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Characterization and analysis of human papillomaviruses of skin warts

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Abstract. We analysed human papillomavirus (HPV) infections in 61 tissue specimens of skin warts of Taiwanese patients by DNA hybridization. The prevalence of HPV infection was 69% by Southern blot hybridization. The typing of HPVs was performed by dot blot hybridization under highly stringent conditions with each probe separately. The prevalence of HPV-1, 2/3, 4, 5, 8, 11, 16 and 18 in skin warts was 13, 7, 16, 2, 0, 5, 2 and 8%, respectively. Chi-squared analysis revealed that there was a correlation between HPV type and copy number. Most HPV-4-induced warts were verruca vulgaris. HPV-1 DNA was detected in verruca plantaris and verruca vulgaris. No specific histopathological features were found to be indicative of the presence or absence of HPV, or of the various types of HPV infection.

Key words: HPV – Skin warts – Dot blot hybridization – Southern blot hybridization

A close clinical relationship between verruca plana, laryngeal papilloma and condylomata acuminata has been known for many years [24]. Copious evidence shows that these conditions are caused by human papillomaviruses (HPVs) [14, 16]. HPVs are naturally occurring DNA tumour viruses that induce epithelial cell proliferation during the course of a productive infection. There are now at least 60 known types of HPV [25]. Each type is distinct, but like all members of the papillomavirus group, they possess an 8-kb virus genome, which is double stranded but contains all of the genetic information on one strand, and is divided into early, late and long control regulatory regions [14].

Among the distinct types of HPVs associated with epithelial or mucosal lesions are types 1 and 4 associated with plantar warts, type 2 with common cutaneous warts, types 5 and 8 with the lesions of epidermodysplasia verruciformis, type 7 with hand warts of patients handling meat [13], type 41 with some cutaneous squamous cell carcinomas [9], types 13 and 32 with oral mucosa [2, 21], types 6 and 11 with laryngeal papilloma, cnodylomata accuminata and penile condyloma, and types 16 and 18 with carcinoma in situ and invasive malignant neoplasm of the cervix [5, 13, 14]. The oncogenic potential of HPVs is reflected by the association of certain mucosotropic HPVs with varying grades of premalignant squamous lesions and invasive carcinoma of the cervix. Thus the HPV type determines, in part, the site of infection, the pathological features, the clinical appearance, and the clinical course of the respective lesion. Therefore, HPV typing could be clinically important for determining the putative biological potential of some productively infected HPV-associated lesions, particularly benign and low-grade premalignant anogenital tract lesions.

HPVs have been grouped for convenience into those associated with lesions of mucosal origin and those mainly found in cutaneous lesions [25]. The study of non-genital lesions has been fragmentary, and there has been no epidemiological study of HPV skin infections in Taiwan. It is important to establish the role of the various types of HPV in the pathogenesis of skin warts including verruca plantaris, verruca vulgaris, and verruca plana identified clinically. In this study, the existence of the HPV genome was detected by Southern blot hybridization with mixed HPV-1, 2, 3, 4, 5, 8, 11, 16 and 18 as probes under less stringent conditions. The HPV types in skin warts were identified by dot blot and Southern blot hybridization under highly stringent conditions. Patient record were reviewed for HPV infection and lesion locations, clinical diagnosis, sex, initial/recurrent lesions and copy number.

Materials and methods

Tissue specimens

Biopsy specimens from skin warts which included 9 cases on the head/neck (including face, scalp, neck and buccal), 4 on the chest/back, 26 on the hand (including hand, thumb, arm, index, wrist, elbow, palm and finger etc), 12 on the leg (including leg, knee and toe) and 10 on the sole were collected from the Dermatology Clinic at the Tri-Service General Hospital, Taipei, Tai-

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wan. Tissues for analysis were obtained at the time of surgical excision. They were frozen in liquid nitrogen and stored at −70 °C prior to isolation of DNA or histopathological examination.

DNA extraction and Southern hybridization

The DNA from the tissue specimens was extracted as described previously [18]. Briefly, the DNA was isolated from the tissue by detergent lysis and protease digestion, followed by phenol and then chloroform extraction. The RNA was removed by digestion with ribonuclease. The DNA was concentrated by ethanol precipitation and 3 µg was digested with restriction endonucleases (Bethesda Research Laboratories) under the conditions specified by the vendor. The digested DNA was fractionated by 0.8% agarose gel electrophoresis, stained with ethidium bromide and examined by UV fluorescence photography. The DNA fragments were transferred to nitrocellulose filters (Schleicher & Schuell; BA 85) [3] which were baked under a vacuum, treated with 10X Denhardt's solution [6] in 6X NaCl/Cit (1X NaCl/Cit = 0.15 *M* sodium chloride/0.015 *M* sodium citrate) for 3 h at 55 °C (less stringent conditions) or 68 °C (highly stringent conditions) prior to hybridization, and incubated at 55 °C or 68 °C for 48 h in 0.6 *M* sodium chloride, 0.06 *M* sodium citrate, 50 m*M* sodium phosphate (pH 7.2), 1X Denhardt's solution and 0.5% sodium dodecyl sulphate (SDS) with 2×10^6 Cerenkof cpm of radioactively labelled HPV DNA (generous gift from Dr. zur Hausen).

Less stringent conditions allow hybridization of weakly homologous regions and so permit detection of multiple HPV types with any one HPV probe [15]. These conditions are employed for screening specimens. Stringent conditions are employed for detection of specific HPV types. For ^{32}P labelling by nick translation, the HPV sequences were separated from the vector by digestion with the appropriate enzyme and recovered after agarose gel electrophoresis. The filters were washed at 55 °C or 68 °C for 4 h in 0.3 *M* sodium chloride, 0.03 *M* sodium citrate, 0.5% SDS, and in 0.15 *M* sodium chloride and 0.015 *M* sodium citrate for 1 h and air dried. The patterns of hybridization were detected by autoradiography using Kodak X-omat film.

Dot blot hybridization

The DNA samples (0.5 µg per tissue specimen) were denatured with NaOH (final concentration 0.3 *M*) for 0.5–1 h and spotted on to nitrocellulose membranes [12]. The filters were then neutralized with 1.5 *M* NaCl, 0.5 *M* Tris, pH 7.4.The nine replica filters containing the DNA samples were hybridized with an HPV probe (HPV-1, 2, 3, 4, 5, 8, 11, 16 or 18) under highly stringent condition. The hybridization, washing and exposing of the filters were carried out as described for Southern blot hybridization.

Histological examination

The specimens were fixed in 4% neutral buffered formalin, dehydrated in a graded series of alcohol, and embedded in paraffin. The histopathological changes of the lesions were routinely evaluated in 4-µm thick sections stained with haematoxylin and eosin (HE) [10, 23].

Results

Detection and typing of HPV infections in skin warts by hybridization

We investigated 61 specimens from patients with skin warts diagnosed clinically as verruca plantaris, verruca vulgaris and verruca plana. The HPV type was determided by Southern blot hybridization. DNA (3 µg per patient) was digested with BamHI, transferred to nitrocellulose filters and hybridized with the mixed HPV-1, 2, 3, 4, 5, 8, 11, 16 and 18 probes under the less stringent conditions as described in 'Materials and methods'. The results show that only 42 of the 61 cases contained HPV DNA (Fig. 1).

The typing of the various HPVs (HPV-1, 2, 3, 4, 5, 8, 11, 16 or 18) was performed by dot blot hybridization under highly stringent conditions (68 °C) with individual HPV probes. Figure 2 shows the data with the HPV-1 probe, the six dots on the top line of the filter from dot 1 to dot 6 were the 10 ng DNA of HPV-1, 2, 3, 4, 5, and 8, respectively (panel A). The results show that a positive signal was present only in HPV-1 DNA dot (panel A) and there were eight positive dots from 61 tissue specimens (panel B). The HPV typing was also done by Southern hybridization from the same filter sequentially hybridized with the different probes under highly stringent conditions. The results of typing by dot and Southern hybridization were the same.

The presence and distribution of the various types of HPV DNA in the warts are shown in Table 1. There were eight cases with HPV-1 infection, four cases with HPV-2 and HPV-3, ten cases with HPV-4, one case with HPV-5, three cases with HPV-11 (including two mixed infections–HPV-11 and 16; HPV-11 and 18), one case with HPV-16 (one mixed infections–HPV-11 and 16) and

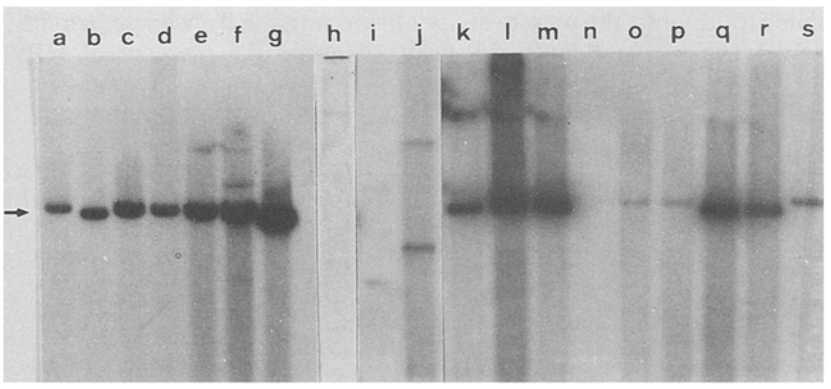

Fig. 1. Southern transfer analysis of 3 µg DNA which was isolated from HPV-positive skin warts, digested with BamHI and incubated with mixed probes containing HPV-1, 2, 3, 4, 5, 8, 11, 16 and 18 at 55 °C. *Lanes a–s* represent positive cases. The *arrow* indicates the 8 kb DNA size. *Lanes a–h* were exposed for 1 day and *lanes i–s* for 3 days at −70 °C

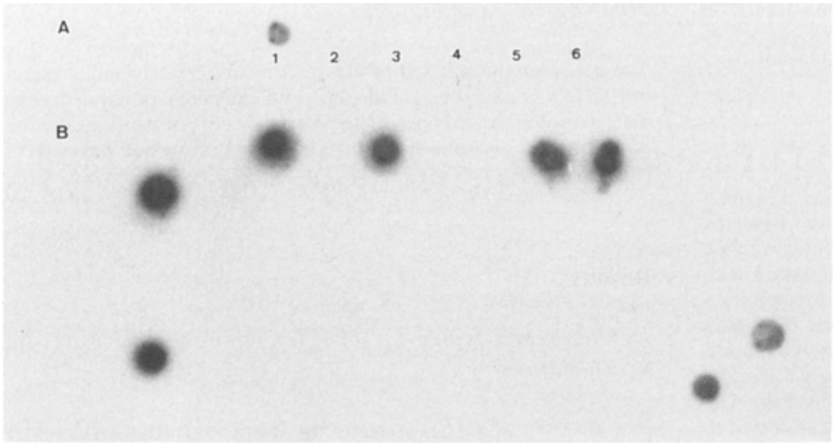

Fig. 2. The typing of HPVs in skin warts with HPV-1 probe by dot blot hybridization under highly stringent conditions. *Panel A*, the six dots on the top line of the filter from *dot 1–6* were the 10 ng DNA of HPV-1, 2, 3, 4, 5 and 8 as positive controls, respectively. *Panel B*, the filter containing 0.5 μg cellular DNA isolated from 61 specimens of skin warts. The filter was hybridized with the HPV-1 probes at 68 °C as described in 'Materials and methods'. The positive dots were identified by comparing the intensity in autoradiograms with the HPV-1- positive dot in *panel A*

five case with HPV-18 (one mixed infection–HPV-11 and 16) and five case with HPV-18 (one mixed infection–HPV-11 and 18). HPV-8 infection was not in the specimens studied, and the HPV type has not yet been identified in 12 cases. The prevalence of HPV-1, 2/3, 4, 5, 8, 11, 16 and 18 were 13, 7, 16, 2, 0, 5, 2 and 8%, respectively. In adition, the filters hybridized separately with HPV-2 and 3 showed considerable sequence homology between them (data not shown). The positive dots of HPV-2 and 3 showed a similar pattern. It was difficult to determine if this represented a mixed infection or cross hybridization.

Autoradiograms (from Southern hybridization) were analysed by densitometric scanning to determine the relative amount of HPV DNA per cell by comparing the intensity shown by known amounts of HPV viral genome. Figure 1 shows that HPV-1 (such as case nos. 3, 4, 13, 50 and 59) and 4 (such as case nos. 1, 18, 33) contained high copy numbers per cell, but the HPV-2 and 3 (such as case nos. 39, 48) contained low copy numbes.

The relationship between HPV type and age, lesion location, clinical diagnosis, sex, initial/recurrent lesion and copy number

The associations between different HPV infections and age, lesion location, clinical diagnosis, sex, initial/recurrent lesion and copy number are shown in Table 2. There was a significant difference between HPV type and copy number as assessed by the Chi-squared analysis an P values (less than 0.05). The most frequent site of infection for HPV-2, 3, 4 and 5 was the hand, and for HPV-1, the sole. Most HPV-4- and HPV-18-induced warts were verruca vulgaris, and HPV-1 DNAs were found in verruca plantaris and verruca vulgaris.

Histological examination

The common histological characteristics of skin warts include foci of vacuolization, hyperkeratosis and papillomatosis (Fig. 3a). In this study, the clinical differentiation of HPV infection by histological examination was usually not possible. Eosinophilic cytoplasmic keratohyalin inclusion bodies were found in four cases (Fig. 3b). Two cases were HPV-1 infections, one case HPV-4 infection and one case uncertain. Three of these four lesions were located on the sole and the other one on the thumb.

Discussion

Much attention has been focused on the association between infection with HPV and the development of uterine cervical neoplasms. Knowledge of the association of skin warts with HPV infection is scarce. HPV infection can cause skin lesions such as verruca plantaris, verruca plana, verruca vulgaris, genital warts and cervical cancer etc. In this study, different kinds of skin lesions were analysed. Many HPV types are associated with cutaneous warts [25]. The common types in skin warts are HPV-1, 2, 3, 4, 5 and 8 [25]. Neither clinical nor histological features could reliably predict the infecting type of HPV. In the present study of skin warts in Taiwanese patients, an attempt was made to assess the presence of HPV

Table 1. The prevalence of the various HPV types in skin warts

	HPV1	HPV2/3	HPV4	HPV5	HPV8	HPV11	HPV16	HPV18	Other types	Total
Positive cases	8	4	10	1	0	3[a,b]	1[a]	5[b]	12	42
Prevalence (%)	13	7	16	2	0	5	2	8	20	69

[a] One specimen positive for both HPV11 and HPV16
[b] One specimen positive for both HPV11 and HPV18

Table 2. The distribution of the various HPV types in 61 patients with skin warts

		HPV1	HPV2/3	HPV4	HPV5	HPV11	HPV16	HPV18	Other types	PHV negative	Total
Age (years)	0–20	3	0	2	1	1	0	1	4	4	16
	21–40	5	4	4	0	1	1	1	7	9	31
	41–60	0	0	2	0	1	0	2	1	1	6
	>61	0	0	2	0	0	0	1	0	5	8
Legion site	Hand	3	2	7	0	1	0	2	4	8	26
	Cheek/Back	0	0	0	0	2	1	0	0	2	4
	Face	1	0	1	0	0	0	2	2	3	9
	Leg	0	1	2	1	0	0	1	2	5	12
	Sole	4	1	0	0	0	0	0	4	1	10
Diagnosis	Verruca vulgaris	3	2	9	1	2	1	4	8	15	43
	Verruca plana	1	1	1	0	1	0	1	1	3	9
	Verruca plantaris	4	1	0	0	0	0	0	3	1	9
Sex	Male	6	3	6	1	3	1	5	9	17	49
	Female	2	1	4	0	0	0	0	3	2	12
Initial lesion[a]		3	3	4	1	2	1	5	10	12	39
Recurrent lesion[b]		5	1	6	0	1	0	0	2	7	22
Copy no.[c]	1–100	3	4	7	1	3	1	4	12	–	
	101–200	5	0	2	0	0	0	1	0	–	
	>201	0	1	1	0	0	0	0	0	–	

[a] Initial lesion: lesion not curetted before
[b] Recurrent lesion: lesion found on the same site where old lesion had been curetted
[c] There is significant difference by the Chi-squared test and P value

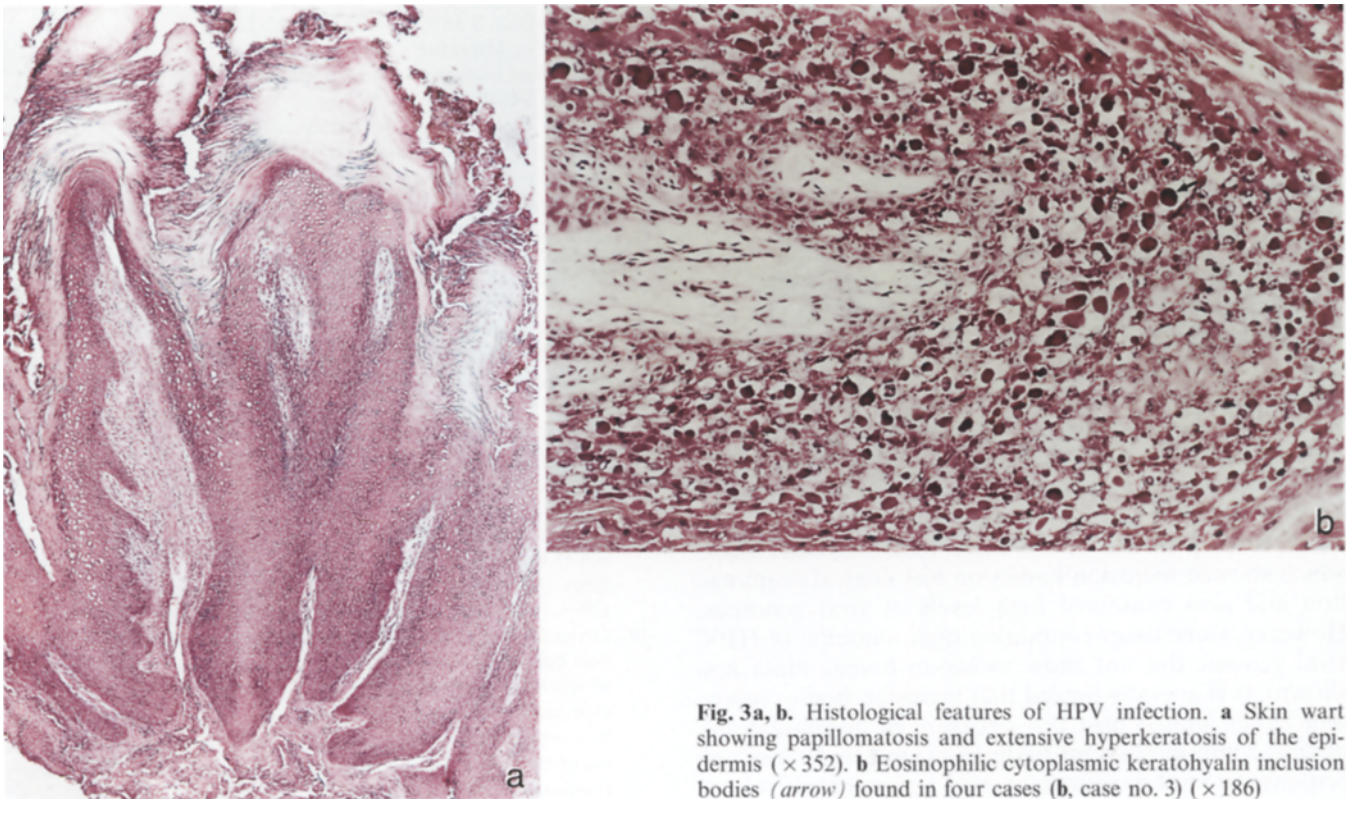

Fig. 3a, b. Histological features of HPV infection. **a** Skin wart showing papillomatosis and extensive hyperkeratosis of the epidermis (×352). **b** Eosinophilic cytoplasmic keratohyalin inclusion bodies *(arrow)* found in four cases (**b**, case no. 3) (×186)

infection, to type the infection as HPV-1, 2, 3, 4, 5, 8, 11, 16 or 18 and to investigate the correlations between HPV type and age, lesion location, clinical diagnosis, sex, initial/recurrent lesion and HPV copy number.

The prevalence of HPV infection in the warts was determined by Southern blot hybridization which was sensitive enough to detect less than a single viral genome per cell by reconstructing one viral genome in ten cell genome equivalents. The prevalence of HPV infection was 69% (Table 1). The prevalence of HPV infection in sole lesions was 89% (8/9 cases) and this is in agreement with previously reported (Table 2), results obtained using the hybridization techniques for detection [4, 11]. However, a prevalence of 50% (15/30 sole lesions) has previously been determined by an immunofluorescence assay using an antibody against PV genus-specific structural viral antigens [16]. The discrepancy in the prevalence results is probably because DNA hybridization is much more sensitive than the immune assay.

Table 1 shows that the most frequent HPV types in skin warts were HPV-1 and 4. These results are different from other reports which revealed HPV-1 and 2 to be the most frequent types [1]. There has been no reported study on genital-associated HPV-11, 16 and 18 infections in skin warts. The results reported here show that seven cases were caused by genital HPVs probably transmitted from genital lesions. The reason for the different prevalence of the various HPVs is not clear, but geographic differences of prevalence in other HPV types has been observed [7, 20]. HPV-1 and HPV-4 were found to be consistently associated with plantar warts and verruca vulgaris, respectively [10]. According to the previous reports, the type of HPV present in warts is age related, with a preponderance of type 1 virus in the youngest, type 2 in the intermediate, and non-1, non-2 viruses in the oldest age group [25]. However, in our study (Table 2), there was no significant correlation between age and HPV type according to the Chi squared analysis.

The copy number of the viral genome in the infected tissues was scored by densitometry of autoradiograms. Plantar warts produced a large number of virus particles that could be purified by banding in CsCl gradients [13]. According to previous studies of HPV-1- and HPV-4-induced warts, the viruses are actively replicating, and the content of viral DNA in skin warts is high [10, 11]. In agreement with our results, as shown in Table 2, most cases with HPV-1 and HPV-4 infection had higher copy numbers of the viral genome per diploid cell than other HPV infections. Inclusion body formation may also indicate the course of viral multiplication within the host cell [10]. Electron microscopy has shown that inclusion bodies represent sites at which virus replication has occurred [17]. In this study, there were only four cases which showed inclusion bodies on histological examination and also contained high levels of viral genomes. However, some cases containing high amounts of HPV viral genome did not show inclusion bodies (data not shown). It is gnerally agreed that inclusion bodies represent either altered host cell structure or accumulations of viral components. Our findings suggest that those cell response to viral product may play an important role in inclusion body formation. We also noticed that three out of four cases containing inclusion bodies were from recurrent lesions at sites where old lesions had been curetted.

Since HPV is transmissible, detection and treatment of HPV infection may play an important role in the prevention of skin and genital warts and cancer. It has been demonstrated that HPV can be transmitted between sexual partners [22] or spread by autoinoculation [19]. HPV transmission between anal condylomata and common hand warts has been reported [8]. It would be important to investigate HPV types in different body areas and analyse factors influencing transmission of HPV between them. We have presented evidence for the papillomaviral aetiology of skin warts, and established the distribution of HPV types at different body site. In the future, a larger number of cases will be studied to identify the transmission pathway of HPVs.

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