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FREQUENCY OF NOTCH 1 MUTATIONS IN ORAL SQUAMOUS CELL CARCINOMA IN TERTIARY CARE HOSPITAL OF PAKISTAN

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Abstract

Introduction: One of the most prevalent cancers in Pakistan is oral sqaumous cell carcinoma (OSCC). Its absence of well-established disease prognostic factors makes community-based screening necessary to find molecular targets for higher risk and better therapy, early identification, rehabilitation, and palliative care. Recently, it was found that NOTCH 1 is a significant oncogene connected to OSCC. **Objective:** The aim of this study was to identify NOTCH 1 mutations in patients with active oral cancer. If these mutations are identified, it will be clear how these genes are involved in the development of oral cancer and will also help researchers identify the genetic mechanism underlying these mutations, and if all known risk factors can be prevented, how these mutations can be prevented. **Methodology:** 63 samples of the said carcinoma with at least 70% tumour cells were obtained, and histopathological analyses for tumour grading were done. Spin column kits were employed and DNA was extracted from the samples. That DNA was employed to amplify the NOTCH1 gene fragment, which was then isolated using an Agarose gel electrophoresis. The satisfactory amplified products were sent for commercial DNA sequencing. The acquired sequences were compared to reference sequences using bioinformatics methods for mutation analysis. **Results:** Among 63 patients in the study, approximately 64.9% had poor to moderately differentiated oral squamous cell carcinoma, and approximately 53.1% had well differentiated disease. Notch mutations were found in F1-6 and F2-13 samples with missense mutations. F1-6 had an exon mutation and F2-13 had an intron mutation. **Conclusion:** Notch 1 mutations in the ligand binding area may serve as functional prognostic markers, and that domain may be employed as a novel therapeutic molecule in oral squamous cell carcinoma.

Keywords: Oral squamous cell carcinoma, NOTCH1, Mutation.

INTRODUCTION

Oral squamous cell carcinoma is the most prevalent type of head and neck cancer in Southern Asia and the main factor in cancer-related death[1]. The Global Cancer Observatory (GCO) estimates that 377,713 OSCC cases were reported globally in 2020[2]. Asia recorded the highest number of cases (248,360), followed by Europe (65,279) and North America (27,469)[3].

Over a five-year period, there were over one million cases of OSCC (959,248), with Asia having the highest incidence. Europe and North America were the next most common regions. It's also vital to keep in mind that in less developed nations, inadequate data documentation may cause OSCC incidence and prevalence to be underreported[4].

A number of risk factors, such as heavy alcohol use and protracted nicotine use, are the first steps in the complex process that leads to OSCC. Genetic alterations that occur in epithelial cells during this phase can lead to the development of multiple neoplastic sites

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throughout the oral cavity or, more frequently, can promote the progression of premalignant lesions that have already started to develop[5]. The most often mutated genes are p53, TP53, CDKN2A, PIK3CA, and CCND1[6]. Different genetic defects brought on by prolonged exposure to carcinogens like alcohol, smoking, viral infections, and inflammation are widely acknowledged as the aetiology of OSCC[7].

It has been found that NOTCH 1 is a significant oncogene or tumor suppressor gene in tumors. Patients with oral cancer may be associated with NOTCH 1 mutations, indicating its prognostic role in cancer[8]. Numerous roles for the Notch pathway are involved in carcinogenesis. Depending on the cellular environment, it may have pro-oncogenic or tumour suppressive properties[9].

In contrast to the oncogenic roles previously identified in malignancies, the NOTCH network has recently been found to have tumor-suppressive actions in a variety of tumour types. The NOTCH signalling pathway can be activated to treat acute myeloid leukaemia (AML)[10].

METHODOLOGY

The Department of Molecular Pathology, DDRRL, at Dow University of Health Sciences carried out this cross-sectional/analytical investigation. After the results of the histopathological study were confirmed, the samples for squamous cell carcinoma were gathered. The DUHS ethical committee approved this study (IRB-1445/DUHS/Approval/2019/110). Samples were obtained from Department of Oral and Maxillofacial Surgery of Dental Hospital, DIKIOHS and Civil hospital Karachi. On the 63 samples that were gathered, histopathology examination was used to grade the tissue. The hispathological analysis was conducted at Dow Diagnostic Research and Reference Laboratory (DDRRL). Then the samples were passed through the inclusion and exclusion criteria for additional verification and analysis.

Total 63 samples of patients with OSCC were gathered with the ratio of 70% tumor enrich content. The sample size calculated from the Open-Epi software on 80% power, 95% confidence interval and 3.1% of OR[11]. The study lasted for 1 year, starting from January 2020 to February 2021. The inclusion criteria were: patients with OSCC with at least 70% tumor content. Exclusion criteria were: the patients having any kind of treatment protocols for example chemotherapy or radiotherapy, diagnosed OSCC patient containing <70% tumor enrichment and pregnant females.

The study parameters were change in protein and mutation (Notch 1) observed along the whole length of test samples and Variation in distribution of oral cancer stage (Poor, Moderate and well differentiated) with respect to age, gender and within region of oral cavity. Biopsies were obtained by the researcher and collected in formalin filled container.

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Thereafter, the biopsied tissues were graded as per Brooders system of classification into:

1) Well differentiated (75 – 100 % of cells show differentiation)
2) Moderate differented (50 – 75% of tumor cells show differentiation)
3) Poorly diffrenteated (25 – 50% of tumor cells show differentiation)
4) Anaplastic (0 – 25% of tumor cells show differentiation)[12]

NOTCH1 gene amplification protocol

In order to extract DNA from FFPE (formalin-fixed paraffin-embedded) tissue specimen, the material must go through deparaffinization before it can be further processed.

Extraction of DNA from (Formalin fixed paraffin-embedded)

As pretreatment for Notch 1 DNA extraction, 1 ml Xylene is added to FFP sample in an Eppendorf, followed by centrifugation for 5 minutes at speed of 12000 rpm. Then 1 ml ethanol is added to precipitate xylene, and centrifugation is done once again at similar speed and time. The consequent solution undergoes incubation in shaking water bath set at 560 C overnight, after adding 180 uL ATL buffer and 20 uL proteinase K.

For DNA extraction, the samples are taken off from water bath and are vortexed for few seconds first, and then once again for 15 seconds after adding 200 uL of ATL buffer and 200 uL of ethanol to the Eppendorf. Thereafter, the solution is transferred into spin column after a spin. It undergoes centrifugation for a minute at 8000 rpm and then the collecting tube is discharged. Then a new collecting tube is employed after addition of 500 uL wash buffer, labelled as 1 in the Eppendorf and it is centrifuged again for a minute at same speed. The collection tube is removed and replaced once again. 500 uL of wash buffer, labelled as 2, is added and it undergoes centrifugation at a speed of 8000 rpm for 3 minutes. Collection tube is again changed, but at this point the new tube is run on vacant spin at 8000 rpm for 1 minute. The collection tube is removed and replaced with an Eppendorf. 50 uL of elusion buffer is added to the spin column membrane and is incubated for a minute. The sample is then centrifuged again at 8000 rpm for a minute. At last, the extracted DNA yield and purity is evaluated by using NanoDrop.

Polymerase chain reaction

Notch 1 gene was amplified employing DreamTaq Master Mix, which was prepared by using 12.5 uL DreamTaq Green, 0.5 uL forward primer, 0.5 uL reverse primer, 8.5 uL water and 3 uL DNA sample. A reaction mixture of 25 µL was prepared. Temperature profile of thermocycler was set for initial denaturation at 950 C for 10 minutes and 1 minute respectively. Annealing was performed at 550 C for a minute, and then elongation was done at 720 C for a minute. Finally, extension was carried out at similar temperature for 7 minutes.

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GEL Electrophoresis

In order to prepare 2% agarose gel, 2 gm of agarose was added to 50 mL 1X TBE buffer and then heated to dissolve agarose. Thereafter 0.5 uL Ethidium bromide was added for observing DNA under UV light. Dissolved agarose solution was then poured into casting tray and combs were placed to form wells. Once the gel had cooled down and solidified, it was taken into electrophoresis chamber that contained 1X TBE buffer. Thereafter, 5 uL PCR product was loaded into the wells along the 100 bp ladder onto the gel and ran at 110V for 45 minutes. Finally, the amplified bands were visualized under UV along a reference ladder.

Purification of PCR product

Following confirmation of product amplification through gel electrophoresis, 100 uL binding buffer was added to 20 uL PCR product. Thereafter the solution was pipetted to a spin column and centrifuged at 10,000 g for a min. After throwing away flow completely, spin column was kept in a new collecting tube and 650 uL wash buffer was added into the column. Thereafter the resultant was centrifuged at 10,000 g for a minute following change of the collecting tube. To eliminate any residual ethanol, the column was centrifuged one more time at full speed for 2-3 minutes. Thereafter the spin column was kept in an Eppendorf and 50 uL elution buffer was added. The material was centrifuged for two minutes at maximum speed after being incubated at room temperature for one minute. The column was thrown away and the PCR product was recovered in an Eppendorf as a purified sample. The resulting sample was kept at -200C until it was processed further.

Table 1: Pre-Treatment reagents for DNA extraction by formalin fixed paraffin embedded

Table 3.2 Reagents for Extraction of Notch 1 DNA

Reagents	Volume of reaction
ATL buffer	200 uL
Ethanol	200 uL
Wash buffer (1)	500 uL
Wash buffer (2)	500 uL
Elusion buffer	50 uL

Table 2: PCR (Polymerase chain reaction) Reagents

Reagents	Volume of reaction
Dream Taq Green	12.5 uL
Forward primer	0.5 uL
Reverse primer	0.5 uL
DNA sample	3 uL
DH_2O	8.5 uL
Total	25 uL

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Table 3: Gel electrophoresis Reagents

Reagents	Volume of reaction
Agargose	2 gm
Ethidium Bromide	0.5 uL
PCR product	5 uL

Table 4: PCR Product purification reagents

Reagents	Volume of reaction
100 uL	Binding buffer
PCR product	20 uL
Wash buffer	650 uL
Elusion buffer	50 uL

Table 5: Conditions for cycling for Amplification of Notch 1 gene exon

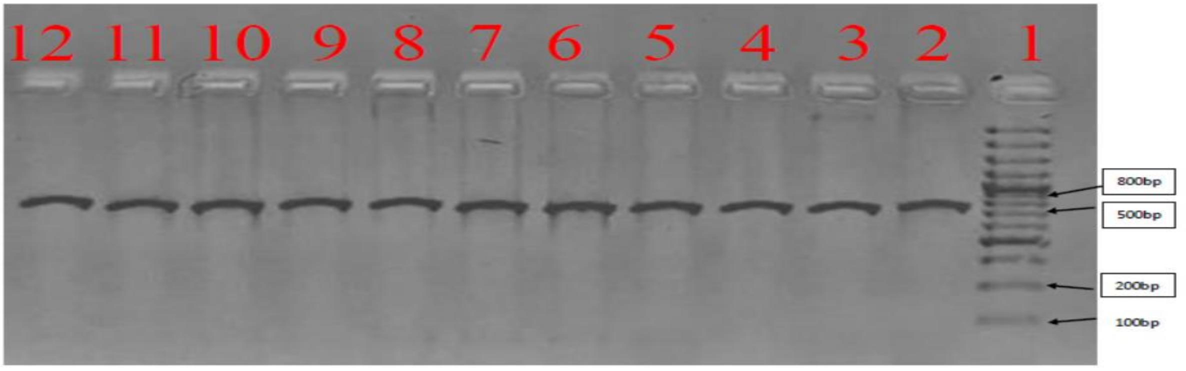
Steps	Temperature	Duration	Cycles
1	95^0 C	10 mins	
2	95^0 C	1 min	
3	55^0 C	1 min	45 cycles
4	72^0 C	1 min	
5	72^0 C	7 mins	

Table 6: Primer sequence used for amplifying Notch-1 gene region

Gene under inspection	DNA ID	Primer name	Sequence (5’ ⟶ 3’)
	S017125-9-1	Ntex6-F	GCACAAAGAGAGGAGGCAAGAC
	S017125-9-2	Ntex6-R	ACAGTCCCTGGGTGAGGTC
NOTCH 1	S017125-9-3	Ntex6-F	GAACGTCCCAAGTGTCAC
	S017125-9-4	Ntex6-R	AAGCAACCCACAGATGTTCC

Table 7: Notch 1 has 34 exons and it’s located on chromosome 9

Sn	Forward primer	Reverse primer	Product size (bp)	Exons coverage
1	GTCAGTACTGTACCGAGGATGTG	CTACCCAGCGAGCACTCATC	692	6 & 7
2	GTGCCAACCCCTGCGAGCATG	CTCACCCGTGGGGCACTCGC	671	8 & 9



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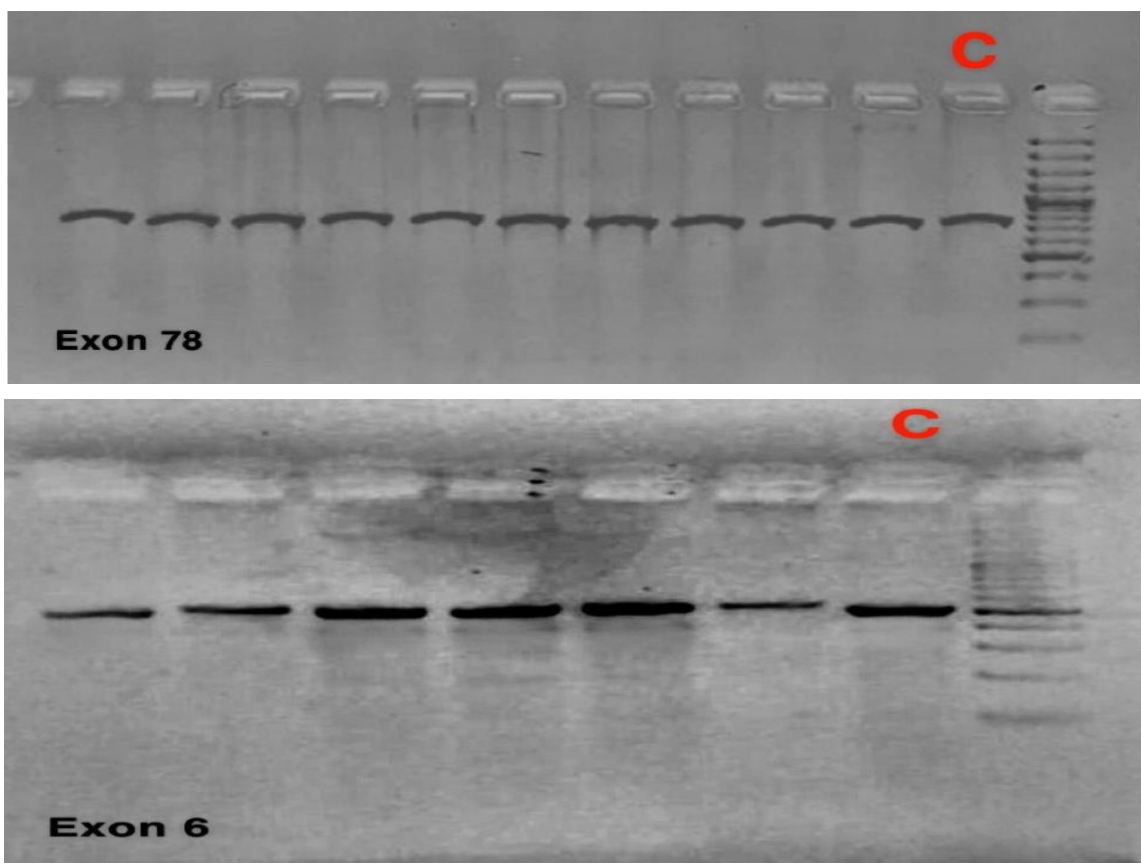

Figure 1: Gel photograph of Notch 1 amplified gene. Agargose gel demonstrating zvarious amplified components. Lane 2 demonstrates positive control.

RESULTS

The number of participants broken down into two age groups is shown in Table 1. a younger age group with participants between the ages of 15 and 39 years, and an adult/older age group with people between the ages of 40 and 80 years. Upon the histological processing of the 63 biopsy samples, it was demonstrated that in the young age group (15-39 years), 14 patients had poor or moderate oral cancer, whereas 7 participants had well-differentiated oral cancer. Whereas, among adult/older age group (40-80 years) there was a rise in the number of OSCC cases, with 25 individuals diagnosed as having poor or moderate type of the disease, and 18 individuals having a well differentiated variety of the disease.

Table 1 lists the number of participants broken down according to different oral sites from where the sample tissue was taken. Three main sites of oral cavity were taken: buccal mucosa, tongue, floor of the mouth. Most of the tissue samples were taken from buccal mucosa followed by tongue and floor of the mouth, respectively. Among the tissue samples of buccal mucosa, 37 had moderately/poorly differentiated oral squamous cell

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carcinoma, and 20 had well differentiated disease. Among samples collected from tongue, only 1 sample was graded as poorly differentiated and three samples had high degree of differentiation. Two samples were collected from floor of the mouth, and they were graded as well differentiated. In table 2, number of participants with OSCC are classified according to sex. Out of total 63 participants, males were 38 in number, of which 31 were graded as moderately/poorly differentiated and 7 as well-differentiated. The number of female participants was 25, of which only 1 case was well differentiated and the other 24 cases were either poorly or moderately differentiated. In order to find out a relationship between parameters of age, gender and oral sites with stage of oral cancer, binary logistic regression was used through SPSS version 23. Table # 4.2 shows the results. With p-Value less than 0.005, the relationship among all parameters was considered statistically significant.

Histological reporting of OSCC samples:

In order to identify the variety of OSCC, H&E staining was performed. Out of total 63 samples, 25 were graded as well differentiated and the rest 38 were either poorly differentiated. The prepared slides were visualized at different magnifications with common feature of infiltrating OSCC visualized in most of histological slides. In slide # 7 and 10, keratin pearls could be visualized. In slide # 8, mitosis was visualized at 130X magnification. Mutational analysis of Notch 1 samples:

Employing PCR, the fragments underwent amplification with the help of specific pair of primers (Table 1 & 2) in order to detect mutations in the Notch 1. Thereafter the amplified fragments were sent for sequencing. The sequences were further purified by BioEdit and then refined. The mutations were visualized in samples labelled as F1-6 and F2-13, with missense mutation. In sample F1-6, exon 6(NG_007458:1:g.32058T>A) carried mutation; whereas in sample F2-13, intron 7-8(NG_007458.1:g.32686G>T) carried mutation. The mutations visualized were shown at bp 258 and bp 309 with encoding of “C” and “T” as observed in graph “1” and “2”. The point mutation can further be seen in output data shown in figure 4 representing sequence alignment through MEGA7.

Table 1: Mutational analysis of Notch 1 samples

Sample	Nucleotide under investigation	Mutation observed	Protein change
F1-6	NG_007458.1:g.32058T>A	Yes Exon 6	NG_007458.1(NOTCH1_i001): p.(Cys321Arg)
F2-13	NG_007458.1:g.32686G>T	Yes Intron 7-8	None

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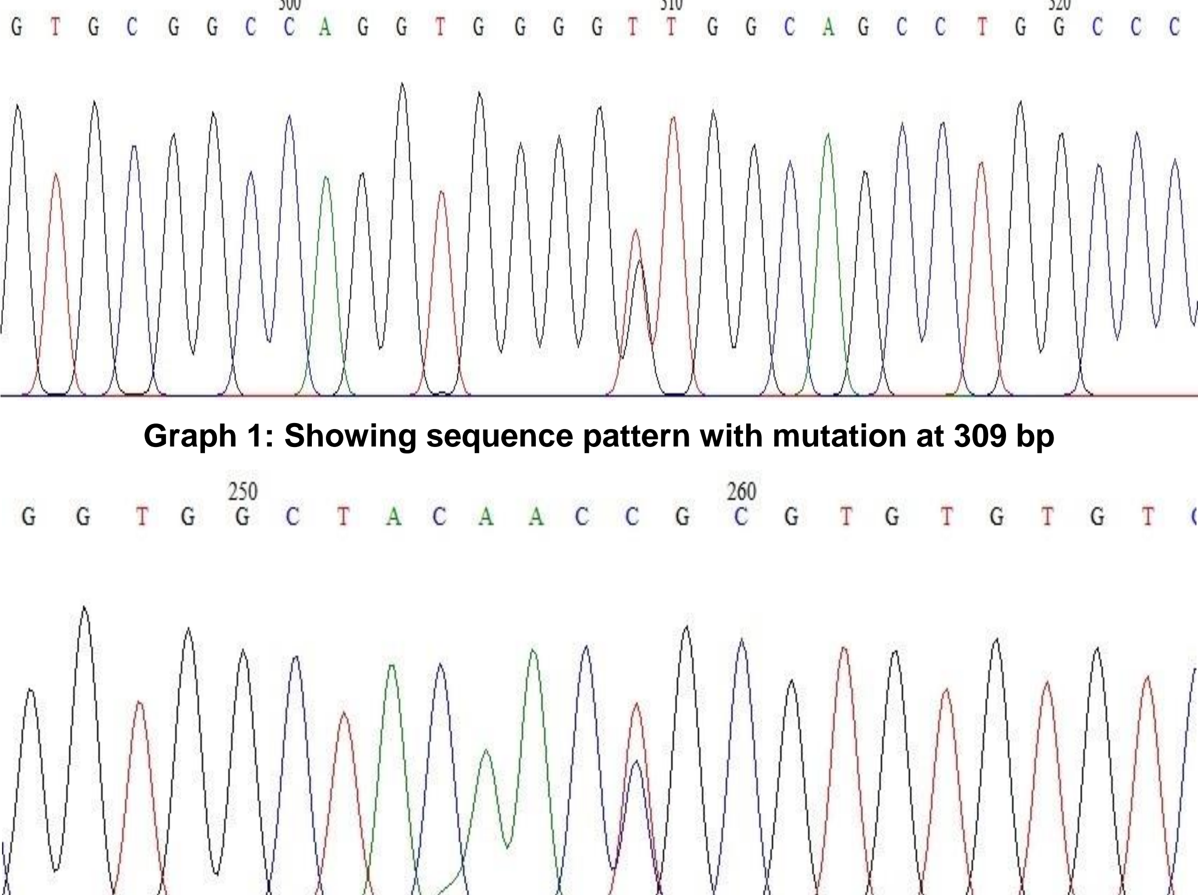
Table 2: Relationship of parameters of age, gender, and site of oral cavity with stage of oral cancer

OSCC GRADING	Poorly differentiated (N=38) (60.3 %)	Well differentiated (N=25) (39.7%)	P-Value	Unadjusted ODDS Ratio (95% CI)	Adjusted ODDS Ratio (95% CI)
Oral cavity site					
Buccal Mucosa (57)	37 (64.9%)	20 (35.1%)		1.0	1.0
Tongue (4)	1 (25%)	3 (75%)	<0.001	1.2(0.9,1.4)	12.6
Floor of the mouth (2)	0 (0%)	2 (100%)			(1.0,154.5)
Age					
15-39 yrs (21)	14 (66.7%)	7 (33.3%)	<0.001	1.0	1.0
40-80 yrs (42)	25 (57.1%)	18 (42.9%)		1.5(0.5,4.4)	2.5 (0.7,8.5)
Gender					
Male (38)	31 (56.4%)	7 (87.5%)	<0.001	1.0	1.0
Female (25)	24 (43.6%)	1 (12.5%)		0.2(0.1,1.6)	0.1 (0.1,1.2)



Graph 1: Showing sequence pattern with mutation at 309 bp

Graph 2: Showing sequence pattern with mutation at 259bp

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DNA Sequences | Translated Protein Sequences

Species/Abbrv	Group Name	Sequence
1. NG_007458.1_		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
2. F-2-2_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
3. F-2-3_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
4. F-2-4_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
5. F-2-5_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
6. F-2-6_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
7. F-2-7_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
8. F-2-8_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
9. F-2-9_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
10. F-2-11_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
11. F-2-12_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
12. F-2-13_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGTTGGCAGCCTGGCCCCAGAACCGATGAG
13. F-2-14_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
14. F-2-15_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
15. F-2-16_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
16. F-2-17_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
17. F-2-18_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
18. F-2-19_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
19. F-2-20_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG
20. F-2-1_F78		TGCCAGCACAGGGGGTGCGGCCAGGTGGGGGTGGCAGCCTGGCCCCAGAACCGATGAG

Figure 2: Multiple sequence alignment of Notch 1 amplified fragment. Mutation can be observed at specimen # 12

DNA Sequences | Translated Protein Sequences

Species/Abbrv	Group Name	Sequence
1. NG_007458.1_		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACTGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT
2. F-1-2_F6		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACTGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT
3. F-1-3_F6		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACTGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT
4. F-1-4_F6		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACTGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT
5. F-1-5_F6		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACTGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT
6. F-1-6_F6		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACCGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT
7. F-1-7_F6		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACTGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT
8. F-1-8_F6		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACTGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT
9. F-1-9_F6		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACTGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT
10. F-1-11_F6		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACTGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT
11. F-1-13_F6		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACTGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT
12. F-1-14_F6		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACTGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT
13. F-1-15_F6		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACTGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT
14. F-1-16_F6		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACTGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT
15. F-1-17_F6		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACTGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT
16. F-1-18_F6		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACTGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT
17. F-1-19_F6		GAACGGCGGGACCTGCCACAACACCCACGGTGGCTACAACTGCGTGTGTGTCAACGGCTGGACTGGTGAGGACTGCAGCGAGAACATT

Figure 3: Multiple sequence alignment of Notch 1 amplified segment. Mutation can be observed at specimen # 6

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DISCUSSION

Oral squamous cell carcinoma is one of the most common cancers. However, well-established factors tied to disease prognosis are deficient[13]. This fact makes community-based screening essential to discover molecular targets for the elevated risk and better therapy, early detection, rehabilitation, and palliative care. These initiatives will help people live longer and have a higher quality of life[14]. Our study demonstrates buccal mucosa to be the most common site for oral cancer. This matches the statement found in the study of Essig H et al, that buccal mucosa is the most common site for oral cancer[15]. Our study also demonstrates that the number of individuals with the disease is almost twice among older age group. This is consistent with a study by Malik et al (2018), in which it was showed that more than 50% of oral cancer cases were found among age group 40 years above[16].

NOTCH1 mutations are most commonly found in head and neck squamous cell carcinoma (HNSCC)[17]. These mutations are found in people with OSCC and HNSCC, especially at the NOTCH1 ligand-binding area. The G1393A (p. A465T) mutation discovered in two Japanese OSCC patients is situated in EGFr12, whereas the other six mutations are found in EGFr1011[18]. An earlier study demonstrated that the G1393A (p.A465T) mutation causes a conformational shift in the NOTCH1 ligand-binding domain employing protein structure modelling. However, other research using cancer cell lines failed to shed light on the functional relevance of NOTCH1 mutations observed in clinical data[19]. In our study mutations observed at genetic sequence from G to T and there is nucleic acid change effect on the protein as well. This is consistent with the study of Yang WF et al. in which they observed NOTCH 1 mutation in individuals with oral cancer and informed that most of the mutations were visualized with substitutions at C > T, followed by G > A and A > G. 7 mutations were found to be nonsense and 35 missense. Yang WF et al. also reported that most of the NOTCH 1 mutations were found at Notch 1 heterodimerization domain that resembled Notch1 mutations found in head and neck cancers. This study's mutation rate, which was 43%, is significantly higher than our study's[20].

CONCLUSION

It is expected that the p.A465T mutation will give rise to alternate NOTCH1 cleavage, leading to structural changes and/or atypical NICD migration. Although derangement in NOTCH 1 pathways and its mutation has been tied to cancer. There are studies that show us that NOTCH1 operates as an oncogene and that the cell proliferation and/or tumorigenicity of OSCC32 is impacted by the p. A465T NOTCH1 mutation in the ligand-binding site. Additionally, our study also revealed that NOTCH1 mutations in the ligand-binding site might be good prognostic markers, and that the domain could be employed as a novel therapeutic molecule in oral squamous cell carcinoma.

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Ethical Approval

Dow University of Health Sciences
Institutional Review Board (IRB)

Ref: IRB-1445/DUHS/Approval/2019/110 **Dated: 04th -November-2019**

Dr. Zia Abbas,
Enrolment# 11/2011/016
MDS Trainee ,
Department of Oral Surgery,
Dr. Ishrat-ul-Ebad Khan Institute of Oral Health Sciences,
Dow University of Health Sciences

Subject: **Institutional Review Board's approval for a research proposal.**

Title of Study: Notch 1 Mutations in Oral Squamous Cell Carcinoma of Pakistani Population.

Enrolment duration: 01-Nov-11 to 31-Oct-19.

CRR Due Date: 03rd November 2020.

Dear Dr.Zia Abbas,

Thank you for submitting the above mentioned study proposal. I am pleased to inform you that the IRB-DUHS has reviewed this proposal in its **150th meeting** held on **07th September, 2019** and gives approval for a period of one year to conduct this study.

It should be noted that project must be submitted and get BASR approval after successful defense on or before 31-Oct-19. Any change in the protocol or extension in the period of study should be notified to the board for approval. Interim report on progress of study should be submitted to IRB from time to time.

Nazli Hossain

Prof. Dr. Nazli Hossain
Meritorious Professor (BPS-22),
Professor of Obstetrics & Gynaecology Unit-II,
Chairperson Institutional Review Board,
Civil Hospital Karachi &
Dow University of Health Sciences,
Karachi.

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