Dental Research Journal

Original Article

The oncogenic role of NOTCH1 as biomarker in oral squamous cell carcinoma and oral lichen planus

Ensieh Sagheb Sadeghi[1], Fatemeh Saeid Nematpour[1], Nooshin Mohtasham[2], Farnaz Mohajertehran[2,3]

[1]Department of Biology, Neyshabur Branch, Islamic Azad University, Neyshabur, [2]Oral and Maxillofacial Diseases Research Center, Mashhad University of Medical Sciences, [3]Department of Oral and Maxillofacial Pathology, School of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran

ABSTRACT

Background: Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer with heterogeneous molecular pathogenesis. Oral lichen planus (OLP) is demonstrated potentially can transfer to OSCC malignant lesions. Unfortunately, there are no definitive prognostic and predictive biomarkers for the clinical management of OSCC patients. The present research is the first study that compared an oral premalignant lesion such as OLP to malignant lesions like OSCC for NOTCH1 expression levels to better understand its oncogenic or tumor suppressive role.

Materials and Methods: In this cross-sectional study, mRNA expression of NOTCH1 was evaluated by quantitative polymerase chain reaction in 65 tissue-embedded Paraffin-Block samples, including 32 OSCC and 33 OLP. Furthermore, we collected demographic information and pathological data, including tumor stage and grade. The association between NOTCH1 and GAPDH gene expressions was determined by Chi-squared, Spearman, and Mann–Whitney tests. A $P < 0.05$ was considered statistically significant for all statistical analyses.

Results: Comparison of OSCC and OLP groups showed a statistically significant difference between the quantitative expression of the NOTCH1 gene ($P < 0.001$). Qualitative gene expression was divided into low expression and high expression. Both study groups demonstrated a statistically significant gene expression difference ($P < 0.001$). There was a statistically significant difference between age and NOTCH1 expression in the OLP group ($P = 0.036$). There was no correlation between NOTCH1 expression and age, gender, tumor grade, and stage.

Conclusion: Since the OSCC is a malignant lesion and the OLP showed the possible nature of malignancy transformation, we can consider the NOTCH1 as a biomarker for the assessment of the tumorigenesis process with a definition of a standard threshold for potentially malignant lesions and malignant OSCC tumors.

Key Words: Biomarkers, NOTCH1, oncogenes, oral lichen planus, squamous cell carcinoma of head and neck

Received: 11-Nov-2022
Revised: 19-Mar-2023
Accepted: 03-Jul-2023
Published: 27-Sep-2023

Address for correspondence: Dr. Farnaz Mohajertehran, Oral and Maxillofacial Diseases Research Center, Mashhad University of Medical Sciences, P.O.Box: 9177948959, Mashhad, Iran. E-mail: mohajertf@mums.ac.ir

INTRODUCTION

Head and neck squamous cell carcinomas (HNSCCs) arise from the mucosal epithelium in the oral cavity, pharynx, and larynx and are known as the 6th most common cancer worldwide. It is

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How to cite this article: Sagheb Sadeghi E, Nematpour FS, Mohtasham N, Mohajertehran F. The oncogenic role of NOTCH1 as biomarker in oral squamous cell carcinoma and oral lichen planus. Dent Res J 2023;20:102.

predicted the HNSCC incidence will increase by 30% (approximately 1.08 million new cases annually) by 2030.[1] The geographical distribution of HNSCC varies across the world. The main risk factors are correlated with exposure to tobacco-derived carcinogens, excessive alcohol utility, and prior infection to human papillomavirus (HPV), primarily HPV-16, and HPV-18. Besides the mentioned risk factors, genetic background plays a pivotal role in the etiopathogenesis of HNSCC.[2] Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer comprised of the mucosal lining of the oral cavity with a poor prognosis and high mortality rate. The heterogeneous molecular pathogenesis of OSCC arises from a wide range of events related to the altered levels of transcripts, proteins, and metabolites.[3] Oral lichen planus (OLP) is one of the premalignant lesions with malignant potential to transform to OSCC that present mainly in the general middle age population, buccal mucosa, and women. OLP is an immune-mediated inflammatory condition that involves the skin and mucous membranes, including oral mucosa.[4,5] OLP is classified into 6 subtypes following the clinical presentation, including reticular, erosive, atrophic, plaque-like, papular, and bullous. Although there are controversial reports about the potentially premalignant transformation of OLP into OSCC, the studies related to cancer development and chronic inflammatory disease support the possible nature of malignancy changes in OLP patients.[6]

Current therapeutic approaches, including radio-chemotherapy, chemotherapy, radiotherapy, and surgery cannot increase the overall 5-year survival rate by more than 50% for HNSCC patients. There was no effective screening strategy for early diagnosis of oral cancer, and careful physical examination remains the primary. Unfortunately, there are no determined definitive prognostic and predictive biomarkers for the clinical management of OSCC patients.[7] It was reported that the detection of standard molecular biomarkers based on involved molecular pathways could be effective for early diagnosis, better prognosis, target therapies, and prolonged survival rate in the other cancer types.[8-11] Understanding molecular pathways which play a pivotal role in the bio pathogenesis of OSCC can help scientists discover reliable biomarkers for early diagnosis.

The NOTCH family genes play the bimodal role as tumor suppressors or oncogenes in the pathogenesis of cancer. The NOTCH1 member is the second major gene after p53 in the molecular pathogenesis of HNSCC.[12] The NOTCH1 gene involves in cell proliferation, self-renewal, angiogenesis, and invasion. During the NOTCH1 pathway, the intracellular domain of NOTCH is activated when the membrane-bound ligands (Delta or DLL) attached to the NOTCH receptor 1–4 members family, then translocate to the nuclear and act as a transcription factor for HES1, HEY, Cyclin D1, and COX-2 expressions and increase keratinocyte differentiation [Figure 1]. NOTCH protein inactivated β-catenin levels by direct interaction. Simultaneous loss of function in both NOTCH1 and FAT1 provides increased β-catenin levels.[13]

Accordance to the NOTCH1 functions, it is expected its expression changes can impact the process of initiation, growth, and development of tumorigenesis in HNSCC patients.[14] In the present study, we evaluated the expression of the NOTCH1 gene in

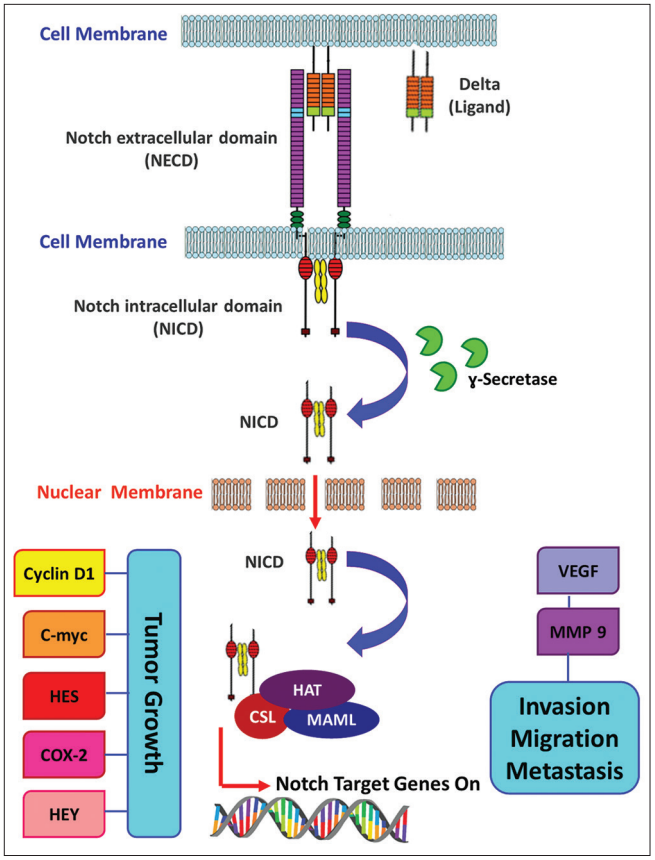

Figure 1: The target genes in the NOTCH1 pathway for tumor growth and metastasis process. NECD: Notch extracellular domain, NICD: Notch intracellular domain, VEGF: Vascular endothelial growth factor, MMP-9: Matrix metallopeptidase 9, CSL: (*D*elta/*S*errate, *L*AG proteins family), HAT: *h*istone *a*cetyl *t*ransferase, MAML: (Mastermind-like Proteins).

OLP patients in comparison to OSCC. This is the first study that evaluated the NOTCH1 expression levels in the possible premalignant nature of OLP in comparison to OSCC malignant lesions. Result of the current study revealed NOTCH1 plays oncogenic or oncosuppressive role and can apply due to the clinical experiments as a target.

MATERIALS AND METHODS

Study participants

This cross-sectional study was conducted on a total of 65 tissue-embedded Paraffin-Block samples, including 32 OSCC and 33 OLP patients with their related healthy surgical margins (control group). The samples were obtained from the Department of Oral and Maxillofacial Pathology at the Dentistry School of Mashhad University of Medical Sciences (MUMS), Iran. The Ethics Committee of MUMS confirmed all experimental processes before the beginning of the current project (IR.MUMS.DENTISTERY.REC.1400.089). The consent form was signed by all study participants before taking a tissue biopsy. The exclusion criteria were patients with malignancy or other lesions in their medical history, and surgery, chemotherapy, and radiotherapy. The samples with no definitive diagnosis and a low quantity of total extracted RNA were excluded, too. Demographic information of patients registered, such as age, sex, drinking, smoking, and drug consumption. Moreover, clinicopathological indices of study patients, such as tumor grade and stage recorded. The tumor stage was determined using the Tumor-Node-Metastasis staging system Grades I and II were considered as early, while Grades III and VI were considered advanced.[15] All tissue samples were fixed in 10% formalin and cut off as 5 μm thickness sections, then they were stained with hematoxylin and eosin for assessment of histopathological grading. For laboratory analysis, each sample was deparaffinized by Xylene and then transferred to 96% Ethanol.

RNA isolation and cDNA synthesis

The total RNA was isolated by High Pure RNA Paraffin Kit (FFPET RNA Tissue, Roche, Germany) following the manufacturer's instructions. The quantification of isolated RNA was assessed by NanoDrop (Thermo Scientific 2000, USA) according to the absorbance ratio of 260 nm/280 nm wavelength. The isolated RNA was stored at −80°C Until the molecular process continued. The cDNA synthesis was performed by AddScript cDNA synthesis kit (Addbio, Korea, REF 22701) following the recommended protocol in 20 μL total volume: 10 μL of 2X Reaction Buffer, 2 μL of 10 mM dNTP mixture, 2 μL of 10X random hexamer primer, 1 μL of 20X AddScript enzyme solution and 5 μL of total RNA (50 ng total concentration) and diethyl pyrocarbonate water mixture. The temperature cycling protocol was priming at 25°C for 10 min, reverse transcription (RT) at 50°C for 60 min, RT inactivation at 80°C for 5 min, and holding at 12°C. The cDNA was stored at −20°C until performing quantitative polymerase chain reaction (qPCR).

Quantitative polymerase chain reaction

The expression of the NOTCH1 gene was assessed by qPCR compared to GAPDH as a housekeeping gene. The qPCR was performed using the Add SYBR Master high ROX (Addbio, Korea, REF 70205HR) by Light Cycler (Roche, Germany). The reactions amplificated duplicate in 20 μL total volume: 10 μL of Add SYBR Master, 0.3 μL of each primer, 2 μL of cDNA, and 7.4 μL of distilled water. The sequence of primers was (F) 5'-CTGGTCAGGGAAATCGTG-3' and (R) 5'-TGGGCAGTGGCAGATGTAG-3' for NOTCH1 gene, and (F) 5'-CCCATCACCATCTTCCAGG-3' and (R) 5'-CATCACGCCACAGTTTCCC-3' for GAPDH gene. The temperature cycling included preincubation at 95°C for 10 min, then 40 cycles for denaturation at 95°C for 30 s, and 60°C for 90 s for annealing. The melting curve was analyzed for assessment of the specific target genes' amplification. The quantification of gene expression was normalized by the ΔΔCT method, and the relative gene expression was evaluated by 2^−(ΔCt) which is commonly used in miRNA expression.

Data analysis

Data analysis was performed by SPSS software (software version 20, SPSS Inc., Chicago, IL, USA). The association between NOTCH1 and GAPDH gene expressions was determined by Chi-squared, Spearman, and Mann–Whitney tests. A $P < 0.05$ was considered statistically significant for all statistical analyses.

RESULTS

In this study, 65 sample biopsies have collected, including a total of 32 women (49.2%) and 33 men (50.8%) with a mean age of 51.54 ± 12.66 and age range of 24–78 years that evaluated for NOTCH1 expression in OLP and OSCC patients. All study

participant information is shown in Table 1. The age range of OLP patients was 48 years old (from 24 to 72 years), and 44 years (from 34 to 78 years) in OSCC patients. The mean age was 46.82 ± 13.66 years old in OLP patients and 56.41 ± 9.48 years in OSCC. The study groups were evaluated for sex and age parameters that demonstrated statistically significant differences for mean age [$P = 0.004$, Table 2].

A comparison of quantitative gene expression showed that the minimum and maximum levels of gene expression were 0.01 and 2.36 in the OLP group, and 1.36 and 11.71 in the OSCC group, respectively. There was a statistically significant difference between the two study groups [$P < 0.001$, Table 3]. If the gene expression showed <2-fold changes considered as low expression, and if the gene expression demonstrated ≥2-fold changes considered as high expression in each study group. One patient (3%) in the OLP group and 29 OSCC patients (90.6%) showed high gene expression. Both study groups demonstrated a statistically significant difference in gene expression [$P < 0.001$, Table 3].

Results of Spearman's test demonstrated a statistically significant difference between age and gene expression in the OLP group [$P = 0.036$, Table 4]. In other words, with increasing age, the gene expression level increased significantly and vice versa. There was not a statistically significant difference between age and gene expression in the OSCC group ($P = 0.206$).

The results of the Mann–Whitney test demonstrated that the minimum level of NOTCH1 expression was 1.36 folds in women and 1.46 folds in men. The maximum level of NOTCH1 expression in men and women was 11.71 folds. The average NOTCH1 expression in men was more than in women, but there was no statistically significant difference ($P = 0.411$).

The results of the Mann–Whitney test showed a minimum level of NOTCH1 expression was 1.36 folds in the early stage and 2.49 folds in the advanced stage. The maximum level of NOTCH1 expression in early and advanced stages was 11.71 folds. The average NOTCH1 expression in the patients with the early stage was fewer than in the advanced stage, but it was not showed a statistically significant difference [$P = 0.119$, Table 5].

The results of the Kruskal–Wallis test showed the minimum level of NOTCH1 expression changes increased by 1.46, 2.49, and 1.36 folds in Grades I, II, and III, respectively. The maximum level of NOTCH1 expression in each grade was 11.71 folds. Minimum and maximum levels of NOTCH1 expression were related to Grades III and II, respectively. Different OSCC grades did not show statistically significant differences for NOTCH1 expression [$P = 0.211$, Table 5]. The melting curves and amplification plots of target gene expressions are illustrated in Figure 2.

Table 1: Study participants' information

Variables	*n* (%)
Groups	
OLP	33
OSCC	32
Age (by year)	
≥60	21 (66)
<60	11 (34)
Sex	
OLP	
Male	16 (48.5)
Female	17 (51.5)
OSCC	
Male	17 (53.1)
Female	12 (46.9)
Grade (OSCC)	
I	20 (62.5)
II	8 (25)
III	4 (12.5)
Stage (OSCC)	
Early	22 (69)
Advanced	10 (31)

OLP: Oral lichen planus; OSCC: Oral squamous cell carcinoma

Table 2: Comparison of average individual age (by maximum and minimum range) and sex by year in study groups

Variables	OSCC (*n*=32), *n* (%)	OLP (*n*=33), *n* (%)	*P*
Age, average±SD	56.41±9.483	46.82±13.660	0.004
Sex			
Female	15 (46.9)	17 (51.5)	0.708
Male	17 (53.1)	16 (48.5)	

OLP: Oral lichen planus; OSCC: Oral squamous cell carcinoma; SD: Standard deviation

Table 3: Comparison of average and NOTCH1 qualitative expression between study groups

Variables	OSCC (*n*=32), *n* (%)	OLP (*n*=33), *n* (%)	*P*
NOTCH1 expression, average±SD	5.71±3.60	0.61±0.67	<0.001
NOTCH1 qualitative expression			
High expression	29 (90.6)	1 (3.0)	<0.001
Low expression	3 (9.4)	32 (97.0)	

OLP: Oral lichen planus; OSCC: Oral squamous cell carcinoma; SD: Standard deviation; NOTCH1: Neurogenic locus notch homolog protein 1

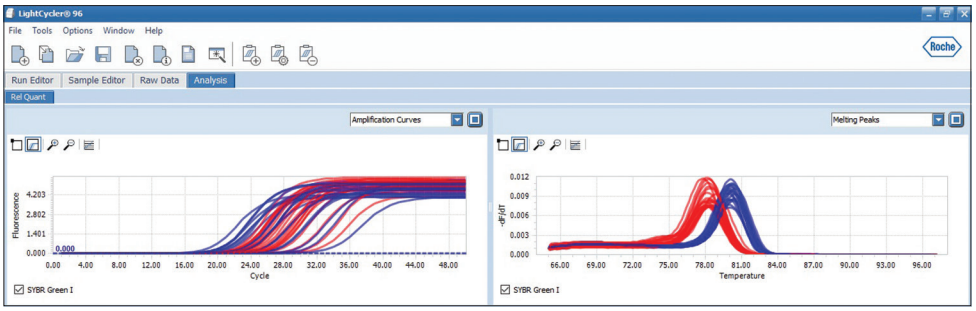

Figure 2: The amplification plot (left) and melting curve (right) of GAPDH (red) and NOTCH1 (blue) expression.

Table 4: Correlation between gene expression and study groups

Variables	OLP	OSCC	Total
Spearman correlation coefficient	0.367*	−0.229	0.371**
P	0.036	0.206	0.002
Count	33	32	65

*The level of significance is taken at 0.05 or 5%; **The level of significance is taken at 0.01 or 1%. OLP: Oral lichen planus; OSCC: Oral squamous cell carcinoma

Table 5: Association of NOTCH1 marker expression with pathological characteristic

Variables	*n*	Gene expression, mean±SE	*P*
Grade			
I	20	5.12±3.12	0.211
II	8	7.75±3.78	
III	4	4.56±4.81	
Stage			
Early	22	4.81±3.13	0.119
Advanced	10	7.67±3.93	

SE: Standard error; NOTCH1: Neurogenic locus notch homolog protein 1

DISCUSSION

In the current study, we evaluated the NOTCH1 expression in 65 tissue-embedded Paraffin-Block samples including 32 OSCCs and 33 OLPs in compared to the GAPDH gene. There was a substantial difference in NOTCH1 expression between OSCC and OLP groups. The overexpression of NOTCH1 more than 3.5 folds in OSCC patients compared to the OLP groups emphasize the oncogenic role of NOTCH1. Because the OSCC is a malignant lesion while the OLP lesion shows the possible nature of malignancy transformation. We can consider NOTCH1 as a biomarker for the assessment of the tumorigenesis process with the definition of a standard threshold for potentially malignant lesions and malignant OSCC tumors.

According to de Freitas Filho *et al.*, upregulation of NOTCH1 expression was observed in approximately 43% of OSCC patients. And also, upregulation of NOTCH1 expression is associated with poorly differentiated, perineural infiltration, and lymph node metastasis. The outcome of their study confirmed the oncogenic role of NOTCH1 in OSCC.[16] They studied 4 subtypes of oral cancer, while we consider OLP as a potentially premalignant lesion and OSCC. They applied the immunohistochemical technique in comparison to qPCR in our study. One of the main points in their investigation was the evaluation of disease-free survival (DFS) and overall survival (OS) rates for NOTCH1 as valuable prognosis factors. The total number of study patients was 63 in their study, similar to our research (n = 65).

A cohort study in the Chinese population evaluated the NOTCH1 expression of 78 OLP patients for potential transformation to OSCC with immunohistochemically staining. They reported 31% of OLP patients showed membranous expression that, 46% of them transformed to OSCC, while 15% of those patients without NOTCH1 expression developed to OSCC. They suggested NOTCH1 can consider as a biomarker for the malignancy potential of OLP.[17] In contrast to the result of our study, they reported expression of NOTCH1 increased by stage in OSCC patients.

In another study, the expression of NOTCH has been assessed in OSCC cell lines (Ca99-2, HSC-2, and HSC-4) and tissue sample biopsies. The qPCR analysis demonstrated the overexpression of NOTCH1, NOTCH2, Jagged1, HES1, and HEY1 in both OSCC cell lines and tissue biopsies. The nuclear aggregation was observed by immunohistochemically staining. Moreover, γ-secretase (GSI X) *in vitro* induction prevented OSCC growth by inhibiting the NOTCH pathway.[18] The result of their experimental study emphasized the oncogenic role of the NOTCH in OSCC pathogenesis and suggested the NOTCH could be considered for future therapeutic approaches.

Whole-exome sequencing (WES) of mouse tongue squamous cell carcinoma SCC demonstrated

mutations in NOTCH1, p53, and Fat1 manifested in early lesions. Moreover, the mutation in the NOTCH1 gene strongly increased immune infiltration, and the clonal diversity following the genetic heterogeneity was higher in moderate dysplasia and invasive SCCs compared to hyperplasia and mild dysplasia.[19] Hence, aggressive tumors presented a more mutational burden. The evaluation of the Indian population with OSCC by WES in 30 tobacco consumers showed a rate of 36% somatic mutations in the NOTCH1 gene in comparison to the total rate that was 11%–15% in HNSCC. They reported eight missense and five nonsense single nucleotide variants in NOTCH1.[20] Their result highlighted the crucial role of NOTCH1 in the pathogenesis of OSCC in the Asian population. According to the mentioned studies that evaluated different variety populations, we can conclude alternation in NOTCH1 expression or the mutations that affect its function can highlight NOTCH1 role as a valuable marker in the prognosis of HNSCC.

In addition to the HNSCC, the oncogenic role of NOTCH signaling has been approved in other cancer types including, hepatocellular carcinoma,[21] gastric carcinoma,[22] gliomas,[23] breast carcinoma,[24] ovarian carcinoma,[25] prostate carcinoma, and colorectal carcinoma.[26]

The bioinformatics analysis of receptors, ligands, and downstream target genes in the NOTCH pathway by GTEx and TCGA-BLCA databases showed all four types of NOTCH receptors (NOTCH 1–4), their ligands (DLL1, 3 and 4) and HES1 differentially expressed in bladder cancer. The high level of NOTCH receptor 2 and 3 expressions was strongly correlated with poor DFS and OS rates.[27]

Besides the database analyzing studies, the proteomic analysis of metastatic oral melanoma by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and in-gel digestion coupled with mass spectrometry showed the peptide mass at 2316 Dalton of NOTCH1 manifested in early-stage and benign oral tumors.[28]

Although many reports confirmed the oncogenic role of NOTCH1 in HNSCC and the other tumor types, results of some studies demonstrated its different role in tumorigenesis, such as tumor suppressive function. Grilli *et al.* showed tumor suppressive role of NOTCH1 in 324 HNSCC samples that NOTCH1 expression strongly correlated with nuclear HES1 and p21 expression. The patients who were triple positive for NOTCH1/HES1/p21 markers had significantly better disease-specific survival and OS rates.[29] The result of *in vivo* study by genetically engineered mouse models showed the inactivation of MAML1, an essential transcriptional coactivator of the NOTCH pathway, induced tumorigenesis, and increased nuclear β-catenin expression. Loss of function in NOTCH signaling approved the tumor suppressive function of NOTCH1 in HNSCC.[30]

The NOTCH1 is known as the pivotal protein regulator of NANOG on mRNA level: Assessment of 120 OSCC patients by Grubelnik *et al.* demonstrated upregulation of NANOG and OCT4 and downregulation of NOTCH1 and AGR2 in metastatic cases in comparison to the nonmetastatic patients. Their result emphasized the pivotal role of protein regulators (like NOTCH1) and microRNAs (such as miR-34a) more than promoter methylation and copy number variation of NANOG.[31] Although some previous studies emphasized on tumor suppressor role of NOTCH1 in OSCC, a novel mutation known as C1133Y was detected in the Chinese population that truncated protein cannot present at the cell surface. In this way, C1133Y mutated NOTCH1 activated the EGFR-PI3K/AKT and improved proliferation and invasion in OSCC.[32] The other study, based on the transfection of mutant NOTCH1 vectors (NOTCH1V1754 L) into OSCC cell lines, approved the activation of the EGFR-PI3K-AKT signaling pathway and presentation of oncogenic phenotype.[33]

The result of the present study highlighted the oncogenic role of NOTCH1 in OSCC pathogenesis. Accordance to the substantial difference in NOTCH1 expression between OLP, OSCC patients, and healthy control, we can say NOTCH1 plays a pivotal role in the bio-pathogenesis of the oral malignancy process. The increased expression in OLP patients is a sign of malignant transformation. It seems that NOTCH1 can consider a biomarker for the evaluation of malignancy transformation. Although the result of our study did not show a statistical difference between grade and stage in NOTCH1 expression, increased expression confirmed the oncogenic role of this gene in the tumorigenesis process of OSCC. Besides, the results of our research demonstrated a statistical correlation between NOTCH1 expression and age. It seems lifestyle and unhealthy environmental factors should be controlled to do not impact the molecular pathways in cells to alter the expression of genes with an oncogenic role.

We propose the detection of other proteins that interact with NOTCH1 to provide more candidates for prognostic and therapeutic approaches such as modifier genes. And also, other molecular regulators such as microRNAs, LncRNAs, and cell surface molecules[34] that target the NOTCH1 pathway can be discovered and evaluated for reducing the oncogenic function of NOTCH1.

CONCLUSION

There was a substantial difference between the expression of NOTCH1 in OSCC and OLP patients in comparison to the healthy group. According to the result of the current study and previous reports, it seems NOTCH1 can be considered as a biomarker in the oral malignancy transformation procedure. The role of NOTCH1 as an oncogene in OSCC pathogenesis requires far more experimental evidence.

Acknowledgments

The authors appreciate the Research Council of MUMSs, Faculty of Dentistry, and Neyshabur Islamic Azad University for their financial support.

Financial support and sponsorship

Nil.

Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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