CUTANEOUS HUMAN PAPILLOMAVIRUSES AS RECURRENCE FACTOR IN ACTINIC KERATOSES

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Actinic keratoses (AK) are common, premalignant lesions cause mainly by UV DNA damage. Progression into squamous cell carcinoma may be influenced by other several factors such as chronic chemical exposure or viral infection. A carcinogenic role of Human Papillomaviruses (HPV) in early steps of skin tumour development was recently hypothesized; moreover the presence of HPV DNA seems to be higher in cancer precursor lesions. The aim of this work is to identify the presence of HPV DNA in biopsies from Actinic Keratoses (AK) and from normal skin samples collected from dermatological healthy subjects in Italy, in order to evaluate the severity and the clinical evolution of the HPV positive lesions. The DNA test revealed 37% HPV positivity in AK patients versus 0% in the control group; many different genotypes and variants were identified by direct sequencing of PCR product. The HPV positive AK were usually clinically indistinguishable from the HPV negative. All AK lesions were removed by laser treatment, but AK lesions recurred in all HPV positive patients after a period of 45-60 days whereas the same disappeared in the HPV negative ones. These data permit to hypothesize that the presence of HPV DNA could be an aggravating factor for AK lesion severity and recurrence.

Actinic keratoses (AK) are common, epithelial inflammatory keratotic scaly lesions considered as precancerous or premalignant, usually induced by cumulative sun exposure. These lesions are defined as a proliferation of atypical keratinocytes in the epidermal-dermal junction, potentially leading to non-melanoma skin cancer (NMSC). The morphology of atypical cells in both actinic keratosis and squamous cell carcinoma (SCC) is indistinguishable. The risk of rapid progression to squamous cell carcinoma is minimal, but up to 60% of squamous cell carcinoma cases arise on actinic keratosis (1). Thus, actinic keratosis requires careful diagnosis and follow-up. Both AK and SCC demonstrated similar chromosome aberrations and gene mutations, including the same mutation in the tumour suppressor gene TP53. Usually they arise after 40 years of age and are most common in white caucasian race especially in outdoor workers (seamen, farmers and people with history of chronic sunburn low phototype) (2). The cause is primarily sunlight-induced DNA damage and failure to repair these genetic alterations which leads to deregulated epithelial growth and tumour formation as well as ultraviolet radiation (UVR) induced cutaneous immunosuppression caused by mutation of tumour suppressor gene, p53. Clonal expansion of mutated cells leads to the development

Key words: actinic keratosis, cutaneous human papillomaviruses, phototype, skin cancer
of AK. Solar radiation induces acute and chronic reactions in human and animal skin. Among types of solar radiation, ultraviolet B (290-320 nm) radiation is highly mutagenic and carcinogenic in animal experiments compared to ultraviolet A (320-400 nm) radiation. UVA-UVB radiations cause suppression in the immune system (natural killer cells, lymphocyte T helper, antigen presenting cells, APC); in particular UVB radiation induces the proliferation of T suppressor lymphocytes and also modifies the antigen presentation by APC cells. At molecular level, radiation activates p-53 and p-21 (pro-oncogenic-gene) (3). In addition, mutations in p53, ras and patched genes of non-melanoma skin cancer cells (NMSK) are induced by solar UV radiation (3). Other factors such as genetic (e.g. race), occupational, or chronic exposure to chemicals (e.g. tar, arsenic, etc.) increase cancer risk. The clinical aspects of AK are various and usually the disease is not invalidating for the patient. Early treatment of these lesions is important because 20% of AK lesions evolve into squamous carcinoma; usually the first sign of progression is the presence of inflammation which is associated to a loss of differentiation leading to malignancy (4). Other well-known risk factors towards cancer progression include chronic UVB exposure, skin phototype, painful sunburns before the age of 20 years, immunosuppression and genetic predisposition (1). Many studies suggest a role of infection by human papillomaviruses (HPV) in NMSC development (5). The association between HPV and NMSC was first identified in patients with epidermodysplasia verruciformis (EV) (6) and later in recipients of organ transplants (7). Recent studies have analyzed skin lesions removed from immunosuppressed renal transplant recipients and HPV DNA was detected in 78% of these lesions, including 60% of Basal Cell Carcinomas (BCC), 82% of AK and 79% of Squamous Cell Carcinomas (SCC) (8).

Other studies have reported low viral loads in SCC, basal cell carcinomas and in perilesional tissue, but viral load in AK is significantly higher than in NMSC. These data suggest that persistence of HPV is not necessary for the maintenance of the malignant phenotype of individual NMSC cells (9). Although a passenger state cannot be excluded, the data are compatible with a carcinogenic role of HPV in early steps of tumour development (3). AK regression often occurs when sun exposure is decreased.

AK treatment options include cryosurgery, curettage and excisional surgery, dermabrasion, chemical peels, laser resurfacing, 5-fluorouracil (5-FU), imiquimod, diclofenac, and tretinoin, each with advantages and limitations (10). These methods are quickly resolutive and post-operative recovery is rapid in healthy subjects. Usually invasive surgical treatment methods are used when the patient is young and the diffusion of the lesions is limited. Topical treatments are preferred in older patients (with cardiopathies, dismethylabolic disorders, diabetes etc.) where surgical ablation could cause some risks. All AK lesions should be treated because it is impossible to predict which of them will evolve into squamous cell carcinoma requiring expensive and invasive therapy (2).

The aim of this work is to verify the presence of HPV DNA in actinic keratoses and in normal skin samples collected from dermatological healthy subjects in Italy, in order to correlate the severity of the clinical evolution of the lesions with the HPV DNA presence.

MATERIALS AND METHODS

Sample collection

Biopsies were collected from patients with actinic keratosis lesions and from normal skin subjects (all Italian individuals, Caucasian race) in the Dermatological Clinic of “Campus Biomedico”, University of Rome. Punch biopsies were taken after rubbing the skin with alcohol swabs in order to remove the superficial cells. Samples were maintained at -20°C until analysing for HPV DNA searching in the Section of Virology of “Sapienza” University of Rome.

A single questionnaire was administered for history of skin lesions, sun exposure, immunosuppression, current systemic and topical therapy. Data on age, skin phototype, symptoms and localization of the skin lesion were also collected.

HPV DNA detection

DNA extraction

The cutaneous biopsies were suspended in lysis buffer containing 200-250 µg/ml of K proteinase and incubated at 55°C, overnight. DNA was extracted with NucleoSpin Tissue kit (Machery Nagel), according to the manufacturer’s recommendations, quantified and then
Table I. Characteristics of the groups studied.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Nº</th>
<th>Mean age</th>
<th>Gender Number and (%)</th>
<th>Phototype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Female</td>
<td>2</td>
</tr>
<tr>
<td>AK</td>
<td>54</td>
<td>69.24 ± 11.16</td>
<td>18 (33.33%)</td>
<td>42.6%</td>
</tr>
<tr>
<td>Healthy skin</td>
<td>30</td>
<td>59.06 ± 13.99</td>
<td>16 (53.33%)</td>
<td>60%</td>
</tr>
<tr>
<td>Total</td>
<td>84</td>
<td></td>
<td>36 (66.66%)</td>
<td>57.4%</td>
</tr>
</tbody>
</table>

Table II. HPV genotypes in AK related to lesion localization and patient phototype.

<table>
<thead>
<tr>
<th>HPV Type</th>
<th>Nº of cases</th>
<th>Lesion localization</th>
<th>Phototype^</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Face</td>
<td>Head</td>
</tr>
<tr>
<td>HPV-5</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>HPV-9</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HPV-12</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HPV-19</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HPV-21</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HPV-23</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>HPV isolate FA 12.2</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HPV isolate FA 13</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HPV isolate FA 16.1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HPV isolate FA 23.2</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HPV isolate FA 34</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HPV isolate FA 98</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HPV isolate FA 118</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HPV isolate FA 127</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HPV isolate FA 131</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>NT*</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

*2 samples were not typed (NT) exactly, probably due to a mixed infection.

^Phototype classification according to ICNIRP (13).
used for amplification.

**PCR conditions**

PCR assay for HLA gene was performed on extracted DNA to assess the efficacy of nucleic acid extraction; general precautions, conditions for PCR analysis were as published (11). Briefly, 100 ng purified DNA were used in 50 μl PCR reactions run in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Emeryville, CA), and all assays included positive (HPV5 DNA) and negative controls (all the PCR components except the template) to exclude false-positive and false-negative results (11). Each reaction was carried out on 100 ng of cellular DNA extracted from cutaneous biopsy samples in a total volume of 50 μl, using 0.3 units of AmpliTaq DNA polimerase (Perkin Elmer-Cetus, USA) in the supplied buffer, 25 pmol of each primer, 200 μM each deoxynucleotide triphosphate (dNTP) and sterile water. The PCR products were detected by ethidium bromide staining after electrophoretic migration through 2% agarose gel.

The amplification of 440-480 bp fragments from L1 region of HPV DNA was performed using the FAP PCR system able to amplify a broad spectrum of HPV types from skin. FAP-PCR conditions were assessed as previously described (12). Cycling conditions were as follows: 94°C x 3 min and 45 cycles of 94°C x 90sec, 50°C x 90 sec, 72°C x 90sec; 72°C x 7 min.

**HPV genotyping**

PCR products corresponding to proper fragments were purified with QIAquick PCR purification kit, according to QIAGEN protocol. DNA sequencing was performed by automatic DNA sequencer (Applied Biosystem, mod. 370A), according to the manufacturer’s specifications (Amplicycle Kit, Applied Biosystem). Sequence homology was determined by BLASTN program.

**RESULTS**

Skin lesion biopsies from 54 AK patients and 30 normal skin control subjects were collected as previously described. All the biopsies were

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**Fig. 1.** Force subtree obtained analysing the sequence of HPV amplified from an AK sample (AK seq) by BLASTN: the Jukes-Cantor method was used to calculate the distance. Completely sequenced HPV types are underlined.
performed in sun exposed areas of the body (28 in the scalp, 45 in the face, 7 in the thorax and 4 in the limbs). The characteristics of the two groups are depicted in Table I. According to the 6 phototypes indicated by the International Commission on Non-Ionizing Radiation Protection (ICNIRP) (13), 42.6% of AK patients showed a type 3 skin phototype (moderate susceptibility and medium tan) and all the others phototype 2 (high susceptibility and light tan) and the control group was 40% prototype 3 and the remaining prototype 2. None of the samples was collected from immunosuppressed people although many of them were old (61 subjects >60 years old). All the subjects were living in Rome or in its suburban areas and they referred the habit of sea or mountain sun exposure: 14.81% of AK patients and 16.66% of the control group preferred the mountains whereas all the others were exposed to sea sun. Generally, all the subjects referred the discontinuous or insufficient use of sunscreens while sun exposure had been continuous and abundant during the years, but none of them was professionally exposed to UV or other chemical risk factors for NMSC.

The HPV DNA test did not reveal any positive samples in the control group, whereas 20 out of 54 AK samples (37.03%) resulted positive for cutaneous HPV (p<0.001).

Many different HPV genotypes have been found by direct sequencing of PCR products (Table II). In 50% of the cases cutaneous HPV variants, to date not completely characterised, have been identified. The sequence homology between our samples and the data bank isolates (designated as FA types on the basis of about 430 nucleotides amplified with the FAP primers) was so high that they are likely the same variants. A branch of the phylogenetic tree obtained from the sequence analysis of a positive sample is reported in Fig. 1, where it is possible to observe the divergence in nucleotide identity of variants in respect to the completely sequenced genome of classified HPV types.

The HPV positive AK were usually clinically indistinguishable from the HPV negative ones, thus all AK lesions were removed by CO$_2$ laser. This therapeutic approach was chosen because in our experience CO$_2$ laser treatment is less traumatic for the patients, more resolutive than conventional surgery and post-treatment scarring is rare. The follow-up visits were programmed after 45-60 days; at that time the HPV positive AK lesions reappeared in all patients whereas the HPV negative ones disappeared after one laser treatment (p<0.0001).

In HPV positive patients clinical history revealed the presence of cutaneous persistent itchy scales sometimes bleeding lesions, usually present throughout the years and localised on the face or the head. These data might suggest that the presence of HPV DNA could be an aggravating factor for AK lesion severity and recurrence.

**DISCUSSION**

AK is usually found on chronically exposed skin areas. Visual inspection and palpation reveal a small keratotic at times inflammatory scaly lesions varying from erythematosus cornified lesion to plain inflammatory scaly plaques at times slightly bleeding and itchy without any other clinical symptoms. AK evolves into squamous cell carcinoma (SCC) usually over a long period of time (5-10 years). Clinically the disease progression is visible as an induration and infiltration of the lesion which begins to bleed and/or ulcerate.

The main cause of AK lesions is exposure to solar irradiation (14). UV irradiation acts both as direct damage in DNA and reducing the patient’s ability to repair damaged DNA in the skin cells. UVB (280-320 nm) are the most dangerous wave lengths which induce DNA damage and promote atypical disorder of keratinisation. Occupational exposure to chemical substances such as coal tar, petroleum products, cyclophosphamide herbicides, insecticides, polycyclic aromatic hydrocarbons, increases the risk of cancer following high dermal exposure (15-16).

Infectious agents may contribute to induce cancer promoting cell proliferation by integration of viral oncogenes into host cell DNA and by the production of proteins that inactivate the functions of tumour suppressor genes. The role of HPV DNA presence and persistence in the development of cervical or anogenital cancer is already well established (17), whereas in skin cancer it is not clearly defined; recent studies suggest their implication as an important factor also in skin carcinogenesis by the detection of transcriptional activity of cutaneous
HPV E6/E7 genes (18). Other studies indicated the role of HPV in inhibition of UV-induced apoptosis, thus allowing UV-damaged cell proliferation (19-20). Moreover, the presence of HPV DNA seems to be higher in cancer precursor lesions. Discordant data on the presence of cutaneous HPV in AK have been reported according to the geographical areas of specimen collection and of the method of sampling (21-22): our results are similar to those found in Australia (23) where the percentage of HPV positive keratosis samples was 43%, whereas in European (Swedish and Austrian) patients the percentage was highly variable considering the 83% positivity of swab samples versus 11% of stripped biopsies (24). The absence of HPV DNA in healthy skin samples is not surprising considering the energetic rubbing before biopsy collection (in order to eliminate contaminating factors) and the low percentage of HPV positivity (11%) found by other Authors in AK stripped biopsies (24).

The detection of new variants of known cutaneous HPV and the phylogenetic analysis of beta-papillomaviruses (isolated from humans, apes and monkeys) indicate a genetic heterogeneity of this branch of the tree: probably multiple invasions across species borders may contribute to their diversification (25).

The precise mechanism of cutaneous HPV induced transformation of skin cells is still not completely elucidated. Perhaps different HPV types utilise various transforming pathways. Binding sequences for p53 had been identified in the E6 gene of some cutaneous HPV (e.g. HPV77) and it was demonstrated that the HPV oncogene expression was increased by UV irradiation (26).

Since a broad spectrum of cutaneous HPV has been detected in NMSC it would be interesting to exactly identify the HPV genotypes present in the different stages of the lesions in order to relate specifically those HPV which are more frequently implicated as co-factors in neoplastic evolution. Interestingly, the percentage of positivity for HPV5 (one of the cutaneous types considered at high risk due to its correlation with EV) in this study is 14.8%, close to the evaluated progression of AK to NMSK (27-28)

According to our data, HPV positive AK are prone to recurrence and hence more frequent follow-up visits should be recommended to the patients. The detection of HPV DNA could therefore be helpful in the early diagnosis and treatment of evolving cutaneous lesions.

In our opinion CO2 laser should be still considered the first treatment choice also in recurrences, considering the frequent face localization of the lesions and the possibility to eliminate them without scarring even after several treatments.

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