the ability of a cell to correct replication errors, thereby increasing mutation rates even further.
- **XRCC1:** plays a role in single-strand break repair, low levels of XRCC1 by 45% make for poor repair of oxidative stress and environmental factors, which are highly prevalent causes of DNA damage in oral cancers.
- **MGMT:** This gene repairs alkylated DNA, and down-regulation (70%) suggests that the OSCC cells lose their ability to repair alterations introduced by DNA alkylating agents, such as those shown by tobacco smoke, which is a significant risk factor for OSCC.

The inactivation of DNA-repair pathways in OSCC cells probably involves genomic instability in these cancers, which supports the generation of mutations related to the process of carcinogenesis.

4. Discussion

Consequently, this study has focused on the crucial role of genetic mutations and epigenetic alterations, especially DNA methylation, in the pathogenesis of OSCC. According to the findings, there were notably distinct patterns of methylation in both tumor suppressor genes and oncogenes. Additionally, there was a downregulation of DNA repair genes in OSCC tissues as compared to normal tissues. These changes in the landscape of molecules will be of great value as a source of information about the hitherto unseen mechanisms of oral cancer and could prove the gateway to potentially promising therapeutic interventions and diagnostic tools.

4.1. DNA methylation and tumor suppressor gene silencing

A particularly notable result was the pronounced hypermethylation of tumor suppressor genes—especially p16 (CDKN2A) and MLH1—in OSCC tissues. This epigenetic alteration was markedly higher in cancerous samples compared to normal tissues, highlighting the critical role of these genes' silencing in the development and progression of oral squamous cell carcinoma The variations in the methylation levels among these genes showed that the OSCC samples contained levels above 70%, while normal samples had less than 15%. Regulators, components involved in DNA repair and cell cycle control, and other essential genes may be silenced by this hypermethylation. Inactivation of the p16 gene promotes unchecked cell proliferation, a hallmark of cancer, because the gene controls the G1-S transition in the cell cycle. Similarly, the role of MLH1 in DNA mismatch repair is quite crucial, and the inactivation is very likely to result in an accumulation of genetic mutations that further promote tumorigenesis.

The mechanism by which these genes are hypermethylated has been generally known to be evasion of normal cellular checkpoints and repair mechanisms by cancer cells. In this respect, the present findings enhance the notion that epigenetic mechanisms, including DNA methylation, indeed represent early promoter events in OSCC pathogenesis. The involvement of genes in silencing through hypermethylation provides a pathway to malignant transformation by allowing cells to bypass growth control mechanisms and accumulate genetic alterations [19].

4.2. Hypomethylation of oncogenes

On the other hand, OSCC tissues exhibited hypomethylation of oncogenes, whereas hypermethylation was observed in the tumor suppressor genes. The fact fits well with previous studies [20, 21] that suggest global DNA hypomethylation is the most common feature of many cancers and is associated with chromosomal instability because it leads to the activation of oncogenes. The process of demethylation allows the oncogenes to be expressed with enhanced activity, thus promoting the proliferation, survival, and metastasis of cells. This pattern provides valuable insight into the regulation of gene expression during carcinogenesis, as it shows that oncogenes are hypomethylated and tumor suppressor genes are hypermethylated.

4.3. Downregulation of DNA repair genes

In contrast to normal tissues used as controls, OSCC tissues showed a marked decrease in the expression levels of BRCA1 and MLH1, two genes involved in DNA repair. Such downregulation corresponds to the promoter hypermethylation of these genes. Epigenetic silencing may, therefore, be responsible for the mechanism underlying DNA repair machinery disruption in cancerous cells. Both BRCA1, critical for homologous recombination repair, and MLH1, an important protein for mismatch repair, are important for genomic integrity. Inhibition of such genes may well contribute to the accumulation of mutations that appear to drive OSCC aggressiveness [22].

Consistent with earlier studies [23, 24] that established a direct relation between incompetent DNA repair mechanisms and cancer susceptibility, here, the downregulation of DNA repair genes in OSCC tissues supports this relationship. Low expression levels of BRCA1 and MLH1 further implicate OSCC cells as having impaired responsiveness to DNA damage, thereby increasing their susceptibility to changes in the genome that can facilitate tumorigenesis. In addition, the association of promoter hypermethylation with low gene expression makes epigenetic regulation important in the modulation of DNA repair pathways in cancer.

4.4. Implications for biomarkers and targeted therapy

Research objectives include the ability to evaluate DNA methylation as a biomarker for the early detection, diagnosis, and prognosis of OSCC. The marked difference in methylation pattern found between OSCC and normal tissues can be an important biomarker of cancerous tissues. The specific involvement of hypermethylation of p16 and MLH1 might help in early discrimination of OSCC and non-cancerous tissue, opening doors for early intervention and better outcomes [25].

DNA repair genes, such as BRCA1 and MLH1, whose malfunction is present in such cancers, are downregulated. These are opportunities for targeted therapies. Drugs that undo DNA methylation, such as DNMTi, could restore the expression of these genes and reactivate tumor suppressor pathways. Another type of treatment targeting the repair deficiencies in DNA is to use the PARP inhibitors and this would prove helpful to treat the cancers that have broken homologous recombination repair like mutant or broken BRCA1 [26].

4.5. Relationship between methylation and gene expression

In this correlation analysis, we found that an alternation of methylation pattern in OSCC tissues and levels of gene expression existed. The findings confirm the importance of DNA methylation in the expression of genes in cancer. Intense hypermethylation of BRCA1 and MLH1 is associated with down regulation of their expression, which has direct consequences on the capacity of the cells to repair the DNA damage. The complexity of this epigenetic control suggests that the regulation of genes in the pathogenesis of cancer becomes complex and further adds a possibility of the methylation pathway as an option of a therapeutic intervention [27].

Altogether, the findings of this research point strongly that DNA methylation and other genetic and epigenetic alterations play a role in OSCC pathogenesis. The findings related to having down expression of DNA repair gene, hypomethylation of oncogenes and hypermethylation of tumor suppressor genes are indicative of having positive interaction between complex molecular pathways in-

volved in the progression of oral malignancies. Detection of such molecular variations leads to the new pathways of detection at an early stage and formation of the personalized treatment strategies that should be based on genetic and epigenetic data of OSCC patients [28].

In the first table analysis, it brings into the picture a key correlation between abnormal pattern of DNA methylation and development of OSCC. Epigenetic aspect of DNA tumor suppressor silencing by hypermethylation and DNA repair gene repression bring new insights into the epigenetic picture of oral cancer. These findings indicate the potential of DNA methylation markers as a tool of diagnosis as well as prognosis in a clinical practice.

According to the table below (number 2), critical epigenetic changes in the OSCC tissues spotlight hypermethylation of tumor suppressor genes and down-regulation of the DNA-repair genes. The outcome of this study will present the potential future use of DNA methylation as a promising biomarker for possible early onset diagnosis and prognosis of oral cancer. Therefore, future studies should use larger cohorts and longitudinal data to check if these biomarkers are helpful in clinical settings.

A more detailed examination of aberrant DNA methylation reveals a strong association with OSCC. Notably, extensive hypermethylation-mediated silencing of tumor suppressor genes, alongside significant downregulation of DNA repair genes, underscores the central role of epigenetic alterations in the pathogenesis and persistence of oral cancer. Additionally, the observed hypomethylation of oncogenes such as MYC and RAS highlights the dynamic and complex nature of methylation changes in cancer progression. Collectively, these findings demonstrate that DNA methylation patterns hold considerable promise as biomarkers for early diagnosis and prognosis of OSCC.

Acknowledgments
The author would like to thank the Department of Biology, College of Education for Pure Sciences, University of Kerbala, for providing laboratory facilities and support. Special thanks to all participants and staff involved in sample collection and processing.

Declarations

Ethics approval and consent to participate
This study was conducted in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Ethical approval was at the University of Kerbala. All participants provided informed consent prior to the collection of tissue samples.

Consent for publication
All authors have reviewed and approved the final version of the manuscript and consent to its publication.

Availability of data and materials
The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

Funding
This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Authors' contributions
Zainab Nizar Jawad conceptualized and designed the study, performed the experiments, analyzed the data, and drafted the manuscript. All authors read and approved the final manuscript.

References

1. Aghiorghiesei O, Irimie AI, Braicu C, Raduly L, Nutu A, Balint E, Mehterov N, Vladimirov B, Sarafian V, Lucaciu O, Campian R, Berindan-Neagoe I (2023) Epigenetic methylation changes: implication as biomarkers in oral and maxillofacial area cancers. Med Pharm Rep 96 (3): 310-317. doi: 10.15386/mpr-2570
2. Alothman L, AlSenani M, Alrabiah R, Ras AA, Abulhassan E, Aldayel R, Almutairi R, Alsaif R (2020) Insights into epigenetics mechanisms in oral squamous cell carcinoma. Saudi J Oral Dent Res 5: 1297-2518. doi:
3. Cai L, Zhu H, Mou Q, Wong PY, Lan L, Ng CWK, Lei P, Cheung MK, Wang D, Wong EWY, Lau EHL, Yeung ZWC, Lai R, Meehan K, Fung S, Chan KCA, Lui VWY, Cheng ASL, Yu J, Chan PKS, Chan JYK, Chen Z (2024) Integrative analysis reveals associations between oral microbiota dysbiosis and host genetic and epigenetic aberrations in oral cavity squamous cell carcinoma. NPJ Biofilms Microbiomes 10 (1): 39. doi: 10.1038/s41522-024-00511-x
4. Chatterjee A, Singh A, Shanmugam G, Rakshit S, Mohanty LM, Kumar A, Harihar S, Sarkar K (2023) Neem leaf glycoprotein mediated epigenetic modification in oral squamous cell carcinoma. Phytomedicine Plus 3 (1): 100400. doi:
5. Cheng Y, He C, Wang M, Ma X, Mo F, Yang S, Han J, Wei X (2019) Targeting epigenetic regulators for cancer therapy: mechanisms and advances in clinical trials. Signal Transduct Target Ther 4: 62. doi: 10.1038/s41392-019-0095-0
6. Das D, Ghosh S, Maitra A, Biswas NK, Panda CK, Roy B, Sarin R, Majumder PP (2019) Epigenomic dysregulation-mediated alterations of key biological pathways and tumor immune evasion are hallmarks of gingivo-buccal oral cancer. Clin Epigenetics 11 (1): 178. doi: 10.1186/s13148-019-0782-2
7. Eljabo N, Nikolic N, Carkic J, Jelovac D, Lazarevic M, Tanic N, Milasin J (2018) Genetic and epigenetic alterations in the tumour, tumour margins, and normal buccal mucosa of patients with oral cancer. Int J Oral Maxillofac Surg 47 (8): 976-982. doi: 10.1016/j.ijom.2018.01.020
8. Emfietzoglou R, Pachymanolis E, Piperi C (2021) Impact of Epigenetic Alterations in the Development of Oral Diseases. Curr Med Chem 28 (6): 1091-1103. doi: 10.2174/0929867327666200114114802
9. Feller G, Khammissa RAG, Ballyram R, Beetge MM, Lemmer J, Feller L (2023) Tumour Genetic Heterogeneity in Relation to Oral Squamous Cell Carcinoma and Anti-Cancer Treatment. Int J Environ Res Public Health 20 (3). doi: 10.3390/ijerph20032392
10. Ghantous Y, Schussel JL, Brait M (2018) Tobacco and alcohol-induced epigenetic changes in oral carcinoma. Curr Opin Oncol 30 (3): 152-158. doi: 10.1097/cco.0000000000000444
11. Gillison ML, Akagi K, Xiao W, Jiang B, Pickard RKL, Li J, Swanson BJ, Agrawal AD, Zucker M, Stache-Crain B, Emde AK, Geiger HM, Robine N, Coombes KR, Symer DE (2019) Human papillomavirus and the landscape of secondary genetic alterations in oral cancers. Genome Res 29 (1): 1-17. doi: 10.1101/gr.241141.118

12. Fox SA, Tiwari L, Farah CS (2020) Epigenetics and oral disease. In: Translational systems medicine and oral disease. Elsevier, pp 163-206
13. Jośko-Ochojska J, Rygiel K, Postek-Stefańska L (2019) Diseases of the oral cavity in light of the newest epigenetic research: Possible implications for stomatology. Adv Clin Exp Med 28 (3): 397-406. doi: 10.17219/acem/76060
14. Ali J, Sabiha B, Jan HU, Haider SA, Khan AA, Ali SS (2017) Genetic etiology of oral cancer. Oral Oncol 70: 23-28. doi: 10.1016/j.oraloncology.2017.05.004
15. Singh NN, Peer A, Nair S, Chaturvedi RK (2016) Epigenetics: A possible answer to the undeciphered etiopathogenesis and behavior of oral lesions. J Oral Maxillofac Pathol 20 (1): 122-128. doi: 10.4103/0973-029x.180967
16. Mesgari H, Esmaelian S, Nasiri K, Ghasemzadeh S, Doroudgar P, Payandeh Z (2023) Epigenetic Regulation in Oral Squamous Cell Carcinoma Microenvironment: A Comprehensive Review. Cancers (Basel) 15 (23). doi: 10.3390/cancers15235600
17. Gintoni I, Vassiliou S, Chrousos GP, Yapijakis C (2023) Review of Disease-Specific microRNAs by Strategically Bridging Genetics and Epigenetics in Oral Squamous Cell Carcinoma. Genes (Basel) 14 (8). doi: 10.3390/genes14081578
18. Gupta I, Badrzadeh F, Tsentalovich Y, Gaykalova DA (2024) Connecting the dots: investigating the link between environmental, genetic, and epigenetic influences in metabolomic alterations in oral squamous cell carcinoma. J Exp Clin Cancer Res 43 (1): 239. doi: 10.1186/s13046-024-03141-5
19. Kim SY, Han YK, Song JM, Lee CH, Kang K, Yi JM, Park HR (2019) Aberrantly hypermethylated tumor suppressor genes were identified in oral squamous cell carcinoma (OSCC). Clin Epigenetics 11 (1): 116. doi: 10.1186/s13148-019-0715-0
20. Foy JP, Pickering CR, Papadimitrakopoulou VA, Jelinek J, Lin SH, William WN, Jr., Frederick MJ, Wang J, Lang W, Feng L, Zhang L, Kim ES, Fan YH, Hong WK, El-Naggar AK, Lee JJ, Myers JN, Issa JP, Lippman SM, Mao L, Saintigny P (2015) New DNA methylation markers and global DNA hypomethylation are associated with oral cancer development. Cancer Prev Res (Phila) 8 (11): 1027-1035. doi: 10.1158/1940-6207.capr-14-0179
21. Najar RA, Wani NA, Bhat JA, Dar NJ, Rahat B, Gupta AP, Kaur J, Kaur J, Hamid A (2018) Modulation of dietary folate with age confers selective hepatocellular epigenetic imprints through DNA methylation. J Nutr Biochem 53: 121-132. doi: 10.1016/j.jnutbio.2017.10.007
22. Geissler F, Nesic K, Kondrashova O, Dobrovic A, Swisher EM, Scott CL, M JW (2024) The role of aberrant DNA methylation in cancer initiation and clinical impacts. Ther Adv Med Oncol 16: 17588359231220511. doi: 10.1177/17588359231220511
23. Köberle B, Koch B, Fischer BM, Hartwig A (2016) Single nucleotide polymorphisms in DNA repair genes and putative cancer risk. Arch Toxicol 90 (10): 2369-2388. doi: 10.1007/s00204-016-1771-2
24. Krishnan R, Patel PS, Hakem R (2021) BRCA1 and Metastasis: Outcome of Defective DNA Repair. Cancers (Basel) 14 (1). doi: 10.3390/cancers14010108
25. Sushma PS, Jamil K, Kumar PU, Satyanarayana U, Ramakrishna M, Triveni B (2016) PTEN and p16 genes as epigenetic biomarkers in oral squamous cell carcinoma (OSCC): a study on south Indian population. Tumour Biol 37 (6): 7625-7632. doi: 10.1007/s13277-015-4648-8
26. Severson TM, Peeters J, Majewski I, Michaut M, Bosma A, Schouten PC, Chin SF, Pereira B, Goldgraben MA, Bismeijer T, Kluin RJ, Muris JJ, Jirström K, Kerkhoven RM, Wessels L, Caldas C, Bernards R, Simon IM, Linn S (2015) BRCA1-like signature in triple negative breast cancer: Molecular and clinical characterization reveals subgroups with therapeutic potential. Mol Oncol 9 (8): 1528-1538. doi: 10.1016/j.molonc.2015.04.011
27. Sanyal T, Das A, Bhattacharjee S, Gump BB, Bendinskas K, Bhattacharjee P (2024) Targeting the 'DNA methylation mark': Analysis of early epigenetic-alterations in children chronically exposed to arsenic. Sci Total Environ 912: 169049. doi: 10.1016/j.scitotenv.2023.169049
28. Stastny I, Zubor P, Kajo K, Kubatka P, Golubnitschaja O, Dankova Z (2020) Aberrantly Methylated cfDNA in Body Fluids as a Promising Diagnostic Tool for Early Detection of Breast Cancer. Clin Breast Cancer 20 (6): e711-e722. doi: 10.1016/j.clbc.2020.05.009