Arch Dermatol Res (2000) 292 : 275–278 

ORIGINAL PAPER

**B. T. Baldursson · H. Beitner · S. Syrjänen**

# Human papillomavirus in venous ulcers with and without squamous cell carcinoma

Received: 16 August 1999 / Revised: 18 January 2000 / Accepted: 27 January 2000

*Abstract* **Malignant transformation of chronic wounds is a well-known, albeit rare, phenomenon. We examined archival paraffin blocks of samples of squamous cell carcinoma (SCC) in chronic venous leg ulcers previously taken from 23 patients and of chronic noncancerous venous leg ulcers from 35 patients for the presence of human papillomavirus (HPV) DNA. The methods used were the polymerase chain reaction (PCR) with GP05+/06+ (mucosal) and nested PCR with CP65/70 and CP66/69 (EV-associated) primers. A subsequent nonradioactive Southern blot hybridization was used to confirm the specificity of the PCR. With PCR three samples were positive on the gel, and with Southern blotting, a further seven samples were positive, to give a total of ten samples. All of the positive samples were from the noncancerous ulcers and with the primers GP05+/06+. HPV infection is probably not the carcinogen responsible for the malignant transformation of venous leg ulcers. The difference in positivity between the ulcers and the SCCs was statistically significant ($P = 0.01$) and raises the question as to whether HPV-positive cells are eliminated in the interaction between the SCC and the immune system. Further studies on the carcinogenic effects of chronic proliferation and the role of HPV infection therein, are needed.**

B. T. Baldursson (✉)
Department of Dermatology Gävle Hospital,
801 87 Gävle, Sweden
e-mail: baldur.baldursson@lg.se,
Tel.: +46-26-154208, Fax: +46-26-154261

H. Beitner
Department of Dermatology Karolinska Hospital,
Stockholm, Sweden

S. Syrjänen
Department of Oral Pathology, Institute of Dentistry,
University of Turku, Turku, Finland

**Key words** Squamous cell carcinoma · Venous leg ulcers · HPV · PCR · Nested PCR · Southern blotting · 'Hit and run'

## Introduction

Chronic venous leg ulcers are a common problem, affecting 0.2% of the total population and 3–4% of the population over 65 years of age [1]. Venous ulcers often become chronic; the median duration of the ulcer diathesis was 13.5 years in one study of venous ulcer patients [2].

Malignant transformation is a known but rare complication of chronic leg ulcers [3]. The appearance of a squamous cell carcinoma (SCC) in a leg ulcer is easily overlooked and diagnosed only as a deterioration of the ulcer, until a rapidly growing exophytic tumour or other clinical signs appear. This exophytic growth is keratinized and sometimes verrucous. SCC in venous leg ulcers can be fatal, especially if poorly differentiated [4]. The aetiological factors of the carcinogenesis of this disease are unknown. Ultraviolet (solar) radiation, generally the overwhelming cause of skin SCC, seems to be unlikely because of the assumed limited exposure of ulcer sites [5].

Human papillomavirus (HPV) is an established aetiological agent of cervical cancer. HPV has also been detected in SCC of the skin in immunocompetent patients, albeit in varying proportions [6–9]. Precursor lesions of SCC have also been studied with regard to HPV, with diverging results [10–12]. There are two disease entities that have been associated with HPV and carcinogenesis of skin, i.e., epidermodysplasia verruciformis (EV) and SCC of kidney transplant recipients [13–15]. The former is linked to an inherent defect in cellular immunity resulting in widespread verrucous lesions which might become malignant especially in sun-exposed areas, whereas in kidney transplant recipients immunosuppressive treatment itself makes the patients prone to develop skin warts and even cancers [13, 14]. Also, serological studies have shown a correlation between seropositivity for HPV types 16 and 33 and noncervical genital cancer [16]. HPV type

16 has been found in periungual SCC and in Bowen's disease, suggesting an autoinoculation from a genital lesion [17–19]. Today HPV is detected mainly by PCR with subsequent hybridization and/or cloning and sequencing [20]. Analysis methods utilizing nested PCR have demonstrated increased sensitivity compared to standard PCR [6, 13].

The target of HPV is thought to be solely the basal cells of squamous epithelia. Thus, an intact epidermis protects, whilst even superficial trauma exposes the basal cells to infection [13]. At the margin of a healing ulcer there is a horizontal growth of a single or a double layer of epidermal cells migrating over the ulcer bed. Such proliferating keratinocytes might be suitable targets for HPV infection in a similar manner to basal cells devoid of the protection of the stratum corneum.

In this study, we sought to determine whether HPV, a potential carcinogen, is present in SCC arising from chronic venous ulcers. For this purpose we studied samples of chronic venous ulcers that were malignantly transformed and of non-malignant venous ulcers.

## Materials and methods

### Materials

An epidemiological and archival search revealed paraffin blocks of 31 samples of SCC in venous leg ulcers from 23 patients and 50 samples of non-malignant chronic venous leg ulcers from 35 patients. The patients are described in detail elsewhere [4]. Recut H&E-stained sections were used to confirm the diagnosis and the presence of the ulcer margin. Additional 4-μm thick sections were cut to give an area of 1 $cm^2$. The sections were deparaffinized, dehydrated and then lysed with 1 ml of a solution of 10 m*M* Tris (pH 8.3), 400 m*M* sodium chloride, 1% SDS, 2 m*M* EDTA and 0.3 mg/ml proteinase K overnight at 37 °C. Proteins were precipitated by adding 300 μl of saturated sodium chloride (approximately 6 *M*). After centrifugation DNA was precipitated from the supernatant with ethanol [21].

### PCR

HPV DNA was detected with PCR using general primer sets GP05+/GP06+ and CP65/CP70. A second nested PCR was performed with CP65/CP70 as external primer and CP66/CP69 as internal primer [13]. The degenerated primers of CP65/CP70 amplify the complete set of EV HPV types and the CP66/CP69 primers are reported to be reliable for their nested amplification [13]. GP05+/GP06+ target the L1 region in mucosal HPV types [22]. The reaction was done with 300 ng genomic DNA in a 50 μl reaction volume. The PCR solution contained 5 μl of 10 × PCR buffer (50 m*M* potassium chloride, 10 m*M* Tris-HCl, pH 8.8, 1.5 m*M* magnesium chloride, 0.1% Triton X-100), 1 U AmpliGold Taq polymerase enzyme (Perkin Elmer, Norwalk, CT, USA), 200 μ*M* of each deoxynucleotide triphosphate, 20 pmol of the primers and sterile water. The reaction was done in a thermal cycler (Perkin Elmer Cetus) with 40 cycles with the following cycle profile after initial denaturation at 95 °C for 10 min: denaturation at 94 °C for 60 s, annealing at 40 °C for 60 s, elongation at 72 °C for 120 s, and final extension at 72°C for 7 min. In the nested PCR, 3 μl of the first-step PCR was used as input. In this PCR, 30 cycles of amplification (1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C) were performed. Beta-globin was amplified to ensure that the samples were appropriate for PCR. Positive controls for HPV were biopsy sections from condyloma and EV, and DNA extracted from SiHa cells. No DNA was added to the PCR reaction of the negative controls. The PCR products were electrophoresed in 3% agarose gel (NuSieve, FMC BioProducts, Rockland, ME, USA). Bands were visualized by ethidium bromide staining.

The specificity of the PCR amplification was confirmed by subsequent Southern blot hybridization. PCR products were transferred to a nitrocellulose membrane (Gore Screen Plus, Biotechnology Systems, Boston, MA, USA). The hybridization was done at 55 °C for 2 h and the background was blocked with salmon sperm (2 mg/ml). The membranes were hybridized with both low-risk (HPV 6, 11, 40, 42, 43, 44) and high-risk (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) HPV-oligoprobe mixtures [23, 24]. The probes were labelled with digoxigenin according to the manufacturer's instructions (DIG Oligonucleotide 3′-End Labelling Kit, Boehringer Mannheim, Germany). The hybridization was carried out at 55°C overnight. After three washes with (2 × SSC, 1% SDS) buffer for 30 min each, the filters were incubated in the blocking reagent (Boehringer Mannheim) at 53 °C for 30–60 min and positive hybridization was detected with antidigoxigenin Fab fragments conjugated to alkaline phosphatase at room temperature for 40 min. Positive signals were visualized with the chemiluminescence substrate (DIG Luminescent Detection Kit, Boehringer Mannheim) at 37 °C for 5 min in darkness. The filters were fixed at 37 °C for 5–15 min. Chemiluminescence was recorded on X-ray film (DuPont, Cronex 4) with exposure times of 10 to 60 min.

Fisher's exact test was used to test the null hypothesis that the cancer specimens and the ulcer specimens had the same probability of reacting positively in the analyses [25].

## Results

PCR with mucosal primers (GP05+/GP06+) was negative in the SCC samples. Of the noncancerous ulcer samples, three were positive on the gel, and after hybridization seven additional HPV samples were positive (see Table 1). Low-risk HPV types were found in eight samples and high-risk types in two. None of the specimens was shown to contain HPV DNA with the primers targeting the EV HPV types (CP65/CP70). The PCR was therefore repeated using the nested method with CP65/CP70 as external primer and CP66/Cp69 as internal primer. This also came out negative for all samples. Altogether, 10 of 35 leg ulcer patients (28%) harboured HPV in their chronic ulcers, all of them mucosal. Only in two cases was the viral load high. The difference between the positivity of the ulcers and the SCCs was statistically significant ($P = 0.01$).

**Table 1** HPV DNA-positive samples from patients with chronic venous ulcers

| Patient no. | PCR positivity on gel GP05+/06+ | Results after Southern blot confirmation |
|---|---|---|
| 5155 | + | Low risk |
| 7213 | – | Low risk |
| 1023 | + | Low risk |
| 1235 | – | Low risk |
| 1097 | – | Low risk |
| 1034 | + | Low risk |
| 2238 | – | High risk |
| 3643 | – | High risk |
| 1358 | – | Low risk |
| 304 | – | Low risk |

## Discussion

This study was designed to investigate the role of HPV in SCC arising from chronic venous leg ulcers. There are no previous studies available focusing on this topic. HPV DNA has been found in SCC of the skin of immunocompetent patients [6, 13]. In 80% of SCCs in kidney transplant recipients, different HPV types belonging mostly to the EV group have been found by nested PCR [13]. HPV has also been detected in a verrucous carcinoma of the leg and foot [26, 27].

Our results indicated that HPV seems not to be the cause of SCC in venous leg ulcers. The study showed, however, that HPV can infect chronic ulcers without any clinical signs of HPV infection. Benign mucosal HPV types seem to be the most usual infection in chronic leg ulcers.

The difference between the observed HPV DNA in the ulcer samples and the SCC samples was interesting and clearly statistically significant. One hypothesis might be that the cancer defence mechanisms have eradicated all HPV, including "innocent bystanders". This opens the way to speculations on "hit and run" HPV infections in these SCCs [28].

As analytical methods have become increasingly sensitive, it has become clear that HPV infection alone is seldom sufficient for malignant transformation of keratinocytes. HPV DNA has been found even in clinically normal skin [6]. Our results are in accordance with current concepts maintaining that, apart from HPV, either a compromised immune system or ultraviolet light is generally needed in the malignant transformation of the skin, and SCC in venous ulcers can be seen as a prototype of non-ultraviolet-induced malignancy [15]. Concerning the findings of HPV in chronic leg ulcers, further investigations may be necessary to elucidate the interaction between HPV and the keratinocytes at the ulcer margin that are in a constant state of proliferation interrupted by repetitive relapses because of infections or other factors. Such research might even help improve our understanding of the cellular mechanisms in chronic ulcers.

**Acknowledgements** The technical assistance of Ms Sarita Järvinen is gratefully acknowledged. The language help provided by Mrs Carol Scinto is also acknowledged. This study was supported by the Forum for Research and Development, Gävle Hospital, and the Finsen-Welander Foundation.

## References

1. Nelsén O, Bergkvist D (1995) Leg ulcers: definitions, classification, differential diagnosis and epidemiology. In: Treatment of venous leg ulcers (workshop recommendations). The Norwegian Medicines Control Authority. The Swedish Medical Products Agency, pp 38–56
2. Nelsén O, Bergkvist D, Lindhagen A (1994) Venous and non-venous leg ulcers: clinical history and appearance in a population study. Br J Surg 81: 182–187
3. Baldursson B, Sigurgeirsson B, Lindelöf B (1995) Venous leg ulcers and squamous cell carcinoma: a large-scale epidemiological study. Br J Dermatol 133: 571–574
4. Baldursson B, Hedblad M-A, Beitner H, Lindelöf B (1999) Squamous cell carcinoma complicating chronic venous leg ulceration: a study of the histopathology, course and survival in 25 patients. Br J Dermatol 140: 1148–1152
5. Marks R (1996) Squamous cell carcinoma. Lancet 347: 735–738
6. Astori G, Lavergne D, Benton C, Hökmayr B, Egawa K, Garbe C, Villiers E-M de (1998) Human papillomaviruses are commonly found in normal skin of immunocompetent hosts. J Invest Dermatol 110: 752–755
7. Pierceall WE, Goldberg LH, Ananthaswamy HN (1991) Presence of human papillomavirus type 16 DNA sequences in human nonmmelanoma skin cancers. J Invest Dermatol 97: 880–884
8. Guitart J, Bergfeld W, Tuthill RJ, Tubbs RR, Zienowicz R, Fleegler EJ (1990) Squamous cell carcinoma of the nail bed: a clinicopathological study of 12 cases. Br J Dermatol 123: 215–222
9. Smith SE, Davis IC, Leshin B, Fleischer AB, White WL, Feldman SR (1993) Absence of human papillomavirus in squamous cell carcinomas of non-genital skin from immunocompromised renal transplant patients. Arch Dermatol 129: 1585–1588
10. Lu S, Syrjänen K, Havu VK, Syrjänen S (1996) Failure to demonstrate human papillomavirus (HVP) involvement in Bowen's disease of the skin. Arch Dermatol Res 289: 40–45
11. Lu S, Syrjänen K, Havu VK, Syrjänen S (1995) No evidence of human papillomavirus DNA in actinic keratosis. Arch Dermatol Res 287: 649–651
12. Trowell HE, Dyall-Smith M, Dyall-Smith D (1991) Failure to detect human papillomavirus in solar keratoses of Australian patients (letter). Int J Dermatol 30: 225
13. Berkhout RJM, Tieben LM, Smits HL, Bavinck JN, Vermeer BJ, Schegget J ter (1995) Nested PCR approach for detection and typing of epidermodysplasia verruciformis-associated human papillomavirus types in cutaneous cancers from renal transplant recipients. J Clin Microbiol 133: 690–695
14. Androphy EJ (1994) Molecular biology of human papillomavirus infection and oncogenesis. J Invest Dermatol 103: 248–256
15. McKenna DB, O'Connor D, Kay E, Leader M, Murphy GM (1997) The role of human papillomavirus in skin cancer – current status. J Eur Acad Dermatol Venereol 9: 103–110
16. Björge T, Dillner J, Anttila T, Engeland A, Hakulinen T, Jellum E, Lehtinen M, Luostarinen T, Paavonen J, Pukkala E, Sapp M, Schiller J, Youngman L, Thoresen S (1997) Prospective seroepidemiological study of role of human papillomavirus in non-cervical anogenital cancers. BMJ 315: 646–649
17. Moy RL, Elezri YD, Nuovo GJ, Zitelli JA, Bennett RG, Silverstein S (1989) Human papillomavirus type 16 DNA in periungual squamous cell carcinomas. JAMA 261: 2669–2673
18. Forslund O, Nordin P, Andersson K, Stenquist B, Hansson B-G (1997) DNA analysis indicates patient-specific human papillomavirus type 16 strains in Bowen's disease on fingers and in archival samples from genital dysplasia. Br J Dermatol 136: 678–682
19. Uezato H, Hagiwara K, Ramuzi ST, Khaskhely NM, Nagata T, Nagamine Y Nonaka S, Asato T, Oshiro M (1999) Detection of human papilloma virus type 56 in extragenital Bowen's disease. Acta Derm Venereol 79: 311–313
20. Villiers E-M de, Lavergne D, McLaren K, Benton EC (1997) Prevailing papillomavirus types in non-melanoma carcinomas of the skin in renal allograft recipients. Int J Cancer 73: 356–361
21. Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for salting out nucleic DNA from human nucleated cells. Nucleic Acids Res 16: 1215
22. Atula S, Grenman R, Kujari H, Syrjanen S (1999) Detection of human papillomavirus (HPV) in laryngeal carcinoma cell lines provides evidence for a heterogeneic cell population. Eur J Cancer 35: 825–832
23. Jacobs MV, Roda Husman AM de, Brule AJ van den, Snijders PJ, Meijer CJ, Walboomers JM (1995) Group-specific differentiation between high- and low-risk human papillomavirus genotypes by general primer-mediated PCR and two cocktails of oligonucleotide probes. J Clin Microbiol 33: 901–905

24. Jacobs MV, Snijders PJ, Brule AJ van den, Helmerhorst TJ, Meijer CJ, Walboomers JM (1997) A general primer GP5+/GP6(+)-mediated PCR-enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in cervical scrapings. J Clin Microbiol 35: 791–795
25. Altman DG (1991) Practical statistics for medical research. Chapman & Hall, London
26. Garven TC, Thelmo WL, Victor J, Pertschuk L (1991) Verrucous carcinoma of the leg positive for human papillomavirus DNA 11 and 18: a case report. Hum Pathol 22: 1170–1173
27. Noel JC, Peny MO, Detremmerie O, Verhest A, Heenen M, Thiry L, De Dobbeleer G (1993) Demonstration of human papillomavirus type 2 in a verrucous carcinoma of the foot. Dermatology 187: 58–61
28. Iwasaka T, Hayashi Y, Yokohama M, Hara K, Matsuo N, Sugimori H (1992) 'Hit and run' oncogenesis by human papillomavirus type 18 DNA. Acta Obstet Gynecol Scand 71: 219–223