Molecular Grading of Oral Squamous Cell Carcinomas Infected with EBV

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Molecular Grading of Oral Squamous Cell Carcinomas Infected with EBV

Theresia Indah Budhy*

Abstract

Background: Squamous Cell Carcinoma (SCC) is a type of cancer that is often found in oral cavity and areas of the head and neck. Viruses are major etiological factors through production of factors that can disturb proliferation and apoptosis regulators such as p53i, c-myc and bcl-2. This study aimed to determine the molecular grading of oral squamous cell carcinoma (OSCCs) infected with the Epstein-Barr Virus (EBV). **Methods:** Twenty-seven OSCC patients underwent biopsy to detect EBV infection through in situ hybridization for RNA EBV (EBER) and immunohistochemical analysis of latent membrane protein-1 (LMP-1) and EBV nuclear antigen-1 (EBNA-1). To assess molecular grades, cell proliferation and apoptosis regulator expression i.e. inactive p53 (p53i), c-myc and bcl-2, were immunohistochemically analysed. **Results:** The cases were divided into two groups; infected and non-infected by EBV. Regression analysis showed that only EBNA-1 expression could affect p53i expression. Based on regression equations molecular grading of OSCCs infected by EBV was divided into three: Grade I (low), EBNA-1 expression was 7.60, and p53i expression was 9.74-17.5; Grade II (medium), EBNA-1 expression was 7.61-19.7, and p53i 17.5-30.1; Grade III (high), EBNA-1 expression was 19.71, and p53i $\geq$ 30.1. **Conclusion:** In OSCC infected with EBV, only EBNA-1 expression can influence p53i expression.

Keywords: EBNA-1- LMP-1- OSCC- p53i- bcl-2

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Introduction

The incidence of oral cancer in Indonesia is still quite high and annualy increasing (Budhy et al., 2001). Oral Squamous Cell Carcinoma (OSCC) is a type of cancer that is often found in oral cavity or in head and neck (Kumar et al., 2005). The causal factors of this disease are considered complex and therefore needs to be concerned (Agar and Patel, 2014; Regezi et al., 2016). One of the causes is Epstein Bar Virus (EBV) (Higa et al., 2002; Acharya et al., 2015; Kikuchi et al., 2017).

EBV belongs to the family of Herpersviridae, which has spread throughout most parts of the world (Kieff, 1996; Arvin et al., 2007; Kikuchi et al., 2017). The viral genome is hidden constantly in cells with strong expression of EBV proteins, nuclear antigen complex (EBNA), membrane protein latent (LMP-1 and LMP-2) and EBV-RNA (EBER) (Budhy, 2005; Xia et al., 2015; Kikuchi et al., 2017). The expression of EBV protein then will affect the regulatory process of proteins in the cells (Sitki-Green et al., 2004). If the presence of EBV disrupts the regulatory process contributing to cell cycle control, such as the expressions of inactive p53 (p53i), c-myc and bcl-2, both cell apoptosis and cell proliferation will be disrupted. This situation can cause the cells to transform into malignant ones, known as carcinogenesis (Muller and Vousden, 2014).

Carcinogenesis, according to Kumar et al. (2005), may occur due to disruption in the mechanisms of regulatory genes related to both cell apoptosis and cell proliferation, namely p53. p53 gene is a tumor suppressor gene that plays a role in the regulation of apoptosis when damages occur (Liu et al., 2015; Yoon et al., 2015; Muñoz-Fontela et al., 2016). Carcinogenesis, however, is not only determined by p53 gene, but also influenced by oncogenes. One of the oncogenes is c-myc which functions as a transcription factor and is mostly associated with cell transformation. As for apoptosis, it is influenced not only by bcl-2 gene, but also by p53i gene. Thus, the expressions of the proliferation and apoptosis of the regulatory molecules can determine the level (grade) of carcinogenesis. This study, therefore, aimed to determine the molecular grading pattern of OSCC infected by Epstein-Barr Virus (EBV), by analyzing at the expressions of p53i, c-myc and bcl-2 genes.

Materials and Methods

This study was an observational analytic study. The protocol of this study was approved by The Ethic

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Committee of Universitas Airlangga Faculty of Dental Medicine No 074/HRECC.FODM/VII/2017. Biopsy of 27 subjects with OSCC were performed in Intergrated Surgery Center Building of RSUD Dr. Soetomo. Inform consents were obtained from all of the subjects.

Examination of EBV infection

The biopsy results were immediately put into a deep freezer with the temperature of -70°C. EBER were identified using in-situ hybridization method. LMP-1 and EBNA was examined using immunohistochemistry. The staining process was conducted using monoclonal antibodies of LMP-1 and EBNA-1 from Dako (Agilent, Santa Clara, USA) with Avidin Biotin Complex technic from Novo Castra Biotin System (Leica Biosystems Newcastle Ltd, Newcastle, UK) in order to make the staining results stronger and clearer due to the amplification. The assessment of microscopic staining results was conducted qualitatively by examining the intensity of color absorption, as well as quantitatively by counting the number of cells manually that positively absorb the color. The present of EBER, LMP-1, and EBNA-1 considered as OSCCinfected by EBV group, while the absent of those proteins considered as non-infected group.

Expression of p53i, c-myc, and bcl-2

The expression of p53i, c-myc, and bcl-2 were examined using immunohistochemistry in both groups. The staining process was conducted using monoclonal antibodies of inactive p53i, bcl-2, c-myc from Dako (Agilent, Santa Clara, USA) with Avidin Biotin Complex technic from Novo Castra Biotin System (Leica Biosystems Newcastle Ltd, Newcastle, UK). The assessment of microscopic staining results was also conducted qualitatively and quantitatively.

Data analysis

The data was analyzed using SPSS version 10 (SPSS Inc, Massachusetts, USA). Multivariate Analysis of Variance (MANOVA) was used to prove the difference of the expressions of p53i, c-myc, ad bcl-2, followed by analysis of the molecular grading pattern.

Then, Independent T test was performed to analyze EBER, LMP-1, EBNA-1, and bcl2 in both infected and uninfected OSCC since the data were normally distributed. Meanwhile, to analyze p53i and c-myc expressions, Mann Whytney test was conducted since the data was not normally distributed. Univariate Linear Regression then was carried out to reveal the effects of EBER, LMP-1, and EBNA-1 on p53i expression.

Results

Based on Table 1, the standard deviation (SD) was generally very high. There were significant differences in EBER, LMP-1, EBNA-1, and Bcl-2 between the infected (n=17) and non-infected groups (n=10). Meanwhile, there were no significant differences in p53i expression and C-myc between the infected and non-infected groups. Next, the regression test was performed to analyze the effects of EBER, LMP1, and EBNA-1 expressions on p53i, c-myc, and bcl-2 expressions. Results of the regression test can be seen in Table 2.

Based on the results of the regression test, only EBNA-1 expression affected p53i expression. As a result, regression equation test was conducted. Results of the regression equation test can be seen as follows.

p53i = 9.574 + 1.043 (EBNA-1)

Based on the regression equation above, it may be said that an increase of 1.043 EBNA-1 expression can enhance p53i expression by a unit of expression. Consequently, to

Table 1. EBER, LMP-1, EBNA-1, p53i, c-myc, and bcl-2 Expressions in both Infected and Non-infected OSCC

Variables	Groups		p-value
	OSCC infected by EBV (n=17)	Non-infected OSCC (n=10)	
	Mean±SD	Mean±SD	
EBER	28.75±25.79	0.56±1.67	0.004*
LMP-1	17.50±20.49	0.00±0.00	0.014*
EBNA-1	11.88±13.28	0.00±0.00	0.014*
p53i	29.38±28.86	18.33±16.96	0.307
c-myc	30.63±24.07	16.11±20.28	0.141
bcl-2	1.88±7.50	6.67±5.00	0.003*

* significantly different if $\alpha<0.05$

Table 2. The Effects of EBV Virus Molecules (EBER, LMP-1, EBNA-1) on the Carcinogenesis Molecules (p53i, bcl-2, c-myc)

	p53i		bcl-2		c-myc	
Molecules	p-value	B	p-value	B	p-value	B
EBER	0.155	0.282	0.761	0.021	0.215	0.297
LMP-1	0.387	0.237	0.260	0.108	0.891	0.045
EBNA-1	0.007*	1.043	0.230	0.151	0.994	0.03

*significantly affecting if P<0.05; B is regression coefficient

Table 3. The Molecular Grading Pattern of SCC Based on EBNA-1 and p53i Expressions

Grade	EBNA-1	p53i
I	0-7.60	9.74-17.50
II	7.61-19.70	17.51-30.12
III	19.71	≥30.13

determine the molecular grading pattern of OSCC, Grades I, II, and III were identified based on the effects of EBNA-1 expression on p53i expression (see Table 3).

The molecular grading pattern of OSCC indicates the levels of carcinogenesis in the oral cavity caused by EBV infection. Grade I described a low molecular stage, grade II showed a moderate molecular stage, while grade III illustrated a high molecular stage of oral cavity cancer infected with EBV. In other words, the expression of EBNA-1 from 0 to 7.6, for instance, affected p53i expression by 0-25.0 times. As a result, it can be said that the higher the expression of EBNA-1 is, the higher the expression of p53i will be.

Discussion

p53 gene is a tumor suppressor gene that has a very important role in cell division and apoptosis processes (Liu et al., 2015; Yoon et al., 2015; Muñoz-Fontela et al., 2016). In this research, the mean amount of inactive p53 (p53i) expression found in OSCC infected with EBV was 29.38. Meanwhile, in OSCC non-infected by EBV, the mean amount of inactive p53 (p53i) expression was 18.33. The SD was generally very high, possibly due to very hetrogen factors triggering carcinogenesis (Rowe and Rickinson, 2001).

Furthermore, the results of this research also showed that the mean amount of bcl-2 expression in OSCC infected with EBV was 1.88, while in OSCC non-infected with EBV it was 6.67. This indicates that EBV infection can make p53 inactive, thus decreasing the activation of bcl-2. The activation of bcl-2 is aimed to open a pore in mitochondria so that cytochrom C can be removed and caspase cascade can be stimulated (Amir, 2014; Luna-Vargas and Chipuk, 2016). In other words, in EBV infection condition, OSSC can make p53 gene defective, hereby becoming inactive. This condition then causes cell division not to be able to stop and cell apoptosis not to happen, which leads to carcinogenesis. Based the results in this study, it is also known that there was no significant difference in p53i and c-myc between OSCC infected with EBV and OSCC non-infected with EBV. It means that the role of suppressor gene that is not active also can affect the activation of c-myc expression as oncogen or transcription factor. This condition plays a role in cell division either infected or not.

In addition, the expression of c-myc found in OSCC infected with EBV in this research was 30.63, while in OSCC non-infected with EBV it was 16.11. Another gene that also contributes to carcinogenesis due to EBV infection is bcl-2 gene (Kieff, 1996; Kvansakul and Hinds, 2015). The expression of bcl-2 in OSCC infected with EBV in this research was 1.88, smaller than in OSCC non-infected with EBV, about 6.67.

Based on the results of the analysis above, the regression analysis then was performed to determine the effects of EBV infection on carcinogenesis. The results of the regression analysis indicated that only EBNA-1 induced p53i. Therefore, it can be assumed that EBV expression plays an important role in carnogenesis, but only EBNA-1 has direct effect. Meanwhile, EBER and LMP-1 are assumed to have no direct effect. This is supported by the opinion of Rowe and Rickinson (2001) that the expression of EBNA-1 is associated with certain types of tumors. Allegedly, one of them is oral carcinoma.

Moreover, since only EBNA-1 expression affected p53i expression in this research, EBNA-1 could be assumed to be able to make p53 expression inactive. The significant difference found between LMP-1 and EBER in both infected and noninfected groups indicates that LMP-1 and EBER have a role in carcinoma in the oral cavity. It is similar to previous study which showed that EBER and LMP-1 are found in almost all tumors (Rowe and Rickinson, 2001). LMP-1, according to MeckesRaab-Traub (2011), can even trigger the transformation of epithelial cells to be malignant by inhibiting cancer cell apoptosis. An important target of LMP-1 is mediated transcriptional upregulation of EGFR, a member of ErbB receptor tyrosine kinase family. EGFR is a growth factor receiver that activates multiple signaling pathways and often causes mutations to cause cancer.

Nevertheless, further researchers are still necessary to be conducted to determine the correlation of the molecular grading patterns and clinical staging in OSCC infected with EBV.

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