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Search for accumulation of p53 protein and detection of human papillomavirus genomes in sebaceous gland carcinoma of the eyelid

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Abstract Twenty-one Japanese patients with sebaceous carcinoma of the eyelid were investigated for tumour incorporation of human papillomavirus (HPV) types-6, 11, 16, 18, 31, and/or 33 DNA by in situ hybridization with fluorescein isothiocyanate-labelled DNA probes, and for p53 protein accumulation by immunohistochemical analysis with an antibody to p53 protein. Thirteen tumours (61.9%), including 9 cases of multiple infections, were positive for HPV DNA. Positive signal in the nucleus was observed not only in the cancer cells, but also in the cells of surrounding normal sebaceous glands and epidermis. Positive nuclear staining of cancer cells with the antibody to p53 protein was detected in 12 cases (57.1%). p53 protein accumulation was more frequently observed in the clinically advanced cases, occasionally in association with recurrence and/or metastasis. Among the 12 p53-positive cases, 7 were also positive for the presence of HPV DNA. HPV infections exist in a high percentage of sebaceous carcinomas of the eyelid in Japan; the overexpression of p53 protein may be important in both carcinogenesis and progression.

Key words Human papillomavirus · p53 protein
Sebaceous gland carcinoma

Introduction

Sebaceous carcinoma, a rare tumour in general, is now recognized to occur with significant frequency on the eyelid. Of eyelid tumours, basal cell carcinoma (BCC), sebaceous (gland) carcinoma, and squamous cell carcinoma (SCC) are most common and account for more than 95% of the total [18]. Unlike BCC, which is the most common eyelid malignancy, ocular sebaceous carcinoma often metastasizes in its early clinical course and sometimes causes death [4, 15, 19].

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While sebaceous carcinoma occurs worldwide, a higher incidence is evident in the Far East. In the West the incidence ranges from 1.0% to 6.4% [30, 35] but reaches 28% of lid cancers and 9.6% of all lid tumours in Shanghai [21], and 12.8%–26.9% in Japan [1, 40]. In a comparative study of sebaceous carcinomas between Shanghai and Boston, the incidence in the Shanghai group was 32.7%, compared with only 1.5% in Boston [22]. Regional or racial predisposition may thus have a significant influence on the incidence of sebaceous carcinoma.

A number of factors were investigated in our patients. Human papillomaviruses (HPVs) are associated with many benign and malignant epithelial lesions [26] and can be separated into two distinct groups on the basis of their clinical associations. The first group, including HPVs-6 and 11 is generally associated with benign anogenital warts, which infrequently progress to cancer and have been referred to as "low-risk" viruses. HPVs-16, 18, 31, and 33 of the "high-risk" group are associated with high risk lesions for malignant progression and with almost all cervical carcinomas [12].

The *p53* gene encodes a nuclear phosphoprotein which can suppress cellular proliferation in its native form [8]. Mutations and/or deletions in the *p53* gene are turning out to be one of the most common genetic alterations in human cancers [13] and overexpression of p53 protein has been identified as a frequent genetic change in a variety of tumour types [14, 28, 32].

The E6 protein encoded by HPVs forms complexes with p53 protein and promotes the degradation of p53 [33]. In a previous report of cervical carcinoma [7], no mutant *p53* gene or protein has been detected in the tumours despite of the presence of HPV E6 gene. To date, however, some investigators have shown the co-localization of accumulated p53 protein and HPV DNA in identical tumours [6, 10].

Sebaceous carcinomas of the eyelid in Japanese patients were screened for the presence of HPV DNAs, including "low-" and "high-risk" groups by in situ hybridization, and immunohistochemically for p53 protein expression using a monoclonal antibody recently developed.

Table 1 Clinical findings and outcome of 21 patients with sebaceous carcinoma of the eyelid (*M* Male, *F* female, *RU* right upper eyelid, *LU* left upper eyelid, *RL* right lower eyelid, *LL* left lower eyelid, *y* years, *m* months, *Rec* recurrent tumour, *NK* not known, *LN* lymph node, + present, – not present)

Case	Age (years)	Sex	Site	Size (cm)	Follow-up date	Recurrence and/or metastasis
1	63	M	RU	2.5×1.5	10 m	(–)
2	51	F	LL	1.2×0.5	6 y	(–)
3	58	F	RL	1.1×0.9	3 y, 4 m	(+) (intraorbital)
4	54	M	RL	NK	4 y	(+) (intraorbital, sinus, and regional LN)
5	72	F	LL	0.4×0.3	2 y, 5 m	(–)
6	60	F	RU	0.6×0.4	2 y, 7 m	(–)
7	79	M	LL	0.8×0.5	8 m	(–)
8	83	F	LU	2.0×1.7	10 m	(–)
9	55	M	RU	1.5×0.7	9 m	(–)
10	75	M	RL	0.8×0.6	3 m	(–)
11	80	F	Rec	NK	6 y	(+) (intraorbital and sinus)
12	52	M	RU	0.9×0.4	1 y, 2 m	(–)
13	65	F	RL	0.7×0.5	6 m	(–)
14	69	M	LL	1.0×0.5	2 y, 5 m	(–)
15	58	F	LL	0.6×0.6	1 y, 8 m	(+) (local)
16	71	F	LU	NK	1 m	NK
17	72	F	LU	1.0×1.0	5 y, 3 m	(–)
18	82	F	LL	NK	NK	NK
19	64	F	LU	NK	NK	NK
20	76	F	RU	NK	1 y, 7 m	(–)
21	56	F	LL	NK	1 y, 10 m	(–)

Table 2 Positivity for human papillomavirus (HPV) DNA probes and anti-p53 protein antibody in each sebaceous carcinoma of the eyelid

Case	"High-risk" HPV types-16, –18, –31, and/or 33	"Low-risk" HPV types-6 and/or –11	Overexpression of p53 protein
1	+ (16, 18, 33)	+ (11)	–
2	–	–	–
3	+ (16, 18, 31, 33)	+ (6)	+
4	+ (16, 18, 31, 33)	+ (6, 11)	+
5	–	–	+
6	+ (16,33)	–	–
7	+ (33)	+ (6)	+
8	+ (16, 18, 31, 33)	+ (6)	+
9	+ (16, 18, 31, 33)	+ (11)	+
10	+ (16, 18, 33)	–	–
11	+ (16, 18)	–	+
12	+ (16)	–	–
13	–	+ (11)	+
14	–	–	–
15	–	–	+
16	–	–	+
17	–	–	+
18	–	–	–
19	–	–	–
20	+ (16, 33)	+ (11)	+
21	+ (16, 31, 33)	–	–
Total positive	12/21 (57.1%)	8/21 (38.1%)	12/21 (57.1%)

Materials and methods

A total of 21 cases of sebaceous carcinoma of the eyelid in Japan were investigated in this study; these consisted of 20 primary lesions and 1 recurrent one. Patients included 7 men and 14 women, between 51- and 83-years-old (Table 1). All specimens examined were fixed in 10% buffered formalin, processed routinely, and embedded in paraffin. Fresh tumour tissues were not available for the present study.

In situ hybridization was performed with fluorescein isothiocyanate (FITC)-labelled DNA probes specific for each HPV type-6, 11, 16, 18, 31, or 33 DNA (DAKO Japan, Kyoto, Japan), using an in situ hybridization kit (DAKO) as follows. Dewaxed sections were digested with pre-warmed pepsin/hydrochloric acid (HCl; 0.8% pepsin in 0.2 N HCl) for 10 min at 37° C. This was followed by simultaneous denaturation of the cellular DNA and each FITC-labelled HPV probe for 6 min at 90° C, and then by hybridization for 60 min in a pre-warmed (37° C) humid chamber. Next, a 58° C-heated DAKO stringent wash solution (×50) containing a blocking agent was mounted on each section for 30 min to remove excess probe and to block non-specific binding sites on the tissue. After washing with TRIS-buffered saline (TBS), each section was treated with alkaline phosphatase-labelled rabbit anti-FITC anti-

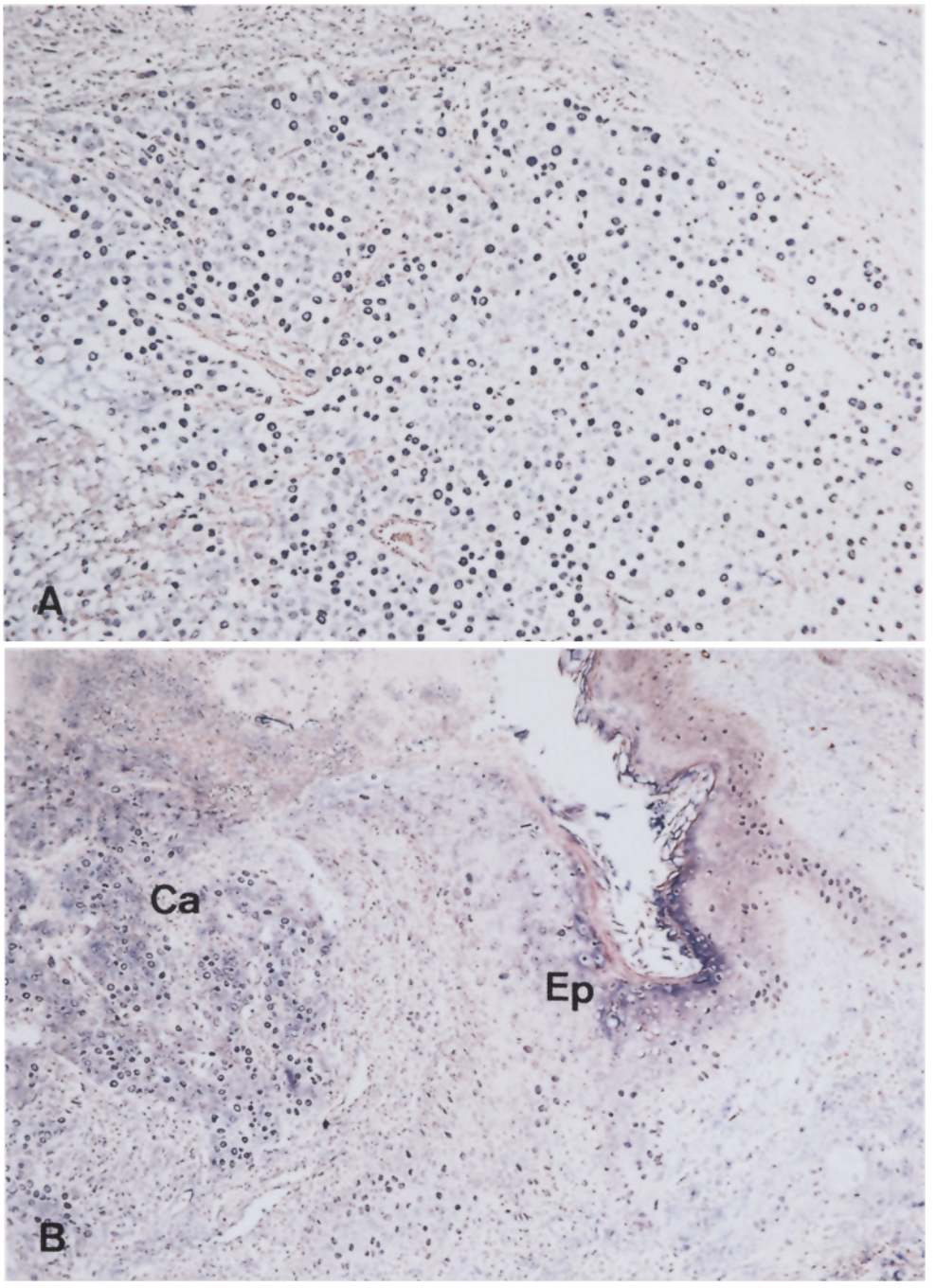


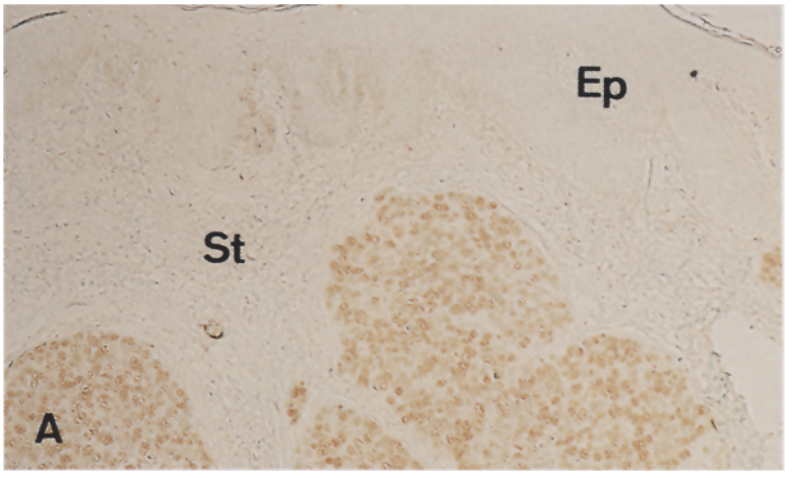
Fig. 1A, B Detection of human papillomavirus (HPV) DNAs by in situ hybridization. **A** Dense strong reaction of cancer cell nuclei with HPV type-18 DNA probe. (×120). **B** Focal positive reaction of the nuclei of normal epidermis (*Ep*), adjacent to cancer (*Ca*), with HPV type-16 DNA probe (×90)

body for 20 min. Then, after TBS washing, the slides were immersed in nitroblue tetrazolium chloride-5-bromo-4-chloro-3-indolyl-phosphate substrate for 1–2 h in the dark at room temperature. The specificity of the DNA probes used in this study was checked by using positive or negative control sections of various kinds of tissues. Positive reaction was characterized by strong uniform nuclear staining.

p53 protein overexpression was assessed by immunohistochemical examination with a monoclonal antibody of DAKO-p53 *Protein*, DO-7 (DAKO) at 1: 30 dilution as reported previously [9]. In brief, dewaxed sections were treated in de-ionized water (heated at 95±5° C) by microwave oven for 5 min, as shown to be effective in the retrieval of masked epitopes of many antigens [34]. Then, the avidin-biotin complex procedure using streptavidin-biotin complex peroxidase kit (Histofine SAB-PO kit; Nichirei, Tokyo, Japan) was performed following directions in the kit manual. Whereas, control sections were run in parallel, and these included replacement of the specific antibody with 0.1 M phosphate-buffered saline (pH 7.4), and staining of known positive and negative tissues.

Results

In 13 of 21 cases (61.9%), numerous nuclei of the cancer cells were positive for HPV DNA types-6, 11, 16, 18, 31, and/or 33 (Fig. 1A). The infection patterns of HPV DNA in each case are shown in Table 2. The "high-risk" group was detected in 12 of 21 patients (57.1%), and among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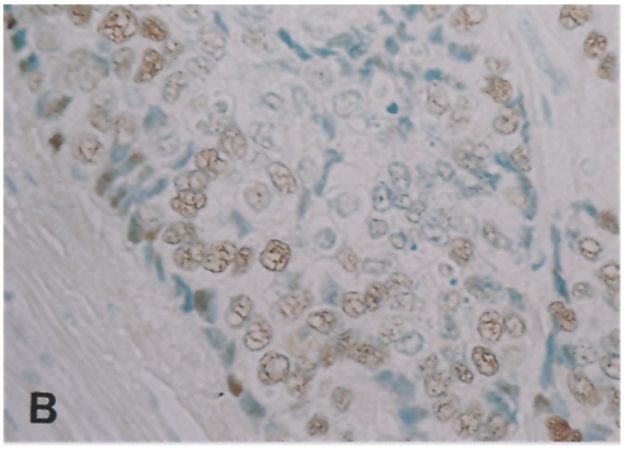
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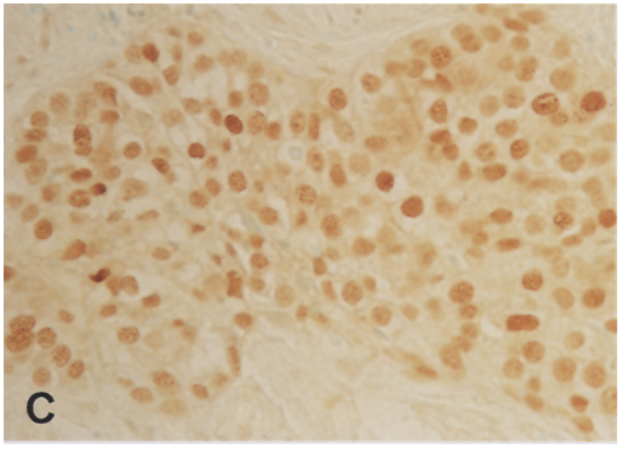
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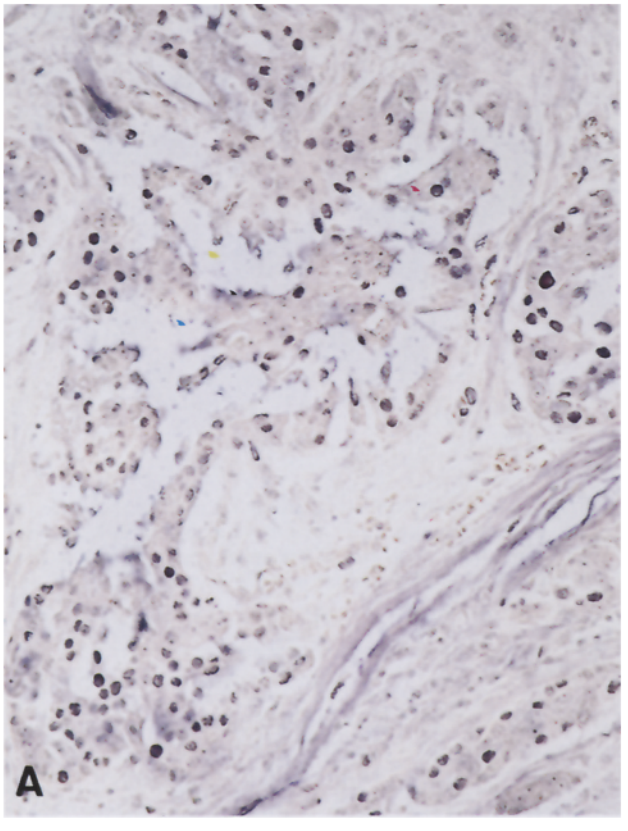
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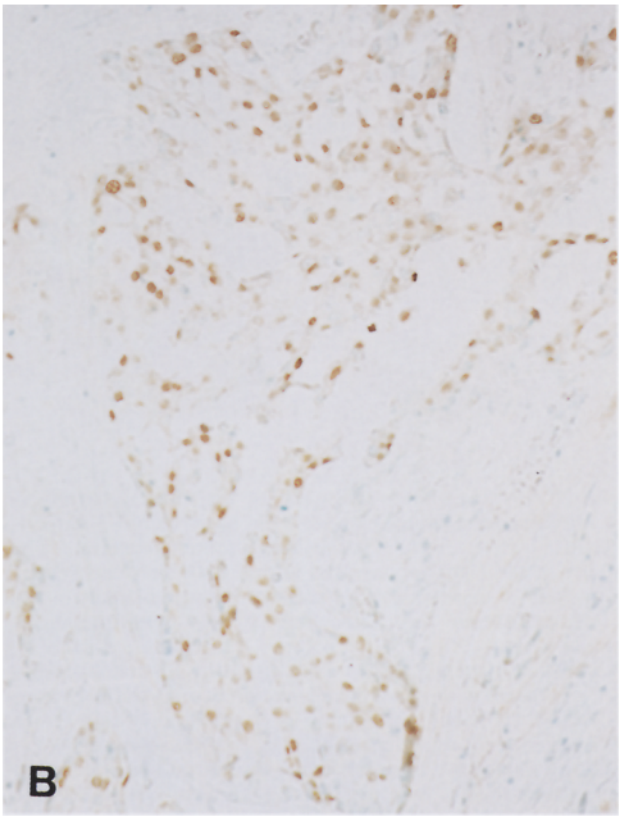
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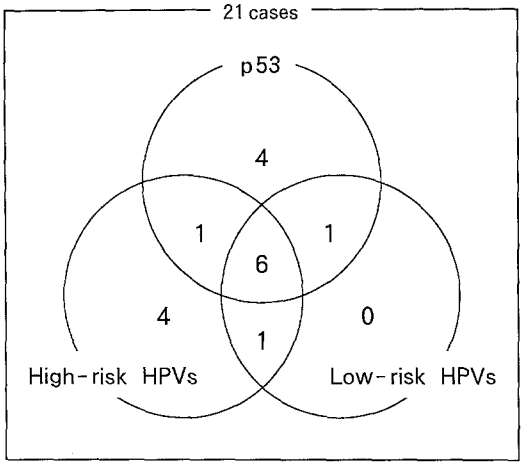

Fig. 3 Relationship between HPV infection and overexpression of p53 protein in sebaceous carcinoma of the eyelid

them, 10 cases showed multiple positivities for "high-risk" types of HPV DNA. Otherwise, although 8 cases in total were positive for "low-risk" group, "high-risk" group was simultaneously detected in 7 cases. Positive signal was observed as a strong stain in the nuclei of the cancer cells. The distribution patterns of cancer cells positive for HPV showed either "diffuse" or "focal" in parts, and positive nuclei were also focally detected in surrounding normal sebaceous glands and epidermis (Fig. 1B). The positive pattern of cancer cells for each HPV type was similar, although the proportion of positive cells varied among the tumours examined.

A positive reaction of cancer cells with anti-p53 antibody was detected in 12 of 21 cases (57.1%) as shown in Table 2. In all of the positive cases, immunoreactivity was restricted to the nucleus of the cancer cells in various populations (Fig. 2A, B). Although positive reaction product was observed only in nuclei, the degrees of the positivity in number and intensity varied in each case. There were no positive reactions in non-cancer tissues, including stroma, inflammatory cells, normal epidermis and adnexa.

In the 4 recurrent and/or metastatic lesions, nuclear positivity of the cancer cells was detected in every lesion, as in their 3 primary lesions available for the present study. It is of interest that especially in metastatic foci, positive nuclei were observed more frequently in number and much stronger in intensity than those of each primary lesion (Fig. 2C).

Fig. 2A–C Immunohistochemical staining of p53 protein with DO-7. **A** Positive reaction of the cancer cell nuclei, in sharp contrast to negative normal epidermis (*Ep*) and stroma (*St*). (×80). **B** The coarsely granular positive staining of most of the pleomorphic cancer cell nuclei. (×300). **C** Intense staining of almost all of cancer cell nuclei in a metastatic focus. (×300)

Fig. 4A, B Doubly positive staining of the cancer cell nest in serial sections with both HPV type-18 DNA probe by in situ hybridization (**A**), and anti-p53 antibody by immunohistochemistry (**B**). (×190)

Figure 3 showed the relationship between the detection of HPV DNAs and the overexpression of p53 protein in each case examined. Six cases (28.6%) were doubly positive. Interestingly enough, recurrent and/or metastatic foci, except for a locally recurrent lesion, were positive for both p53 antibody and HPV DNA (Fig. 4A, B). Among 8 cases positive for the "low-risk" HPV DNA, 7 were also associated with "high-risk" group, and only 1 remaining case was showed the overexpression of p53 protein. Both HPV DNA and p53 antibody were negative in 4 cases (19%).

Discussion

Infection with "high-risk" HPVs has been detected in a high percentage of patients with several types of cancer, including cervix [27], oesophagus [9, 37], larynx [25], and urinary bladder [2, 10], suggesting that this may be a risk factor in carcinogenesis. In sebaceous carcinoma, there are regional or racial predispositions [22], with a higher incidence in Orientals, including the Japanese. We used in situ hybridization techniques to search for HPV DNAs in surgically resected tumour tissues of sebaceous carcinoma. We found that HPV DNAs type-6, 11, 16, 18, 31, and/or 33 were detected in 13 of 21 patients, viruses of the "high-risk" group were demonstrated in 12 cases. Multiple HPV DNAs were found in 10 cases. Although "low-risk" HPV DNAs were detected as well as "high-risk" HPV and HPV DNAs were detected in both cancer tissues and surrounding normal tissues, the frequent detection of HPV DNAs in sebaceous carcinoma suggests that HPV infection may contribute to its incidence. From the distribution patterns of the positivity in both cancer tissues and normal tissues, we could not determine whether HPV infections were likely to be causative or not, but associations of HPV with sebaceous carcinoma have not been reported so far.

p53 protein expression and mutation of the *p53* gene have been demonstrated in many kinds of tumours, especially in association with tumour progression and a poor prognosis. There has been no previous report on the investigation of p53 protein expression in sebaceous carcinoma of the eyelid, however, we detected the accumulation of p53 protein in 57.1% of these sebaceous carcinomas immuno-histochemically. All lesions, recurrences and metastases showed positive staining with antibody to p53 protein. Interestingly, metastatic lesions showed much stronger and more wide spread positivity than the primary ones. In the previous reports of other skin tumours, using the polymerase chain reaction followed by direct DNA sequencing, the presence of *p53* gene mutations was detected in 50% of BCC [29] and 58% of SCC [5]. However, p53 protein expression was observed in 10 of 26 SCC immunohistologically and was more frequently observed in recurrent and metastatic lesions [38]. In another study, detectable p53 protein was found in 56% of SCC and 42% of BCC [31]. In our research, overexpression of p53 protein in sebaceous carcinoma was

found in more than 50%, which was equal to or a little more frequent than those of SCC and BCC cited above. In addition, we found the accumulation of p53 protein in primary foci, and gradually increased overexpression of p53 protein in cases of clinically advanced tumours. With the development of the tumour, overexpression of p53 protein increased, as observed in malignant melanoma [16]. These results indicate the possibility that p53 protein accumulation is strengthened in tumour progression.

Previous studies have shown that the accumulation of p53 protein correlates with the presence of *p53* gene mutations, allowing an immunohistochemical evaluation of the role of *p53* mutations in various tumours of the lung [14], colon [32], and oesophagus [3]. However, some reports have shown that there appears to be no clear association between abnormalities of *p53* gene and p53 overexpression [36] and that some *p53* mutations were found in human and animal tumours without expression of p53 mRNA and protein [17, 20]. False positivity due to infrequent non-mutational stabilization of p53 protein has also been observed in a few tumour cell lines [39]. It is clear that mutations do not directly reflect p53 overexpression as assessed by immunohistochemistry. Although we cannot exclude the possibility of altered phosphorylation or stabilization of p53 proteins, or overexpressed wild-type p53 protein, our immunohistochemical results suggest that the overexpression of p53 protein might be a factor of carcinogenesis in sebaceous carcinoma and have some relevance to progression of sebaceous carcinoma.

We also detected "high-risk" HPV DNAs together with the accumulation of p53 protein in sebaceous carcinoma of the eyelid. Loss of wild-type p53 function is one of the critically important stages in carcinogenesis in many tumours [23]. However, *p53* mutation has been identified in cervical carcinoma simultaneously with HPV 18 infection [24]. Recently, the coexistence of overexpressed p53 protein and HPV infection was reported in both cervical and urinary bladder carcinomas in relation to prognosis [6, 10]. Our data make it clear that the accumulation of p53 protein and/or the presence of HPV DNAs may contribute to the carcinogenic process.

The aetiology of sebaceous carcinoma is unknown, but a minority of individuals have a history of radiation exposure [4]. Several investigators have emphasized more frequent occurrence in Orientals [22]. The association between sebaceous gland tumour (adenoma, epithelioma, or carcinoma) and internal malignancy (colorectal, genitourinary, et al.) is commonly referred to as the Muir-Torre syndrome [11]. These observations suggest that genetic changes and environmental carcinogens may both be related to the occurrence of sebaceous carcinoma. Our study demonstrates that the overexpression of p53 protein may be an important factor in carcinogenesis and HPV infection might be one of the aetiological factors.

This is the first study to reveal the presence of HPV DNAs and the overexpression of p53 protein in sebaceous carcinoma. Our findings indicate that these may be important factors, in carcinogenesis and as indicators for progression.

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