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Viral DNA detection and *RAS* mutations in actinic keratosis and nonmelanoma skin cancers

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Summary

Background Actinic keratosis (AK) is a well-established precancerous skin lesion that has the potential to progress to squamous cell carcinoma (SCC). Basal cell carcinoma (BCC) is a locally aggressive slowly growing tumour that rarely metastasizes. A number of viruses have been proposed to play a role in the development of nonmelanoma skin cancers (NMSC), but the most plausible evidence to date suggests that cutaneous human papillomavirus (HPV) is the key instigating factor.

Objectives To evaluate the prevalence of HPV, cytomegalovirus (CMV), herpes simplex virus (HSV) and Epstein–Barr virus (EBV) and investigate their relationship with the presence of *RAS* gene mutations in cutaneous lesions obtained from nonimmunosuppressed patients.

Methods HPV, CMV, HSV and EBV detection was performed using polymerase chain reaction (PCR) in skin biopsies (26 AK, 12 SCC and 15 BCC samples) that were collected from immunocompetent patients. The *RAS* mutation incidence was also investigated in all cutaneous lesions by use of PCR/restriction fragment length polymorphism and direct DNA sequencing.

Results Seventeen out of 53 (32%) skin lesions were found to be positive for HPV DNA. The highest incidences of HPV infection were five of 15 (33%) in BCC and four of 12 (33%) in SCC specimens. The HPV incidence was eight of 26 (31%) in AK and eight of 53 (15%) in normal skin tissue. Twelve out of 53 (23%) skin lesions were CMV-positive. The highest incidence of CMV infection was six of 15 (40%), observed in BCC specimens. The CMV incidence was two of 26 (8%) in AK and four of 12 (33%) in SCC. No normal skin biopsy was found to be positive for CMV. All cutaneous samples were negative for HSV and EBV DNA, as assessed by our PCR-based assays. Only three samples, one AK (4%), one BCC (6%) and one SCC (8%), were found to carry a G>T transversion at the second position of *HRAS* codon 12. Both *HRAS* mutant SCC and BCC biopsies were HPV- and CMV-positive, as well.

Conclusions HPV DNA is detected in NMSC, AK and normal skin biopsies. Our results also indicate that CMV is involved in NMSC at higher levels than in premalignant lesions, whereas the virus was not detected in normal skin biopsies. HSV and EBV do not appear to be involved in the pathogenesis of cutaneous lesions. Moreover, we suggest that the *HRAS* codon 12 mutation is not a very common event in AK or NMSC. Finally, both viral infection and *HRAS* activation appear to represent independent factors in the aetiology of NMSC, samples of which were obtained from immunocompetent patients.

Nonmelanoma skin cancer (NMSC), squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), are the most frequently occurring types of cancer among European populations.[1–3] The risk of SCC is strongly associated with chronic sun exposure, and SCCs appear primarily on sun-exposed parts of the body. Actinic keratoses (AKs) are premalignant precursors to SCCs and share many histological similarities with them.[4] Indeed, AKs are currently commonly considered to be a vari-

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ant of SCC *in situ*. Clinically, AKs appear primarily on chronically sun-exposed areas,[5,6] and sites adjacent to AKs may contain significant histological alterations, suggesting extensive preneoplastic alterations in sun-damaged skin.[7]

A number of viruses have been proposed in the development of NMSC, but the most plausible evidence to date is that for human papillomavirus (HPV).[8] According to tissue tropism and oncogenic potential, which reflect differences in nucleic acid composition, viruses are classified into the following subgroups: cutaneous, cutaneous involved in epidermodysplasia verruciformis (EV), cutaneous and mucosal, and mucosal of low and high risk.[9] Most studies examining the involvement of papillomaviruses in the development of cutaneous carcinomas have been performed on lesions from patients with EV or from immunosuppressed patients, e.g. renal allograft recipients.[10] HPV in cutaneous malignancy is also implicated in the development of cutaneous cancers on sun-exposed sites.[10,11] A possible role for EV and other HPV types in the carcinogenic process has been investigated, but results have shown enormous variation in the frequency and type of HPV DNA detected, depending on the method employed. Furthermore, the sensitivity and specificity of the detection methods have improved considerably, leading to the identification of many more HPV types; there are now more than 130, of which 82 have been fully characterized.

CMV has been associated with cervical, prostate and colon carcinoma.[12–14] It was suggested to be strongly involved in the development of cutaneous SCC and BCC,[15] but it has not been confirmed by other studies, nor has its involvement been studied in AK.

EBV is a herpesvirus that has been linked to several human malignancies.[16] It is present in the tumour tissue of nasopharyngeal undifferentiated carcinoma and various lymphoid malignancies.[16–20] Apart from Zafiropoulos *et al.*,[15] no other studies have been conducted regarding the prevalence of EBV in SCC, BCC or AK.

HSV has been linked to cervical carcinoma.[21] However, the virus has not been shown to be involved in the pathogenesis of NMSC, although a causal relationship between HSV infection and cutaneous SCC was hypothesized in the past.[22]

The *RAS* family genes (*KRAS*, *HRAS* and *NRAS*) encode GTP-binding proteins of 21 kDa (p21) located on the inner surface of the plasma membrane. Early observations identified that these molecules have an impact on cell transformation and tumorigenesis. Cumulative evidence strongly supports the involvement of activated *RAS* genes in various human malignancies.[23,24]

Recent studies have shown that aberrant proto-oncogene expression due to point mutation, gene amplification or deletion may be involved in ultraviolet (UV) carcinogenesis.[25,26] It has been shown that about 40% of human skin cancers occurring on sun-exposed body sites contained mutations in codon 12 of the *HRAS* oncogene and that only one of 40 human skin tumours contain a mutation at codon 12 of the *KRAS* oncogene.[27] However, the frequency of occurrence of *RAS* gene mutations in premalignant lesions and NMSC is often contradictory, depending on the detection methods or geographical criteria employed.[27–30] Moreover, their correlation with viral infection remains unclear.

The aim of the present study was to investigate the incidence of HPV, CMV, HSV and EBV DNA detection in a group of cutaneous lesions obtained from immunocompetent patients. Specifically, we screened 26 AK, 12 SCC and 15 BCC samples, applying commercially available polymerase chain reaction (PCR) assays. Moreover, we examined the same samples for the presence of mutations in codons 12 and 13 of the *RAS* family genes (*KRAS*, *HRAS* and *NRAS*), using PCR/restriction fragment length polymorphism (RFLP) and DNA sequencing assays.

Materials and methods

Tumour specimens and DNA extraction

Twenty-six AK, 12 SCC and 15 BCC and normal tissue paired samples were obtained from nonimmunosuppressed patients treated at 'A. Sygros' Hospital (Athens, Greece) with the approval of the donors and the institute's ethics committee. All samples were histopathologically examined prior to DNA extraction by an experienced pathologist. Extreme effort was made to avoid any adjacent normal tissue and to isolate areas of tissue containing > 70% tumour cells. Clinicopathological characteristics of the patients are depicted in Table 1. Immediately following dissection, the specimens were stored at −80 °C until DNA extraction. The tissues were digested with 0·1 mg mL^{-1} proteinase K (Promega, Madison, WI, U.S.A.) and 400 μL of digestion buffer containing 150 mmol L^{-1} NaCl, 400 mmol L^{-1} Tris–HCl, 60 mmol L^{-1} ethylenediamine tetraacetic acid (EDTA) and 15% sodium dodecyl sulphate pH 8·0 in a 1·5-mL Eppendorf tube. Samples were then incubated at 60 °C for 2–3 days. Fresh proteinase K was added three times daily. The samples were extracted once with phenol/chloroform and once with chloroform. DNA was precipitated with the addition of 20 μL of 5 mol L^{-1} NaCl and 1 mL ethanol, recovered with centrifugation for 15 min at 4 °C, washed once with cold 70% ethanol and resuspended in 50 μL double distilled water. Genomic DNA was extracted from blood samples using the standard phenol/chloroform and ethanol

Table 1 Patient clinicopathological characteristics

	Cutaneous lesions		
Patients	AK (n = 26)	SCC (n = 12)	BCC (n = 15)
Sex (M/F)	16/10	7/5	10/5
Age (years)	63–92	63–88	65–82
Mean ± SD	76 ± 7·2	75 ± 7·5	75 ± 7·5
UV exposure			
High[a]	25	10	13
Low[b]	1	2	2

[a]Location on the face. [b]Location on other parts of the body. AK, actinic keratosis; SCC, squamous cell carcinoma; BCC, basal cell carcinoma; UV, ultraviolet.

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precipitation protocol, and was analysed as a matched control DNA.

Viral-specific polymerase chain reaction amplification

Amplification of HPV, CMV, HSV and EBV viral DNA was performed by multiplex PCR reactions. Specifically, for the detection of HPV two pairs of primers were used (Biotypap, Biotools, Madrid, Spain). The first primer pair hybridized with common sequences to the HPV genotypes (L1 and L2 genes) and, therefore, indicated the presence of HPV (450 bp). The second primer pair hybridized with specific sequences for oncogenic HPV genotypes (E6 and E7 genes) (250 bp). Amplified HPV PCR products were then subjected to a restriction fragment analysis, able to detect 32 alpha-HPV sequences (6, 11, 13, 16, 18, 30, 31, 32, 33, 34, 35, 39, 40, 42, 43, 44, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 62, 64, 66, 67, 68 and 69) according to the manufacturer (Biotypap, Biotools). In order to verify the presence of generic HPV (450 bp), we applied a second PCR reaction using the SPF primer pair that also targets the L1-conserved region and yields a PCR product of 65 bp, as previously described.[31] CMV-specific detection was performed using PCR primer sets specific for the *MIEIA* gene of the CMV genome, as described in the CMV Early Complete kit (Nanogen Advanced Diagnostics Srl, Torino, Italy). HSV and EBV DNA were detected using specific primers (HSV 430/720 IC and EBV 290; Sacace Biotechnologies Srl, Caserta, Italy). Reactions were performed along with an internal/amplification control, according to the producer's specifications. Qualitative analysis of the results was based on the presence or absence of specific bands of amplified DNA on agarose gel (2%). Each PCR reaction contained two negative controls. The positive samples underwent repeated testing for verification of the results.

RAS mutation analysis

RAS genes were amplified using the following primers: *KRAS*, forward, 5′-ACTGAATATAAACTTGTGGTAGTTGGACCT-3′ and *KRAS*, reverse, 5′-TCAAAGAATGGTCCTGGACC-3′ (PCR product: 157 bp); *HRAS*, forward, 5′-GAGACCCTGTAGGAGGACCC-3′ and *HRAS* reverse, 5′-GGGTGCTGAGACGAGGGACT-3′ (PCR product: 312 bp); *NRAS* forward, 5′-AACTGGTGGTGGTTG-GACCA-3′ and *NRAS* reverse, 5′-ATATTCATCTTACAAAGT-GGTCCTGGA-3′ (PCR product: 83 bp). PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 37 cycles of denaturation at 94 °C for 35 s, annealing at 60 °C for 40 s, and elongation at 72 °C for 40 s. The reaction ended with a final extension at 72 °C for 10 min. PCR products were analysed on a 2% agarose gel and were photographed on a UV light transilluminator.

RFLP analysis was as follows: for *KRAS* and *NRAS*, 10–40-μL aliquots of the amplification products were digested for 16 h with 30 U *Bst*NI. For *HRAS*, 10–40-μL aliquots of the amplification products were digested for 16 h with 30 U *Msp*I. RFLP products were analysed on a 3% agarose gel and photographed on a UV light transilluminator. DNA from the SW480 cell line bearing a homozygous mutation in *KRAS* codon 12, and EJ, which is mutant at the same codon of the *HRAS* gene, were used as positive controls, respectively.

The PCR products were resolved through 2% agarose gel and purified (Qiagen, Hilden, Germany) to remove unincorporated primers and dNTPs. The sequencing reaction contained 4 μL Big Dye Terminator ready-reaction mix (PE ABI, Warrington, U.K.), 2 μL of the purified PCR product and 1·6 pmol of the antisense sequencing primer, in a total reaction volume of 10 μL. Reaction conditions were: 96 °C for 10 s, 50 °C for 10 s and 60 °C for 4 min, for 25 cycles. Sequencing products were precipitated with isopropanol to remove unincorporated dye terminators and resuspended in 10 μL of loading buffer (formamide : dextran sulphate/EDTA, 5 : 1). Products were run on a 377 ABI PRISM automatic sequencer and analysed with Sequencing Analysis software (PE ABI).

Statistical analysis

Statistical analyses were performed with SPSS 11.5 (SPSS, Chicago, IL, U.S.A.). Statistical significance was set at the 95% level (P-value < 0·05).

Results

Seventeen out of 53 (32%) skin lesions were found to be positive for generic-HPV DNA. The highest incidences of generic-HPV DNA detected in the present study were five of 15 (33%), observed in BCC, and four of 12 (33%), detected in SCC specimens. One of the BCC biopsies presented a 250-bp band (specific for an oncogenic alpha-HPV type) apart from the 450-bp band (specific for a generic-HPV type) in the multiplex PCR reaction (Fig. 1). Further RFLP analysis revealed that this sample was positive for the HPV-18 type. The generic-HPV incidence was eight of 26 (31%) in AK and eight of 53 (15%) in the normal skin biopsies. However, none of these samples was positive for any oncogenic alpha-HPV type. Apart from the above-mentioned HPV 18-positive BCC sample, the RFLP assay did not detect any alpha-HPV type for any

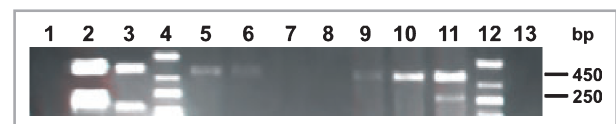

Fig 1. Human papillomavirus (HPV) detection for the nonmelanoma skin cancer samples using two pairs of primers. The 450-bp band indicates the presence of generic HPV types, while the second 250-bp band is specific for oncogenic alpha-HPV types. Polymerase chain reaction products were electrophoresed through a 3% agarose gel. Lanes 5, 6, 9 and 10: samples positive for the generic HPV types. Lane 11: oncogenic alpha-HPV-positive basal cell carcinoma sample (HPV-18 type). Lanes 1 and 13: negative controls. Lane 2: positive control (250 and 450 bp). Lanes 3, 4 and 12: DNA ladders.

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of the rest of the cutaneous samples. Further genotyping of the generic-HPV-positive samples, in order to classify them into cutaneous (beta- or gamma-HPV) types and cutaneous wart-associated HPV types (mu-HPV, nu-HPV, species A2 and A4 of alpha-HPV), was not performed.

Twelve out of 53 (23%) skin lesions were CMV-positive. The highest incidence of CMV infection was six of 15 (40%), observed in BCC specimens. The CMV incidence was two of 26 (8%) in AK and four of 12 (33%) in SCC. No normal skin tissue was found to be positive for CMV DNA.

All cutaneous biopsies were negative for HSV and EBV DNA, as assessed by the PCR-based assays that we applied. Moreover, all matched control DNA samples from blood were negative for the all of the viral DNAs studied.

Only three samples, one AK (4%), one BCC (6%) and one SCC (8%) biopsy, were found to carry a G>T transversion at the second position of *HRAS* codon 12 (leading to an amino acid substitution of Gly to Val). The results were verified with both PCR/RFLP and antisense *HRAS* primer sequencing (Fig. 2). No *HRAS* codon 12 or 13 was detected in the normal skin biopsies or the matched control DNA samples extracted from blood. Finally, all cutaneous and blood samples were characterized as wild type in codons 12 and 13 of *KRAS* and *NRAS* genes. Both *HRAS* mutant SCC and BCC biopsies were HPV- and CMV-positive, as well. Of note is that the only BCC sample with an *HRAS* codon 12 mutation was positive for HPV-18.

Viral infections did not show any statistically significant correlation with the histological type, age, site of lesion or sex. Furthermore, no significant correlation was established between viral infection and *RAS* point mutation. The results are collectively shown in Table 2.

Discussion

The vast majority of studies regarding viral detection have been conducted in patients with EV, as well as in immunosuppressed patients, i.e. renal transplant recipients.[32] The purpose of the present study was to detect the presence of a group of viral DNAs in premalignant and malignant skin lesions obtained from nonimmunosuppressed hosts and investigate their relationship with the presence of *RAS* gene mutations.

In the present study, we detected a frequency of 33% HPV-positive BCC samples. Only one of these cases (20%) was characterized as an oncogenic alpha-HPV type (HPV-18). However, because the RFLP assay that we used is specific for only 32 alpha-HPV types (6, 11, 13, 16, 18, 30, 31, 32, 33, 34, 35, 39, 40, 42, 43, 44, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 62, 64, 66, 67, 68 and 69) and not for cutaneous HPV or cutaneous wart-associated HPV types, we could not differentiate the HPV type for the rest of the cases. This inability to differentiate the HPV type along with the relatively small sample number shows a weak point of the present study. Cutaneous HPV types and cutaneous wart-associated HPV types have previously been reported to induce NMSC.[33–36] However, we strongly verified the presence of generic-HPV DNA in the cutaneous samples by performing a secondary PCR reaction with the SPF primers.[31] More comprehensive

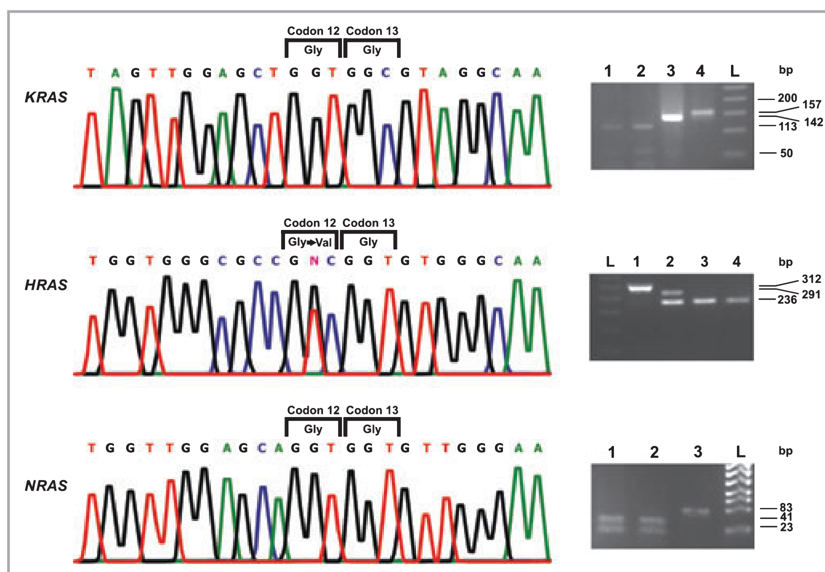

Fig 2. Sequencing electropherograms (left column) and polymerase chain reaction (PCR)/restriction fragment length polymorphism analysis (right column) of the same patients showing the detected DNA sequences of *KRAS*, *HRAS* and *NRAS*, in codons 12 and 13. *KRAS* amplification products (157 bp) were digested with the restriction enzyme *BstNI* and electrophoresed on a 3% agarose gel. L, 50-bp ladder; lanes 1 and 2, *wt* samples; lane 3, homozygous *mut*-positive control from the SW480 cell line; lane 4, undigested *KRAS* PCR product. *HRAS* amplification products (312 bp) were digested with the restriction enzyme *MspI* and electrophoresed on a 3% agarose gel. L, 50 bp-ladder; lane 1, undigested *HRAS* PCR product; lane 2, heterozygous *mut* sample; lanes 3, 4, *wt* samples. *NRAS* amplification products (83 bp) were digested with the restriction enzyme *BstNI* and electrophoresed on a 3% agarose gel. L, pUC/*MspI* ladder; lanes 1 and 2, *wt* samples; lane 3, undigested *NRAS* PCR product.

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Table 2 Viral infection and RAS mutation percentages

	HPV status of the tissue		CMV status of the tissue		HSV status of the tissue		EBV status of the tissue		Samples bearing RAS codon 12 mutations (%)		
	Number infected (%)	Number noninfected (%)	Number infected (%)	Number noninfected (%)	Number infected (%)	Number noninfected (%)	Number infected (%)	Number noninfected (%)	KRAS	HRAS	NRAS
Histology											
AK	8 (31)	18 (69)	2 (8)	24 (92)	0 (0)	26 (100)	0 (0)	26 (100)	0/26 (0)	1/26 (4)	0/26 (0)
SCC	4 (33)	8 (66)	4 (33)	8 (66)	0 (0)	12 (100)	0 (0)	12 (100)	0/12 (0)	1/12 (8)	0/12 (0)
BCC	5 (33)	10 (67)	6 (40)	9 (60)	0 (0)	15 (100)	0 (0)	15 (100)	0/15 (0)	1/15 (6)	0/15 (0)
Normal skin	8 (15)	45 (85)	0 (0)	53 (100)	0 (0)	53 (100)	0 (0)	53 (100)	0/53 (0)	0/53 (0)	0/53 (0)
UV exposure											
High[a]	20 (83)	42 (51)	10 (83)	50 (53)	0 (0)	66 (62)	0 (0)	66 (62)	0 (0)	3 (100)	0 (0)
Low[b]	4 (17)	40 (49)	2 (17)	44 (47)	0 (0)	40 (38)	0 (0)	40 (38)	0 (0)	0 (0)	0 (0)
Sex											
Male	10 (42)	40 (49)	5 (42)	46 (49)	0 (0)	48 (47)	0 (0)	48 (47)	0 (0)	1 (33)	0 (0)
Female	14 (58)	42 (51)	7 (58)	48 (51)	0 (0)	55 (53)	0 (0)	55 (53)	0 (0)	2 (66)	0 (0)

[a]Location on the face. [b]Location on other parts of the body. HPV, human papillomavirus; CMV, cytomegalovirus; HSV, herpes simplex virus; EBV, Epstein–Barr virus; AK, actinic keratosis; SCC, squamous cell carcinoma; BCC, basal cell carcinoma.

experiments, such as the analysis of viral expression and viral load and/or DNA *in situ* hybridization, need to be performed in the future to highlight further the link between viral infection and NMSC. Generic-HPV DNA was also present in SCC, AK and even in normal skin tissue at similar frequencies (33% and 31%, respectively). However, an even higher prevalence of HPV infection has been widely reported for immunosuppressed patients (75%) compared with immunocompetent ones.[33,34,37–40] The aforementioned frequencies were also verified by another PCR reaction using the SPF primer set.[31] As the presence of multiple HPV types in skin lesions has been reported in the past,[33,34] further investigation for the double detection of cutaneous (beta- and gamma-HPV) and cutaneous wart-associated HPV types should be performed in the present cutaneous samples.

The importance of a co-carcinogen in the induction of skin cancer is suggested by the oncogenic mechanism of the EV HPVs whereby DNA from EV-specific HPV types persists episomally within primary cutaneous tumours rather than being incorporated into cellular DNA. This incorporation usually occurs in genital high-risk HPVs.[41] It has been speculated that the combination of an oncogenic HPV, i.e. HPV-18 and a putative oncogenic HPV, i.e. HPV-8, considered nonpathogenic for the general population, results in the development of clinical disease through the mechanisms of virus/virus interaction.[11] Other HPV types have also been reported in the past. It was previously shown that HPV-38 was detected in ~ 50% of skin carcinomas.[42] An Australian study further demonstrated HPV-38 DNA in 16% of BCCs.[35] One possible model that has been reported by Harwood and Proby[36] regarding the significance of HPV in the development of BCC is that the viruses have a promoter effect, acting in conjunction with specific tumour initiators or other promoters, the most important of these being UV radiation. Of particular interest is that several reports have indicated a possible link between HPV and skin cancer formation in patients with psoriasis.[39,43] However, conflicting studies do not support a specific causal role for HPV in psoriasis, suggesting rather that psoriatic skin may be more permissive for viral presence than normal skin.[44] Alternatively, as HPV DNA has also been detected in normal skin, it has been suggested that the virus is simply a coincidental cutaneous 'passenger'.[36] Nonetheless, further studies are required in order to elucidate the strong association between HPV and NMSC.

CMV is a beta herpesvirus that infects 50–90% of adults and establishes latency. In nonimmunosuppressed patients, it is reactivated and can even cause focal colonic epithelial lesions. CMV proteins have been shown to promote mutagenesis, modulate cell cycle progression, angiogenesis and cell invasion.[45,46] Detection of CMV has been reported in colorectal cancer.[47,48] Moreover, the viral genome of CMV was reported along with the expression of viral proteins IE1-72 in a tumour cell-specific pattern in colorectal polyps and adenocarcinomas, but not in adjacent non-neoplastic tissue.[49] These data strongly suggested an important role for CMV in

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the multi step model for the transformation of a normal cell to a malignant one.

Our results confirm the considerable frequency of CMV infection in the group of patients with NMSC, as previously reported.[15] In the present study we show that CMV must be involved in NMSC at higher levels than in premalignant lesions, whereas the virus was not detected in normal skin biopsies. It is known that CMV can infect epithelial cells but its ability to transform them is not established. Because in NMSC the major aetiological factor is considered to be the UV-induced mutation, it could be argued that the CMV infection with its proven mutagenic capacity[50] contributes to genomic instability thereby increasing the possibility of deregulating the cell cycle genes. Further studies are needed to test the above hypothesis for NMSC based on clinical observations.

The contribution of *HRAS* in the development of skin cancer has led to much controversy.[29,51] Furthermore, it is suggested that *HRAS* mutations are caused by UV radiation, but this point is often argued. *RAS* gene mutation frequencies were previously analysed at length and it was proposed that the *RAS* oncogene significantly contributes to skin cancer development.

In the present study, we detected relatively low percentages of *HRAS* oncogene activation in both AK and NMSC lesions, through mutation at codon 12 (4% for AK, 8% for SCC and 6% for BCC), whereas no mutation was detected in codons 12 or 13 for *KRAS* and *NRAS* genes, in either of the cutaneous lesions studied. Activated *HRAS* could play a role during the early stages of SCC or BCC development. However, the low incidence detected suggests that this does not appear to be very significant. These mutation rates are in agreement with recent reports, suggesting an overall mutation frequency of 10–20% for AKs, SCCs and BCCs.[27,29,30,51–54] On the other hand, a high mutation frequency and prevalence of *NRAS* mutations has been reported for patients with xeroderma pigmentosum, which is consistent with an even more important role of UV-induced lesions and a different mutation profile in these patients.[55]

The HPV-5 and HPV-8 types have been shown to give rise to transformed cell lines, in collaboration with the activated *HRAS* gene.[56] Other EV-associated HPV types (HPV-22 and -25) and cutaneous HPV types (HPV-2 and -3) have been associated with NMSC and benign lesions of the skin.[39] In the present study, both NMSC biopsies that were detected to bear a mutant *HRAS* gene were HPV- and CMV-positive as well. Of note is that the only BCC sample bearing the *HRAS* codon 12 mutation was positive for HPV-18. However, other HPV types such as HPV-5, -8 or -38, were not detected, probably due to currently putatively uncharacterized types from the vast range of HPVs. Furthermore, the vast majority of the HPV- and CMV-positive AK and NMSC tissues were not found to carry any *HRAS* mutation. Therefore, we assume that *HRAS* codon 12 mutations exist without a direct link with the presence of HPV or CMV infection, and that viral infection and oncogene activation appear to represent two independent factors in the aetiology of AK and NMSC obtained from immunocompetent patients.

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