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# UNIVERSITÀ DEGLI STUDI DI TORINO

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## **Analysis of human beta papillomavirus and Merkel cell polyomavirus infection in skin lesions and eyebrow hair bulbs from a cohort of chronic lymphocytic leukaemia patients**

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**Short title:**  $\beta$ -HPV and MCPyV infection in skin lesions from CLL patients

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**What's already known about this topic?**

Individuals with compromised immune surveillance, including organ transplant recipients (OTRs) and chronic lymphocytic leukaemia (CLL) patients, are at increased risk of developing skin cancer.

Active beta papillomavirus ( $\beta$ -HPV) infection has been demonstrated in skin lesions from OTRs.

**What does this study add?**

Detection of  $\beta$ -HPV and Merkel cell polyomavirus DNA in the skin lesions and hair bulbs from CLL patients without any evident reactivation at skin tumour sites indicates a coincidental rather than causal infection.

## Summary

*Background.* Research consistently demonstrates an increased incidence of skin cancer in the immunocompromised host, including patients with chronic lymphocytic leukaemia (CLL) and organ transplant recipients (OTRs). Active human beta papillomavirus ( $\beta$ -HPV) infection has been found in OTR skin lesions, suggesting their possible involvement in skin carcinogenesis. Although less frequent, Merkel cell polyomavirus (MCPyV) has also been reported in cases of skin cancer.

*Objectives.* To investigate: i) potential correlations between patient clinical features and skin cancer development; and ii) the presence of  $\beta$ -HPV and MCPyV DNA, and viral protein markers in skin lesions and hair bulbs from CLL patients.

*Methods.* The clinical features of 293 patients with CLL were analysed according to the presence or absence of skin lesions.  $\beta$ -HPV and MCPyV infection was investigated in skin lesions and hair bulbs from the study cohort by both PCR analysis and immunohistochemical screening.

*Results.* No significant correlations were observed between any of the analysed haematological parameters and the development of skin cancer. PCR analysis revealed the presence of  $\beta$ -HPV and MCPyV DNA in skin tumours, and 83% of positivity for MCPyV DNA in hair bulbs, while systematic immunohistochemical analysis of all the lesions failed to detect any expression of the viral proteins  $\beta$ -HPV E4, L1 or MCPyV LTA<sub>g</sub>.

*Conclusion.* Overall, our data indicate that carriage of  $\beta$ -HPV and MCPyV in the lesional skin and hair bulbs from CLL patients, without any evident reactivation at skin tumour sites, represents coincidental rather than casual infection. This contrasts with our previous findings in relation to OTR-derived skin lesions.

## Introduction

Chronic lymphocytic leukaemia (CLL) is one of the most common adult haematological malignancies.<sup>1</sup> CLL patients exhibit a status of chronic immunodeficiency that shows some similarities to that typically observed in solid organ transplant recipients (OTRs) treated with immunosuppressive agents.<sup>2</sup> Specifically, the risk of developing skin cancer is increased 8- to 13-fold in CLL patients and 65- to 250-fold in OTRs, making cutaneous neoplasms the most frequent type of malignancy reported in both settings.<sup>2-4</sup>

An increasing body of evidence suggests that human beta papillomaviruses ( $\beta$ -HPVs) are implicated in the pathogenic mechanisms underlying the development of skin cancer in patients with immune dysfunction.<sup>5</sup>  $\beta$ -HPVs (e.g., HPV5 and 8) are evolutionarily distinct from the genus

Alpha and appear to cause widespread unapparent or asymptomatic infections in the general population. The relationship between  $\beta$ -HPV infection and skin cancer has been clearly defined in patients with congenital immune defects, including epidermodysplasia verruciformis (EV), an autosomal recessive disease characterised by a predisposition to infection by specific  $\beta$ -HPV types.<sup>6,7</sup> Despite these findings, a causal role of these viruses has been difficult to verify in non-EV patients because of their ubiquitous prevalence in the general population and their absence in some cancers. In recent studies, we demonstrated productive  $\beta$ -HPV infection in actinic keratosis and the adjacent pathological epithelium in skin cancer lesions from OTRs, thus pointing to  $\beta$ -HPV reactivation in this setting and its possible involvement in the process of skin carcinogenesis.<sup>8</sup>

A causal role of Human Polyomaviruses (HPyVs), another family of small DNA tumour viruses, in the pathogenesis of skin cancer has also been envisaged.<sup>9</sup> DNA of a novel HPyV, namely Merkel Cell Polyomavirus (MCPyV), was detected in the majority of investigated cases of Merkel cell carcinoma (MCC), a rare form of neuroendocrine carcinoma of the skin.<sup>9</sup> MCPyV DNA has also been reported in skin cancer cases from both immunocompetent and immunosuppressed patients (OTRs).<sup>9,10</sup>

The aims of this study were i) to correlate patient clinical features with the presence or absence of skin cancer in a cohort of 293 CLL patients; and ii) to evaluate the presence of  $\beta$ -HPV and MCPyV infection in CLL patient skin lesions and hair bulbs using both PCR analysis and immunohistochemistry.

## **Materials and methods**

### **Study population and skin tumour specimens.**

This study was based on a consecutive series of 293 CLL patients admitted to the Division of Haematology between June 1985 and February 2011. The mean patient follow-up period was 78 months. All patients provided informed consent in accordance with the local institutional review board requirements and Declaration of Helsinki guidelines. CLL diagnosis and the analysis of clinical and biological variables were performed as previously described.<sup>11</sup>

### **$\beta$ -HPV and MCPyV DNA detection**

DNA extraction from FFPE blocks and hair samples was performed as previously reported.<sup>6</sup> A nested PCR method was used to detect  $\beta$ -HPV DNA.<sup>12</sup> For  $\beta$ -HPV genotyping, second-step PCR amplicons were purified and sequenced after cloning using the CloneJET PCR Cloning Kit

(Fermentas). For MCPyV DNA analysis, MCV-qPCR primers that amplify a 177 bp product within the *stAg* gene were used in an adapted protocol previously described.<sup>13</sup>

### **Immunohistochemistry**

Consecutive 5 µm sections, cut from FFPE skin biopsy blocks, were dewaxed and rehydrated. Polyclonal antibodies against  $\beta$ -HPV E4 and L1 and the staining procedures used are described elsewhere.<sup>7,8</sup> For MCPyV LTA<sub>g</sub> detection, we used the primary mouse monoclonal antibody CM2B4 (Santa Cruz Biotechnology).<sup>14</sup> For assessment of histological features, hematoxylin and eosin staining was performed.

### **Statistical analysis**

Categorical and continuous variables were analysed as previously described.<sup>11</sup> Statistical significance was considered for  $p \leq 0.05$ .

## **Results**

The clinical features of the study population, stratified according to the presence or absence of skin cancer, are presented in Table 1. Forty of the 293 CLL patients (14%) developed at least one skin lesion, with a total number of 96 lesions identified. Histologically, the skin cancer lesions were diagnosed as follows: squamous cell carcinoma (SCC),  $n=16$ ; basal cell carcinoma (BCC),  $n=35$ ; actinic keratosis (AK),  $n=17$ ; Bowen's disease (BD),  $n=3$ ; keratoacanthoma (KA),  $n=3$ ; seborrheic keratosis (SK),  $n=13$ ; wart,  $n=2$ ; non-epithelial tumours,  $n=6$  (melanoma,  $n=2$ ; fibroxanthoma,  $n=1$ ; Kaposi's sarcoma,  $n=3$ ). The proportion of males was significantly higher among patients with skin cancer. No significant correlation was found between any of the analysed haematological parameters and the presence or absence of skin cancer. Skin cancer developed after CLL diagnosis in 29 patients, with a mean time from CLL diagnosis of 63 months; while 11 patients were diagnosed with skin cancer prior to CLL diagnosis (mean time, 80 months). The majority of skin lesions occurred in sunlight-exposed body sites, mostly the head and neck region ( $n=51$ , 53%).

DNA was extracted from at least one biopsy for each histological type per patient, providing a total of 50 samples, and subjected to  $\beta$ -HPV genotyping. As shown in Table 2, the majority of the benign lesions (namely warts and SK) showed higher percentage of positivity in comparison with tumours with an overall positivity of 38%.

Next, to investigate whether productive  $\beta$ -HPV infection could be visualised by means of viral protein detection (as previously reported for skin lesions from OTRs), tissue sections from all 50 FFPE blocks were stained with antibodies against  $\beta$ -HPV E4 and L1 proteins. No lesion

displayed any positivity for either marker and tissue histology did not reveal any virus-related cytopathic effects. The same samples were also screened for MCPyV DNA by PCR analysis. Eleven specimens (22%) were positive, distributed among the lesion types as follows: 6 AK, 2 KA, 1 SCC, 1 BD and 1 wart. Once again, all the specimens were screened for the expression of viral protein MCPyV LTA<sub>g</sub>, but none of them displayed any positive immunostaining for this viral marker.

Since plucked eyebrow hair bulbs were also available for 46 patients of the study population (14 with and 32 without skin lesions), we extracted the DNA and ran PCR analysis for MCPyV and  $\beta$ -HPV DNA, applying an approach similar to that used for the skin biopsies. MCPyV DNA was found in 38 specimens (83%), while  $\beta$ -HPV DNA was present in all of them.

## Discussion

The biological aggressiveness of skin cancer with respect to multiplicity and recurrences was confirmed in this CLL series, since 50% of the patients with skin cancer experienced multiple lesions (up to 18 in one case), and one patient developed a multi-relapsing BCC (5 relapses) on the forehead that was treated by Mohs surgery.

While PCR analysis revealed the presence of  $\beta$ -HPV and MCPyV DNA in a significant proportion of tumours (38% and 22% respectively), systematic immunohistochemical analysis of all the lesions failed to detect any expression of the viral proteins  $\beta$ -HPV E4, L1 or MCPyV LTA<sub>g</sub>. In addition, the prevalence of MCPyV DNA in hair bulbs was higher than those reported in HIV patients (83% v.s. 50% respectively).<sup>15</sup>

Using antibodies against  $\beta$ -HPV E4, L1 or MCPyV LTA<sub>g</sub>, we showed that all the skin lesions from our study cohort of CLL patients were negative indicating that when the viral DNA was found in these tumours it was most likely a coincidental infection consistent with their ubiquitous asymptomatic presence in the general population. Although skin cancer arising in CLL patients may share some common risk factors with OTRs, they do not seem to be associated with  $\beta$ -HPV infection as reported in OTRs.<sup>8</sup>

## Supporting Information

**Supplementary materials and methods:** Diagnosis of chronic lymphocytic leukaemia (CLL);  $\beta$ -HPV and MCPyV-DNA detection in formalin-fixed paraffin-embedded (FFPE)



biopsies and plucked eyebrow hair bulbs; Supplementary references.

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