Detection of Epstein-Barr and Human Papilloma Viruses in the Middle Ear Squamous Cell Carcinoma

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Abstract The uncommon ear tumor of middle ear squamous cell carcinoma (MESCC) is thought to be associated with the history of long-term chronic otitis media in the most cases. The main etiologic factor of MESCC is still unclear and may be multifactorial. Infections of Epstein-Barr virus (EBV) and Human Papillomavirus (HPV) are considered as one of the etiologic factor of MESCC. Previous studies have shown that the EBV and HPV have been detected in MESCC. Although the EBV and HPV have been implicated in human malignancies, their roles in pathogenesis of MESCC have not been elucidated. There has never been report on the presence of EBV and HPV in Indonesian MESCC. This study aimed to determine the presence of EBV and HPV in MESCC. Seven paraffin-embedded tissues of specimen from biopsy were analyzed for the presence of EBV and HPV by immunohistochemistry, stained using polyclonal antibody anti EBNA1 and anti HPV. The samples consisted of 4 (57 %) males and 3 (43 %) females with age range of 26–87 years old. Immunohistochemistry result demonstrated that EBV was detected in three of seven (43 %) and HPV in two of seven (29 %) samples. Coexistence of the presence of EBV and HPV were found in one of seven (14 %) sample. The presence of EBV and HPV in MESCC suggests that viral infection may play an important etiologic role in the carcinogenesis of middle ear.

Keywords Middle ear squamous cell carcinoma · EBV · HPV · Immunohistochemistry

Introduction

The incidence of head and neck cancer worldwide is about 1 per 100,000 and tends to increase every year [1]. In 2000, global mortality rate due to head and neck cancer ranked eighth among overall cancer death. About 480,000 new cases detected and 320,000 people died because of the cancer, thus average mortality rate was 7.3 and 3.2 per 100,000 men and women, respectively, and the incidence rate was 8.8 and 5.1 per 100,000 men and women, respectively [1]. Among all head and neck cancers, head and neck squamous cell carcinoma (HNSCC), which might involve oral cavity, oropharynx, hypopharynx, and larynx, is the most commonly found histopathologically. HNSCC ranks sixth among all malignancies worldwide [2]. Epidemiology and laboratory data have shown that infection of Epstein-Barr virus (EBV) and Human papilloma virus (HPV) have a role in development of cancer in HNSCC [3, 4].

EBV bears highest malignancy potential among all herpesvirus [4]. EBV shows affinity to B cell lymphocyte. EBV was the causative agent of oral hairy leukoplakia in immunocompromised patients and was able to self-replicate in epithelial cells [5], but whether EBV was able to infect epithelial cells in immunocompromised patients remained unclear. Like other viral agents, tumor genesis effect of EBV is mediated by its proteins, which will disrupt cell cycle regulation. In addition, the latent membrane protein (LMP1) and Epstein-Barr nuclear antigen 5 (EBNA5) will inhibit p53-mediated apoptosis, and EBNA5 also interacts with retinoblastoma (Rb) protein [4].

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HPV is a circular DNA virus from Papovaviridae family whose role has been implied in the genesis of benign and malignant tumors in various sites covered by mucous membrane or skin, such as cervix and anogenital region [6]. About 70 types of HPV have been discovered. HPV can be classified into three risk type: low, medium, and high risk, according to the transformation. Results from molecular studies have shown specific evidence about HPV involvement in tumor genesis. Epidemiology and molecular biology studies have also proven the association between HPV infection and anogenital and also head and neck cancer epithelial tumor [3, 6]. The most common sites of cancer where HPV DNA was detected were oral cavity, pharynx, and larynx [3, 7].

Middle ear squamous cell carcinoma (MESCC) was first introduced by Politzer [8]. MESCC is a rare cancer but is the most commonly found cancer in middle ear [9]. Incidence of MESCC was very low and estimated about 0.8–1 per 10⁶ every year worldwide [10], or about 1 case per 8000–10,000 patients admitted to hospital with otologic disorders [11]. One of main predisposition factors in MESCC was a history of chronic otitis media with duration about 20 years in 40–85 % cases [12, 13]. The mechanism contributing to MESCC development with the history of chronic otitis media is still unclear. Several carcinoma cases were also associated with previous cholesteatoma [14]. By polymerase chain reaction, Bergmann et al. [15] detected the presence of HPV DNA in 36 % cholesteatoma cases. Other factor contributing in the tumor genesis was a history of radiotherapy for head and neck cancer and exposure to radium [16]. Due to its rare incidence, there is lack of research which is able to explain the development and mechanism of carcinogenesis in MESCC. A research in Taiwan had shown that type 16 and 18 of HPV DNA were found in middle ear carcinoma with the prevalence of 89 % [12, 17]. Leung et al. [18] reported that EBV was found in middle ear carcinoma, particularly in one of nonkeratinizing squamous cell carcinoma and one of undifferentiated carcinoma. Since both viral infections played a role in carcinogenesis and were found in MESCC, there was no study in Indonesia. This study was proposed to determine the presence of EBV and HPV in MESCC by immunohistochemistry.

Materials and Methods

This study was approved by the Ethics Committee of Faculty of Medicine. Study design was retrospective with specimen samples were obtained from patients diagnosed as middle ear squamous cell carcinoma with either local or general anesthesia from 2007 to 2008. This study was conducted in Department of Otolaryngology Head and

Neck Surgery of Sardjito Hospital Yogyakarta and Anatomical Pathology Department of Faculty of Medicine, Universitas Gadjah Mada Yogyakarta Indonesia. Inclusion criteria of this study included age above 15 years, clinical symptoms of middle ear tumor confirmed with CT-scan, register data of patients from 2007 to 2008, results of histopathology examination were in accordance with squamous cell carcinoma, no previous chemotherapy and/or radiotherapy and/or surgery. The exclusion criteria was incomplete data and severe complication of other diseases.

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue of 4–5 µm thick sections were cut. The sections were deparaffinized in xylene and rehydrated through graded concentration of alcohol. Antigen retrieval was performed in a microwave oven for two cycles of 10 min. Endogenous peroxidase activity was blocked by incubating the slides in 1.5 % hydrogen peroxide in absolute methanol at room temperature for 10 min. Each primary polyclonal antibodies was performed by anti EBNA1 (Abcam, USA) to detect EBV and anti HPV antibody (Thermo Scientific, USA). Primary polyclonal antibodies were applied for 1 h at room temperature, then sections were washed three times with 0.05 mol/l Tris-buffered saline (TBS), pH 7.2 and then incubated with 50 µl of polymerized horseradish peroxidase antimouse immunoglobulin G for 30 min at room temperature. Then, sections were washed three times with 0.05 mol/l TBS and the reaction products were visualized with diaminobenzidine (DAB kit, Thermo Scientific, USA). Sections were then counterstained with H&E, dehydrated and evaluated under a light microscope. As a negative control, samples were incubated using 0.01 mol/l TBS instead of a primary antibody. EBNA1 and HPV were scored only according to the presence (1+) or absence (0) of nuclear and/or cytoplasmic expression. Positive samples were defined as having EBNA1 staining of >10 % and HPV staining of >1 % of the tumor area.

Results

Characteristic of Patients

Patients' age ranged between 26 and 87 years old and consisted of 4 (57 %) males and 3 (43 %) females. Six patients were histopathologically diagnosed as well-differentiated squamous cell carcinomas and one patient as poor-differentiated squamous cell carcinoma. According to MESCC stage from Pittsburgh TNM system [19], four patients presented to the clinic with stage IV, while three patients with stage III. No patient in this research came with stage I and II. Clinical and histopathological data were shown on Table 1.

Table 1 Histopathological data and the presence of EBV and HPV in 7 patients with MESCC

No	Sex	Age (year)	Histopathology	Stage	EBV	HPV
1.	M	41	Well-differentiated SCC	IV	+	–
2.	M	63	Well-differentiated SCC	III	–	–
3.	F	60	Well-differentiated SCC	IV	+	+
4.	M	75	Poor-differentiated SCC	IV	+	–
5.	F	26	Well-differentiated SCC	IV	–	+
6.	F	62	Well-differentiated SCC	III	–	–
7.	M	87	Well-differentiated SCC	III	–	–

Stage according to TNM system from University of Pittsburgh [19]

SCC squamous cell carcinoma, *M* male, *F* female

Detection of EBV and HPV

Previous study reported the detection of EBV and HPV in MESCC on DNA level by using PCR or in situ hybridization [12, 17, 18]. To detect EBV on DNA level, several researchers used one of EBV genes called EBER, while the presence of HPV may be performed with specific probe for type 16 and 18. In this study, paraffin-embedded tissues were analyzed on the protein level by immunohistochemistry method with anti EBNA1 (EBV) and anti HPV (recognizes viral capsid peptide) polyclonal antibodies. The reason of using anti EBNA1 instead of EBER was due equal expression level between EBNA1 and EBER. In addition, EBNA1 is a multifunctional protein which has important roles in the latent phase, and is needed for replication and maintenance of viral duplication. EBNA1 expression was found in three of seven (47 %) MESCC specimens with the histopathology features of two well-differentiated carcinoma and one poor-differentiated carcinoma. Immunohistochemistry result showed that EBNA1 expression was detected not only in nucleus, but also in cytoplasm and scattered in colony-like pattern in tumor tissue as well as in nasopharyngeal carcinoma tissue (Fig. 1).

HPV expression was found in two of seven (29 %) well-differentiated MESCC specimens. Hematoxylin-eosin result showed that koilocyte cells were unequally distributed and in very few amount. The expression of HPV was visualized in a very few amount and was not found in all koilocyte cells (Fig. 2). This was contrary to the cervical tumor, in which the expression of HPV and koilocyte cells pattern were detected in abundant amount in tumor cells. Both expressions of EBV and HPV were found in one of seven (14 %) well-differentiated SCC patient. Surprisingly, this result showed the first documented double infection of EBV and HPV in MESCC.

Fig. 1 Immunohistochemistry result with polyclonal antibody anti-EBNA1 in patient 4 (Table 1). The arrow showed visualized EBNA1 s with brown-color in nucleus and cytoplasm of squamous cell carcinoma tissue (Magnified 10 × 40)

Discussion

Incidence of middle ear tumor is an aggressive malignancy despite its rare occurrence. Incidence in population and prevalence among other head and neck tumors was very low [10, 11]. The most patients present with advanced stage with symptoms such as severe headache and paresis of facial nerve. The tumor spread to all direction, especially through direct invasion or surrounding bone erosion. Lymphatic metastasis was rarely found unless in advanced stage and distant metastasis was also very rare [20]. Pathogenesis in MESCC is still unclear. A few possible etiology factors had been reported. Radiation was one of the main factor implied to cause middle ear tumor [16]. Previous study reported the association between a history of prolonged chronic otitis media with MESCC [12, 17]. HPV infection with high risk type (type 16 and 18) was

Fig. 2 Immunohistochemistry result with polyclonal antibody anti-HPV in patient 3 (Table 1). The arrow showed the presence of HPV in koilocyte cells visualized in squamous cell carcinoma tissue. Head arrow showed that not all koilocytes were present in cancer cells (Magnified 10 × 100)

found in MESCC with various prevalence rates [12, 17]. EBV DNA was also detected in middle ear carcinoma where histopathological appearance resembled nasopharyngeal carcinoma [18]. Based on those results, we investigated the presence of EBV and HPV infection in MESCC.

About 90 % nasopharyngeal carcinoma is associated with the presence of EBV in tumor tissue. EBV infection is one of the main causative agents of nasopharyngeal carcinoma. The virus enters human body and replicates in oropharyngeal epithelial cells, is persistent, latent, and long life [21, 22]. Embriologically, middle ear mucous epithelial cells are similar with those in nasopharynx. If the tumor morphology of the two sites are similar, the presence of EBV in middle ear tumor tissue is very likely. This might be proved with the detection of EBNA1 in three (47 %) of seven MESCC. The question raised to determine the common presence of EBV in nasopharyngeal carcinoma but rare in the middle ear cavity is still remain unknown. Even though EBV-related carcinoma is rarely found unless in nasopharynx, previous study reported the presence of EBV in salivary glands [23], nose, and paranasal sinuses [24, 25], tonsils [26], saccus lacrimalis [27], thyme [28], and lungs [29]. This study demonstrated middle ear cavity as a possible site where EBV plays a role in the carcinogenesis of middle ear tumor although the mechanism still has to be investigated.

There are at least 70 pathogenic types of HPV already identified in human. Mostly they are found in benign proliferation, but several of them play a role in malignant tumors, especially cervical, anogenital, skin, oral and nasal cavity, and laryngeal carcinoma [31]. The most commonly found HPV DNA in benign papillomatous lesions are type 2 and 11 [32], while type 16 and 18 are

found in malignancies in oral cavity and sinonasal tract [33], larynx [34], and esophagus [35]. Although MESCC is a part of head and neck region, it is not associated with HPV infection. This study, although not specifically define which type of HPV on DNA level, HPV protein was found in 2 of seven (29 %) MESCC patients. As well as detection of EBNA1 in MESCC, the presence of HPV protein is able to explain the role of HPV in carcinogenesis of MESCC like other organs in head and neck region.

Despite the type of HPV was not analyzed in this study, it could be assumed based on previous findings that type 16 and 18 of HPV were the most commonly found in carcinoma compared to other high risk types (type 31, 33, 52b, and 58). HPV-16 was the highest virus prevalence found in uterine cervix and is often associated with squamous cell carcinoma [12, 30]. HPV-18 was the type of virus most often associated with adenocarcinoma and small-cell neuroendocrine carcinoma in cervix and rarely associated with squamous cell carcinoma [31, 32].

In a study performed in Taiwan [12, 17], HPV DNA was found in high prevalence, 11(78.6 %) of 14 cases with dominated HPV-16 compared to HPV-18. The study tried to find the correlation between the histories of chronic otitis media with MESCC. This study only found 2 (29 %) of 7 cases. The real difference was the detection on molecular level. The presence of HPV DNA in tumor tissue cannot yet imply the role of HPV to the molecular carcinogenesis mechanism. In HPV-associated malignant transformation, viral DNA integrates to cellular DNA, leaving a wide deletion sector from the viral genome. Late genes (L1 and L2) and sometimes early genes (E1 and E2) usually disappeared, leaving E6 and E7. The transcription process of E6 and E7 to be protein in tumor indicates their role in malignant transformation [33]. E6 and E7 expression disturbs normal regulation of cell proliferation mediated by p53 and pRb, and thus results in uncontrolled growth and becomes potential mediator of malignant transformation [34].

The coincidence of EBV and HPV was shown in this study, where both viral proteins were found in one of seven (14 %) MESCC. The presence of both viruses supported the role in carcinogenesis of middle ear and may affect prognosis through their molecular mechanisms, such as the status of p53 and pRb proteins which maintain the cellular homeostasis against malignancy processes.

Conclusion

This is the first research report showing the presence of EBV and HPV on protein level in MESCC in Indonesian population. The role of both viral infections is probably an etiologic component and plays an important function in middle ear carcinogenesis. Further investigation is

necessary to define MESCC pathogenesis in relation to EBV and HPV infections in molecular level.

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Compliance with Ethical Standards

Conflict of interest The authors confirm that they have no potential conflicts of interests that could inappropriately influence or bias this work.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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