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ORIGINAL ARTICLE

Absence of nucleotide alteration in region of exon 34 of *NOTCH1* and *NOTCH2* receptor genes analysed in oral cancer samples: a preliminary observation

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Keywords

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Abstract Oral cancer is one of the common cancer cases identified in the developing countries. Genetic mutation and overexpression of certain genes and proteins have been associated in the development of this cancer. Notch signalling pathway is normally involved in controlling the development process of vertebrates and invertebrates; however, deregulation of this pathway was found to be responsible in the formation of certain cancers including oral cancers. Activation of this pathway requires binding of the ligands to its receptors. Four *NOTCH* receptors (*NOTCH 1, 2, 3* and *4*) have been identified in mammals. Disruptions within these molecules might interfere with the normal functions of Notch signalling pathway. Hence, this study was conducted to detect mutations of *NOTCH1* and *NOTCH2* receptor genes which might be occurring in the oral cancer cases obtained from the local population. DNA extracted from fresh-frozen tissue biopsy of the tongue and buccal mucosa from 10 confirmed cases of oral cancer were subjected for polymerase chain reaction (PCR) amplification using the specific sets of primers. The PCR products were sent for sequencing before final results were analysed. Due to time and cost limitation, only two out of four *NOTCH* receptor genes; *NOTCH1* and *NOTCH2,* were used in this analysis. The results revealed absence of nucleotide changes for both *NOTCH* receptor genes amplified from these oral cancer samples. More samples and further analysis looking into other regions in these genes are required to conclude the involvement of *NOTCH* receptor genes mutation in causing oral cancer.

Introduction

Oral cancer is a common cancer observed world-wide and is more prevalent in developing countries, such as Malaysia. Together with pharyngeal cancer, it has become the 6th most common cancer in the world with annual estimated incidence of 275,000 cases (Warnakulasuriya, 2009). In Malaysia, Indians and indigenous people of Sabah and Sarawak have the highest risk and prevalence of precancer and oral cancer (Zain, 2001).

Becoming a part of group of cancers called head and neck cancers, this epithelial neoplasia generally begins as a focal clonal overgrowth of altered stem cells near the basement membrane, expanding upward and laterally, replacing the normal epithelium. They can be classified into three; based on the histopathological and clinical features which are benign, pre-cancerous (leukoplakia and eryhtroplakia) and malignant. About 15% of leukoplakias and 51% of erythroplakias tend to develop into cancer whereby they are found to be malignant during biopsy by demonstrating histologic progression to squamous cell carcinoma (Reichart and Philipsen, 2005). Squamous cell carcinoma becomes the most common types of malignant lesions; representing 94% of all types of malignant tumours (Neville *et al.,* 2002). In Asian, the common site of oral cancer detected was at the buccal mucosa due to betel nut and tobacco chewing (Warnakulasuriya, 2009).

Besides multiple risk factors such as tobacco smoking and betel nut chewing, studies have shown that mutation or over expression of certain genes and proteins maybe associated with oral cancer

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development (Neville *et al*., 2002). One of the many signalling pathways which have been found to be associated with the incidence of cancer development is Notch signalling pathway (Leethanakul *et al.,* 2000). Notch signalling pathway plays a crucial role during embryonic development in both vertebrates and invertebrates (Artavanis-Tsakonas *et al.,* 1999). Activation of this pathway requires binding of the ligands to its receptors. Four *NOTCH* receptors (*NOTCH 1, 2, 3* and *4*) and five ligands (JAG1, JAG2, DLL-1, DLL-3 and DLL-4) have been identified in mammals (Lai, 2004). NOTCH receptors are heterodimeric proteins which are cleaved into an extracellular, a transmembrane and intracellular subunits. The intracellular subunit consists of a RAM domain, ankyrin repeats, a transactivation domain and a PEST domain (Mumm and Kopan, 2000; Lai, 2004). When activated, the intracellular subunit translocates into the nuclei and regulates the transcription of many genes. Activation of Notch signalling pathway regulates multiple cellular processes including cell proliferation, differentiation and apoptosis (Leong and Karsan, 2006).

Overexpression of NOTCH proteins have been implicated in tumorigenesis and contribute to the pathogenesis of hematopoetic and solid malignancies (Leong and Karsan, 2006). Somatic mutation in *NOTCH1* was detected in 56% of T-cell acute lymphoblastic leukemia (T-ALL) mainly in the DNA sequences encoding for N-terminal heterodimerization domain (exon 26, HD^N), C-terminal heterodimerization domain (exon 27, HD^C) and exon 34 of PEST domain (Weng *et al.,* 2004). Besides hematopoetic malignancies, deregulation of NOTCH receptors (NOTCH1, 2, 3 and 4) has also been identified in other malignancies (Leong and Karsan, 2006, Lee *et al.,* 2007). Interestingly, gene expression analysis also shown that most of head and neck squamous carcinoma overexpressed members of Notch signalling pathways molecules, suggesting contribution of these genes in squamous cell carcinogenesis (Leethanakul *et al*., 2000).

Given that deregulation in *NOTCH1* receptor has been identified in many cancers, it is possible that NOTCH1 gene mutation might be present in oral cancer cases. Also, because NOTCH2 has the similar structure and mechanism of action with NOTCH1, it would be interesting to analyse if mutation in *NOTCH2* is also responsible for the pathogenesis of oral cancer. Therefore, the objective of this preliminary analysis was to identify and analyse presence of mutation in *NOTCH* receptor genes (*NOTCH1* and *NOTCH2*) at exon 34 which might be occurring in the cases of oral cancer from our local population.

Material and methods

Ten fresh-frozen biopsy tissue samples obtained from patients diagnosed with oral cancer from Hospital Universiti Sains Malaysia (HUSM) were included in the study. The biopsy samples included in this analysis were taken from the most common site of lesion of oral cancer which was the buccal mucosa, tongue and floor of the mouth. Ethical approval was obtained prior to conduct of the study.

Molecular analysis

a. DNA extraction

The DNA was extracted from the tissue samples using the commercially available DNA extraction kit (GeneALL, USA) following the protocol provided by the manufacturer. The DNA obtained was stored in -20^{o}C for further use.

b. DNA amplification

The extracted DNA obtained from 10 oral samples cases were subjected for polymerase chain reaction (PCR) amplification using primers specific for *NOTCH1* and *NOTCH2* receptor genes at exon 34. The primers were selected based on the findings that some *NOTCH1* gene mutations have been detected within the PEST domain (exon 34) (Weng *et al.,* 2004). The sequences for these primers were according to Lee *et al.* (2007).

The primers were generated by Sigma-Aldrich (Malaysia). The PCR mixture was prepared in a total volume of 25 µl reaction mixture containing 20 mM $MgCl_2$ in 10X PCR buffer, 10 mM dNTP mix, 10 µl of each forward and reverse primer, 0.625 µl of DNA Polymerase (5U/µl) (YEAtaq, Yeastern Biotec. Co. Ltd, Korea) and 3 µl of DNA template. The PCR cycle used was similar for both *NOTCH1* and *NOTCH2* primers; 94 ^{o}C for 5 minutes for initial denaturing process followed by denaturing step at 94 ^{o}C for 30 seconds, annealing at 63.5 ^{o}C for 20 seconds and elongation at 72 ^{o}C for 30 seconds. The cycle was repeated for 30 times followed by a final elongation step at 72 ^{o}C for 5 minutes (MJ

Research, PTC-200, Peltier Thermal Cycle, USA). 3 μl of the PCR product were electrophoresed on 2% agarose gel in TBE buffer at 90V (ELITE 200 Electrophoresis System, UK) and visualised under UV after SYBr green (1 μl) staining of the gel. Production of a discrete single band at 175 bp (*NOTCH1*) and 195 bp (*NOTCH2*) on 2% agarose gel indicates the amplification of the specific *NOTCH* receptor genes analysed.

The DNA bands were then excised and purified using purification kit following the manufacturer's protocol (NOVAgen SpinPrep PCR Clean-Up Kit, Germany) and were sent for sequencing (First Base Company, Applied Biosystem, USA). The sequencing results were viewed using Sequence Scanner V.1.0 (Applied Biosystem, USA).

Results

All 10 samples of oral cancer samples were of suitable quality and quantity for molecular analysis. Final diagnosis obtained from histopathological reports (HPE) showed that of the 10 samples, all were squamous cell carcinoma, with 5 of them were well-differentiated, 1 was moderately differentiated and only 1 were verrucuous carcinoma-moderately differentiated.

Table 1 represents the samples with its corresponding histopathology examination (HPE) diagnosis and the sequencing results of *NOTCH1* and *NOTCH2* receptor gene analysed. All samples were successfully amplified using the PCR conditions stated (Figures 1 and 3). Sequencing analysis revealed no nucleotide changes occurred in the regions of *NOTCH1* and *NOTCH2* analysed (Table 2). The DNA sequence was confirmed through BLAST search for database using *Homo sapiens NOTCH1* (Figure 2) and *NOTCH2* (Figure 4) as reference sequences.

Table 1 Primer sequences of *NOTCH1 and NOTCH2* receptor genes used in this study according to Lee *et al.* (2007)

Gene	Sequences	Size (bp)
NOTCH 1 Exon 34-1	F : 5'-AGCAGGTGGAGCCACAAAAC-3' R : 5'-GCTCGGCTCTCCACTCAGG-3'	170
NOTCH 2 Exon 34-1	F : 5'-CCCCCATTGTGACTTTCCAG-3' R : 5'-GAGCTACCTGCCCGTCCTG-3'	195

Discussion

Oral cancer patients develop a series of premalignancies and squamous cell carcinoma over a number of years, with most of them are genetically related, being derived by separate mutations within the same abnormal mucosal field, or altered by exposure to carcinogens or growth promoters in tobacco or alcohol (Mao *et al.*, 2004). Loss of heterozygosity (LOH) studies showed that a field of altered mucosa that is polyclonal involved in development of oral cancer.

Table 2 Results on mutational analysis of *NOTCH 1* and *NOTCH 2* Exon-34-1 in oral cancer samples analysed

Samples No.	Age / gender	Histopathology examination (HPE) diagnosis	Site of lesion	Sequence alteration	
				NOTCH 1 **Exon 34-1**	***NOTCH 2*** **Exon 34-1**
S2	72/M	SCC	Base of tongue	No	No
S5	42/M	SCC	Lt. buccal mucosa + Lt. Mandible	No	No
S9	57/M	SCC	Lt. lateral border of tongue + soft palate	No	No
S10	65/M	SCC-well differentiated	Tongue	No	No
S12	41/M	SCC-well differentiated	Tongue	No	No
S14	63/M	SCC-well differentiated	Rt. hard palate + maxilla	No	No
S15	48/M	SCC-moderately differentiated	Lt. lateral border of tongue	No	No
S18	71/F	SCC-well differentiated	Rt. cheek	No	No
S21	74/F	Verrucuous Ca-well differentiated	Lt. buccal mucosa + upper and lower lip	No	No
S31	39/F	SCC-well differentiated	Lt. lateral border of tongue	No	No

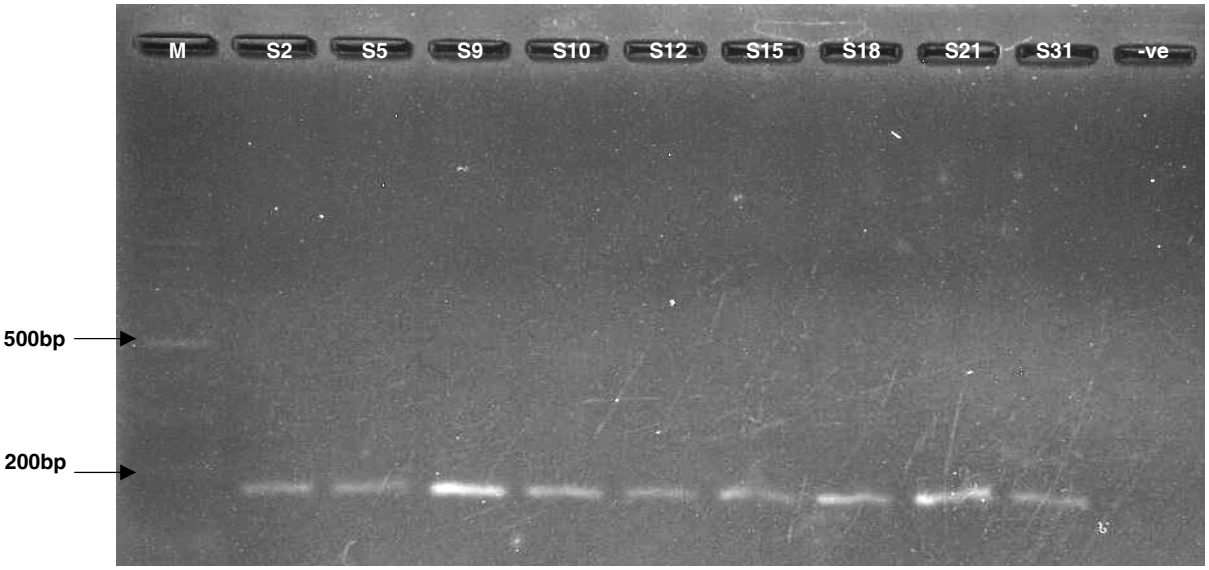
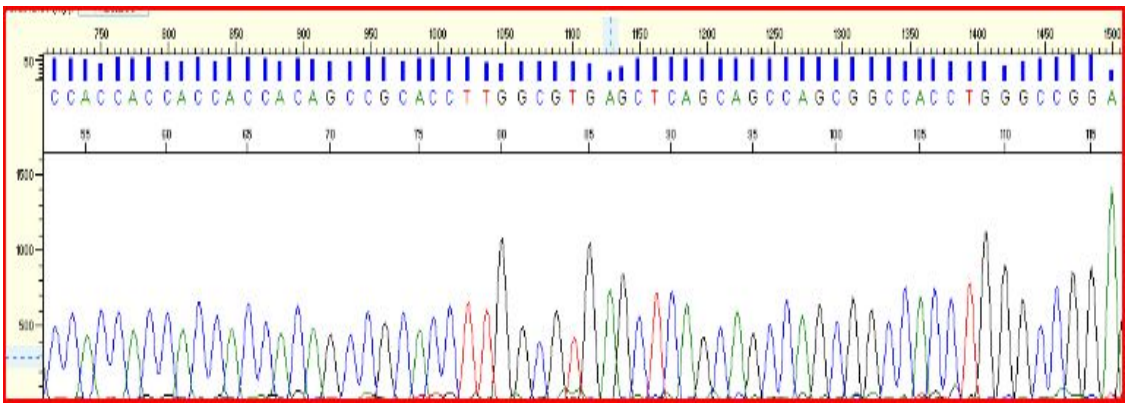


Figure 1 Analysis of PCR products amplified using *NOTCH1*(Exon-34-1) primers. PCR products of DNA obtained from oral cancer samples amplified using primers for *NOTCH1* were amplified at 175 bp. Re-substitution of DNA template with water acted as negative control (-ve), M=DNA ladder.



```
>ref|NG_007458.1| Homo sapiens Notch homolog 1, translocation-associated (Drosophila)
(NOTCH1) on chromosome 9
Length=58343

 Score =  239 bits (129),  Expect = 9e-61
 Identities = 129/129 (100%), Gaps = 0/129 (0%)
 Strand=Plus/Plus

Query  11     CTgcagccagcaaacatccagcagcagcaaagcctgcagccgccaccaccaccaccacag  70
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  54237  CTGCAGCCAGCAAACATCCAGCAGCAGCAAAGCCTGCAGCCGCCACCACCACCACCACAG  54296

Query  71     ccgcaccTTGGCGTGAGCTCAGCAGCCAGCGGCCACCTGGGCCGGAGCTTCCTGAGTGGA  130
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  54297  CCGCACCTTGGCGTGAGCTCAGCAGCCAGCGGCCACCTGGGCCGGAGCTTCCTGAGTGGA  54356

Query  131    GAGCCGAGC  139
              |||||||||
Sbjct  54357  GAGCCGAGC  54365
```

Figure 2 (Top) An example of sequencing analysis result of *NOTCH1* receptor gene obtained from oral cancer samples. (Bottom) An example of sequence alignment between *NOTCH1* receptor gene with the GenBank sources (BLAST). Both analyses show absence of nucleotide sequence alteration between subjects with reference sequence.

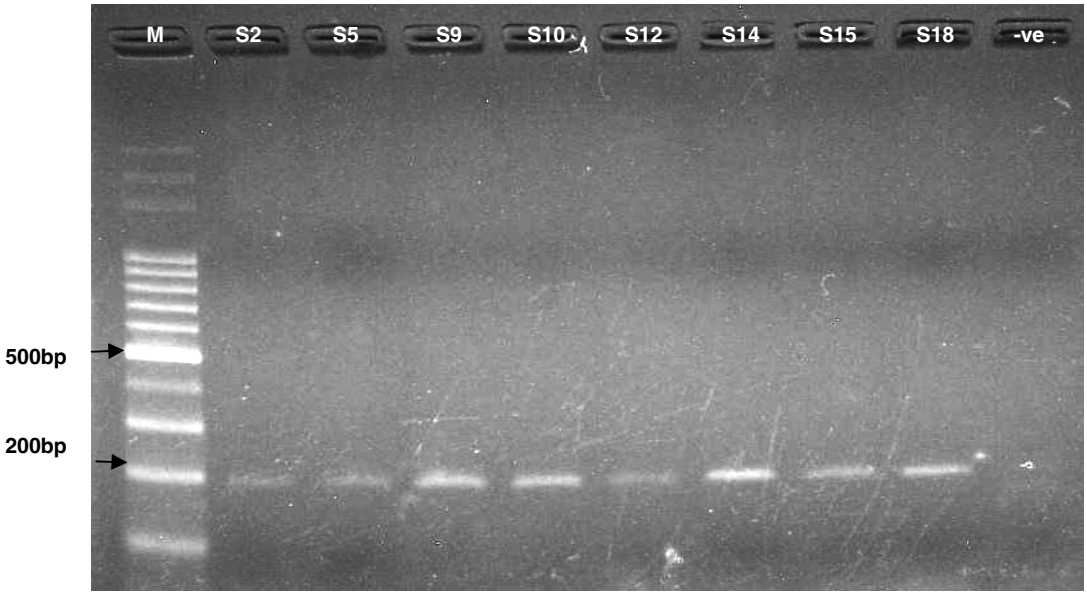
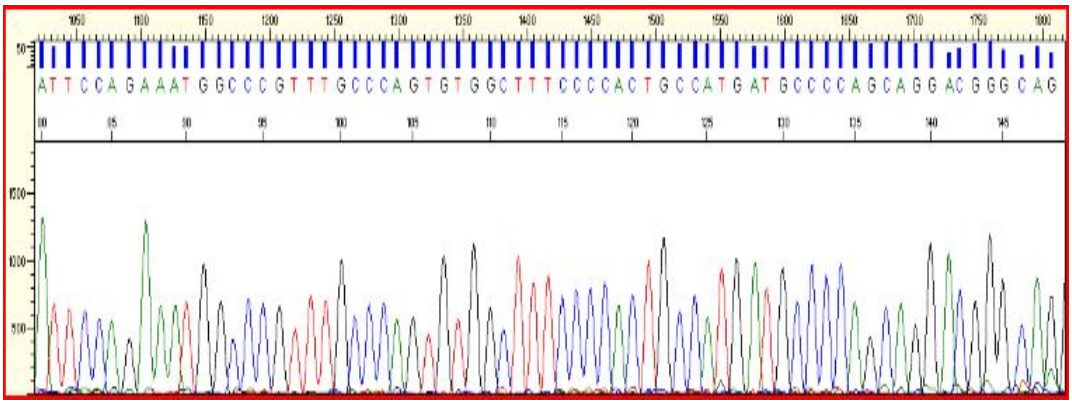


Figure 3 Analysis of PCR products amplified using *NOTCH2* (Exon 34-1) primers. PCR products of DNA obtained from oral cancer samples amplified using primers for *NOTCH2* were amplified at 195 bp. Re-substitution of DNA template with water acted as negative control (-ve), M=DNA ladder.



```
>ref|NG_008163.1| Homo sapiens Notch homolog 2 (Drosophila) (NOTCH2) on chromosome
1
Length=165101

 Score =  279 bits (151),  Expect = 6e-73
 Identities = 151/151 (100%), Gaps = 0/151 (0%)
 Strand=Plus/Plus

Query  6       AACCAGCGGGGGCTCCCCAGCCTCAGTCCACCTGCCCTCCAGCTGTTGCGGGCCCCCTGC  65
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  158882  AACCAGCGGGGGCTCCCCAGCCTCAGTCCACCTGCCCTCCAGCTGTTGCGGGCCCCCTGC  158941

Query  66      CCACCATGTACCAGATTCCAGAAATGGCCCGTTTGCCCAGTGTGGCTTTCCCCACTGCCA  125
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  158942  CCACCATGTACCAGATTCCAGAAATGGCCCGTTTGCCCAGTGTGGCTTTCCCCACTGCCA  159001

Query  126     TGATGCCCCAGCAGGACGGGCAGGTAGCTCA  156
               ||||||||||||||||||||||||||||||
Sbjct  159002  TGATGCCCCAGCAGGACGGGCAGGTAGCTCA  159032
```

Figure 4. (Top) An example of sequencing analysis result of *NOTCH2* receptor gene obtained from oral cancer samples. (Bottom) An example of sequence alignment between *NOTCH2* receptor gene with the GenBank sources (BLAST). Both analyses show absence of nucleotide sequence alteration between subjects with reference sequence.

However, most oral carcinoma are clonal suggesting that carcinomas develop from earlier lesions by a succession numbers of genetic changes (Hunter *et al*., 2006). Additionally, mutational and gene expression analysis of known tumor suppressors (e.g: *p53* and *pRb*) and oncogenes (e.g: *ras*, *myc, c-erb*) in the context of early tumorigenesis had provided insight into the role of these genes in cancer progression (Hahn and Weinberg, 2002). Deregulated expression of these oncogenes or mutation in these tumour suppressor genes have been identified in oral carcinomas *(*Neville *et al.,* 2002).

Notch signalling mechanism is responsible in many differentiation process and cell fate decision during embryonic and postnatal development (Artavanis-Tsakonas *et al.,* 1999). Studies have shown that *NOTCH* receptors also play role as oncogene when they are identified in cancer progression of T-cell leukaemia neoplasia (T-ALL) and breast cancer (Stylianou *et al.,* 2006). These suggest that aberrant Notch signalling might be able to promote tumorigenesis. Interestingly, one study had observed abnormal expression patterns of Notch signalling molecules in cases of squamous cell carcinomas thus providing the evidence implicating NOTCH signalling pathway in squamous cell carcinogenesis (Leethanakul *et al*., 2000).

Due to some limitations, this preliminary study was only able to perform analysis on *NOTCH1* and *NOTCH2* receptors (exon 34). *NOTCH1* was selected based on the high frequency of *NOTCH1* gene as a target in cancers (Lee *et al*., 2007). Additionally, presence of mutation in the DNA sequences encoding for PEST domains (Exon 34) of *NOTCH1* receptor gene has been detected in cases of T-cell acute lymphoblastic leukemia (Weng *et al.,* 2004). As for *NOTCH2*, it shares the same structures as *NOTCH1* where it contains the epidermal growth factor (EGF) repeats (slightly different number of EGF repeats) in their ectodomain, the transmembrane and the intacellular segment harbouring the PEST sequence (Radtke and Raj, 2003).

Human NOTCH1; also known as translocation-associated NOTCH homologue (TAN1) was found in a rare subset of T-cell tumors (Radtke and Raj, 2003). This raised some questions whether the NOTCH1 mutation occurs in every solid cancer, as they play important role in tumorigenesis. Consequently, Lee and colleague (2007) performed mutational analysis of NOTCH receptor genes in common solid cancers and acute leukemia cases. No mutation of NOTCH1 was found in breast, lung, colorectal and gastric carcinoma cases although it was detected in a case of T-cell acute lymphoblastic leukaemia (ALL). Interestingly, the same study revealed the involvement of NOTCH2 mutation in breast carcinoma case but not in other solid cancers and leukaemias. They found a nonsense mutation in one of the analysed EXON 34 of NOTCH2 receptor (7198C>T). While deregulation of NOTCH1 was frequently detected in leukaemias, these results suggest that NOTCH1 mutation may not play an important role in tumorigenesis of solid cancers whereas other NOTCH receptors such as NOTCH2 might be involved in the pathogenesis of solid cancers (Lee et al., 2007). However, we failed to detect any nucleotide changes at exon 34 of NOTCH1 and NOTCH2 receptor gene analysed from our oral cancer samples.

We were unable to rule out the involvement of *NOTCH* receptor mutation in causing oral cancer since we only looked at one particular region of the exon (EXON34-1) in both *NOTCH* receptors. Additionally, we only studied 10 oral cancer samples. For more accurate conclusions to define the involvement of this *NOTCH1* and *NOTCH2* receptor genes mutation in causing oral cancer samples, future research needs to embark on a bigger number of samples and wider coverage of the exons involves as well as looking into other receptor genes available (*NOTCH3* and *4*) to increase the probability of finding any mutations. Additionally, other molecules involved in Notch signalling pathway should also be looked at to understand the mechanism of how this crucial signalling pathway controlled the normal development of epithelial cells, among others.

Although *NOTCH* seems to contribute to oncogenesis via many mechanisms, its role in oral cancer development is still unclear. Either alterations in *NOTCH* gene expression or overexpression or its protein might lead to neoplastic transformation *in vivo* is still unclear. However, it is imperative to discover possible targeted structures and proteins of Notch pathway molecules that might be involved in pathogenesis of oral cancer as they had become a therapeutic target for investigators to cure this oral cancer.

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