

# Human papillomavirus type spectrum in normal skin of individuals with or without a history of frequent sun exposure

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Cutaneous human papillomavirus (HPV) has been widely detected in healthy skin. Previous studies have found that UV radiation can activate several HPV types, and a possible role for cutaneous HPV in the development of non-melanoma skin cancer has been suggested. This study investigated the prevalence and type-spectrum of cutaneous HPV in relation to UV radiation by studying forehead skin swab samples from 50 healthy males frequently exposed to the sun and 50 healthy males who were not frequently exposed to the sun. A questionnaire including ethnic background of the participants, history of cancers and a self-assessment of sun-exposure was also conducted and analysed. PCR with the FAP primer pair was carried out to detect HPV DNA in samples. HPV prevalence was higher in individuals who spent more time outdoors and in individuals with a history of skin cancers ( $P=0.044$  and  $P=0.04$ , respectively). Furthermore, individuals wearing sunglasses as a means of sun protection had a lower prevalence of HPV ( $P=0.018$ ). Interestingly, HPV-76 was only detected in the group without frequent sun-exposure ( $P=0.001$ ). These results suggest that increased UV radiation exposure may be a factor leading to a difference in prevalence of cutaneous HPV types.

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## INTRODUCTION

Human papillomaviruses (HPVs) infect cutaneous and mucosal epithelia and are recognized as the causative agents of warts. It has recently become apparent that clinically normal skin harbours many different HPV types, and many of these HPV types have not been described previously (Antonsson *et al.*, 2000, 2003a; Astori *et al.*, 1998). Furthermore, it has been shown that asymptomatic HPV infections are acquired very early in infancy with a broad spectrum of HPV types (Antonsson *et al.*, 2003b), and that the prevalence of HPV DNA in healthy skin increases with age (Antonsson *et al.*, 2000). Moreover, cutaneous HPV infections on healthy skin have often been found to persist over time (Hazard *et al.*, 2006).

Many studies have also suggested a role for HPV in the carcinogenesis of non-melanoma skin cancer (NMSC). Squamous cell carcinoma (SCC) can metastasize, and is known as the unfavourable form of NMSC compared with basal cell carcinoma (BCC). Interestingly, BCCs are found approximately three times more frequently in immunocompetent individuals; however, in immunosuppressed organ-transplant recipients, SCCs are more prevalent (Pfister, 2003). It has been reported that more HPV types have been found in actinic keratosis and SCC skin lesions

than in normal skin (Alotaibi *et al.*, 2006), and a greater variety of HPV types have also been found in immunosuppressed individuals than in immunocompetent individuals (Harwood *et al.*, 2004). Both SCC and BCC develop mostly on sun-exposed sites (Frost & Green, 1994), and some specific types of HPV, including HPV-5, HPV-8, HPV-20 and HPV-77, have been shown to be activated by UV radiation (Akgül *et al.*, 2005; Massimi *et al.*, 2008; Michel *et al.*, 2006; Purdie *et al.*, 1999; Storey *et al.*, 1998). Evidence of UV-induced E6/E7 oncogenicity and transcriptional activity of E6/E7 oncogenes in NMSC has been found (Al Moustafa *et al.*, 2004; Dang *et al.*, 2006; Michel *et al.*, 2006). Furthermore, differences in transforming activity potential between various cutaneous HPV types have been shown, providing further evidence for a role of cutaneous HPV in the development of NMSC (Massimi *et al.*, 2008).

Recent reports have suggested that HPV DNA is more prevalent on sun-exposed sites in both normal skin and skin lesions (Antonsson *et al.*, 2000; Forslund *et al.*, 2003). Given the high rate of sun exposure in Australia, as evidenced by the country having the highest rates of skin cancer in the world, we undertook a population survey of skin HPV frequency in workers with or without a history of frequent sun exposure. This study has identified HPV-76 as a more frequently occurring HPV type in healthy individuals who were not frequently exposed to the sun.

The GenBank/EMBL/DBJ accession number for the partial L1 sequence of the putative new HPV type FAD11 is EU340869.

## METHODS

**Subjects.** Fifty healthy frequently sun-exposed (SE) males and 50 healthy males without frequent sun exposure (NSE) were studied. Thirty-six road workers and 14 vegetation and pest control workers from the Brisbane City Council participated in the SE group. These were individuals who currently work at least 20 h per week in the sun during weekdays and have been working in the sun for 1 year or more. Participants of the NSE group were age-matched to the SE group, and were individuals with indoor jobs who spent an average of 1.1 h per day in the sun during weekdays.

**Questionnaire.** A two-page questionnaire was given to each of the participants. The first section of the questionnaire included questions about the participant's age, sex, ethnic background, skin type and eye and hair colour, to provide a brief personal background and to allow the matching of age and sex of individuals from the two study groups. The second section of the questionnaire involved a more detailed analysis of the participant's history of skin cancer and other skin conditions. History of cancer and organ transplantation was also surveyed to provide information about the immune status of the participants. Questions relating to the participant's family history of skin cancer were also included to give indications as to whether there could be hereditary issues associated with skin cancers. The third section of the questionnaire was a self-assessment of sun exposure. Participants were asked about the number of hours per day spent outdoors, degree of tan, strategies used to protect themselves from the sun, frequency of sunburns as a child and the number of sunburns occurring during the last 12 months.

**Samples.** Samples were collected with pre-wetted (in 0.9% NaCl solution) cotton-tipped swabs (Sarstedt) that were drawn back and forth five times over the forehead skin within an area of  $5 \times 10$  cm. The swabs were suspended in 1 ml 0.9% NaCl solution (Baxter) in a 1.5 ml Eppendorf tube, and analysed within 48 h of collection. All samples were left at room temperature for as little time as possible, and samples that were not analysed immediately were kept at 4 °C.

**PCR.** PCR using the FAP primer pair is able to detect most cutaneous HPV types (Forslund *et al.*, 1999). The 25 µl PCR contained 5 µl neat sample, 0.75 µM of each primer (FAP59/FAP64) (Forslund *et al.*, 1999), 3.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP (Fisher), 0.2% BSA (Sigma), 0.625 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and GeneAmp PCR buffer II (Applied Biosystems). The PCR was incubated for 10 min at 94 °C, followed by 45 amplification cycles of 94 °C for 90 s, 50 °C for 90 s and 72 °C for 90 s. Distilled water was used as a negative control; a patient sample containing HPV-18 was used as a positive control. A PCR using Human L1-F and Human L1-R primers (Deragon *et al.*, 1990) was carried out on all samples to ensure that they contained human DNA and that no PCR-inhibiting agents were present. All reactions were undertaken in a Thermo Hybaid PxE PCR machine. PCR products were analysed using agarose gel electrophoresis. Agarose gels (1.5%) were made with 1 × TAE buffer and stained with 10 mg ethidium bromide ml<sup>-1</sup>.

**Cloning and sequencing.** FAP PCR products of HPV-positive samples were cloned into the pCR-script SK(+) cloning vector (Stratagene) or the pCR 2.1-TOPO cloning vector (Invitrogen). Plasmid DNA of bacterial colonies with inserts of HPV DNA was purified using a Wizard mini-prep purification kit (Promega). The plasmid DNA was sequenced with T7 or T3 primer for the Stratagene reactions and M13 Forward or M13 Reverse primer for the Invitrogen reaction. Sequencing reactions were performed using the CentriSep machine, ABI PN#401762. The GenBank BLAST search (<http://www.ncbi.nlm.nih.gov/blast>) (Altschul *et al.*, 1997) was used to compare sequences and to identify HPV types. One to three clones were analysed per HPV-positive sample.

**Statistical analysis.** The  $\chi^2$  test was used to compare the prevalence of HPV DNA among SE and NSE individuals and to compare the distribution of HPV types detected. Fisher's exact test was used to identify frequently occurring HPV types, and to determine the prevalence of multiple HPV types. A *P*-value less than 0.05 was considered statistically significant in the  $\chi^2$  test and Fisher's exact test. Odds ratios and 95% confidence intervals for the outcome variable HPV DNA positivity and a number of independent variables from the questionnaire were calculated using logistic regression. Variables with *P*<0.1 in univariate analysis were included in a multiple logistic regression analysis, and a *P*-value less than 0.05 in the multiple regression analysis was considered statistically significant.

**Ethical approval.** This research has been approved by the University of Queensland Medical Research Ethics Committee under the approval number 2006000670.

## RESULTS

### Participants' background

Fifty SE and 50 NSE forehead skin swabs were collected together with results of a questionnaire assessing ethnic background, history of cancer and a self-assessment of sun-exposure. The age of the 100 participants ranged from 20 to 68 years, with a mean age of 40 years (Table 1). More than 80% of the participants were Caucasians; the rest of the participants were indigenous Australians, Chinese, Indian and Maori. Sixty-one per cent of the participants had fair skin, 32% had olive skin, 4% had freckled skin and 3% had dark skin. The SE group spent an average of 8.5 h in the sun every day, with a range of 4–13 h, while the NSE group spent an average of 1.1 h in the sun each day, ranging from <1 to 4 h. In addition, participants from the SE group had worked under the sun for an average of 18 years ( $18.1 \pm 11.4$ ).

**Table 1.** Participant background

Parameter	SE (n=50)	NSE (n=50)
Age range (mean ± SD)	20–66 (40.24 ± 11.49)	22–68 (40.42 ± 11.65)
Caucasian ethnicity	43 (86%)	39 (78%)
Fair skin type	30 (60%)	31 (62%)
Hours spent outdoors per day (mean ± SD)	4–13 (8.52 ± 1.98)	<1–4 (1.12 ± 0.77)

## HPV DNA prevalence between the two study groups

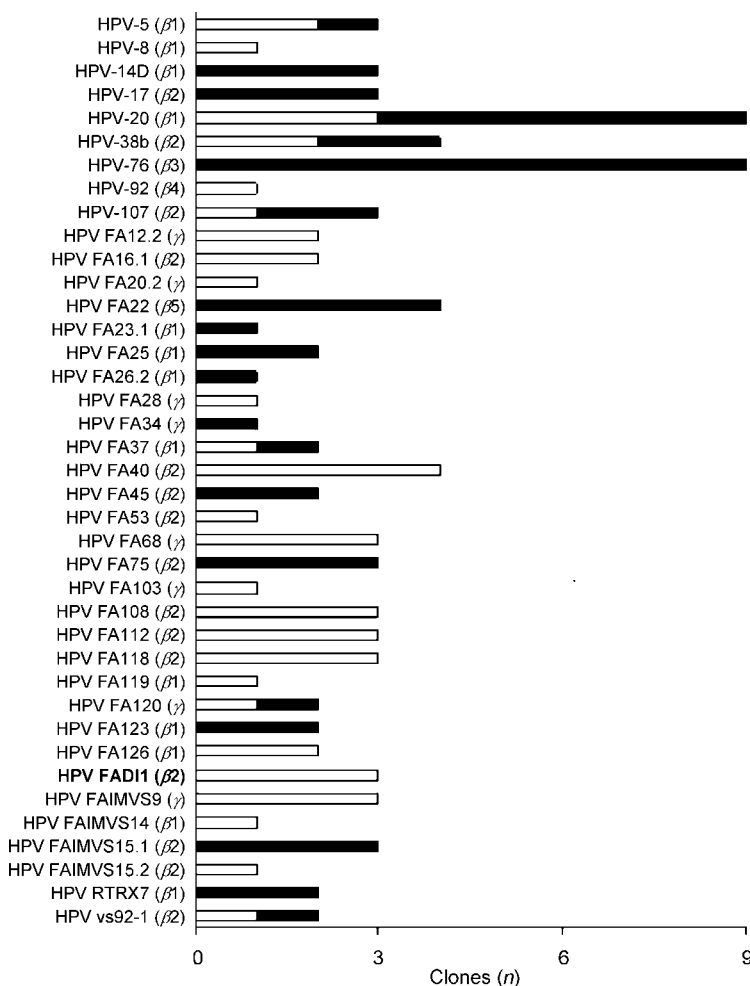
Twenty-seven of the 50 (54 %) SE individuals and 24 of the 50 (48 %) NSE individuals tested positive with the FAP PCR. However, the difference in HPV DNA prevalence between the two study groups was not significant ( $P=0.54$ ). All samples tested positive for human L1 DNA by PCR, indicating that they all contained human cells and that no PCR-inhibitory agent was present in the samples.

## HPV type determination and analysis

In order to determine the identity of each HPV type present in our samples, DNA sequence analysis was performed. Twenty-one of the 27 SE HPV DNA-positive samples and 21 of 24 NSE HPV-DNA-positive samples could be cloned and sequenced, and a wide diversity of HPV types was found (Fig. 1). A total of 48 sequences were identified in the SE group, and 50 sequences were identified from the NSE group. We had aimed to sequence three clones from each HPV DNA-positive sample. However, of 51 HPV-DNA-positive samples, 25 samples yielded three HPV sequences, six samples had two HPV

types sequenced, 11 samples had one HPV type sequenced and nine samples were not able to yield the sequence of any HPV type. Twenty-six HPV types were detected from the SE group, including one new HPV type. This new HPV type was 437 bp in length, and was 81 % similar to HPV FA53. Our new putative HPV type was named FAD11 (where FA stands for the primer pair used and DI for the institute, i.e. Diamantina Institute, Brisbane, Australia). Twenty HPV types were detected from the NSE group; no new HPV type was found.

Seven HPV types were found in both the SE and NSE groups (HPV-5, HPV-20, HPV-38b, HPV-107, HPV FA37, HPV FA120 and HPV vs92-1). However, each group had unique HPV types. The SE group had 19 distinctive types, while 13 were found only in the NSE group. Thirteen HPV types were detected only once in the SE group and seven HPV types were detected only once in the NSE group. Significantly, HPV-76 was found exclusively in the NSE group ( $P=0.001$ ). Although the HPV types FA22 and FA40 were also detected in only one group each (NSE and SE, respectively), the differences were not statistically significant ( $P=0.053$  in each case). HPV-20 was the most commonly detected type that was found in both study groups.



**Fig. 1.** HPV types and number of clones detected from the SE (open bars) and NSE (filled bars) groups. The putative new HPV type identified in this study is indicated in bold.

A broad spectrum of types from both the  $\beta$  and  $\gamma$  cutaneous HPV species was found in this study, but the differences in the distribution of each species were found not to be significant ( $\chi^2$  test,  $P=0.28$ ). Analysis of the number of HPV types detected per sample revealed that individuals who were frequently sun-exposed were more likely to carry multiple types of HPV; however, the result was not statistically significant (Fisher's exact test,  $P=0.11$ ). Furthermore, none of the samples contained more than two HPV types in either of the groups.

### Analysis of HPV DNA prevalence in relation to variables from the questionnaire

According to the questionnaire, none of the participants had a history of organ transplantation, hence all participants were considered immunocompetent. In order to analyse the variants in relation to prevalence of HPV DNA, univariate analysis and multiple regression analysis were carried out. Referring to the statistical analysis, HPV DNA prevalence was not related to family history of skin cancer ( $P=0.76$ ) or history of moles, warts or other skin conditions such as eczema and dermatitis ( $P=0.56$ ,  $P=0.41$  and  $P=0.39$ , respectively) (Table 2). Individuals with various ethnic backgrounds, skin types, degree of tan,

different number of recent sunburns and frequency of sunburns as children also had similar HPV DNA prevalence. Furthermore, use of an increased number of several common sun-protection methods did not show any relationship to HPV prevalence ( $P=0.59$ ). These sun-protection methods included wearing a hat ( $P=0.52$ ), wearing protective clothing ( $P=0.93$ ) and using sunscreens ( $P=0.43$ ).

While six variables from the questionnaire had a  $P$ -value of  $<0.1$ , only four were continued for multiple regression analysis as one was highly correlated with another variable ('history of skin cancer' was highly correlated with 'number of skin cancers'), and one was not collected on one of the sampling groups (i.e. number of years working outdoors as, by definition, members of the NSE group did not work outdoors). The four variables analysed further were 'older age', 'number of skin cancers', 'number of hours spent outdoors' and 'wearing of sunglasses'. The multiple regression results showed that a greater number of previous skin cancers and a greater number of hours spent outdoors increased the HPV DNA prevalence ( $P=0.04$  and  $P=0.044$ , respectively; Table 3); HPV DNA prevalence was more than threefold associated with having a prior history of skin cancer in the SE compared with the NSE group. Eleven of

**Table 2.** Univariate results of HPV DNA prevalence in relation to variables from the questionnaire

Variable	Odds ratio	95 % Wald confidence limits	P-value
SE group	1.27	0.58–2.79	0.54
Older age*	1.03	0.99–1.06	0.083
Caucasian ethnicity	1.82	0.64–5.16	0.26
Fair skin type	1.16	0.52–2.59	0.71
History of skin cancer	4.21	1.09–16.1	0.036
Number of skin cancers†	3.39	1.23–9.31	0.018
History of moles	0.76	0.3–1.91	0.56
History of warts	0.67	0.26–1.72	0.41
History of other skin conditions	0.64	0.23–1.76	0.39
Family history of skin cancer	0.86	0.32–2.26	0.76
Hours spent outdoors†	1.12	0.98–1.28	0.083
Years working outdoors†	1.1	1.02–1.19	0.0063‡
Degree of tan§	1.18	0.77–1.81	0.42
Sun protection methods†	0.9	0.63–1.29	0.59
Wearing a hat	0.73	0.27–1.93	0.52
Wearing protective clothing	0.96	0.41–2.25	0.93
Wearing sunglasses	0.42	0.16–1.11	0.083
Use of sunscreen	1.4	0.59–3.28	0.43
Frequency of sunburns as a child†	1.13	0.77–1.68	0.51
Number of recent sunburns†	1.06	0.93–1.21	0.35

\*Age was classified as 20–24, 25–29, 30–34, 35–39, 40–44, 45–49, 50–54, 55–59, 60–64 or 65–69 years. 'Older age' tested groups of increasing age against HPV DNA prevalence.

†Increasing number for each of these variables tested against HPV DNA prevalence.

‡Although the  $P$ -value is significant, it was only measured in the SE group. Hence, multiple regression analysis could not be applied on this variable in Table 3.

§Measured as light, medium or dark. An increased degree of tan was tested against HPV DNA prevalence.

||Included a maximum of five choices: wearing a hat, protective clothing, sunglasses, use of sunscreen and use of zinc. Use of zinc was not included as an independent variable because only a few people from the SE group used it.

**Table 3.** Multiple regression analysis of the questionnaire in relation to HPV DNA prevalence

Variable	Effect on HPV DNA prevalence	Odds ratio	95 % confidence limits	P-value
Number of skin cancers	Increased	3.07	1.05–9.00	0.04
Hours spent outdoors	Increased	1.16	1.00–1.35	0.044
Wearing sunglasses	Decreased	0.27	0.09–0.80	0.018

the 14 individuals who had a history of skin cancer had NMSC (79 %), two had both melanoma skin cancer (MSC) and NMSC (14 %) and one did not know which type of skin cancer he had (7 %). However, analysis of the relationship between HPV prevalence and MSC or NMSC could not be carried out due to the relatively small sample size. The multiple regression analysis also showed that individuals using sunglasses to protect themselves from the sun had a lower HPV DNA prevalence ( $P=0.018$ ). However, increasing age, which was significantly associated with HPV DNA prevalence on its own (univariate analysis,  $P=0.083$ ), was not significantly related to HPV DNA prevalence in combination with other factors in the multiple regression analysis ( $P=0.25$ ).

## DISCUSSION

Previous studies have shown the widespread nature of HPV in clinically normal skin (Antonsson *et al.*, 2000, 2003a; Astori *et al.*, 1998) and that HPV DNA can persist in normal skin for years (Hazard *et al.*, 2006). In order to determine the differences in prevalence of HPV in normal skin in relation to levels of sun-exposure UV radiation, we investigated the presence of HPV DNA in forehead skin swabs from 50 healthy frequently sun-exposed (SE) men and 50 healthy men who were not frequently sun-exposed (NSE).

Firstly, we noted no significant difference in overall HPV DNA prevalence between the SE group and the NSE group. One explanation for this may be the variation in sun protection practices between age groups in the SE group (e.g. we noted that younger participants tend to wear sunglasses and protective long-sleeved clothes more than older workers). Therefore, different levels of sun protection method may have confounded the results. A previous study found increasing HPV prevalence with older age (Antonsson *et al.*, 2000). However, statistical analysis in this study did not show a significant increase in HPV prevalence with increasing age using multiple regression analysis ( $P=0.25$ ). Furthermore, our measure of sun exposure was based on reported self-assessment during the interview. However, given this, it was shown that there was a direct relationship between increased time spent outdoors and detection of HPV DNA (Tables 2 and 3). These data indicate that the more years spent working outdoors and the longer the time spent outdoors, the greater the risk of increased cutaneous HPV in normal skin. The logical explanation for these findings is that UV

radiation plays a role in cutaneous HPV prevalence in normal skin, and that the variable 'number of hours spent outdoors' could be a better measure of sun exposure than the binary variables SE and NSE to determine any difference between these two groups.

Healthy individuals carry multiple HPV types (Antonsson *et al.*, 2000, 2003a, b; Hazard *et al.*, 2006); therefore, we analysed the presence of multiple HPV infections in both study groups. Our study shows that multiple HPV types were more common in individuals who are frequently exposed to the sun ( $P=0.073$ ). One explanation for this could be immune suppression induced by increased exposure to UV radiation (Pamphilon *et al.*, 1991; Parrish *et al.*, 1983). However, a more thorough understanding of the cutaneous HPV transmission pathway and virus–host interactions would be needed in order to identify the mechanisms of HPV persistence in human skin.

Overall, a large spectrum of HPV types from both the  $\beta$  and  $\gamma$  HPV groups was detected. Although there was no significant difference in HPV group distribution between the two study groups, a greater number of HPV types from the  $\beta_2$  and  $\gamma$  group were detected within the SE group. A recent publication (Forslund *et al.*, 2007) suggested that cutaneous  $\beta_2$  HPV species predominate in sun-exposed skin in SCC. In our study, HPV-76, which belongs to the  $\beta_3$  group, was only detected within the NSE group. Future research could involve growing HPV-76 and HPV types from the  $\beta_2$  and  $\gamma$  HPV group in raft culture (Bell *et al.*, 1983; Regnier *et al.*, 1981), so the mechanism of action of these HPV types in skin tissues can be investigated.

UV radiation damages DNA by inducing mutations and impairing apoptosis, leading to cancer formation (Ruhland & de Villiers, 2001). It has also been found to have suppressing effects on the immune system through skin-mediated responses (Kripke, 1988; Morison, 1989; Noonan & De Fabo, 1992). Increased presence of HPV DNA was detected in the forehead skin swabs of individuals with increased numbers of previous skin cancers, suggesting that increased numbers of previous skin cancers may be a risk factor for cutaneous HPV. It has been found that people who are often exposed to the sun with poor sun protection methods are more likely to develop both MSC and NMSC (Dummer & Maier, 2002), with Caucasians who have fair skin types having a higher risk (Neugut *et al.*, 1994). Our result did not show a higher prevalence of HPV DNA in Caucasians and individuals with fair skin type, and most participants who had a history of skin cancer had NMSC.

Cutaneous HPV DNA has been found to be more common on skin than in NMSC biopsies, suggesting that cutaneous HPV may be necessary for NMSC development, but not essential once the tumour has formed (hit-and-run) (Boxman *et al.*, 2000; Forslund *et al.*, 2004; Purdie *et al.*, 1993). However, the role of HPV in NMSC is still controversial.

Surprisingly, our results show that individuals who wore sunglasses had lower HPV DNA prevalence in forehead skin swabs, while other sun protection methods did not show any benefit in terms of lower HPV DNA prevalence. Suppression of immune functions can occur through an eye-brain-mediated response (Denis *et al.*, 1993; Jankovic, 1994; Moore & Klein, 1974; Roberts, 1995), so one explanation for this result would be that sunglasses decrease the amount of UV light reaching the eyes and therefore the pineal gland. This would result in an upregulation of melatonin and thus reduce any immune suppression caused by UV radiation. The eye-brain mechanism is also age-dependent, due to variations in the filtering characteristics of the lens throughout life (Dillon, 1991). A decrease in immune responsiveness with age has been suggested to be the result of a decrease in melatonin production (Caroleo *et al.*, 1992; Maestroni & Conti, 1991; Maestroni, 1993). These findings may also explain why increasing prevalence of HPV DNA was detected with increasing age in a previous study by Antonsson *et al.* (2000). Additional studies to elucidate the mechanism(s) of immune enhancement caused by the blockage of UV radiation detection from the eyes in relation to HPV prevalence should be carried out to determine whether sunglasses are protective against HPV.

This study investigated HPV prevalence and type spectrum in healthy males who are frequently sun-exposed in comparison to healthy males who are not frequently sun-exposed. Our results suggested that UV radiation plays a role in cutaneous HPV prevalence in normal skin, and an increased number of skin cancers may be a risk factor for cutaneous HPV. Furthermore, HPV-76 was only detected among the NSE group. Elucidation of the transmission mechanism of cutaneous HPV would result in greater understanding of this virus.

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