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Assessing DNA methylation of ATG 5 and MAP1LC3Av1 gene in oral squamous cell carcinoma and oral leukoplakia- a cross sectional study

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ABSTRACT

Background: The progression and pathogenesis of oral cancer is greatly impacted by epigenetic modifications, such as DNA methylation. Autophagy, is an adaptive mechanism used to maintain the survival and integrity of cells. Oral squamous cell carcinoma is linked to a number of autophagy indicators, although it is yet unknown if DNA methylation of autophagy-related genes promotes the development of oral leukoplakia (OL), oral squamous cell carcinoma (OSCC).

Aim: Our study was aimed to assess, compare and evaluate the DNA methylation of ATG5 and MAP1LC3Av1 genes in oral leukoplakia, oral squamous cell carcinoma.

Materials and methods: This cross-sectional study was designed with sample size of 48 tissues which was clinically and histopathologically diagnosed as OL, OSCC and normal tissue. The samples were divided into three groups (Group A, Group B, and Group C; (n = 16 each). Following histopathological confirmation, the tissue was stored in the RNA reagent, then subjected to DNA extraction, methylation-sensitive polymerase chain reaction (MS-PCR). DNA methylation of the ATG5 and MAP1LC3Av1 genes were assessed.

Results: Shapiro-Wilk and Kolmogorov-Smirnov tests showed that the values were normally distributed. Both the ATG5 and MAP1LC3Av1 genes were methylated in OSCC, OL tissues compared to normal tissues. A statistically significant results was seen among the three study groups.

Conclusion: A significant difference was noted in the hypermethylation status of the promoter regions of the ATG5 and MAP1LC3Av1 genes. This provides some insight into their crucial role in the development of tumors. Future research with larger sample is needed to assess its potential clinical implications in oral carcinoma.

1. Introduction

Oral cancer accounts 30–40 %, the sixth most common cancer globally. In India, its prevalence rate is 15.62 %, and more than one-third of oral cancers are diagnosed in advanced stages.[1,2] The risk factors for oral squamous cell carcinoma (OSCC) include smoking and smokeless tobacco use, alcohol use, poor oral hygiene and genetic alterations.[3,4]During development and tissue homeostasis, epigenetic mechanisms are essential for controlling gene expression and maintaining cellular identity. Disturbance of an epigenetic network by external factors potentially leads to malignancy. Key epigenetic processes include RNA-mediated silencing, histone modification, and DNA methylation.[5] DNA methylation in CpG islands is strongly associated with tumor development, leading to gene silencing.[6]

DNA methylation causes cancer by two different mechanisms: either it causes somatic mutations or it regulates gene expression.[7] First, it has been discovered that hypomethylation of a proto-oncogene promoter sequence can result in enhanced oncogenic activity from that locus, and hypermethylation of a tumor suppressor promoter area can lead to lower expression of the tumor suppressive transcript. Therefore, an important event in the pathophysiology of cancer may be the gene regulatory activity of DNA methylation. The identification of methylated CpG islands in biological samples such as serum or sputum, may be helpful in the early diagnosis of malignancies.[8]

Eukaryotic cells endure various stresses and stimuli, disrupting homeostasis.[9] To counter and adjust to such stress, cells have a variety of intracellular defensive mechanisms at their disposal. Autophagy is a cellular defense mechanism that preserves cell survival and integrity by degrading intracellular components within autophagosomes that are transported to lysosomes for breakdown. Early in cancer, autophagy

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suppresses tumors by degrading harmful substances,[10]later it promotes tumor viability, complicating treatment. Therefore, autophagy in cancer cells is called a "double-edged sword" Genes known as autophagy -related genes (ATGs) systematically regulate autophagy mechanism. Literature suggest that DNA methylation of autophagy-associated genes exhibit carcinogenic action, which causes tumor development. Patients who carry these genetic traits have been shown to have poor clinical outcomes and survival rates. There are currently 41 known ATG genes identified. Of these 41 genes, ATG5 and MAP1LC3Av1 is found to be a major gene for autophagy while ATG5 proteins is essential for the creation of autophagic vesicles and autophagy process can be completely inhibited or downregulated upon taking down or eliminating ATG5, indicating that ATG5 is essential to autophagy where as "Microtubule associated protein 1 light chain 3 A variant 1(MAP1LC3Av1) and are gene regulates autophagosome formation. Autophagosomes are needed to facilitate cytoplasmic material transport for degradation.[11,12] Recently, ATG 5 and MAP1LC3Av1 was found to be inactivated in various cancer cell lines and clinical squamous cell carcinoma tissues due to aberrant DNA methylation induced by unknown factors.

However,in OSCC and OL it has not yet been determined if DNA methylation of ATG 5 and MAP1LC3Av1 gene promotes the tumor growth. Based on the background analysis, a hypothesis was formulated to assess the methylation silencing of ATG5 and MAP1LC3Av1 genes in OSCC and OL and thereby leading to tumor progression.

2. Methodology

2.1. Study design

With proper ethical clearance from the institutional Review Board and Ethics committee (SRMDC/IRB/March 2021/MDS/No.902), a cross-sectional observational study was designed and conducted in department of Oral medicine and Radiology. The study was registered in the Clinical Trial Registry – India (CTRI/2023/10/058654).

2.2. Study samples

The sample size (n = 48) was calculated based on the study by Muhammed et al. (2017)[13] with a power of 90 %.The participants satisfying the inclusion criteria of histologically confirmed and diagnosed of oral leukoplakia and oral squamous cell carcinoma were included in the study and divided into three groups (n = 16) each: Group A (healthy individuals),Group B (clinically and histologically diagnosed of oral leukoplakia (OL) patients), and Group C (clinically and histologically diagnosed oral squamous cell carcinoma (OSCC) patients). Following Helsinki Declaration guidelines, informed consent was obtained after thoroughly explaining the study's objectives, procedures, risks, and advantages to the study participants. A detailed history was recorded which included demographic data and habit history. Clinical staging was performed according to Amagasa et al. (2006) for oral leukoplakia patients, and malignant lesions were staged according to the new AJCC -TNM staging system (2020) and the control group samples were normal healthy tissue collected from the orthodontic extraction site, impaction sites, and flap surgeries. The biopsy samples were obtained by incisional biopsy. A part of the sample was processed for histopathological confirmation of the disease, while the other part was stored in the RNA reagent at −20 °C was subjected to further processing and DNA extraction.

3. Methods

3.1. DNA extraction

1X PBS (phosphate-buffered saline) was used to wash and remove RNA traces, and then lysed in 500 µl of lysis buffer containing 0.1 % SDS, 25 mM EDTA, and 75 µg/100 µl proteinase K in 200 mM Tris-Cl (pH 8) at 57 °C for 12 h with agitation (Sigma Aldrich, St. Louis, MO, USA). Following lysis, the DNA was extracted following phenol and chloroform-isoamyl alcohol protocol. The genomic DNA obtained was suspended in 100 µl of Tris-EDTA, and the DNA concentration was determined using a fluorometer.Following DNA extraction methylation-sensitive PCR (MS-PCR) technique was used in our study because in this method optimization is easy and is as reliable as bisulfite modification assays.Bisulfite modification assays are difficult to optimize and are time consuming.

3.2. Methylation -sensitive restriction digestion

A total of 250 ng of genomic DNA was digested with methylation-sensitive *Hpa*II enzyme (New England Biolabs, Ipswich, MA, USA). *Hpa*II recognizes CCGG motifs mainly in gene promoters, and nicks the first C, causing DNA fragmentation. Methylated Cs within CCGG motifs, such as those in the ATG5 or MAP1LC3Av1 promoters, inhibits *Hpa*II activity, thus preserving the CCGG sequence. This differential cleavage allows methylation to be distinguished from unmethylated genome regions, aiding epigenetic studies.

3.3. Methylation sensitive polymerase chain reaction (PCR)

The promoter sequence of the ATG5 and MAP1LC3Av1 genes were analyzed with Methylation finder software. Prediction analysis of the ATG 5 and MAP1LC3Av1 promoter and the methylation finder program identified CpG rich islands in the 500 base pair regions upstream of the transcription start site (Fig. 2(A & B)). Primers were designed flanking the CpG islands identified in the above analysis (Table 1).

Chromosomal DNA from a healthy control was used to optimize the amplification of CpG sites with respective primers (Fig. 1). The following amplification condition was used. After an initial denaturation 94 °C, samples were subjected to 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C.

Gel electrophoresis:

Amplified PCR products were analyzed by running them in a 1.5 % agarose gel at 100 V for 30 min with 1X TAE (Tris acetate EDTA) buffer. The DNA bands were visualized by staining the gel with ethidium bromide (a DNA intercalating agent that fluoresces when excited by UV in the range of 302–364 nm), and images were captured with a gel documentation unit. The detection of a PCR amplified band in a sample indicated hypermethylation of the promoter region of the ATG5 or MAP1LC3Av1 gene, while the absence of a band indicated an unmethylated promoter. (Fig. 3A & 3B).

3.4. Statistical analysis

Shapiro-Wilk and Kolmogorov-Smirnov test results showed the values were normally distributed. As a result, parametric data analysis was used. To determine the frequency and percentage of ATG5 and MAP1LC3Av1 gene methylation among oral squamous cell carcinoma and oral leukoplakia samples, descriptive data were compared to those of normal, healthy samples. Chi-square tests were utilized to compare the percentage of ATG 5 and MAP1LC3Av1 gene methylation in the study group and control group. SPSS (IBM SPSS Statistics for Windows, Version 26.0, Armonk, NY: IBM Corp., Released 2019) was utilized for the data analysis with p-value <0.05 is statistically significant.

Table 1
Primers used for Methylation sensitive polymerase chain reaction (PCR).

ATG5 LEFT PRIMER	GGTTTGGTCGCGAGTTCAAG
ATG5 RIGHT PRIMER	CTCCTTCCTACCACACCTACC
MAP1LC3Av1 LEFT PRIMER	CCTGGGCGTTCATTTCTAGC
MAP1LC3Av1 RIGHT PRIMER	ACGTCAGGTCACAACATTCC

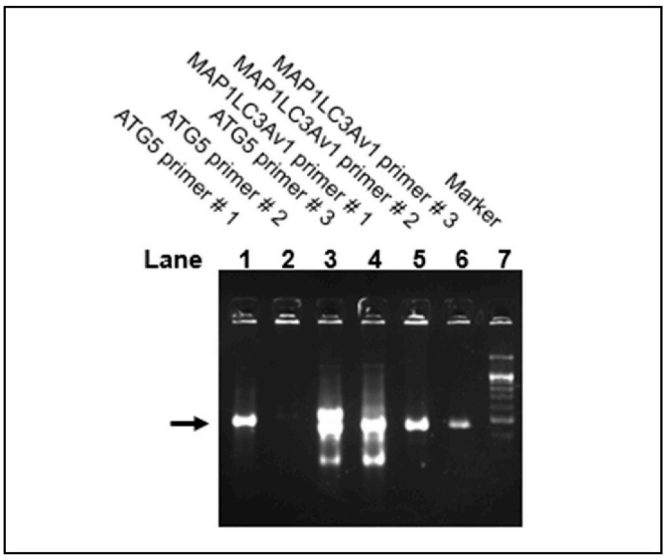

Fig. 1. PCR amplification of ATG5 and MAP1LC3 promoters in normal control DNA showed a clear single band with ATG5 primer#1 and MAP1LC3 primer#2. Hence these primers were used for methylation analysis.

4. Results

Epidemiological and clinical characteristics: The demographic data of the 48 patients revealed a median age of 35 years (range: 35–75 years). The study included both males (63.5 %) and females(36.5 %) of which the majority were males. The clinical characteristics of the 16 patients with OL and OSCC are summarized in Table 2. While examining the lesion location, nearly 11(68.75 %) patients had leukoplakia in the buccal mucosa, followed by 3 in the gingiva and least in the alveolus and commissure of the lip. According to the histopathological findings, nearly 35.25 % of the patients were diagnosed with severe dysplasia whereas 25.65 % with moderate dysplasia, and 37.24 % with mild dysplasia of the lesion. Among the patients with OSCC 43.75 % had lesions in the buccal mucosa, followed by lesions in the tongue (24.5 %), gingiva (13 %), alveolus (6.25 %) and floor of mouth (6.25 %). Regarding histopathological diagnosis, nearly 28.25 % of the patients were diagnosed with well -differentiated lesions,30.75 % were diagnosed with moderately differentiated lesions and 31 % were diagnosed with poorly differentiated lesions.

DNA methylation of the ATG5 and MAP1LC3Av1 genes in oral squamous cell carcinoma and oral leukoplakia tissues.

To examine the DNA methylation of ATG5 and MAP1LC3Av1 in the development of oral squamous cell carcinoma and oral leukoplakia methylation-sensitive-PCR was performed to evaluate DNA methylation in OSCC, OL, and normal tissues. In 48 tissue samples, methylation of the ATG5 gene was not detected in any of the normal patients, 2 in leukoplakia patients, 6 in OSCC patients, and MAP1LC3Av1 gene methylation was not detected in any of the normal patients, 3 in leukoplakia patients, 7 in OSCC patients (Graph 1). When assessing the statistical significance, the p-value was <0.05, indicating significant difference among the study groups(Table-3).The site methylation status was assessed, which revealed that nearly 35 % of the patients had OSCC and OL in the buccal mucosa, and poorly differentiated patient with OSCC was significantly associated with hypermethylation (p-value:>0.05).For the oral leukoplakia tissue sample, patients with severe dysplasia had a significantly greater number of methylation genes than did those without dysplasia.

5. Discussion

Oral cancer, especially oral squamous cell carcinoma (OSCC), frequently occurs in the head and neck region.The Global Cancer Observatory (GCO) recently released data showing that 377,713 instances of OSCC were registered worldwide in 2020. Early detection remains critical for improving the prognosis and outcomes of individuals affected by this condition. Challenges in planning the treatment for oral leukoplakia and OSCC arise due to its diverse nature and genetic variability of the disease and according to the literature the five-year survival rate of OL is 40.9 % and OSCC is 20.7 %.

With the latest molecular research, it is possible to precisely recognize the malignant transformation of oral lesions, which decreases the incidence of oral lesions and improves the clinical assessment and classification of these lesions.[14] In their 2023 review, Mesgari, H. et al., highlighted epigenetics as crucial in OSCC.[15] Epigenetic mechanisms regulate gene activity without altering DNA sequence DNA methylation, histone modifications, and miRNAs are key in this process. Methylation at the C-5 position of cytosine rings in higher eukaryotic genomes is a significant epigenetic marker, gene expression at the C-5 position.The literature suggest that OSCC is associated with abnormal DNA methylation of tumor suppressor genes (TSGs),such as DAPK, and p16.[16,17] These methylation changes may silence these genes, promoting

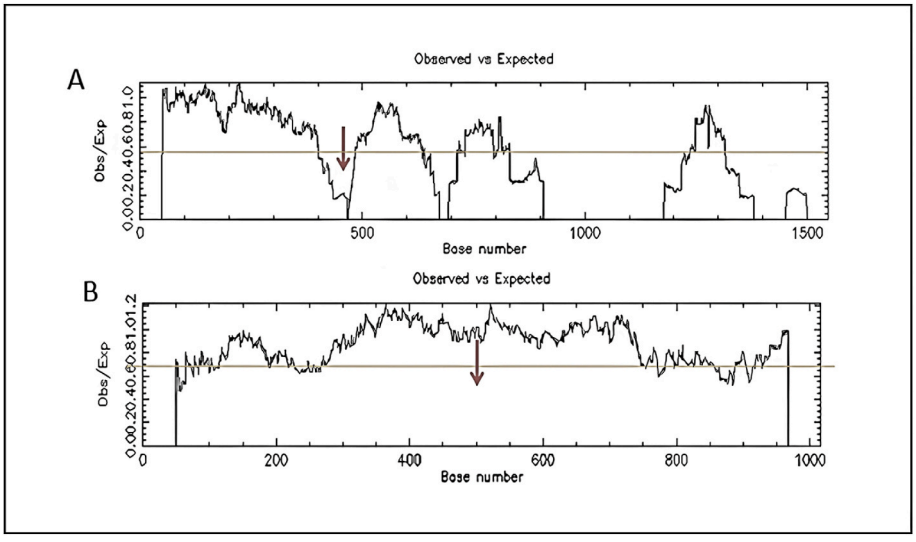

Fig. 2. (A&B) arrow shows the promoter sequence of ATG 5 & MAP1LC3Av1 which showed a concentrated CpG island pool between bases 0 to 500 upstream of transcription start. Line indicates observed versus expected CG ratio was greater than 0.6,which confirmed the presence of CG rich promoter region.

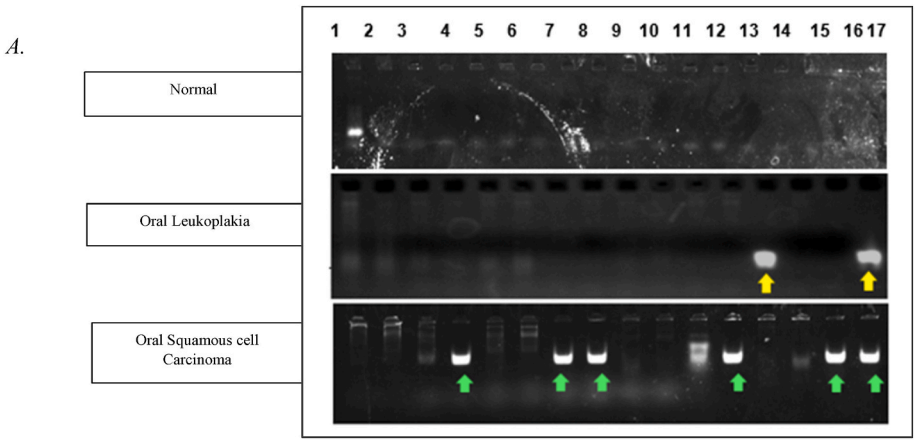

Fig. 3. A.ATG5 specific amplification by Methylation Sensitive PCR on NORMAL, LEUKOPLAKIA and OSCC samples showed no amplification in normal samples. Yellow and green arrows showed amplification indicated hypermethylation of the ATG5 gene promoter region in leukoplakia sample and OSCC samples A faint background amplification was seen in a few samples, but this does not indicate hypermethylation.

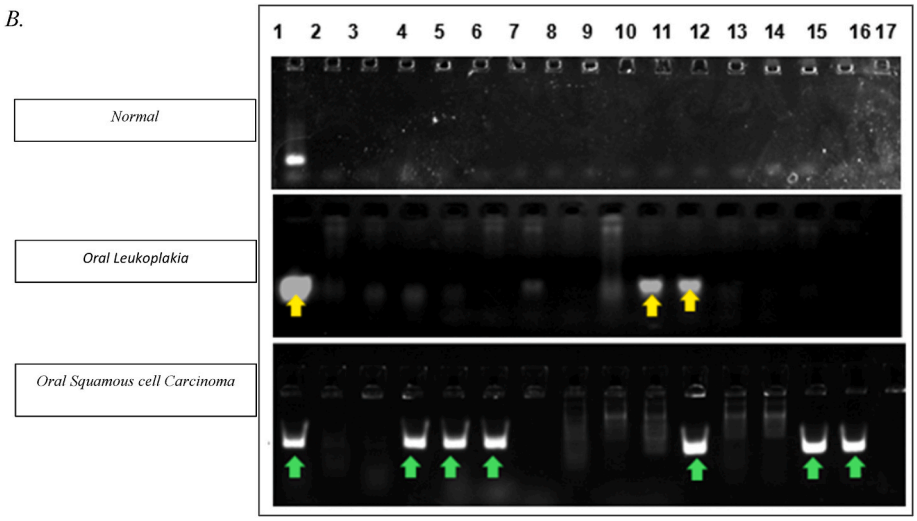

Fig. 3. B.MAP1LC3Av1 specific amplification by Methylation Sensitive PCR on NORMAL, LEUKOPLAKIA and OSCC samples showed no amplification in normal samples. Yellow and green arrows showed amplification indicated hypermethylation of the MAP1LC3Av1 gene promoter region in leukoplakia sample and OSCC samples A faint background amplification was seen in a few samples, but this does not indicate hypermethylation.

uncontrolled cell division and tumor progression. DNA methylation is one of many epigenetic changes that has been connected to many autophagy-related markers in various carcinoma types. Thus, using a methylation-sensitive enzyme-based approach, the present study assessed the DNA methylation status of autophagy-related genes (ATG5 and MAP1LC3Av1) in OL and OSCC tissues compared with that in normal tissue.

The current study included a population aged 25–80 years. Oral cancer mostly affects adults older than 40 years in approximately 95 % of cases, with an average age at diagnosis of 60 years. The greatest number of study participants were(n = 20) male since the use of tobacco is more prevalent among men in India.[18,19] According to a study by Rossi, R. et al.,[20] both clinically healthy mucosa and patients with oral leukoplakia had positive methylation status; hence, our study also included leukoplakia tissue samples in comparison with OSCC and normal tissue samples. Tang et al.,[21] investigated the expression and prognostic importance of Beclin-1 and ATG5, two autophagy-related (ATG) proteins,in human OSCC tumors. In our present study,the ATG5 gene promoter was hypermethylated in two OL tissue samples and six OSCC tissue samples but not in any normal tissue samples, according to our analysis of the epigenetic modification (DNA methylation) of the gene. The p-value (<0.05) showed a statistically significant difference between the OL and OSCC samples, suggesting that OSCC tissues has more ATG5 gene methylation silenced than OL or normal tissues. Various studies have suggested that one of the important regulators of autophagosome formation is microtubule-associated protein 1 light chain 3 (MAP1LC3/LC3).According to Bai et al. (2012)[22] the majority of primary esophageal tumor samples and human cancer cell lines have silenced MAP1LC3Av1 gene by methylation of the gene. They also proved that methylation status replacement of LC3Av1 was sufficient to stop tumor growth in an esophageal squamous cell carcinoma (ESCC) cell line. Our study analyzed the epigenetic alteration (DNA methylation) of the MAP1LC3A variant 1gene,and the results showed MAP1LC3Av1 gene promoter hypermethylation in three OL tissue samples and seven OSCC tissue samples and no hypermethylation in normal tissue samples. There was a statistically significant difference in the p-value (<0.05) between the OL and OSCC samples indicating that there was greater methylation silencing of the MAP1LC3Av1 gene in OSCC tissues than in OL and normal tissues.Astudy by Muhammed et al. (2017)[13] also assessed Helicobacter pylori infection induced methylation silencing of host autophagy-related (ATG) genes, and results found of all 34 ATG genes, MAP1LC3A variant 1 is essential for autophagy and

Table 2
Shows Oral leukoplakia and Oral squamous cell carcinoma groups based on site and histopathological diagnosis.

Site	Oral Leukoplakia						Oral squamous cell carcinoma					
	Mild Dysplasia		Moderate Dysplasia		Severe Dysplasia		Well Differentiated		Moderately Differentiated		Poorly Differentiated	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
Alveolus	0	0	1	6.25	0	0	0	0	0	0	1	6.25 %
Buccal Mucosa	4	25	3	18.75	4	25	4	25	2	12.25	1	6.25
Tongue	–	–	–	–	–	–	0	0	2	12.25	2	12.25
Gingiva	1	6.25	0	0	2	12.25	1	6.25	0	0	1	6.25
Floor of the Mouth	0	0	0	0	0	0	0	0	1	6.25	0	0
Commissure Of lip	1	6.25	0	0	0	0	0	0	0	0	0	0

Graph 1. Methylation silencing of ATG5 and MAP1LC3Av1 among study groups.

Helicobacter pylori-induced methylation silencing of MAP1LC3Av1 may impair autophagy, facilitating gastric carcinogenesis which is consistent with our study results.

Abe et al. (2016)[23] investigated aberrant promoter methylation in OL patients with a high risk of malignant transformation. A significantly greater number of genes related to oral leukoplakia in patients with dysplasia than in patients without dysplasia were differentially expressed. Our study also categorized the methylation status of the ATG5 and MAP1LC3Av1 genes based on the severity of dysplasia, and the results revealed that significant methylation occurred in OL tissue samples from patients with severe dysplasia. In the OSCC tissue samples, patients who had poorly differentiated OSCC were significantly more likely to have hypermethylation (p-value <0.05). The results of this study have clinical significance since the hypermethylation profile can be utilized for monitoring the activity of genes related to autophagy in premalignant OL and OSCC lesions. Our study identified the DNA methylation of autophagy-related genes (ATG5 and MAP1LC3Av1) for the first time and promoter hypermethylation in OSCCs, and OLs compared to normal tissues, which suggests that the ATG5 and MAP1LC3Av1 genes may be used as cancer predictive biomarkers in oral leukoplakia and oral squamous cell carcinoma.

6. Conclusion

In conclusion, hypermethylation of the ATG5 and MAP1LC3Av1 genes may has significant role in the progression of premalignancy to malignancy in both oral leukoplakia and oral squamous cell carcinoma, which differ significantly from normal tissue. The DNA methylation patterns of the ATG5 and MAP1LC3Av1 genes offer several advantages and could be used as biomarkers for the early diagnosis of OSCC. This provides some insight into their crucial role in the development of tumors. To evaluate its possible clinical significance in oral cancer and to corroborate the findings, additional investigation larger sample size.

Limitations and future aspects

One of the study's limitations was the small sample size, which was due to time and financial restrictions and because it was a pilot study and self-funded. Future modifications could include increasing the sample size, performing a multicentric study, dividing the samples according to clinical grade, and calculating the tumor invasion index.

Consent for publication

Proper consent is obtained from the participants for the publication of study results without revealing personal information.

Availability of data and materials

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

Declarations

Ethics approval was obtained from the Institutional Review Board

Table 3
Methylation silencing of ATG 5 and MAP1LC3Av1among study groups.

	Methylation	Normal		Leukoplakia		OSCC		P-value
		Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	
ATG 5	PRESENT	0	0	2	12.5	6	37.5	0.028*
	ABSENT	16	100	14	87.5	10	62.5	
MAP1LC3Av1	PRESENT	0	0	3	18.75	7	43.75	0.021*
	ABSENT	16	100	13	81.25	9	56.25	

(IRB) (SRMDC/IRB/2021/MDS/No.902) and consent to participate was obtained. The study has been registered in the Clinical trial registry of India(CTRI/2023/10/058654). Following the guidelines of the Helsinki declaration, participants who volunteered for the study and met the inclusion criteria were chosen. Informed consent was obtained from the participants of the study.

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Declaration of competing interest

There are no conflicts of interest.

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Abbreviation

OSCC	Oral Squamous cell Carcinoma
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
ATG	Autophagy Related genes
MAP1LC3Av1	Microtubule-associated protein 1 light chain 3 A variant 1
WHO	World health Organization
AJCC	American Joint Committee on Cancer
OL	Oral Leukoplakia

References

1. Sung H, Ferlay J, Siegel RL, et al. Global cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Ca - Cancer J Clin*. 2021;71:209–249.
2. Ganesan A, Kumar NG. Assessment of lipid peroxides in multiple biofluids of leukoplakia and oral squamous cell carcinoma patients-a clinico- biochemical study. *J Clin Diagn Res*. 2014 Aug;8(8):ZC55–Z58.
3. Pulte D, Brenner H. Changes in survival in head and neck cancers in the late 20th and early 21st century: a period analysis. *Oncol*. 2010;15(9):994–1001.
4. Raja N, Ganesan A, S KV, K A, Chandrasekar Lakshmi K. Awareness of prebiotics and probiotics in oral lesions among dental postgraduates -A questionnaire-based study. *Biomedicine*. 2023;43(3):921–924.
5. Reid BC. Visual screening for oral cancer may reduce oral cancer mortality in high-risk adult populations through early diagnosis and treatment. *J Evid Base Dent Pract*. 2013;13:174–176.
6. Kim Samaranayake, Pradhan S. Epigenetic mechanisms in mammals. *Cell Mol Life Sci*. 2008;66:596–598.
7. Bird AP. CpG-rich islands and the function of DNA methylation. *Nature*. 1986;321:209–213.
8. Lai WKM, Pugh BF. Understanding nucleosome dynamics and their links to gene expression and DNA replication. *Nat Rev Mol Cell Biol*. 2017;18:548–562.
9. Kong WJ, Zhang S, Guo C, Zhang S, Wang Y, Zhang D. Methylation-associated silencing of death-associated protein kinase gene in laryngeal squamous cell cancer. *Laryngoscope*. 2005;115:1395–1401.
10. Kuma A, Hatano M, Matsui M, et al. The role of autophagy during the early neonatal starvation period. *Nature*. 2004;432:1032–1036.
11. Matsushita M, Suzuki NN, Obara K, Fujioka Y, Ohsumi Y, Inagaki F. Structure of Atg5.Atg16, a complex essential for autophagy. *J Biol Chem*. 2007;282:6763–6772.
12. Rubinsztein DC, Codogno P, Levine B. Autophagy modulation as a potential therapeutic target for diverse diseases. *Nat Rev Drug Discov*. 2012;11:709–730.
13. Muhammad JS, Nanjo S, Ando T, et al. Autophagy impairment by Helicobacter pylori-induced methylation silencing of MAP1LC3Av1 promotes gastric carcinogenesis. *Int J Cancer*. 2017 May 15;140(10):2272–2283.
14. Bray F, Jemal A, Grey N, Ferlay J, Forman D. Global cancer transitions according to the Human Development Index - a population-based study. *Lancet Oncol*. 2012;13:790–801.
15. Cramer JD, Burtness B, Ferris RL. Immunotherapy for head and neck cancer: recent advances and future directions. *Oral Oncol*. 2019;99, 104460.
16. Mesgari H, Esmaelian S, Nasiri K, Ghasemzadeh S, Doroudgar P, Payandeh Z. Epigenetic regulation in oral squamous cell carcinoma microenvironment: a comprehensive review. *Cancers*. 2023;15:5600–5601.
17. Moyer AM, Nowsheen S, Liu T, et al. DNA methyltransferase expression in triple-negative breast cancer predicts sensitivity to decitabine. *J Clin Investig*. 2018;128:2376–2388.
18. Ramesh Nandhini, Krithika Chandrasekar Lakshmi, Kannan Asokan, Anuradha Ganesan, Aniyan Yesoda K. Expression of sirtuin 3 in oral cancer and oral leukoplakia: a cross-sectional observational study. *J Int Oral Health*. 2022;14(3):290–297.
19. Saravana Murthy P, Kannan A, Ganesan A, Lakshmi KC, Aniyan Kumbalaparambil Y. Evaluating the expression of microRNA-15a-5p and YAP1 gene in oral squamous cell carcinoma in comparison with normal tissue: a cross-sectional study. *J Oral Pathol Med*. 2023:1–8.
20. Rossi R, Gissi DB, Gabusi A, et al. A 13-gene DNA methylation analysis using oral brushing specimens as an indicator of oral cancer risk: a descriptive case report. *Diagnostics*. 2022 Jan 23;12(2):284.
21. Tang D, Kang R, Livesey KM, et al. Endogenous HMGB1 regulates autophagy. *J Cell Biol*. 2010 Sep 6;190(5):881–892.
22. Bai H, Inoue J, Kawano T, et al. A transcriptional variant of the LC3A gene is involved in autophagy and frequently inactivated in human cancers. *Oncogene*. 2012;31:4397–4408.
23. Abe M, Yamashita S, Mori Y, et al. High-risk oral leukoplakia is associated with aberrant promoter methylation of multiple genes. *BMC Cancer*. 2016;16:350.