Original Article

Salivary Ki-67 and Micronucleus Assay as Potential Biomarker of OSCC in Betel Nut Chewers

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Abstract

Introduction: Oral cancer is often associated with various factors, such as betel nut consumption, which usually causes specific premalignant lesions. The most common oral cancer is oral squamous cell carcinoma (OSCC) which has a low 5-year survivor rate because early detection of the malignancies is not widely used and not routinely carried out in dental practice. Early detection of malignancy can be done by measuring the salivary Ki-67 level and micronucleus assay from the buccal smear. **Aims and Objectives:** The study aimed to examine the potency of the salivary Ki-67 level and micronucleus assay for early detection of OSCC in betel nut chewers. **Materials and Methods:** This study was conducted in 17 betel nut chewers and 17 healthy people as a control group. Saliva was collected with the passive drooling technique and then analyzed using Enzyme-linked immunosorbent assay (ELISA). Buccal smears were taken, then a cytological slide was made and stained using Papanicolaou. **Settings and Design:** This study was a cross-sectional analytic survey that was conducted in the Banyumas District of Indonesia with a post-test- only control group design. **Statistical Analysis:** The statistical analysis used is a non-parametric test using Mann–Whitney and Kruskal–Wallis tests. **Results:** There was a significant difference between the Ki-67 level and micronucleus in the betel nut chewers group and the control group. There was a significant difference between Ki-67 and micronucleus levels in the various types of oral lesions that were found in the betel nut chewer's group. **Conclusion:** Examination of Ki-67 and micronucleus assay is effective as an alternative early biomarker for OSCC.

Keywords: Betel nut, Ki-67, micronucleus, oral squamous cell carcinoma, saliva

Introduction

Cancer is a disease that occupies the first position, in that, it can cause death before the age of 70 years. A survey has been conducted by World Health Organisation (WHO) and the results show that there are approximately 18.1 million new cancer cases found every year and approximately 9.6 million deaths from cancer took place in 2018. Cancer of the lips and oral cavity ranks 18th as cancer with new cases and highest deaths were in 2018.[1]

The incidence of oral cancer is often associated with various factors such as chronic irritation, poor oral hygiene, viral infections, malnutrition, and lifestyle. The etiological factors associated with lifestyle include tobacco, betel nut, and alcohol consumption. In several studies that have been carried out in the Southeast Asian region, it is known that the habit of chewing betel nut is a risk factor that can increase the incidence of cancer in the oral cavity.[2] The most common cancer of the lips and oral cavity is oral squamous cell carcinoma (OSCC). This cancer has a low five-year survivor rate, which is estimated at 40–50% because early detection of malignancy is not widely carried out.[3,4]

Delay in diagnosing OSCC occurs because the early primary lesions of OSCC do not cause any symptoms or complaints in the patients and rarely do routine mucosal examinations.[3] Many methods of early detection of oral malignancies have been developed, especially non-invasive examinations for patients using various light-based examinations and

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nanotechnology.[5] Early detection of malignant conditions can be checked by cytology examination to detect DNA damage. One of the tests that can be performed is the examination of the micronucleus.[6] A biomolecular examination can see the progression of malignancy and can be done by examining various markers of cell proliferation such as Ki-67 protein from the saliva that can be associated with increased severity of dysplasia.[7]

Because early detection of oral malignancy conditions is not widely used as a routine examination of the oral mucosa, researchers are interested to find an alternative early detection of oral malignancy that is easy and inexpensive to do. The aim of this study is to examine the potency of the salivary Ki-67 level and micronucleus assay for the early detection of OSCC in betel nut chewers.

Materials and Methods

This research was approved by the Ethics Committee, Faculty of Medicine, Jenderal Soedirman University with certificate number 042/KEPK/III/2020 and approval date June 8, 2020. This study was held in Banjarsari Wetan Village, Sumbang District, Banyumas Regency, Central Java, Indonesia. The design of this study was quantitative research that used analytical survey methods conducted in one time or cross-sectional studies.[8]

The research subjects consisted of two groups, the first group was betel nut chewers and sample size estimation was calculated using Slovin's formulation. The first and second groups consisted of 17 people. The second group was of healthy people as the control group and the sample estimation was determined using quota methods which adjust the amount of the first group. Research subjects were selected according to the inclusion criteria adjusted to the calculation of the number of samples. First of all, the research subjects were explained the procedures, objectives, and benefits of the study, and the signing of the informed consent was used as evidence of the research subject's willingness. An intraoral examination was then carried out to know the condition of the oral cavity and to detect oral lesions that resemble malignancy as an effect of betel nut consumption.

The buccal mucosal smear was collected using a cytobrush that was applied to the patient's buccal mucosa with a 360° circular motion that can be seen in Figure 1. The cytobrush was then rotated on the end of the slide that had been given a drop of the saline solution. Then the cytobrush was applied to all parts of the object-glass with minimal pressure. The object-glass was fixed by dropping 96% absolute alcohol solution. the slide was sent to the laboratory to be stained by the Papanicolaou stain. The cytological slides that had been stained were observed under a light microscope and an Optilab Pro camera and the micronucleus cells were counted in every 500 cells.

The saliva specimens were collected by passive drool method until they reached a volume of 10–15 mL shown in Figure 2. The collected specimens were then tightly closed and given an identity, stored in an icebox with a temperature of approximately −20 to −40°C. The specimens were taken to the laboratory for measurements of Ki-67 levels. The specimens were centrifuged at 3.000 rpm for 20 min and the supernatant formed from the specimens was carefully collected. Ki-67 levels were detected by ELISA test using the BT-Lab Human Ki-67 ELISA Kit protein and the absorbance was observed at a wavelength of 450 nm. The absorbance value of Ki-67 was converted into levels of Ki-67 with the help of the online site *elisaanalysis.com*.

Statistical analysis was performed using the software Statistical Package for Social Sciences (SPSS) version 22. Normality test was done using Shapiro–Wilk, however, data were not normally distributed and data analysis was continued with a non-parametric test. The non-parametric test used in this study is Mann-Whitney U-test and Kruskal–Wallis test.

Results

Intraoral examination of the betel nut chewer group found various oral lesions, namely exogenous pigmented lesions, hairy tongue, and various other lesions that resembled leukoplakia and lesions that resembled lichenoid reactions shown in Figure 3.

Salivary Ki-67 levels

Levels of Ki-67 in saliva were measured by ELISA testing and the results can be seen in Table 1. Based on Table 1, it is known that the average Ki-67 level was higher in the betel nut chewers group when compared to the control group. Ki-67 levels increased approximately three-fold in the betel nut chewers group. The Shapiro–Wilk normality test was carried out and the results obtained were $P = 0.00$ ($P < 0.05$), so it was

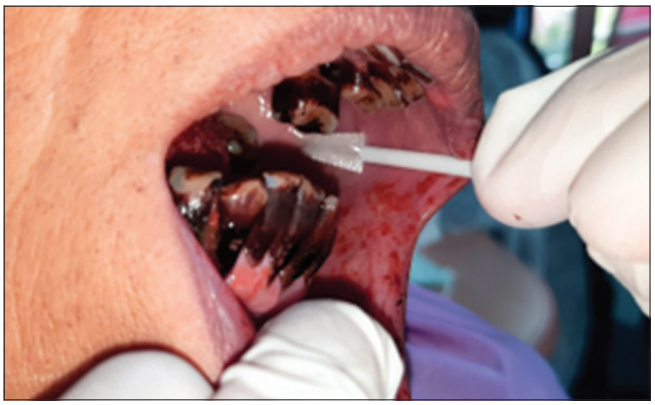

Figure 1: Cytological swab using cytobrush

Table 1: Calculation of Ki-67 levels

Group	Number of sample (*n*)	Mean (ng/mL) ± SD
Betel nut chewer's group	17	30,02±17,44
Control group	17	10,37±2,48

concluded that the data were not normally distributed. The data transformation had been carried out but it still showed the data was not normally distributed so that further tests were carried out using non-parametric tests.

The non-parametric Mann–Whitney test showed a significance value of $P = 0.00$ ($P < 0.05$), so it can be concluded that there was a significant difference between the Ki-67 levels in each group.[9] Kruskal–Wallis bivariate test was conducted to determine whether there was a difference in the mean Ki-67 levels in various types of oral lesions that were found. Oral lesions are divided into four categories, namely no lesions, exogenous pigmented lesions, lesions resembling leukoplakia, and lesions resembling lichenoid reactions. The Kruskal–Wallis test shows a P value = 0.00 ($P < 0.05$), so it can be concluded that there is at least a difference between the two types of lesions. The *post hoc* follow-up test was in the form of a Mann-Whitney test and the following significance values were obtained shown in Table 2.

Based on Table 2, it is known that the Ki-67 level were significantly different between the type without lesions with pigmented lesions, lesions resembling leukoplakia, and lesions that are resembling a lichenoid reactions. The type of lesions resembling leukoplakia did not have a significant difference in the level of Ki-67 when compared with the lesions resembling lichenoid reaction. pigmented lesions and lesions resembling lichenoid reaction showed a significant difference in Ki-67 levels.

Micronucleus assay buccal mucosal smear

In the micronucleus observations, the cells counted were micronucleus with a maximum size of one-third of the normal nucleus with a texture and similar color to the nucleus. Micronucleus can be seen in Figure 4.

The calculation of the mean micronucleus in the group of people with the habit of chewing and the control group is shown in Table 3.

Based on Table 3, the mean number of micronucleus in the betel nut chewers group increased 3.5-folds when compared to the control group. The data analysis begins with the Shapiro–Wilk normality test and the P value is obtained = 0.008 ($P > 0.05$) and it is concluded that the data are not normally distributed. Data transformation has been carried out but the data are still not normally distributed so that the advanced test chosen is the non-parametric test.

The non-parametric Mann–Whitney test was used to determine the difference in the number of micronucleus in each group and the p P value was obtained = 0.00 ($P < 0.05$), so that it can be concluded that there was a significant difference between the number of micronucleus in the betel nut chewer's group and the control group. Kruskal–Wallis non-parametric bivariate was then carried out to determine whether there was a significant difference between the types of oral lesions found and the number of micronuclei and the result was $P = 0.00$ ($P < 0.05$). it can be concluded that there is at least a difference between the two types of lesion. Follow-up test *post hoc* of Mann–Whitney test was conducted to find which types of lesions were significantly different and the results obtained can be seen in Table 4.

Based on Table 4, it is known that the number of micronuclei is significantly different in normal conditions or without lesions when compared with all types of lesions, both pigmented lesions, leukoplakia-like lesions, and lesions resembling a lichenoid reaction. This is evidenced by the P value < 0.05. The number of micronuclei did not differ significantly in lesions resembling leukoplakia with lesions resembling lichenoid reaction. This is indicated by a significance value of $P = 0.671$ or $P > 0.05$.[9]

Correlation of Ki-67 salivary levels with the number of micronucleus of buccal mucosal smear

The correlation of Ki-67 salivary levels and the number of micronucleus from buccal mucosal smear was tested using Spearman's rank correlation test. The associative test was chosen because the Ki-67 and micronucleus data were not normally distributed even though the data had been transformed. In Spearman's 'rank correlation test, the result

Figure 2: Saliva collection with passive drooling method

Table 2: *Post hoc* Mann-Whitney test of Ki-67 levels for each type of lesion

Oral lesions	*P*			
	Exogenus pigmented lesion	Lesion resembling leukoplakia	Lesion resembling lichenoid reaction	No lesion
Exogenus pigmented lesion		0,086	0,019*	0,001*
Lesion resembling leukoplakia	0,086		0,734	0,002*
Lesion resembling lichenoid reaction	0,019*	0,734		0,000*
No lesion	0,001*	0,002*	0,000*	

* Mean differ significantly

was $P = 0.00$ ($P < 0.05$), it could be concluded that the levels of Ki-67 and micronucleus had a significant correlation. The coefficient correlation in the test has a value of 0.964 and it can be concluded that the Ki-67 and micronucleus levels have a positive correlation with a very strong level because they are in the range of 0.76–0.99.[9]

Discussion

Ki-67 levels

The mean of Ki-67 levels in the betel chewers group was three-fold higher than the control group. This proved that the consumption of betel nut in the longterm can cause changes in DNA, which is indicated by the increased levels of Ki-67 in saliva.[10] The Ki-67 expression will increase in conditions of high basal cell proliferation activity due to chronic irritation and physical trauma, and consumption of betel nut for a long time can trigger microtrauma in the oral mucosa due to friction from the betel nut material.[11]

The Ki-67 levels differ significantly in normal conditions when compared to various types of oral lesions. The Ki-67 expression will increase up to fourfolds in the condition of premalignant lesions when compared to normal mucosa.[12] Ki-67 expression will also increase based on the degree of dysplasia of a lesion. The Ki-67 expression also increases significantly to the severity of clinical symptoms in patients.[11,13]

This study found significant differences in Ki-67 levels in the lesions resembling lichenoid reaction. The lichenoid reaction lesion had the appearance mimicking oral lichen planus (OLP), but the ability of OLP to be a malignancy is still debated. Some OLP lesions are thought to be malignant if the biopsy results show dysplasia.[14] The lesions mostly have progression to malignancy in lesions resembling leukoplakia.[15]

Measurement of Ki-67 have been done in many other study using immunohistochemical testing of biopsy as the gold standard examination with a cut-off point of 15%,[16] while in this study, Ki-67 level was measured using ELISA test. Until now, the normal range of Ki-67 has not been found through ELISA examination. Previous research conducted the calculation of Ki-67 levels from blood serum in patients with breast cancer where the Ki-67 levels found in breast cancer patients were approximately 33.47 ng/mL and the maximum Ki-67 levels in the control group of 10.78 ng/mL.[17]

Table 3: Calculation of the average micronucleus each 500 cells

Group	Number of sample (*n*)	Mean (ng/mL) ± SD
Betel nut chewer's group	17	28,30±6,02
Control group	17	8,67±3,81

Number of micronucleus

The increase of the mean number of micronucleus in the betel nut chewer's group was 3.5-folds higher when compared to the control group and it can be concluded that betel nut is carcinogenic and can trigger DNA damage although the pathogenesis is still unclear.[18] The appearance of the micronucleus is closely related to the presence of genomic instability and DNA damage. Irreversible damage of DNA can lead to aberrations in both the chromosomes and chromatids and can lead to the formation of polycentric chromosomes or acentric chromosome fragments. Another mechanism that triggers micronucleus formation is the dysfunction of the spindle and centromere so the mitotic process is disrupted.[19]

Micronucleus examination of the buccal mucosal smear is widely used as biomonitoring to detect genotoxic exposure and the risk of certain types of cancer.[20] The micronucleus will increase by 2.2-folds in oral leukoplakia conditions compared to normal conditions, while in other precancerous conditions, the micronucleus can increase by 3.3-folds compared to normal conditions.[21]

In this study, the number of micronuclei differed significantly between normal conditions without lesions when compared with the other lesions. The number of micronuclei also differed significantly between the types of pigmented lesions with lesions resembling leukoplakia and lesions resembling lichenoid reaction. Leukoplakia lesions progressed to a malignant state with a percentage of 17%.[15] A lichenoid reaction lesion may accompany the initial condition of Oral Submucous Fibrosis (OSF), but the ability to progress to malignancy needs to be further examined through tissue biopsy to determine the definitive diagnosis and degree of dysplasia of the case.[22] These results prove that micronucleus examination can help early detection of lesions that are suspected of progressing to a malignant condition.

Table 4: Mann-Whitney post-test, number of micronucleus in each type of lesion

Oral lesions	*P*			
	Exogenus pigmented lesion	lesion resembling leukoplakia	Exogenus pigmented lesion	no lesion
Exogenus pigmented lesion		0,049*	0,019*	0,001*
Lesion resembling leukoplakia	0,049*		0,671	0,002*
Lesion resembling lichenoid reaction	0,019*	0,671		0,000*
No lesion	0,001*	0,002*	0,000*	

* Mean differ significantly

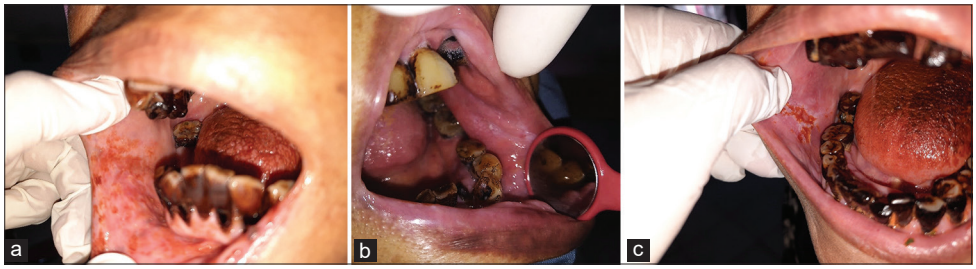

Figure 3: (a) Exogenous pigmentation lesions (b) Leukoplakia-like lesions (c) Lesions resembling lichenoid reactions

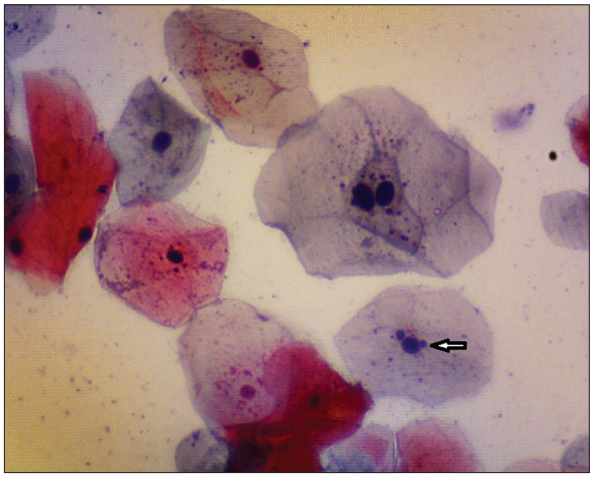

Figure 4: Micronucleus

Ki-67 correlation in saliva and micronucleus buccal mucosal smear

The statistical analysis in this study using Spearman's Rank showed a very strong correlation between level of Ki-67 and number of micronucleus, and it can be interpreted that with increased level of Ki-67, the number of micronucleus will also increase. Ki-67 and micronucleus are biomarkers that can assess the risk of premalignant lesions in the oral cavity.[9]

Acute depletion of the Ki-67 and p53 proteins are synergistically capable of triggering an increase in the levels of the chromosome bridge at the anaphase stage which causes the formation of the micronucleus. In various studies, it is known that Ki-67 levels will increase with tumor severity, but Ki-67 also has an important role in maintaining the integrity of the chromosome structure. In the mitotic phase, Ki-67 will envelop the mitotic chromosomes whose function is to protect the nuclear structure as a building block for daughter cells. The presence of Ki-67 and p53 depletion simultaneously will cause disturbances in the anaphase stage which is usually characterized microscopically by the formation of the micronucleus. Ki-67 and p53 depletion will increase exposure to genotoxic agents that can trigger irreversible damage of DNA.[23]

In this study, Ki-67 levels were less able to detect the progression of malignancy in detail based on the type of lesion due to the gold standard of Ki-67 examination performed by immunohistochemical tests. On immunohistochemical examination, Ki-67 can detect the progression of malignancy according to the degree of dysplasia and the type of lesion.[12] In this study, the micronucleus examination was able to detect the risk of progression of malignancy based on the type of lesion quite well.

Limitations and future prospects

1. The absence of a biopsy examination to determine a definitive diagnosis of the various oral lesions found so that the risk of progression to malignancy based on the degree of dysplasia cannot be synchronized with the biomolecular tests performed in this study.
2. Further research should include biopsy examination with larger sample size.

Conclusion

Examination of Ki-67 levels in saliva and micronucleus cytology examination of the buccal mucosal smear is effective as an alternative biomarker for early malignant conditions. However, until now, normal range of Ki-67 levels in the saliva and the number of micronuclei thad not been established.

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Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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