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Original Article

Relationship between p21 and p53 Expression, Human Papilloma Virus Infection and Malignant Transformation in Sinonasal-inverted Papilloma

H. Katori*, A. Nozawa†, M. Tsukuda*

*Department of Otolaryngology, Yokohama City University Medical Center, Yokohama, Japan; †Department of Pathology, Yokohama City University Medical Center, Yokohama, Japan

ABSTRACT:
Aims: **To identify the relationship between p21 and p53 expression, human papilloma virus (HPV) infection and malignant transformation in sinonasal-inverted papilloma.**
Material and methods: **Nasal tissues, exophytic papilloma, inverted papilloma (IP) with dysplasia, IP with carcinoma and invasive squamous cell carcinoma (SCC) were stained with the monoclonal antibodies p21 and p53. *In-situ* hybridisation for HPV DNA was also carried out for types 6/11, 16/18 and 31/33.**
Results: **Significant increased staining of p21 and p53 was observed in IP with severe dysplasia, IP with carcinoma and invasive carcinoma compared with control nasal mucosa. A significant increase of dysplasia was observed in IP in the HPV 6/11 and 16/18-positive group, compared with the HPV 6/11 and 16/18-negative group. Significant decrease in expression of p21 and p53 was observed in HPV 16/18-positive IP compared with HPV 16/18-negative IP.**
Conclusions: **Our data raise the possibility that testing for p21, p53 and HPV may help to screen out papilloma lesions with a potential for dysplasia or carcinoma. Katori, H. *et al*. (2006). *Clinical Oncology* 18, 300–305**

Key words: Human papilloma virus (HPV), inverted papilloma, malignancy, p21, p53

Introduction

Inverted papilloma (IP) is a rare benign tumour of the nasal cavities and paranasal sinuses. IPs are epithelial in origin, and are characterised by a hyperplastic epithelium with an endophytic type of growth, whereas the basement membrane is intact and morphologically normal [1]. The true incidence of IP is unknown; however, 0.5–4% of all nasal tumours and 2–3% of all nasal polyps are believed to be IP [2]. IP recurs in nearly 15% of cases, and is associated with squamous cell carcinoma (SCC) in about 5–21% of cases [2,3].

Although multiple studies have investigated IP, their mechanisms of growth and malignant transformation have not yet been fully described. Bacterial and viral infections, chronic inflammatory conditions, allergies, tobacco and occupational exposures have all been cited as possible causes. Increasing evidence is now pointing towards a viral origin, human papilloma virus (HPV) [4]. HPV infection can be found in benign and malignant squamous tumours in a variety of body sites, such as the uterine cervix and anogenital tracts [5]. In head and neck regions, HPV DNA is detected in more than 90% of juvenile and adult-onset papillomas, whereas the viral genomes, including oncogenic types, are also found in 15–38% of malignant lesions [5,6]. HPV E6 and E7 oncoproteins are capable of functionally inactivating the cell-cycle regulators, such as p53 and cyclin D1, resulting in the disruption of normal cell-cycle control [7].

The mechanism of action of HPV in oncogenesis has been attributed to its rendering ineffective the p53 tumour suppressor gene of the host cell. In fact, mutation of the p53 gene is currently the most commonly identified gene mutation in human neoplasia [8]. Interestingly, several studies on anogenital tract malignancies suggest an inverse correlation between the presence of HPV and the presence of p53 mutations [9]. To date, the role of HPV and p53 expression in IP lesions has not been fully explained.

p21 (p21WAF1) has been implicated in the mechanisms of cell-cycle arrest at G1 phases through p53-dependent or independent pathways, resulting in terminal differentiation in several cell lineages [10]. Although recent studies have revealed functional significance for p21 in the biological behaviour of several human malignancies, little is known about changes in expression of these proteins in sinonasal tumours, including IP and SCC [11].

In this study, we investigated immunohistochemical alterations of cell-cycle-related molecules, including p21 and p53, in exophytic papilloma, IP and SCC of the sinonasal epithelium. The results were compared with data for cell proliferation and HPV infection.

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Patients and Methods

Recruitment

From 1998 to 2004, patients with sinonasal papilloma and invasive SCC attending the Department of Otolaryngology, Yokohama City University Medical Center, Yokohama, Japan, were selected. These included seven patients with exophytic papilloma, 29 with IP and 12 with invasive SCC.

Tissue was taken from each patient after informed consent. Inferior turbinate tissue from 10 patients was used as control tissue. The tissue was fixed in 10% neutral-buffered formalin, embedded in paraffin and routinely processed for histological examination. More than two doctors independently graded haematoxylin- and eosin-stained sections of the IP for dysplasia. The following microscopic changes seen within the IP were used to established the diagnosis of dysplasia: slight dysplasia (little or no hyperplasia with hyperkeratosis in the cell of the basal layer of the IP); moderate dysplasia (irregular stratification, mitosis and loss of polarity in the cell of the basal and suprabasal layer of the IP); and severe dysplasia (individual cell keratinisation, increased mitosis in the surface layer of the IP, occasional atypical mitosis and dysplasia changes in all of the layer) [12].

Immunohistochemistry

All immunohistochemical staining was carried out on formalin-fixed, paraffin-embedded tissue. The 4-μm-thick paraffin sections were deparaffinised and dehydrated, and then microwaved for 10 min at 500 W in citrate buffer (pH 6.0) to retrieve antigens. The sections were then treated with 1% hydrogen peroxide for 30 min, and incubated with rabbit anti-mouse-immunoglobulin antibody (1 : 25 dilution) to block immunoreactivity of the primary antibody with intrinsic mouse immunoglobulin at 37°C for 1 h. After rinsing the sections in phosphate-buffered saline, the sections were incubated at 4°C overnight with the primary antibody and treated with streptavidin–biotin complex peroxidase kit (LSAB, DAKO, Carpentaria, CA, USA) according to the manufacturer's instructions. Finally, after counterstaining with Meyer's haematoxylin and mounting with Malinol (Muto chemical, Tokyo, Japan), the stained cells were observed by light microscopy. Specific primary antibodies for p21 (sc-6246, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1 : 1000) and p53 (DO-1) (sc-126, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1 : 500) were used. As a positive control, we used laryngeal carcinoma tissue that showed positive staining internally. As a negative control, samples were examined without the primary antibody.

Analysis of Immunohistochemistry

Cells positive for p53 and p21 expression were counted by light microscopy. Two observers independently counted up to 500 cells and calculated the percentage of positive cells in nuclear staining. Positivity for p21 and p53 was instead evaluated using differences in staining intensity (negative, low, moderate and high) and heterogeneity (heterogeneous or homogeneous). Staining intensity was determined by comparing normal squamous epithelium on the same slide. The staining intensity of basal cells was regarded as 'low' and the highest staining intensity of parabasal cells that showed heterogeneous p21 and p53 expression was regarded as 'moderate'. Cells with low and moderate staining intensity were observed under physiological conditions. A staining intensity stronger than that of the parabasal cells was regarded as 'high'. p21 and p53 expression was determined as follows: 0, no nuclear staining; 1+, low staining intensity; 2+, moderate to high staining intensity with a heterogeneous staining pattern; and 3+, moderate to high staining intensity with a homogeneous staining pattern [13].

In-situ *Hybridisation*

In-situ hybridisation for HPV DNA was carried out for types 6/11, 16/18 and 31/33 on a paraffin-embedded, formalin-fixed tissue section using Rembrandt® Dish and AP Detection Kit of HPV typing (Pan Path, Amsterdam, Netherlands). The 4-μm-thick paraffin sections were deparaffinised and dehydrated. The tissue sections were treated with 1.25 mg/ml of pepsin for 30 min at 37°C. Then the slides with digoxigenin labelled HPV 6/11, 16/18 and 31/33 DNA probes were heated at 95°C for 10 min. Hybridisation was then carried out for 2 h at 37°C. The slides were washed with phosphate-buffered saline and treated with alkali phosphates conjugated anti-digoxigenin antibody for 30 min at 37°C to detect digoxigenin hybrid. After counterstaining with nuclear fast red and mounting with PermaFluor Aqueous Mounting Medium (Thermo, Waltham,

Table 1 – Correlations of p21 expression and control, exophytic papilloma, inverted papilloma and invasive squamous cell carcinoma

p21 staining intensity	Control	Exophytic papilloma	Inverted papilloma				Invasive squamous cell carcinoma
			Slight dysplasia	Moderate dysplasia	Severe dysplasia	With carcinoma	
0	9	6	6	5	3	2	6
1+	1	1	1	3	2	3	3
2+	0	0	0	0	2	1	2
3+	0	0	0	0	0	1	1