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Research Report

Immunodetection of *ras*P21 and c-myc oncogenes in oral mucosal swab preparation from clove cigarette smokers

Silvi Kintawati
Department of Oral Biology
Faculty of Dentistry Padjadjaran University
Bandung - Indonesia

ABSTRACT

Background*: Smoking is the biggest factor for oral cavity malignancy. Some carcinogens found in cigar will stimulate epithel cell in oral cavity and cause mechanism disturbance on tissue resistance and produce abnormal genes (oncogenes). Oncogenes ras and myc are found on malignant tumor in oral cavity which are associated with smoking.* ***Purpose****: This research is to find the expression of oncogenes rasP21 and c-myc in oral mucosa epithelial of smoker with immunocytochemistry reaction.* ***Methods****: An oral mucosal swab was performed to 30 smokers categorized as light, moderate, and chain, and 10 non smokers which was followed by immunocytochemistry reaction using antibody towards oncogene rasP21 and c-myc is reacted to identify the influence of smoking towards malignant tumor in oral cavity. The result is statistically analyzed using Kruskal-Wallis test.* ***Result****: Based on the observation result of oncogene rasP21reaction, it shows that there is significant difference between non smoker group and light smoker, compared to moderate and chain smoker group ($p < 0.01$). On the other side, the observation result of oncogene c-myc indicates that there is no significant difference between the group of non smokers and the group of light, moderate, and chain smokers ($p > 0.05$).* ***Conclusion****: The higher the possibility of oral cavity malignancy and that the antibody for rasP21 oncogene can be used as a marker for early detection of oral cavity malignancy caused by smoking.*

Key words*: smokers, rasP21, c-myc, oncogene, immunohistochemistry*

Correspondence: Silvi Kintawati, c/o: Bagian Biologi Oral, Fakultas Kedokteran Gigi Universitas Padjadjaran. Jl. Sekeloa Selatan I Bandung, Indonesia. E-mail: letisrei@bdg.centrin.net.id

INTRODUCTION

Today, malignant tumor is one of the most dangerous causes triggering death for human. Tumor in oral cavity is one of malignant tumors that is rapidly increased. Even though its cause is not definitely known, it has been claimed that there are multi-factors causing malignant tumor in oral cavity, for examples environmental predisposition factor and genetic factor. Of various factors causing malignant tumor in oral cavity, smoking is the most common cause.[1,2] Some carcinogens found in cigar will stimulate epithel cell in oral cavity and cause mechanism disturbance on tissue resistance. The growth of malignant tumor is related to the epithel change that is triggered by the number of carcinogens and the length of carcinogen development. The longer the carcinogens are developed, the more the risk of getting malignant tumor.[2,3]

Malignant tumor is clinically difficult to be detected in early stage, so it is mostly known at acute stadium. To socialize early diagnosis of malignant tumor, immunocytochemistry reaction is undergone by mucosal swab technique. On mucosal swab technique, it is known that in oral cavity there are changes of mucosal cells caused by development of irritation substance, like tobacco.[4,5] Carcinogen can produce abnormal genes (oncogene), yielding specific anti-gen. Specific anti-gen examination is done by using antibody based on reaction of anti-gene–antibody known as immunocytochemistry reaction.

Besides oncogene EGF-R and c-erbB, other oncogenes found on malignant tumor in oral cavity are myc and *ras*

which are associated with smoking. It has been reported that *ras*P21 produced from lineage protein of gene *ras* is found on dysplasia and on early stage of tumor. Meanwhile on malignant tumor, c-myc level is augmented.[6,7]

Based on the background mentioned above, this study is conducted to detect the expression growth of oncogene myc (c-myc) and *ras* (*ras*P21) on the cells found in oral cavity of cigarette smokers whose mucosa has been swabbed. By considering that smoking is one of the greatest factors causing malignant tumor in oral cavity,[2,8] increased the expression of these oncogenes–c-myc and *ras*P21–on cigarette smokers is possibly found. The reason is that those smokers have consumed many cigars.

MATERIALS AND METHODS

The research conducted by the writer was laboratory research using *ex post facto* model. The samples of the research were 30 smoker employees of Faculty of Dentistry, Padjadjaran University. The criteria for sample were 1) male; 2) aged above 40 years old; 3) they had been smokers for 5 years or more; 4) clinically there was no disorder of oral cavity. The smokers were classified into light smokers (less than 10 cigars in one day), moderate smokers (11-20 cigars in one day), chain smokers (more than 20 cigars in one day). As comparison, 10 non smokers were selected from employees.

One-way swabbing from inner part to outer part in mucosa of oral cavity for three times was conducted for smokers and non smokers using wooden spatula. After the result of one-way swabbing was smeared on glasses, it was dried for 1 hour. Then, ethyl-alcohol was reacted on it for 1 hour. Reacting immunocytochemistry was the next step, using Dako LSAB (Labeled Streptavidin Biotin/LSAB method) Kit Peroxidase.

Antibody used in this research was antibody towards *ras*P21 and c-myc. Monoclonal antibody *ras*P21was obtained from "Oncogene Science", being dissolved 1 : 20. Meanwhile, c-myc was taken from "Oncogene Science" clone 9E10; being dissolved 1 : 10. LSAB kit peroxidase was gained from Dako KO-681.

The process: After the result of mucosal swab technique was dried in open air and reacted with ethyl-alcohol for one hour, it was incubated by methanol containing H_2O_2 2% for 20 minutes. Next, it was rinsed using alcohol 90% for 5 minutes, alcohol 80% for 5 minutes, alcohol 70% for 5 minutes and put in "Tri Buffer Saline" (TBS) for 3 × 5 minutes. Then, it was incubated using blocking reagent for 5 minutes and later incubated in each primary antibody (*ras*P21 and c-myc) for 30 minutes; rinsed with TBS for 3 × 5 minutes; incubated by linking antibody for 10 minutes; rinsed with TBS for 3 × 5 minutes; incubated by Strep-avidin Biotin for 30 minutes; rinsed with TBS for 3 × 5 minutes; incubated by chromogene substance, diaminobenzidine (DAB), for 5 minutes. After the color was formed, it was rinsed by flowing water, and counter-stain was given on it, using Meyer's hematoxylin for 2 minutes.

The result of immunocytochemistry reaction was observed by light microscope using 400 times enlargement. The observation of oncogene *ras*P21 and c-myc level was based on percentage (the number of positive cells on each oncogene and the number of all cells on each result of swabbing mucosa in oral cavity), which was shown on the following equation:

$$\frac{\text{Number of positive cells}}{\text{Number of all cells (in 1 result)}} \times 100\%$$

The result of reaction would be stated positive for *ras*P21 and c-myc if the color on cytoplasm cell turned brown. It would be stated negative if the color did not turn into brown.

RESULT

The data were taken from 30 smoker-employees and 10 non smoker employees in Faculty of Dentistry Padjajaran University, who had been treated with the mucosal swab technique for oral cavity, and then had been tested with immunocytochemistry reaction by using *ras* P-21 and c-myc antibodies, shown with the following table 1 and table 2.

Table 1. The estimation of the expression level of *ras* P-21 oncogenes (%)

Groups of Smokers / ras P-21 Expression	Non-Smokers	Light Smokers	Moderate Smokers	Chain Smokers
Subject 1	0	0	2.624	2.901
Subject 2	0	0	3.921	3.010
Subject 3	0	0.381	4.230	4.067
Subject 4	0	0.852	6.245	7.425
Subject 5	0	1.131	6.786	9.197
Subject 6	0.200	1.142	10.600	11.573
Subject 7	0.394	2.385	12.170	12.197
Subject 8	1.122	2.842	19.372	21.171
Subject 9	2.621	4.110	23.410	30.072
Subject 10	4.860	5.054	23.951	30.870
$\overline{X}$	0.9197	1.7897	11.3309	13.2483

By using Kruskal-Wallis test, the estimation of the expression level of *ras* P-21 oncogenes between non-smoker group and the other three groups, light smoker groups, moderate smoker groups, and chain smoker

groups did show any significant difference ($p < 0.01$). In addition, Mann-Whitney test was also used in order to determine pairs of different groups. The result of Mann-Whitney test shows that there was no significant difference ($p > 0.05$) between light smoker group and non smoker group. However, there was significant difference ($p < 0.01$) between non-smoker group and other two groups, moderate smoker group and chain smoker group. There was also significant difference ($p < 0.01$) between light smoker group and other two groups, moderate smoker group and chain smoker group. Nevertheless, there was no significant difference between moderate smoker group and chain smoker group ($p > 0.05$).

Table 2. The estimation of the expression level of c-myc oncogenes (%)

Groups of Smokers / c-myc Expression	Non-Smokers	Light Smokers	Moderate Smokers	Chain Smokers
Subject 1	0	0	0	0
Subject 2	0	0	0	0
Subject 3	0	0	0	0
Subject 4	0	0	0	0
Subject 5	0	0	0	0
Subject 6	0	0	0	0
Subject 7	0	0	0	0
Subject 8	0	0	0	0.198
Subject 9	0.112	0.972	0.670	1.023
Subject 10	0.193	1.091	1.840	1.240
$\overline{X}$	0.0305	0.2063	0.2510	0.2461

By using Kruskal-Wallis test, the estimation of the expression level of c-myc oncogenes between non-smoker group and the other three groups, light smoker groups, moderate smoker groups, and chain smoker groups did not show any significant difference.
($p > 0.05$).

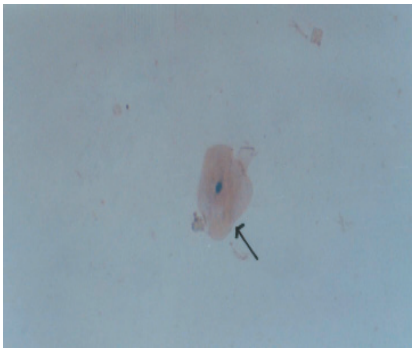

Figure 1. Positive cell towards *ras* P-21 oncogenes 200×.

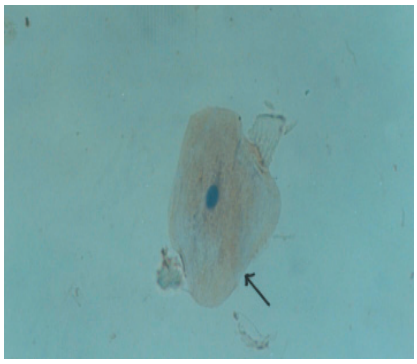

Figure 2. Positive cell towards *ras* P-21 oncogenes 400×.

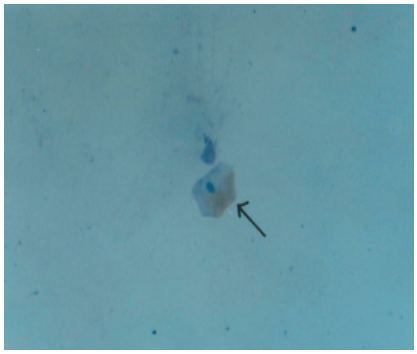

Figure 3. Positive cell towards c-myc oncogenes 200×.

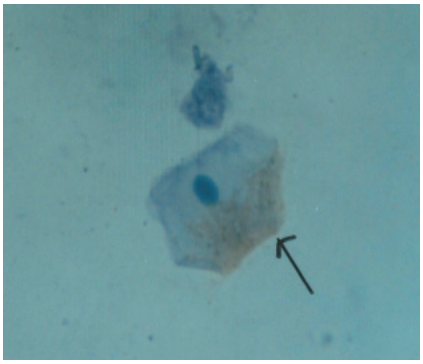

Figure 4. Positive cell towards c-myc oncogenes 400×.

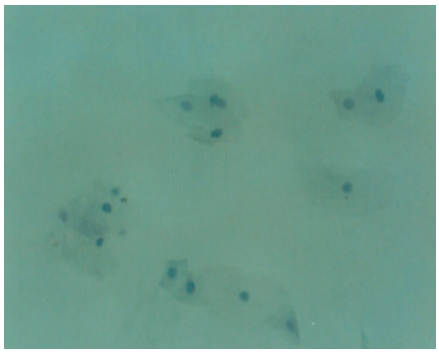

Figure 5. Negative cell towards *ras* P-21 and c-myc oncogenes 200×.

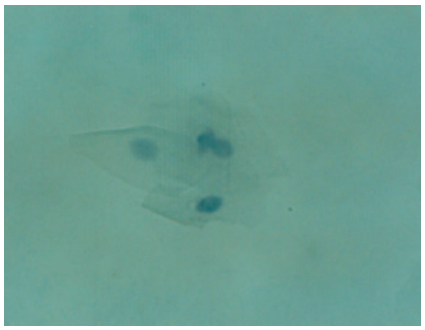

Figure 6. Negative cell towards *ras* P-21 and c-myc oncogenes 400×.

DISCUSSIONS

It has been clearly analyzed that cigarette smoke consists of many harmful chemicals, as a result, smoking habit can cause many defects in oral cavity like malignant tumor.[9] The malignant tumor in oral cavity, which is often suffered, is squamous cell carcinoma.[8,10] Based on many researches, there is a strong relation between smoking habit and the malignant tumor in oral cavity.[9,11] According to a research in America, the risk of smokers in suffering the malignant tumor is even six times as high as that of non-smokers.[12] Moreover, the risk of suffering the malignant tumor in oral cavity is higher for patients above 40 years old, as well as for chain smokers.[10]

Many carcinogens contained in cigarette can stimulate epithel cells in oral cavity and cause trouble in the defense mechanism of tissues. The development of the malignant tumor is related to the change of epithel caused by the number and the period of the spread carcinogens. The longer the period of the spread carcinogens and the bigger the number of them, the higher the risk of getting the malignant tumor is.[2,3]

The mucosal swab technique for oral cavity can become a routine diagnostic examination for detecting the defects in oral cavity since this examination is sensitive, fast, and unpainful.[4,13]

In this research, the mucosal swab technique for oral cavity is taken in order to take and analyze mucosa cells of oral cavity. As we know that, carcinogenes of cigarette can create abnormal genes while oncogenes can produce specific antigens. Thus, the examination of specific antigens can be done by using antibodies based on antigen-antibody reaction known as immunocytochemistry reaction.

In this research, the immunocytochemistry reaction is used in order to know whether the level of *ras* P-21 oncogene expression and the level of c-myc oncogene expression in mucosal cells swabbed from oral cavity are related to the number of clove cigarettes consumed. The result of this immunocytochemistry reaction then shows that there is a relation between the level of *ras* P-21 oncogene expression and the number of clove cigarettes consumed. However, it does not show any relation between the level of c-myc oncogene expression and the number of clove cigarettes consumed.

Ras P-21 protein is a product of *ras* gene family, mammal gene, which is often related to the malignant tumor. *Ras* P-21 is a phosphor protein bound by plasma membrane with 188–189 amino acid residues, and has intrinsic GTPase activities. *Ras* P-21 also has the same biological characteristics as protein-G, known as "signal transducer" from membrane receptors to cytoplasm effectors. In other words, *ras* P-21 has a function as a controller of the information exchange from membrane to nucleus. If this *ras* P-21 genes is activated by mutation, its function can be disturbed since the P21-GTP complexity becomes ceaselessly active and then causes any possibilities of the neoplastic change. *Ras* P-21 protein mutant has a character of transferring transduction signals from outside cell, especially the signals of developing extra cell into its effectors in nucleus. *Ras* P-21 protein mutant can stimulate tumorgenesis process with its abilities of continuing transferring transduction signals of developing extra cell ceaselessly, so apoptosis can be inhibited. This condition then causes an early stimulation process of tumorgenesis.[14] According to Varghese *et al.*,[6] *ras* P-21 can be found in the malignant tumor in oral cavity. Based on the result of this study, it can be noticed that there is an increasing expression level of *ras* P-21, which is higher for moderate smoker group and chain smoker group than for light smoker group and non-smoker group (Table 1). It indicates that the bigger the number of cigarettes consumed, the higher the level of *ras*P21 oncogenes, causing the inhibition of apoptosis and the possibility of the malignant tumor in oral cavity.

Furthermore, c-myc gene is located on chromosome 8. The level of c-myc expression can increase in malignant tumor in oral cavity, especially in an advanced stage or in relating to poor prognosis.[7] Based on the result of this study, it can be realized that there is no increasing expression level of c-myc oncogenes in mucosal cells swabbed from oral cavity either for light smoker group, moderate smoker group, chain smoker group, or for non-smoker group (Table 2). The reason is because the level of c-myc expression can increase if the malignant tumor is aggressive, and in an advanced stage,[7] meanwhile in this study the mucosal cell swabbed from oral cavity of light smoker group, moderate smoker group, and chain smoker group, which are clinically diagnosed without any symptoms of the malignant tumor in their oral cavity.

Based on all the results, it can be concluded that there is an activation of *ras* P-21 oncogenes in swab mucosal technique for smokers' oral cavity. As a comparison, the result of this study is the same as the result of Kintawati[15]study, showing that there is an activation of some oncogene and receptor of development factor, like EGF-R and c-erbB oncogenes in swab mucosal technique for smokers' oral cavity. According to some researchers, the oncogene activation which is related to the gene changes can stimulate the malignant tumor including the malignant

tumor of oral cavity.[1,13] This study shows that moderate smoking and chain smoking can stimulate gene mutation which then cause the malignant tumor of oral cavity.

Thus, it may be concluded that *ras* P-21 oncogenes expression has increased in moderate smoker groups and in chain smoker ones. As a result, it can become an indicator for early detection of malignant tumor in oral cavity because of smoking. For this reason. Moderate smokers and chain smokers are recommended to do routine screening in order to detect malignant tumor of mucosa in oral cavity.

REFERENCES

1. Lippman SM, Sudbo J, Hong WK. Oral cancer prevention and the evolution of molecular-targeted drug developement. J Clin Oncol 2005; 23(2): 346–56.
2. Regezi JA, Sciubba JJ, Jordan RCK. Oral pathology. 5th ed. Saunders: Elsevier; 2008. p. 48–52.
3. Abeloff MD, Armitage JO, Niederhuber JE, Kastan MB, McKenna WG. Clinical Oncology. 3rd ed. Philadelphia: Elsevier Inc. 2004; p. 1498–1541.
4. Koss LG. Koss' diagnostic cytology and its histopathologic bases. 5th ed. Philadelphia: Lippincott; 2006. p. 99.
5. Sundbo J, Samuelsson R, Risberg B, Heisten S, Nyhus C, Samuelsson M, *et al*. Risk markers of oral cancer in clinically normal mucosa as an aid in smoking cessation counseling. J Clin Oncol 2005; 23(9): 1927–33.
6. Varghese P, Balaram P, Das BC. Ras p21 and its expression variations in the development of oral squamous cell carcinoma. Ind J Med Resch 2005; 81.
7. Vita M, Henriksson M. The Myc oncoprotein as a therapeutic target for human cancer. Semin Cancer Biol 2006; 16(4): 318–30.
8. Morse DE, Psoter WJ, Cleveland D, Cohen D, Mohit-Tabatabai M, Kosis DL, *et al*. Smoking and drinking in relation to oral cancer and oral epithelialdysplasia. J Cancer Causes Control. 2007; 18(9): 919–29.
9. Study suggests nicotine may promote cancer development. CA Cancer J Clin 2003; (53):66-68. [update 2008]. Available from: http:// www.caonline.amcancersoc.org/. Accesed November, 2006.
10. Slootweg PJ, Everson JW. Tumours of the oral cavity and oropharynx. In: Barners I, Everson JW, Reichart P, Sindransky, eds. Pathology & genetics of head and neck tumours. Lyon: IARC Press; 2005. p. 166–75.
11. Lee CH, Ko YC, Huang HL, Chao YY, Tsai CC, Shieh TY, *et al.* The precancer risk of betel quid chewing, tobacco use and alcohol consumption in oral leukoplakia and oral submucous fibrosis in southern Taiwan. British J Cancer 2003; 88: 366–72.
12. Gosselin BJ. Malignant tumours of the mobile tongue. Available from URL:http//www.emedicine.com/ent/topic256.htm. Accessed January, 2007.
13. Kujan O, Desai M, Sargent A, Bailey A, turner A, Sloan P. Potential applications of oral brush cytology with liquid-based technology: result from a cohort of normal oral mucosa. J Oral Oncology. 2006; 42: 810–18.
14. Diaz R, Lopez-Barcons L, Ahn D, Garcia-Espana A, Yoon A, Matthews J, *et al*. Complex effects of Ras proto-oncogenes in tumorigenesis. Carcinogenesis. 2004; 25(4): 535–39.
15. Kintawati S. Immunodetection of EGF-receptor, c-erbB oncogenes and HSV-1 antigenes in oral mucosal swab preparation from clove cigarette smokers. Padjadjaran J Dent 2008; 20(2): 116–22.