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Isabelle Pélisson · Yvette Chardonnet · Sylvie Euvrard
Daniel Schmitt

Immunohistochemical detection of p53 protein in cutaneous lesions from transplant recipients harbouring human papillomavirus DNA

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Abstract Human papillomaviruses (HPV) are thought to be involved in the malignant evolution of cutaneous lesions from transplant recipients. As E6 proteins from potentially oncogenic HPV types degrade *p53* tumour suppressor gene product in vitro, we analysed p53 protein status in benign, premalignant and malignant skin lesions from grafted patients, to determine whether HPV may interfere with p53 function. With immunohistochemistry, p53 protein accumulation was detected in 70% of skin lesions from grafted patients. p53 immunoreactivity was confined to basal keratinocytes in benign lesions (warts, condylomas), while suprabasal keratinocytes were also stained in premalignant and malignant skin lesions (precancerous keratoses, squamous cell carcinomas). Multiple HPV carriage was detected with in situ hybridization in benign and malignant skin lesions from transplant recipients: low risk HPV types 1, 2, 6, 11 and potentially oncogenic HPV types 5, 16, 18 were frequently found. There was no clear correlation between p53 detection and the presence of the HPV types under study. The frequent detection of p53 protein in cutaneous lesions from grafted patients is suggestive of p53 protein accumulation interfering with normal function. Our results may reflect the presence of mutated p53 proteins due to the mutagenic effect of ultra-violet (UV), or wild-type p53 protein accumulation in response to UV-induced DNA damage, or may be produced by the interaction with HPV-encoded E6 proteins.

Key words Transplant recipients · Human papillomaviruses · p53 protein · Ultra-violet

Introduction

The long term immunosuppression required to prevent allograft rejection is associated with a high rate of skin disorders and human papillomavirus (HPV) infections. Organ transplant recipients successively develop numerous benign, premalignant and malignant skin lesions on sun-exposed areas in which histological signs of viral infection and HPV DNA are detected [3, 7, 13]. This suggests that HPV may be involved in the evolution of cutaneous lesions from grafted patients towards malignancy.

Studies on anogenital cancers have pointed out the potential role of HPV in human carcinogenesis. However, clinical and experimental data suggest that HPV infection is not sufficient to induce cancer [47], and additional alterations of host cells may be required for the malignant progression of HPV-infected cells. Alterations of *p53* tumour suppressor gene are the most common genetic alterations detected in human tumours [9], and it has been shown that E6 oncoproteins encoded by potentially oncogenic HPV types 16 and 18 bind and promote the degradation of *p53* tumour suppressor gene product in vitro [30]. Analysis of cervical carcinoma cell lines showed that *p53* gene and protein are mutated in HPV-negative cell lines, whereas HPV-positive cell lines contain wild type *p53* sequences and low levels of normal p53 protein [11, 31, 44]. These data strongly suggest that, like *p53* gene mutations, interaction of E6 proteins from potentially oncogenic HPV types with p53 protein may interfere with p53 function and lead to cell cycle alterations responsible for tumour progression.

In this study, we analysed p53 protein expression by immunohistochemistry, and the presence of HPV DNA by in situ hybridization in benign, premalignant and malignant cutaneous lesions from transplant recipients. Our aim was to analyse p53 protein status in grafted patient skin lesions, and to determine whether the presence of HPV interfered with p53 expression in these lesions.

Materials and methods

We analysed 40 skin biopsies from renal and cardiac transplant recipients, and 14 lesions from non-immunocompromised control patients. Seven samples of normal unexposed skin and four samples of foreskin from non-grafted patients were also used as controls. Each biopsy was divided into two parts: one part was fixed

I. Pélisson · Y. Chardonnet (✉) · S. Euvrard · D. Schmitt
INSERM U346 affiliée CNRS, Pavillon R,
Hôpital Edouard Herriot, F-69437 Lyon Cedex 03, France

in Bouin's solution, and embedded in paraffin for histological examination after haematoxylin-eosin staining. The other part was snap frozen and stored in liquid nitrogen. Five micron serial cryostat sections were then prepared on aminopropyl-triethoxysilane (Aldrich Chemie, Steinheim, Germany) precoated slides, and fixed for 10 min in cold acetone before immunostaining or in situ hybridization.

For the immunohistochemical detection of p53 protein frozen sections were incubated for 15 min in 0.15% hydrogen peroxide to quench endogenous peroxidase activity, and washed twice in phosphate-buffered saline (PBS; bioMérieux, Marcy l'Etoile, France). The biotin-streptavidin-peroxidase staining was then performed using Dako LSAB kit and amino-ethylcarbazole (Dako, Carpinteria, Calif.). In preliminary assays, three monoclonal antibodies (mAb) purchased from Oncogene Science (Manhasset, NY) were tested: PAb 240 and PAb421 mAb were diluted 1:50 and PAb 1801 was diluted 1:100; they were incubated for 30 min onto sections to detect p53 protein. PAb 240 mAb does not bind to wild type p53 protein, but reacts with a conformational epitope that results from different activating mutations on mutant p53 proteins [14]. PAb 421 and PAb 1801 mAb recognize two different epitopes shared by wild type and mutant p53 proteins, respectively located at the carboxy and the amino end of the proteins [1]. In controls, PBS was applied onto sections instead of primary antibody.

Plasmid DNA probes for HPV types 1a, 2a, 5, 6a, 11a, 16 and 18 were prepared and purified throught caesium chloride gradients and labelled using a nick-translation kit (BRL, Gaithersburg, Md.) and biotinylated 11-dUTP (Sigma, St Louis, Mo.). The conditions for HPV DNA detection by in situ hybridization were described previously [16]. Briefly, hybridization was performed under stringent conditions with 50% formamide in the hybridization mixture, at −19° C. The DNA-DNA hybrids were revealed with a three-step reaction and streptavidin-alkaline phosphatase complex. The specificity of HPV typing with in situ hybridization was assessed on typical cutaneous and mucosal lesions previously shown to contain the different HPV types under study with Southern blotting and the polymerase chain reaction (PCR), as describing by Soler et al. [34]. CaSki and HeLa carcinoma cell lines respectively containing 600 copies of HPV type 16 and 10–50 copies of HPV type 18 were also used as positive controls. In negative controls, the specific probe was omitted or replaced by an heterologous probe; cutaneous lesions unrelated to HPV infection were also used.

Results

Typical histological signs of HPV infection were seen in most premalignant and malignant skin lesions from transplant recipients, with vacuolized keratinocytes containing basophilic inclusions in the upper epidermal layers, either in the centre or on the border of the lesions. Squamous cell carcinomas (SCC) often retained architectural features of pre-existing viral warts.

In preliminary experiments, we tested the three mAb to p53 protein on serial frozen sections of two

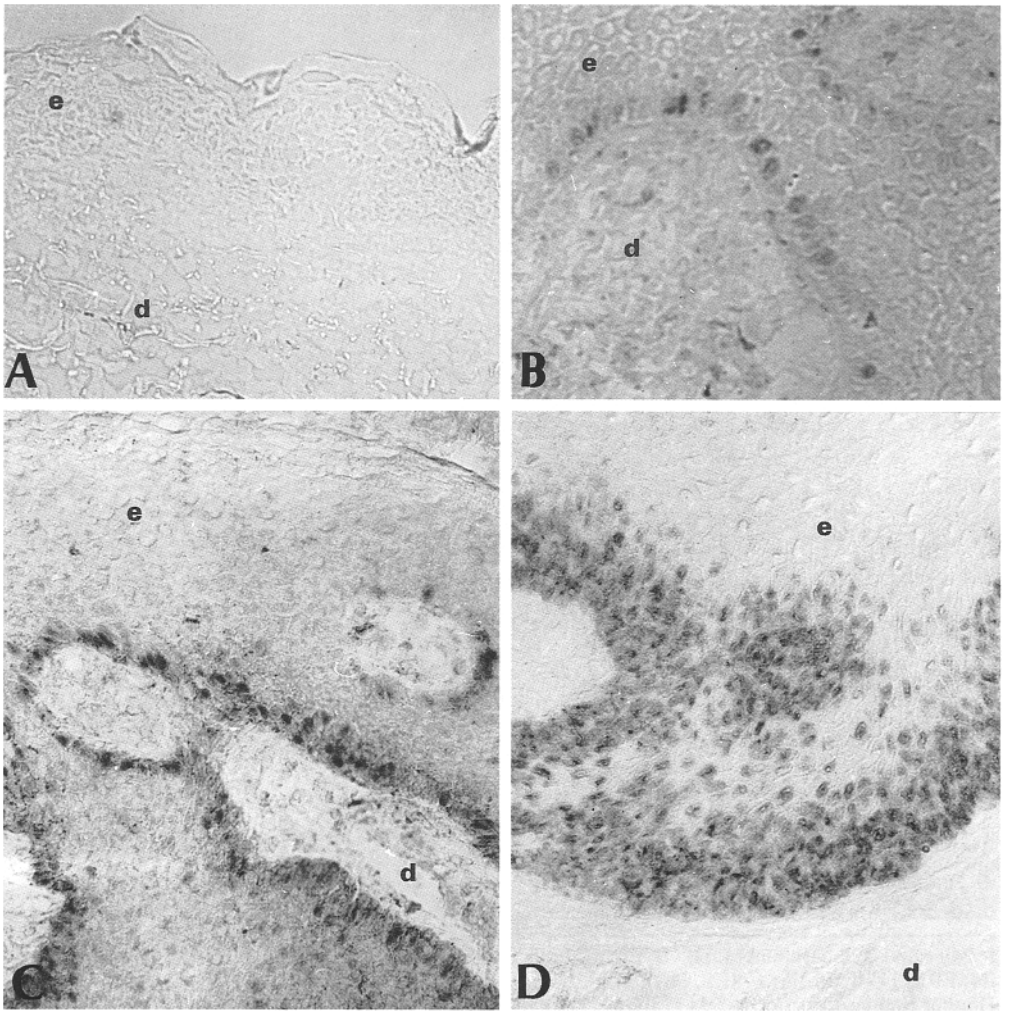

Fig. 1A–D Immunohistochemical detection of p53 protein in skin samples. **A** Absence of p53 protein staining in normal skin (×320). **B** Common wart from a grafted patient (×320): p53 is detected in few isolated basal keratinocytes. **C** Anal condyloma of a non-immunocompromised control patient (×200): nuclear p53 protein is found continuously along the basal cell layer. **D** Precancerous keratosis from a grafted patient (×200): a strong p53 immunoreactivity is detected in the basal and several suprabasal cell layers. (*d* dermis; *e* epidermis)

Table 1 Detection of p53 oncoprotein by immunohistochemistry and human papillomavirus (*HPV*) DNA by in situ hybridization in benign cutaneous lesions (*NTR* non-transplant recipient control patients, *TR* transplant recipients)

Lesions		Case number	Localisation of p53 protein detection	Detection of HPV types
Common warts	NTR	1	Basal cells (foci)	–[a]
		2	Basal cells (1 focus)	2
		3	Few isolated basal cells	–
		4	Few isolated basal cells	2
		5	Few isolated basal cells	2
		6	–	–
		7	–	1
	TR	1	Basal cells (foci)	1, 18
		2	Basal cells (foci)	2, 11
		3	Few isolated basal cells	–
		4	Few isolated basal cells	2, 5
		5	Few isolated basal cells	2, 16
		6	Few isolaed basal cells	–
		7	–	–
		8	–	16
Anongenital condylomas	NTR	1	All basal cells	6, 11
		2	All basal cells	6
		3	All basal cells	–
		4	Few isolated basal cells	–
		5	–	6
	TR	1	All basal cells	5, 6
		2	All basal cells	5, 6,11
		3	Basal cells (foci)	–
		4	–	16
		5	–	5, 11
Normal skin	NTR	7	–	–
Normal foreskin	NTR	4	–	–

[a] Absence of signal

Table 2 Detection of p53 oncoprotein by immunohistochemistry and of HPV DNA by in situ hybridization in premalignant and malignant cutaneous lesions

Lesions		Case number	Localisation of p53 protein detection	Detection of HPV types
Precancerous keratoses	TR	1	All basal and suprabasal cells	1, 16, 18
		2	Basal and suprabasal cells (large zone)	–[a]
		3	Basal and suprabasal cells (large zone)	–
		4	Basal and suprabasal cells (large zone)	–
		5	Basal cells (large zone)	–
		6	Basal cells (1 focus)	11, 18
		7	–	5, 11
		8	–	–
		9	–	–
Bowen's disease	NTR	1	All basal and suprabasal cells	–
		2	Basal cells (1 focus)	–
	TR	1	Few isolated basal cells	1, 16, 18
Basal cell carcinomas	TR	1	Basal and suprabasal cells (foci)	18
		2	Basal cells (1focus)	1, 16
		3	–	–
		4	–	18
Squamous cell carcinomas	TR	1	Basal and suprabasal cells (large zone)	–
		2	Basal and suprabasal cells (foci)	16, 18
		3	Basal and suprabasal cells (foci)	16, 18
		4	Basal cells (large zone)	1, 16
		5	Basal cells (large zone)	5, 18
		6	Basal cells (foci)	1, 2, 6
		7	Basal cells (foci)	–
		8	Basal cells (1 focus)	–
		9	Basal cells (1 focus)	11, 18
		10	Basal cells (1 focus)	1, 18
		11	–	18
		12	–	–
		13	–	–

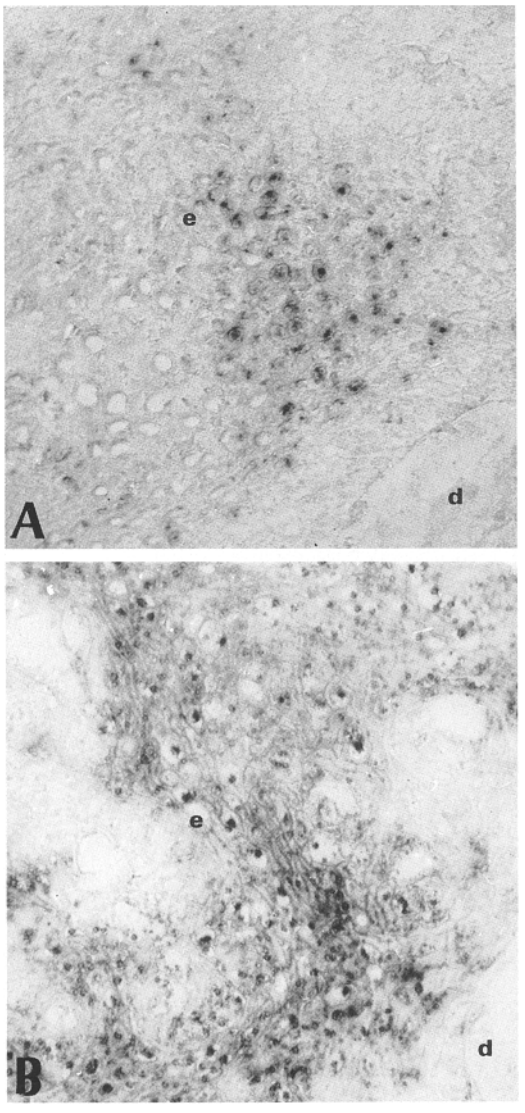

Fig. 2A, B Detection of human papillomavirus (HPV) DNA by in situ hybridization. **A** Detection of HPV type 11 DNA in a focus of few keratinocytes in a wart from a grafted patient (×200). **B** Detection of HPV type 18 DNA in a squamous cell carcinoma from a grafted patient (×200). (*d* dermis; *e* epidermis)

normal skin samples and five biopsies of cutaneous SCC from grafted patients (data not shown). PAb 421 mAb consistently gave a strong cytoplasmic signal in basal and suprabasal keratinocytes in all the biopsies. With PAb 240 and PAb 1801 mAb, no signal was observed in normal skin samples, while nuclear p53 protein was detected in foci of basal cells in three of the five SCC with PAb 1801, and in two of five SCC with PAb 240. p53 immunoreactivity was located in the same areas of the biopsies with the two mAb, but the signal was weaker with PAb 240. Furthermore, PAb 240-stained sections showed a high non-specific diffuse background in dermis and epidermis. Following these preliminary results, we used PAb 1801 antibody to detect p53 protein in our series of skin lesions from grafted and control patients.

With PAb 1801 mAb, p53 immunoreactivity was always located in basal and/or suprabasal cell nuclei in skin lesions, whereas no signal was detected in seven biopsies of normal unexposed skin (Fig. 1A) and four foreskin samples.

In benign warts and anogenital condylomas, the same p53 staining pattern was observed in grafted and control patients (Table 1). p53 protein was undetectable in 4 of 15 common warts; it was found in few isolated basal cells in 7 of 15 samples (Fig. 1B) and located in foci of basal cells in the 4 other benign warts. p53 protein staining was continuous in the basal cell layer in five of ten anogenital condylomas (Fig. 1C); two other samples exhibited either foci or few isolated positive cells, and the last three specimens were negative.

In premalignant and malignant skin lesions from grafted and control patients, p53 immunoreactivity was not always restricted to basal cells: two or three suprabasal cell layers were also stained in some cases (Table 2), but no signal was ever seen in superficial keratinocyte layers. p53 protein was detected in six of nine precancerous keratoses; the signal was found in a large part of the dysplastic epithelium of five positive samples (Fig. 1D), while adjacent normal skin was negative. Among 13 SCC from transplant recipients tested, 10 showed a positive reaction; in most of these cases, p53 staining was confined to large foci of lesional keratinocytes while the other parts of the tumours were negative. Similar patterns of immunoreactivity to p53 protein were observed in a few Bowen's disease and basal cell carcinoma biopsies tested.

When p53 immunoreactivity was compared in benign, premalignant and malignant skin samples, about 70% of biopsies reacted to p53 protein in each group of lesions, but the proportion of positive cells and the signal intensity varied. Precancerous keratoses showed the most intense staining with a high proportion of positive basal and suprabasal cells, whereas benign warts often displayed a faint signal restricted to few isolated basal cells. The proportion of p53-immunoreactive keratinocytes was intermediate in SCC with several foci of positive basal and suprabasal cells, and in anogenital condylomas with a continuous signal restricted to basal cells.

HPV DNA was detected in 31/54 lesions under study (Tables 1, 2), whereas no HPV DNA was found in normal skin and foreskin biopsies, and in cutanous lesions unrelated to HPV infection. HPV types 16 and 18 DNA was consistently detected in CaSki and HeLa control cells respectively. The analysis of typical control lesions in which HPV DNA had previously been typed with Southern blotting and PCR confirmed the specificity of the different HPV probes with in situ hybridization.

The analysis of 12 benign skin samples from non-immunocompromised control patients showed that common warts contain HPV types 1 or 2 (4 of 7 cases), while anogenital condylomas harbour HPV types 6 or 11 (3 of 5 cases). These benign lesions showed a strong signal in many intermediate and upper epidermal cell layers; they did not react with the other probes tested.

In skin samples from transplant recipients, low risk HPV types 1, 2, 6, 11 and potentially oncogenic HPV types 5, 16, 18 were detected in benign (Fig. 2A), premalignant, and malignant (Fig. 2B) cutaneous lesions. Several HPV types were often found in foci of epidermal cells located in different areas of the same biopsy; in these multiple infections, benign and potentially oncogenic HPV types were usually associated.

There was no significant correlation between the presence of the different HPV types under study and the detection of p53 protein in skin lesions: the biopsies in which p53 protein was detected haboured either benign and/or potentially oncogenic HPV types, or no HPV DNA. Similarly, among 15 lesions without p53 immunoreactivity, 8 contained benign and/or potentially oncogenic HPV types, and no HPV DNA was detected in the 7 other cases.

Discussion

In this study, we analysed p53 protein expression and HPV infection in benign, premalignant and malignant skin lesions from grafted patients. We found that p53 protein was frequently detected by immunohistochemistry in these lesions, which show typical histological signs of HPV infection and an uncommon distribution of HPV DNA types.

Immunohistochemistry has been widely used to analyse *p53* tumour suppressor gene product. Normal p53 protein has a very short half-life and the detection of p53 protein with immunohistochemical techniques in human tissues provides strong evidence of the presence of stabilizing mutations responsible for p53 protein accumulation [21]. It has been shown that the accumulation of p53 protein, as demonstrated by immunohistochemistry, accounts for the presence of *p53* gene mutations in many human tumours including lung [19], ovarian [22] and breast [40] cancers, but recent data suggest that the detection of p53 protein by immunohistochemistry is not always related to the presence of *p53* gene mutations in non-melanoma skin cancers [46]. However, immunohistochemistry may be a useful technique to assess functional alterations of p53 due to wild type or mutated p53 protein accumulation [45].

We first used three commercially available mAb on normal skin and SCC biopsies. We eliminated PAb 421; this consistently gave a strong non-specific cytoplasmic signal in basal and suprabasal epidermal keratinocytes. Indeed, this antibody is known to recognize a cross reactive epitope present on keratin polypeptides [4] typically expressed in basal and suprabasal cell layers of the epidermis. PAb 240 mAb was also eliminated as it gave a diffuse background in the epidermis. Moreover, the analysis of five SCC with PAb 1801 and PAb 240 mAb showed more positive cells with a stronger signal and less background with PAb 1801; thus PAb 1801 mAb was chosen to analyse p53 protein expression in our series of skin samples. Working on cutaneous basal cell carcinomas in similar conditions, Shea et al. [32] recently observed that p53 nuclear immunoreactivity was more frequent with PAb 1801 than with PAb 240 mAb. This may reflect the accumulation of mutant p53 proteins missing the conformational epitope recognized by PAb 240 antibody, or an increased amount of wild type p53 protein in skin lesions.

When benign warts from both grafted and control patients were analysed with PAb 1801 mAb, nuclear p53 protein was detected in foci of basal keratinocytes in 4 of 15 cases while a faint signal was seen in most lesions (7 of 15 samples). Two previous studies showed that p53 protein was undetectable in skin warts [23, 29]; however, in these reports, p53 immunostaining was performed on paraffin-embedded biopsies. Several antibodies commerically available are now reported to work on paraffin-embedded material; this provides the advantage of well-preserved tissue morphology and offers the possibility of retrospective studies. However, their use can lead to false negative results unless special treatment of the sections is performed before the staining. For example, trypsin digestion [6] or the use of a target unmasking fluid [41] have proved to increase the sensitivity of p53 detection in many paraffin-embedded tumour tissues, giving concordant results with frozen section immunostaining of the same samples. Thus, the higher incidence of p53 immunoreactivity in our series of skin warts, when compared with studies on paraffin-embedded skin biopsies, may be due to the higher sensitivity of frozen section immunostaining to detect low levels of p53 protein accumulation. This was confirmed by the analysis of premalignant and malignant skin lesions: the percentage of p53-positive SCC, which only showed foci of positive keratinocytes, was higher in our study than in other investigations on dewaxed sections [23, 29, 39]. In contrast, the incidence of p53 protein detection in precancerous keratoses with a strong p53 immunoreactivity was not significantly different in our study to that in previous reports on paraffin-embedded material [33], suggesting that the two techniques are equivalent in detecting high levels of p53 protein.

The high frequency of p53 protein detection in our report suggests that *p53* gene may frequently be mutated in skin lesions from grafted patients. This is supported by several studies that reported a high incidence of *p53* gene mutations in non-melanoma skin cancers [5, 24, 27, 46] and precancerous keratoses [8] from the general population; in these reports, the pattern of *p53* gene mutations was consistent with a mutagenic effect of ultra-violet (UV) radiation. Potentially UV-induced *p53* gene mutations may thus account for p53 immunoreactivity in our study. However, it is likely that p53 detection with immunohistochemistry in 70% of our skin lesions may overestimate the presence of such mutations, since the reported incidence of UV-induced *p53* mutations in non-melanoma skin tumours was lower, ranging from 15% [24] to 58% [5]. Moreover, Ziegler et al. [46] reported that 40% of their skin tumours showed immunohisto-

chemical detectable p53 protein accumulation without *p53* gene mutations. Further analyses are currently in progress to determine whether *p53* gene is mutated in our samples.

In some of our samples, the observed p53 signal may also reflect the accumulation of wild type p53 protein, interfering with its normal function. Hall et al. [17] recently showed that the exposure of normal human skin to UV radiation induces immunohistologically detectable wild type p53 protein accumulation in basal and suprabasal epidermal keratinocytes. Such an accumulation is thought to be involved in the cellular response to UV-induced DNA damage [20]. In our study, most skin lesions were located on highly exposed body sites, and p53 protein was detected in basal and suprabasal epidermal keratinocytes. For ethical reasons, our normal skin controls were obtained from plastic surgery and were located on unexposed body sites. However, p53 immunoreactivity was previously described in foci of basal and suprabasal keratinocytes in few normal sun-exposed skin samples [10]. We believe that UV radiation may be partially responsible for wild type p53 protein accumulation in skin lesions in some of our samples because of the cellular mechanisms involved in the repair of UV-induced DNA damage. However, we also frequently detected p53 protein in basal cells from unexposed anogenital condylomas, suggesting that other factors than UV radiation may lead to p53 protein accumulation in these lesions, and may also be involved in other cutaneous epithelial proliferations.

As potentially oncogenic HPV types 16 and 18 E6 proteins are known to bind p53 protein in vitro [30], and HPV infection is common in cutaneous lesions from grafted patients [3, 42], it was interesting to investigate the presence of HPV DNA in such lesions. In benign lesions from non-immunocompromised control patients, only low risk HPV types 1 or 2 and 6 of 11 were respectively detected in warts and anogenital condylomas as usually described [43]. Benign, premalignant and malignant skin lesions from transplant recipients frequently contained several HPV types infecting different areas of the biopises, as previously shown in our group [34, 35]. Both low risk HPV types 1, 2, 6, 11 and potentially oncogenic HPV types 5, 16, 18 were found in these samples. However, more than 70 HPV types have been described and we can not exclude that other types than those that we tested might infect cutaneous lesions from transplant recipients. The detection of mucosal HPV types 6, 11, 16 and 18 which was previously described in few cases of skin lesions from the general population [2, 15, 28], was a common feature in our grafted patients [37]. HPV type 5, commonly found in skin cancers from patients with epidermodysplasia verruciformis [43], has also been previously found in grafted patient skin lesions [3, 36, 42]. These virological data, together with clinical and histological observations [13], are in favor of a role of HPV in the malignant progression of cutaneous lesions from grafted patients.

The detection of p53 protein in our series of skin samples was not related to the presence or the absence of the different HPV types under study. In particular, potentially oncogenic HPV type 16 and 18 DNAs were unexpectedely present in some biopsies with a strong p53 immunoreactivity. It was shown in vitro that HPV types 16 and 18-encoded E6 proteins promote the degradation of p53 protein [18, 30], suggesting that E6-promoted p53 degradation may be a crucial event in the malignant progression of HPV-infected keratinocytes. This was supported by the analysis of several cervical carcinoma cell lines in which the presence of HPV DNA was associated with low levels of wild type p53 protein, whereas HPV negative carcinoma cell lines contained high levels of mutated p53 protein [31, 38, 44]. Our results did not confirm the hypothesis of p53 protein degradation by HPV types 16 and 18 E6 proteins in skin lesions from grafted patients, although the presence of *E6–E7* genes was demonstrated by PCR in many of our lesions [37]. Similarly, Ogunbiyi et al. [25] recently reported that there was no correlation between p53 immunostaining and HPV type 16 status in anal squamous neoplasia. However, the immunohistochemical detection of p53 protein in our skin samples may reflect the accumulation of wild type p53 protein due to the binding of viral E6 proteins. Indeed, it was shown that E6 proteins encoded by low risk HPV types 6 and 11 can bind p53 protein in vitro, but are defective in inducing p53 degradation [12]. To date, there has been no investigation on the ability of E6 proteins from other HPV types to fix p53 protein. Similar binding capacities, interfering with p53 protein function, may be hypothetized as the region of HPV E6 protein involved in p53 protein binding is conserved among all HPV types [12].

In conclusion, our results show that p53 protein is frequently detected in benign, premalignant and malignant skin lesions from grafted patients. Whether the signal reflects the presence of mutated p53 protein or the accumulation of wild type p53 is unclear and requires molecular analysis of *p53* gene in these samples. However, the immunohistologically detectable p53 protein accumulation is likely to affect p53 function. Multiple HPV type carriage, which is a common feature in grafted patient epithelial proliferations, may interfere with p53 protein function, but our results suggest that the presence of potentially oncogenic HPV types 16 and 18 does not induce p53 protein degradation. Both p53 alterations and HPV infection are likely to play a role, and may cooperate with other genetic alterations [26], in the progression of skin warts towards malignancy in grafted patients.

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