学 位 論 文 の 要 旨

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学　位　論　文　名: Feasibility of HPV16, HPV18, and p16 Expression as Biomarkers for Distinguishing Normal Oral Epithelium from Oral Epithelial Dysplasia and Oral Intraepithelial Neoplasia

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論 文 内 容 の 要 旨

INTRODUCTION

Many oral squamous cell carcinoma (OSCC) lesions develop from potentially malignant disorders (PMDs). Numerous criteria exist for the diagnosis of oral epithelial dysplasia (OED), and there is not always a clear-cut distinction of what represents mild dysplasia consisting of only focal atypia, moderate dysplasia, and severe dysplasia which may present as carcinoma *in situ* (CIS) as PMDs. Furthermore, according to the general rules for clinical and pathological studies on oral cancer, mild and moderate dysplasias are defined as OED, while severe dysplasia is defined as oral intraepithelial neoplasia (OIN). As for CIS and OIN, however, a definitive distinction cannot always be drawn between mild and moderate dysplasias and CIS/OIN.

The identification of human papillomavirus (HPV) in oropharyngeal carcinoma might have prognostic significance, with longer survival and a higher rate of response to therapy in cases positive for HPV. However the detail of HPV identification and the roles of these infections in terms of the prognosis and carcinogenesis still remain unclear especially in OSCC.

In this preliminary study, we thus evaluated the association between the expression of HPV16, HPV18, and p16 and various lesions derived from the oral epithelium, immunohistochemically, testing the hypothesis that the expression of HPV16, HPV18, and p16 could be feasible biomarkers to distinguish PMDs in the oral cavity.

MATERIALS AND METHODS

Participants and samples

All participants with clinically diagnosed OIN, OED, and OSCC underwent a preoperative biopsy located on the tongue, gingiva, buccal mucosa, lip, and palate at the Department of Oral and Maxillofacial Surgery, Shimane University Hospital, Japan from 1980 to January 2014. Normal oral epithelium (NOE) was taken from healthy volunteers.

HPV16, HPV18, and p16 immunohistochemistry

HPV16 and HPV18 expression was determined immunohistochemically using an anti-HPV16 E1+E4 antibody (Abcam, Cambridge, UK; diluted at 1:100) and an anti-HPV18 E6 antibody (Abcam, Cambridge, UK; diluted at 1:500). As a surrogate marker of HPV presence, $p16^{INK4a}$ (VENTANA, AZ, USA, ready to use) was also used. Biopsy specimens (formalin-fixed paraffin-embedded sections) were treated with primary antibody (diluted 1:1,000 overnight at 4°C) after deparaffinization. Immunoperoxidase staining was performed using an EnVision™+ Kit (Dako, CA, USA). Counterstaining was done with Mayer's hematoxylin (MUTO PURE CHEMICALS Co., Ltd., Tokyo, Japan).

HPV16, HPV18, and p16 SIs

All sections were examined using a standard light microscope with a ×40 objective lens. An attached digital camera was used to capture images and estimate the number of HPV16-, HPV18-, and p16-positive cells (at least 100 cells/field). The SI (stained cells / total cells counted × 100 [%]) was expressed as the percentage of positive cells among the total number of cells in the area scored.

Statistical analysis

The results were analyzed using SAS™ version 9.3 (Cary, NC, USA) and R version 3.2.2 (R Foundation, Vienna, Austria). The participants were stratified according to a pathological classification with four levels: NOE, OED, OIN, and OSCC. In addition to analysis by all participants, subgroup analysis in participants with NOE, OED, and OIN was performed. Continuous and categorical variables were summarized as the mean ± standard deviation (SD) and frequency (percentage), respectively. In addition, to construct a clinically useful decision tool for the diagnosis of NOE or OED/OIN, regression tree analysis was performed using a conditional inference method with a splitting criterion of $p < 0.05$. Age, sex, positive/negative, and LIs for HPV16, HPV18, and p16 were used as candidate predictors in the regression tree analysis. A p-value <0.05 was considered significant.

The study protocol was approved by the Ethics Committee of Shimane University and written informed consent was obtained from all subjects.

RESULTS AND DISCUSSION

Participants' backgrounds

The participants comprised 150 cases (84 men, 66 women; mean age, 65.3 years), 41 with OED (mean ± SD: 65.2 ± 12.6 years), 30 with OIN (71.7 ± 10.8 years; trend test: $p = 0.002$) and 67 with OSCC (63.8 ± 15.4 years). NOE was taken from 12 healthy participants (60.7 ± 10.1 years).

Immunohistochemical findings for HPV16, HPV18, and p16

Staining in HPV16-positive cells in OED and OSCC was distributed in the nucleus of dysplastic or tumor cells. Staining in HPV18-positive cells in OED, OIN, and OSCC was distributed predominantly in the nucleus of dysplastic or tumor cells. Staining in p16-positive cells in OED, OIN, and OSCC was distributed predominantly in the nucleus and/or cytoplasm of dysplastic or tumor cells

Expression and SIs

HPV16: The SIs (%) of HPV16 were 0.4 ± 1.7% (mean ± SD) and 0.2 ± 1.2% in OED and OSCC, respectively. There was no trend for HPV16 status and SIs in all cases or in the subgroup including NOE, OED, and OIN.

HPV18: The SIs of HPV18 were 30.2 ± 32.7% (mean ± SD), 56.1 ± 36.0%, 47.1 ± 41.3%, and 29.9 ± 31.4% in NOE, OED, OIN, and OSCC, respectively. There was no trend for HPV18 SIs was provided in all cases, an increasing trend was observed in the subgroup including NOE, OED, and OIN ($p = 0.043$).

p16: The SIs of p16 were 22.0 ± 22.1% (mean ± SD), 22.5 ± 26.8%, and 8.7 ± 20.5% in OED, OIN, and OSCC, respectively. There was no trend for p16 status and SIs in all cases, significant trends were found in the subgroup including NOE, OED, and OIN ($p < 0.001$ and $p = 0.027$, respectively)

Discrimination between NOE and OED/OIN

As a result, 4 stratified groups (100.0%, 93.8%, 60.0%, and 30.0% of OED/OIN) by age (60 years), p16 status, and HPV18 status were provided. In this study, a statistical trend test of each variable was first performed, then followed by the manifest confirmation of statistically significant values, which revealed putative feasible candidates for biomarkers or factors, namely, age, p16, and HPV18, to distinguish NOE from OED/OIN. Further regression tree analysis considering the participants' age revealed that p16 and HPV18 expression and the participants' age (60 years) are feasible biomarkers to distinguish NOE and OED/OIN.

CONCLUSION

This preliminary study showed the expression of p16 and HPV18 and patients' age would be feasible biomarkers to distinguish NOE and OED/OIN.

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論文審査の結果の要旨

子宮頸癌の原因とされるHuman papillomavirus (HPV)と頭頸部癌との関連が近年注目されているが、口腔領域における前癌病変とHPV感染の関連についてはいまだ統一された見解がない。申請者は、口腔粘膜における境界病変と口腔扁平上皮癌でHPV発現に差があり、そのことが診断のための有用なマーカーになるのではないかとの発想に基づいて、本研究を実施した。口腔粘膜病変組織標本150例(正常組織:12例, 口腔上皮異形成症:41例, 口腔上皮内腫瘍:30例, 口腔扁平上皮癌:67例)を対象にHPV16, HPV18, P16の免疫組織化学染色を施行し、各病変内での陽性細胞率について、性別、年齢とともに検討した。 正常組織, 口腔上皮異形成症, 口腔上皮内腫瘍については、HPV18, P16を発現する陽性細胞の率がこの順に有意に上昇した。更に、回帰樹木分析を用いた解析では、年齢、HPV18染色、P16染色が識別因子となり、正常組織と口腔上皮異形成症, 口腔上皮内腫瘍を識別できることを示唆する結果が得られた。

この結果は、将来HPVやP16染色を用いた口腔粘膜病変の鑑別法開発に繋がる成果であり、臨床的意義が高い研究である。