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Research Report

Transformation analysis of oral epithelial dysplasia to carcinoma in-situ and squamous cell carcinoma by p53 expression and gene mutations

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ABSTRACT

***Background**: It is known that oral squamous epithelial dysplasia (SED) and carcinoma in-situ (CIS) are precancerous lesion and it could transform to squamous cell carcinoma (SCC). We had reported p53-Protein Over-Expression and Gene Mutational of Oral CIS, such as basaloid, verrucous, and acanthothic/atrophic types, but demarcated between SED to CIS and CIS to SCC and how their transformation is still unclear. It is considered that their molecular behavior related one another. **Purpose**: To understand the molecular behavior of them we examined p53 exon 5-8 gene mutation and their protein expression in the sequential cases. **Methods**: Using 10 cases formalin–fixed paraffin sections that composed SED appearance, CIS and SCC in the same case were subjected to P53 immunohistochemistry. Then all cases were subjected to p53 gene mutations analysis. By laser capturing microdissection dysplasia part, CIS part and SCC part were cutted, and followed by direct sequencing of PCR product for exon 5-8. **Result**: SED p53-protein over-expression in some cells, and the expression was increased to CIS and SCC. Mutational analysis for p53 gene showed that 60% of p53 gene mutation in CIS also found in SCC, therefore SCC had additional mutation in other exon of p53 gene. While no particular mutations were found in SED part of all cases. **Conclusion**: Carcinoma in-situ is a squamous cell carcinoma eventhough not invasive yet, but squamous epithelial dysplasia is an early step to malignancy. It needs other genes examination to know any genes are involved in the precancerous to cancer transformation process.*

***Key words**: dysplasia, carcinoma in-situ, squamous cell carcinoma, mutation, p53*

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INTRODUCTION

Cancer of oral cavity is one of several cancers in the body and oral squamous cell carcinoma (OSCC) accounts for more than 95% of all cancer in the oral cavity[1] and most of oral cancers arise in the tongue which majority of oral cancer present at an advanced stage III or IV.[2]

Epidemiological study and experimental evidence indicate a causal relationship between some carcinogenic with oral cancer such as chewing tobacco, betel quid chewing, smoking and drinking,[2-6] but the exact cause of cancer is unknown.

Squamous epithelial dysplasia (SED) and carcinoma in-situ (CIS) known as precancerous lesion that composed of dysplastic change in intra epithelial of squamous epithelium.[7] According to carcinoma which arise only limited in intra epithelial, many authors using different term such as severe dysplasia,[8] or squamous intraneoplasia (SIN) high grade.[9] Whereas WHO classified oral CIS as one classification that separate to squamous epithelial dysplasia (SED) classification.[10]

Carcinoma in-situ (CIS) has been defined as a true and non invasive neoplasm lying within an epithelial layer. The neoplastic cells are different from SED, because they have already been proliferated and subsequently differentiated. In epithelial dysplasia, the constituent cells are only proliferated but not differentiated yet.[11]

The p53 gene structure consists of eleven exon which are exon 2-3 as transactivation domain, exon 5-8 as DNA binding domain and exon 10 as oligomerization domain. They are multifunctional such as transcription factor, which regulates cell cycle progression and interacts with several key proteins which involved in DNA replication, transcription and reparation.[12]

Many studies reported that the p53 protein over-expression is frequently found in both malignant and dysplastic lesions which increased due to grades of dysplasia, and may be an early event in multistage carcinogenesis of head and neck cancer.[13–17,18] The abnormal p53 protein expression reported in 10 to 80% of oral SED[19–22,24] that some cases related to p53 gene mutation,[13,14,24,25] and nearly 60% of human cancers are accompanied by mutation in p53 gene,[13] however, in some cases it is possible to observe protein over expression but they did not show any mutation gene or p53 gene mutation.[25]

As reported before, oral CIS have shown p53 gene mutation almost distributed in exon 7 and exon 8, eventhough this lesion is not invasive yet.[26] Based on this appearance, it is speculated that CIS is carcinomatous lesion the same as squamous cell carsinoma (SCC), but wether SED is carcinomatous lesion. It is still unclear, considering their molecular behaviors are the same.

Based on this p53 gene exon 5–8 and its protein expression were evaluated in sequential cases which each case composed of epithelial dysplasia part, carcinoma in-situ and squamous cell carcinoma. The purpose was understand their molecular behavior transformation from dysplasia to CIS and SCC. In this concept, it is considered that all of SCC started from SED and CIS.

MATERIALS AND METHODS

Formalin paraffin-fixed block selected from the surgical pathology files in the Division of Oral Pathology, Niigata University Graduate School of Medical and Dental Sciences, Japan, during sixth year period 2002 to 2004, after critical reviewing of hematoxylin and eosin (HE) stained sections. These researches consisted of ten cases which is each case compose oral epithelial dysplasia/SED (mild and moderate cases), CIS case (basaloid types, verrucous or acanthothic type). Two oral pathologists screened all the specimens, when the diagnoses of SED, CIS and SCC part were not identical those cases would be reevaluated together. All of the specimens were routinely fixed in 10% formalin and embedded in paraffin. Serial 4 μm sections were cut from paraffin blocks. One set of the sections was stained with hematoxylin and eosin and used for reevaluation of histological diagnosis, and the other sets were toluidine blue staining used for microdissection as well as immunohistochemistry for p53 protein.

The antibodies against p53-protein used in this study were mouse monoclonal antibody clone Bp53-11, (IgG_{2a}) (Progen, Progen Biotechnik GmbH, Heidelberg, Germany), that reacted to wild-type and mutant forms of human p53 antigen within N-terminal region epitope aa20-31. For immunohistochemistry staining, tissue sections 4 μm in thickness were taken from tissue blocks. After deparaffinization and dehydration, sections were washed in 0.01 M phosphate buffered saline (PBS). To restore the antigenic sites, sections were autoclaved in 0.01 M citrate buffer (pH 6.0) for 15 min at 121° C and then kept standing for 20 min at room temperature. To block endogenous peroxidase activities, all the sections were quenched with 0.001% H_2O_2 in 100% methanol for 30 min at room temperature and rinsed with PBS containing 0.5% skim milk and 0.05% triton X-100 (PBST). After rinsing in PBST, the sections were incubated in 5% skim milk in PBS containing 0.05% TritonX-100 for 1 hr at 37° C to block non-specific protein bindings. The sections were then incubated with monoclonal primary antibodies against P53-protein were P53-protein/clone p53 Ab-Bp53-11 (1: 100, Progen Biotechnik GMBH, Heidelberg, Germany), for overnight at 4° C. After incubations with the primary antibodies, the sections were rinsed in PBST and then treated with polymer-immune complexes (Envision+ peroxides, rabbit/mouse, Dako, 1:1) for 1 hr at room temperature. The peroxidase reaction products were visualized by incubation with 0.02% 3, 3-diaminobenzidine (DAB, Dohjin Laboratories, Kumamoto, Japan) in 0.05 M Tris-HCl solution (pH 7.6) containing 0.005% H_2O_2. The sections were counterstained with hematoxylin. Cells were regarded as positive for p53 protein if nuclear staining was intense and could be readily visualized at 10 times magnification. Positive staining for p53 protein staining were calculated quantitatively using a micrometer scale 1mm/square at 10 times magnification. Calculation of positive cells had been done three times and the average was taken. For control experiments, the primary antibodies were replaced with pre-immune mouse IgG2a (Dako).

After evaluation of immunostaining pattern for p53, each sample was stained by toluidine blue for laser microdissection (LMD). Appropriate ten location of SED, CIS and SCC part were cutted due to appropriate area and collected in collection tube for extracted DNA using Proteinase K 10% for one night at 37° C. The DNA solution was purified using phenol/chloroform/isoamyl alcohol mixture (25:24:1). PCR amplification was carried out for p53 exons 5–8. All primer sets were designed on intron sequences adjacent to each exon as follow: [exon 5] sense, 5'-GTT TCT TTG CTG CCG TGT TC-3', antisense, 5'-AGG CCT GGG GAC CCT GGG CA-3' spanning 323 bps; [exon 6] sense, 5'-TGG TTG CCC AGG GTC CCC AG-3', antisense 5'-GGA GGG CCA CTG ACA ACC A-3' spanning 223 bps; [exon 7] sense, 5'-CTT GCC ACA GGT CTC CCC AA-3', antisense 5'-TGT GCA GGG TGG CAA GTG GC-3' spanning 196 bps; [exon 8] sense, 5'-TTC CTT ACT GCC TCT TGC TT-3', antisense 5'-CGC TTC TTG TCC TGC TTG CT-3' spanning 201 bps. PCR were performed on a thermal cycler (PC-800, Astec

Co., Ltd., Fukuoka Japan), after a pre-denaturation at 94° C for 5 minutes. The amplification step was carried out for 35 cycles in 100 μl of a PCR reaction mixture containing 5 units of TaKaRa Ex taq polymerase (Takara Biotechnology, Co., Ltd., Otsu, Japan), 10X Ex Taq buffer, 2.5 mM each of dNTP mixtures, and 20 pmol of each sense and antisense primers. The thermal cycling condition was as follow: denaturation at 94° C for 1 min, annealing at 63° C for exon 5, at 60° C for exon 6 and 8, and 62° C for exon 7 for 0.30 sec each, and extension at 72° C for 1 min. The last extension was prolonged by additional 7 min. Amplification products were analyzed by electrophoresis on 3% agarose gel (NuSieve 3:1 agarose, Cambrex BioScience Rockland Inc., Rockland, ME USA) and the band were visualized by ethidium bromide upon exposure to an ultraviolet transilluminator.

All PCR products were subjected to cycle sequencing by using Thermo Sequenase Primer Cycle Sequencing Kit with 7-deaza -dGTP (Amersham Biosciences Corp., Piscataway, USA). The sequence primers were synthesized based on the published data (Lehman TA *et al.*) and labeled with Texas red 5'-end. The labeled primers were as follow: 5'-GTT TCT TTG CTG CCG TGT TC-3' for exon 5; 5'-GCC TCT GAT TCC TCA CTG AT-3' for exon 6; 5'-CTT GCC ACA GGT CTC CCC AA-3' for exon 7; 5'-TTC CTT ACT GCC TCT TGC TT-3' for exon 8. One tube sequencing reaction contained 3 μl of master mixes (appropriate nucleotides/reaction buffer/thermo sequenase DNA polymerase), 2 μl of the template PCR products, which were purified with GFX PCR DNA and gel band purification kits (Amersham Biosciences Corp., Piscataway, USA), 8 μl of distillated water and 2 μl (2pM) of Texas red-labeled primers. Each sequencing reaction added 3 μl of A, C, G, T reagent. After denaturation at 95° C for 30 sec and annealing at 55° C for 30 sec. the reaction products were dissolved in 3 μl loading dye by vortexing and concentrated with vacuum desiccators. Then 3 μl of samples for each lane were loaded on a gel (7% Long Ranger/6.1 M urea/1.2× TBE buffer (10 mM Tris, 10 mM boric acid, and 2 mM EDTA). The electrophoresis was performed in a fluorescent DNA sequencer (SQ-5500-S, Hitachi Ltd., Tokyo, Japan), and the sequencing data were analyzed by using the SQ-5500 analysis software ver.3.03 (Hitachi).

Statistical analysis was perform with calculating the numbers of p53 and Ki-67 positive cells in a square unit 1 mm^2 on a microscope equipped with a micrometer. Ten fields were randomly counted per section at x100 magnification. One-Way ANOVA was used for statistical comparison of cell numbers between each group by using the SPSS software program (SPSS Inc., Chicago, IL, USA).

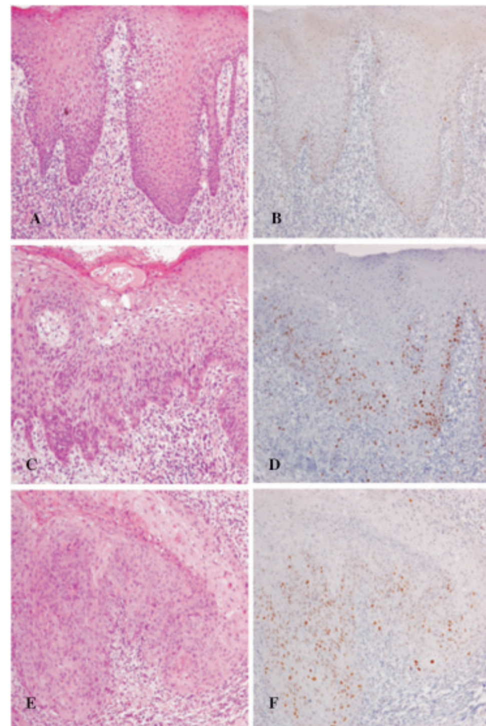

Figure 1. Sequential HE staining (sample 01-803) showed SED, CIS and SCC (A, C, E). Immunohistochemistry of P53 protein found P53 protein over expressed in basal and parabasal layer of SED (B) and CIS (D) and tumor nest of SCC (F) (100×).

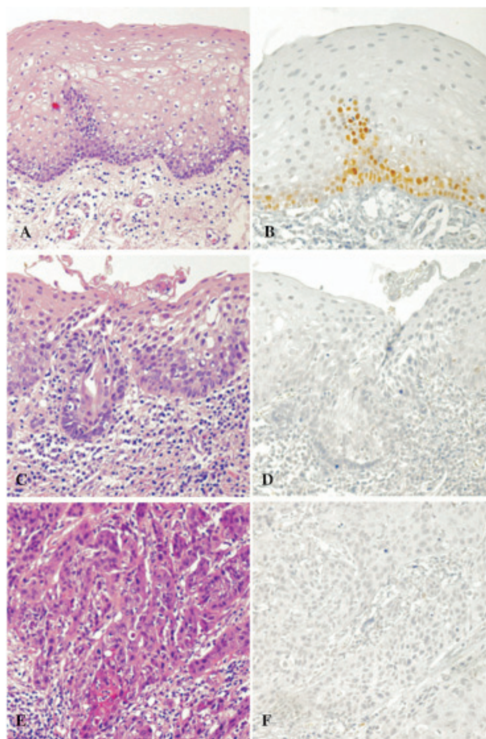

Figure 2. Sequential HE staining (sample 03-969) showed SED, CIS and SCC (A, C, E). p53 protein over expressed in basal and parabasal layer SED (B) but CIS and SCC had not showed p53 protein expression (D, F) (100×).

RESULT

Immunohistochemical staining for p53 protein, when observed, it was found exclusively in the nuclei of epithelial cells of SED, CIS and SCC. Their expression was increased

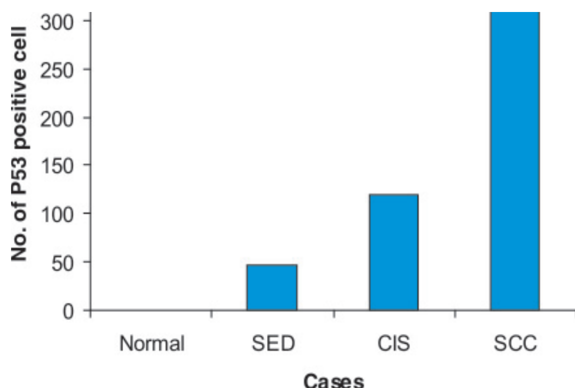

Figure 3. p53-protein expression showed positive cell were icreased significantly from dysplasia to CIS and SCC.

from SED to CIS and SCC (Figure 1, 2). The increasing of p53-protein expression in number was statistically significant ($p < 0.05$). In some cases SED part showed p53-protein expression had not showed p53 protein expression both in CIS and SCC (Figure 2 and Table 1).

Table 1. The p53-protein over expression positive cells in SED, CIS and SCC

No.	No. case	SED	CIS	SCC
1	03-769	125	453	1210
2	02-021	34	100	289
3	03-969	42	0	0
4	03-952	72	0	0
5	02-1409	34	0	0
6	01-803	40	126	200
7	02-205	31	0	0
8	04-369	32	63	505
9	03-573	22	53	38
10	02-1683	23	402	911

All sequential cases of SED, CIS and SCC had shown p53 protein over-expression positive cells collected by laser microdissection. Next step, those cells were sequencing for p53 gene analyses of exon 5-8. The result show 80% of CIS and 90% of SCC cases had p53 gene mutation which mutation type were 89% point mutation and 11% were frame shift/deletion of 13 base pair (Table 2 and Figure 4). Meanwhile, 60% of p53 gene mutation in CIS also found in SCC cases, therefore SCC had additional mutation in other exon of p53 gene (Table 2). Meanwhile no particular mutations were found in SED part of all cases (Table 2 and Figure 5).

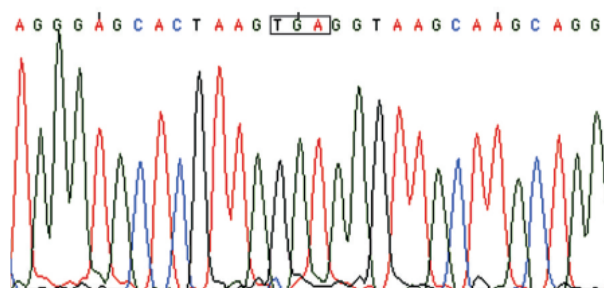

Figure 4. Point mutations of p53 gene found in CIS and SCC which CGA (arginine) to TGA (stop codon) (sample no. 03-969).

DISCUSSION

Oral SED is considered as an early step to malignancy because dysplastic cells could proliferate continuesly to form basaloid cells which replaced normal cell in whole layer that called CIS and this CIS is a step to SCC that has behavior to invade the adjacent tissue or metastases to distant organs.[14,26]

Table 2. p53 gene mutational analysis in SED, CIS and SCC

No.	No. of Case	SED	CIS	SCC
1	03-769	None	E7:248, CGG-CAG	E7:248 CGG-CAG
2	02-021	None	E7:242, TGC-TTC	E7:242, TGC-TTC E5:140, ACC-ATC
3	03-969	None	E6:196, CGA-TGA	E6:196, CGA-TGA
4	03-952	None	None	E5:150-154 Deletion 13 base pair
5	02-1409	None	E8:306, CGA-TGA	E8:306, CGA-TGA
6	01-803	None	E8:282, CGG-TGG	E8:282, CGG-TGG
7	02-205	None	E8:272, GTT-GTG E8:291, AAG-TAG	E8:272, GTT-GTG E8:291, AAG-TAG
8	04-369	None	E7:231, ACC-GCC	E5:139, AAG-AAC E5:183, TCA-CCA E8;303, AGC-AGT
9	03-573	None	E8:282, CGG-TGG	None
10	02-1683	None	None	E8:301, CCA-GCA E8:302, GGG-GAG E8:303, AGC-GAC

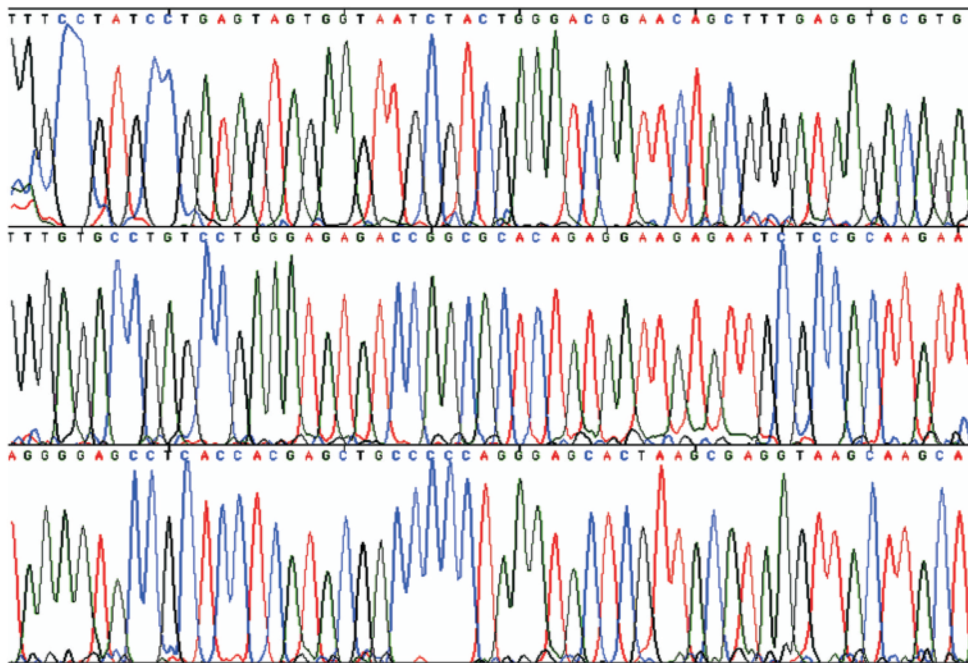

Figure 5. p53 gene analysis result in SED, no mutation were found in exon 5–8 (sample no. 03-769).

Alterations of p53 either at the gene or at the protein expression are a common feature in many human cancers. Some reporting p53 mutation as an early event in the development of oral cancer and others suggesting that it is a late finding.[14, 27] The transformation timing from oral SED to CIS and SCC has not clear yet.

Many study of p53 gene mutation and protein over-expression in oral SED and SCC,[14,17,22] but most of their study used sample from different case/patient. In this present study we use sequential cases included SED, CIS and SCC that were taken from the same case/sample, therefore with this method we can see the molecular behavior concomitantly.

Immunohistochemical study of this present study demonstrated that p53 protein was over-expression from SED to CIS and SCC which was increased according to severity of lesion. It could understand that p53 over expression could be able as a marker of malignant transformation; nevertheless, some case did not show any protein expression. It caused the mutation was stop codon or frame shifts resulting no protein can be detected by antibody. Cruz, *et al*.[22] reported that p53 protein expression in basal cell layer is an early even of malignant transformation.[24]

All of SED and CIS cases showed preservation of basement membrane and without stoma induction,[11] but CIS should get more intention because the basaloid formation intra epithelial more dominant than SED, it is a sign of malignant transformation from precancerous lesion (SED), as Syafriadi and Saku reported basaloid cell is dysplastic cell that can replaced the normal intra epithelium, even though they have been toward keratinization but they are cancerous cells,[27] and potentially transform to SCC which ability to destruct basement membrane and carcinomatous foci perform, that invade to adjacent tissue followed by stromal formation.

In this study showed p53 gene mutation of CIS found in 80% cases and interestingly their mutation particularly same location with SCC cases. It means CIS is cancerous lesion but had not invasive yet as SCC that showed tumor nest formation in the adjacent tissue or metastasis to other organs. Saku,[1] in his report called CIS as superficial carcinoma and tend to recur. Recently, in Japan the superficial carcinomas are increasing in number. However in this study, mutation of p53 gene which involved exon 5 or 6 in SCC cases was also found. It could be considered as progressive process of this lesion. This is supported by many reports demonstrated p53 gene mutation of head and neck SCC showed similarity to oral CIS p53 gene mutation in ths present study,[1,5,24] it suggested SCC was started from CIS.

The SED cases, several cases of CIS and SCC showed p53-protein over-expression but not any mutation of p53 gene exon 5-8, it was suggested that it involved other onco-supressor gene as possible factor, because it had been known that p53 gene produce protein to interact with other gene such as p21, MDM2, p63 or p73.[6,12,13]

The p53 gene mutation in CIS and SCC occurred in various exon but exon 8 more frequently (65% of total mutation cases) and was followed by exon 7 (29% of total mutation cases) and codon 248, 282 in exon 7 and 8 known as “hot spot”.[13, 14] Millward *et al*.,[28] reported the p53 mutation in codon 157, 248, 249 and 273 is linked to the exogenous and endogenous carcinogenic factors such

as found in head and neck, colorectal and breast cancer. In addition, other point mutation in exon 6 and exon 8 codon 196 (CGA→TGA) resulted from arginine to stop codon; codon 291 (AAG→TAG) resulted from lysine to stop codon and codon 306 (CGA→TGA) resulted from Arginine to stop codon also found. These mutations were denoted as hot spots of p53 mutations. Several study showed that arginine residue function is involving in DNA repair,[28, 29, 30] therefore, mutation in this amino acid could make DNA fail to repair their DNA damage.

In conclusion, we have studied a series of oral precancerous lesion (SED) that have shown progression of p53 protein expression to cancerous lesion (CIS and SCC) and the p53 gene mutation location of CIS and SCC are similar and often involved in exon 7-8 moreover, some cases SCC showed additional mutation in exon 5-6 that resulted frame shift or stop codon. The p53 gene mutation that occurred in hotspot codon of CIS and SCC may suggesting is related to extrinsic carcinogenic agent. The progressiveness of SED to CIS and CIS to SCC may be detected by p53 protein expression and gene mutational. We suggested the oral surgeon should give attention to the dysplastic cells nearby the surgical margin that shows p53 protein over-expression. To understand clearly the progression of SCC, more study need to be addressed to other gene which may involve in progression and metastasize of SCC.

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