α- and β-Papillomavirus infection in a young patient with an unclassified primary T-cell immunodeficiency and multiple mucosal and cutaneous lesions

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Abstract: Background The correlation between human papillomavirus (HPV) genotype along with their histopathological and clinical features of skin lesions (from genital and non-genital sites) can present a diagnostic challenge.
Objective In this study, the correlation of HPV infection patterns with pathology and clinics was investigated in lesional and non-lesional body sites from a young patient with primary T-cell immunodeficiency.
Methods HPV infection was evaluated at both DNA and protein levels by PCR and immunohistochemistry.
Results Patient's genital lesions were exclusively caused by alpha-genotypes (high-risk type HPV51 in the anal and low-risk type HPV72 in the penile condylomas); the opposite was true for the skin lesions, which were infected by beta-genotypes only (HPV8 and 24); of which, HPV24 was the predominant type in terms of viral loads and the only one found in productive areas of infection. The patient had already developed high-grade dysplasia in the anal condylomas and showed areas of early stage dysplasia in the lesions caused by the beta-genotype HPV24.
Limitations The basic etiology of the immunodeficiency is not yet defined
Conclusion These findings provide proof of principle that both alpha and beta-genotypes can cause overt dysplastic lesions when immunosurveillance is lost, which is not restricted to Epidermodysplasia verruciformis.

1 **Alpha- and Beta-Papillomavirus infection in a young patient with an unclassified primary T-**
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Alpha- and Beta-Papillomavirus infection in a young patient with an unclassified primary T-cell immunodeficiency and multiple mucosal and cutaneous lesions.

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Capsule summary

The association between β-HPV infection and skin cancer has been established in Epidermodysplasia verruciformis.

This study provides correlations between clinics, pathology and HPV infection patterns for both α and β-genotypes in the skin lesions from a patient with an unclassified primary T-cell immunodeficiency.

Understanding of the natural history and the molecular and cellular pathogenesis of β-HPV-induced skin lesions will aid the development of new diagnostic interventions to predict skin cancer risk in the immunocompromised host, thus not restricted to Epidermodysplasia verruciformis.

1   **Alpha- and Beta-Papillomavirus infection in a young patient with an unclassified primary T-**

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26   **Abstract**

27

28   **Background.** The correlation between human papillomavirus (HPV) genotype along with the

29   histopathological and clinical features of skin lesions (from genital and non-genital sites) can

30   present a diagnostic challenge.

31   **Objective.** In this study, the correlation of HPV infection patterns with pathology and clinics was

32   investigated in lesional and non-lesional body sites from a young patient with a primary T-cell

33   immunodeficiency.

34   **Methods.** HPV infection was evaluated at both DNA and protein levels by PCR and

35   immunohistochemistry.

36   **Results.** Patient's genital lesions were exclusively caused by α-genotypes (high-risk type HPV51 in

37   the anal and low-risk type HPV72 in the penile condylomas); the opposite was true for the skin

38   lesions, which were infected by β-genotypes only (HPV8 and 24); of which, HPV24 was the

39   predominant type in terms of viral loads and the only one found in productive areas of infection.

40   The patient had already developed high-grade dysplasia in the anal condylomas and showed areas

41   of early stage dysplasia in the lesions caused by the β-genotype HPV24.

42   **Limitations.** The basic etiology of the immunodeficiency is not yet defined.

43   **Conclusion.** These findings provide proof of principle that both α and β-genotypes can cause overt

44   dysplastic lesions when immunosurveillance is lost, which is not restricted to Epidermodysplasia

45   verruciformis.

46

INTRODUCTION

Primary immunodeficiencies (PIDs) comprise a rare group of genetic disorders associated with an enhanced susceptibility to specific infections and, in certain cases, an increased incidence of malignancy.[1] Immune dysregulation leads to the reduced clearance of viruses, including human papillomavirus (HPV), which causes proliferative lesions in genital and skin sites that can also progress to cancer.[2-4]

To date, more than 150 HPV types have been completely sequenced, classified into five genera (α, β, μ, ν and γ) and a series of intragenus species, indicated by Arabic numbers, based on sequence analysis; the different types having different life-cycle characteristics and disease associations.[5,6] The most medically important HPVs belong to the genus α, which is divided into cutaneous (which cause common warts) and mucosal types; the mucosal types are further subdivided into high-risk (e.g.HPV16 and 18) and low-risk (e.g. HPV6 and 11) according to their propensity to cause cancer.[7,8] In recent years, it has become clear that many HPV types, including those contained within the β-genus, only result in asymptomatic infections in immunocompetent individuals.[9-10] However, in subjects with impaired immune function, they can cause cutaneous lesions that may become difficult to manage and in some circumstances progress to cancer.[11-13] Specific susceptibility for HPV infection has been extensively reported in patients with Epidermodysplasia Verruciformis (EV)[14-18] and warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome.[4,19,20] EV is a genodermatosis characterized by an increased susceptibility to cutaneous infections with β-genotypes. EV is thought to be an autosomal recessive disease; however, homozygous mutations in *EVER1* or *EVER2* have been identified in approximately 75% of patients clinically diagnosed with EV, leaving a considerable proportion of patients with an unexplained genetic cause. [21-23]

WHIM patients also display a specific and poorly understood susceptibility to α-HPV-induced warts.[1,19,20] Condilomas in the genital region and genital cancers, always caused by α

72 genotypes, have also been reported in these patients. WHIM syndrome is inherited in an autosomal

73 dominant fashion and is caused primarily by heterozygous gain-of-function mutations in the gene

74 encoding the chemokine receptor *CXCR4* .[24,25]

75   More recently, patients with T cell defects associated with mutations in *RHOH* and *MST1*

76 genes have been reported to display an increased susceptibility to β genus HPV infections.[26,27]

77   This study provides correlations between clinics, pathology and HPV infection patterns for

78 both α and β genotypes in the skin lesions of a patient with an unclassified primary T-cell

79 immunodeficiency.

80

81 **MATERIALS AND METHODS**

82 *Genetic analysis*

83   Genomic DNA was extracted from the patient's whole blood samples using the Gentra

84 Puregene Blood Kit (Qiagen). All the coding exons and boundary introns of *EVER1* and *EVER2*

85 genes were amplified as previously described,[12] and the products were sequenced by Primm S.r.l..

86 Each electropherogram was analyzed using the program Chromas Lite, version 2.01 to detect

87 mutations.

88 The genetic analysis of *CXCR4, RHOH*, and *MST1* genes was carried out as previously

89 described.[24,26,27] The PCR products were sequenced using the BigDye Terminator Kit and the

90 sequences analyzed on a 3130 Genetic Analyzer (Applied Biosystems). Written informed consent

91 was obtained by the patient according to the Declaration of Helsinki and approval was obtained

92 from local ethic committee.

93 *FACS analysis*

94   Flow cytometry was performed as previously described.[24] Briefly, Peripheral Blood

95 Mononuclear Cells (PBMC) (1.5×10^6) were resuspended in 200 μl of the appropriate medium with

96 CD3, CD4, CD8, CD145RA, CD45R0, CD31, CCR7, anti-HLA-DR mAbs (5 µg in 200 µl) from

97 Beckton Dickinson..

98 *HPV-DNA detection and Quantitative real-time PCR (Q-PCR)*

99   Swabs and hair bulbs were taken and processed as previously described.[16-18,22,23] α-HPV-

100 DNA genotyping was performed using the CLART® (Clinical Array Technology) Human

101 Papillomavirus 2; Genomica, Madrid, Spain.

102   β-HPV-DNA analysis was performed as previously described[16] using broad spectrum PCR

103 (PM-PCR) in combination with a reverse hybridization system (RHA) [Skin (beta) HPV assay;

104 Diassay BV, Rijswijk, The Netherlands].[28]

105   Type-specific real time Q-PCR protocols were performed on a CFX96 (Biorad) using

106 previously described primers for HPV8, 24[16,29] or the newly designed primers for HPV16, 18, 51, 61,

107 72 (sequences available on request). HPV DNA copy numbers were determined using standard

108 curves as previously described.[16]

109 *DNA-protein (FISH) or protein-protein (IF) double detection or IHC and antibodies*

110   The polyclonal antibodies raised against beta genus HPVE4 and L1 have been previously

111 described (CB & MG manuscript submitted).[17] For anti-E4 and beta L1 costaining, an anti-HPV5E4

112 monoclonal antibody was used. Antibodies to alpha genus L1 were obtained from Dako, MCM7

113 from Neomarkers Fremont, and p16[INK4a] from Santa Cruz Biotechnology.

114   Consecutive 5-µm sections obtained from FFPE tissues were processed for the

115 immunofluorescent detection of viral antigens coupled to DNA-FISH, or for protein-protein double

116 detection as previously described.[16,17,30]

117

118 **RESULTS**

119   The 26-year-old Caucasian male (born 1987) revealed multiple flat, reddish papular (wart-

120   like) lesions across his whole body (Figure 1), with the highest density on the dorsum and forearms;

121   and numerous penile and anal condylomas were also evident (Figures 2 and 3). He is HIV negative.

122   *Immunophenotype abnormalities are compatible with T-cell lymphocytopenia*

123   Immunophenotype analysis of the patient's peripheral blood mononuclear cells (PBMCs)

124   revealed marked lymphopenia with depletion of CD4 at levels as low as 250 cells/ml. In addition,

125   analysis of CD4 subsets revealed a marked reduction of naïve CD4+ CD45RA+CCR7+ cells (3.3%)

126   and of the recent thymic emigrant subset (RTE), (CD45RA+CCR7+CD31+: 1.3%), while central

127   memory (CD45RA-CCR7+: 60.5 %) and effector memory T cells (CD45RA-CCR7-: 36%) were

128   proportionally increased. Likewise, naïve CD8+ cells were decreased with a relative increase in

129   central memory and effector CD8+ cells, indicating a depletion of the naïve compartment for both

130   CD4 and CD8 cells. Analysis of HLA-DR expression by T cells showed that about 50% of them

131   display an active phenotype. The patient's B cells were found to make up 1.3% of total

132   lymphocytes; neutrophils and immunoglobulin levels were in the normal range.

133   *Absence of mutations in genes known to be associated with similar PIDs*

134   Genomic DNA extracted from the patient's blood was used to perform genetic analysis of

135   genes associated with EV, such as *EVER*1 and *EVER2*,[21] or with immunodeficiencies characterized

136   by susceptibility to HPV infections, including *CXCR4, RHOH*, and *MST1*.[24,26,27] Sequence analysis

137   of these genes did not reveal any causative mutation.

138   *Alpha versus Beta genotype distributions*

139   The DNA extracted from swabs obtained from the skin of either affected or unaffected sites,

140   and from plucked eyebrow or inguinal hair bulbs was analyzed by PCR and real time Q-PCR for α

141   and β-HPV genotypes. As shown in Table 1, four α-genotypes were found in hair bulbs from both

142   sites with a very low viral load. By contrast, only HPV8 and 24 β-genotypes were found in these

143   sites with the highest load values reported for HPV24 in affected skin areas (up to 6×10^3

144 copies/cell). In the swabs from the anal condylomas surface, HPV51 gave high viral loads (228

145 copies/cell) followed by HPV61 and 72 (both considered low-risk α-genotypes). HPV72 was also

146 detected in the swabs from penile condylomas. Overall, the patient showed a very clear and

147 consistent HPV signature defined by two β-genotypes, HPV8 and 24, the α-genotypes HPV51 and

148 72 with high viral loads and traces of HPV16, 18, and 61.

149 *Comparison of Alpha versus Beta viral life cycle and their differential modulation of cellular*

150 *markers*

151 Biopsies from anal, penile condylomas, and two wart-like lesions of the skin were available

152 as formalin-fixed paraffin-embedded (FFPE) blocks. To gain further insight the infection pattern

153 and visualize viral life cycle events of α versus β-genotypes, tissue sections from these blocks were

154 co-stained by immunofluorescence (IF) for anti-E4 and anti-L1 antibodies to characterize the

155 expression of viral antigens and for antibodies raised against minichromosome maintenance protein

156 7 (MCM7), a marker of cellular proliferation.[31] Fluorescent *in situ* hybridization (FISH) was carried

157 out for the virus genotypes detected by surface sampling.[32] As shown in Figure 2a right hand

158 column, the anal condylomas showed areas with high-grade dysplasia that displayed p16^{INK4a}

159 staining across basal and suprabasal epithelial layers. FISH analysis for the HPV51 genome

160 revealed many positive nuclei throughout the entire lesion, especially in the areas with lower grade

161 of dysplasia, while HPV16, 18, 61, and 72 genomic probes gave negative results (data not shown).

162 Expression of the late capsid protein L1 was also detected in the superficial layers. As reported for

163 cervical cancer induced by high-risk α-genotypes (e.g. HPV16 and 18), a massive increase of E2F-

164 activated genes was revealed, as visualized by staining for the cellular MCM7 protein, which

165 extended throughout the entire epithelium.[7]

166 Figure 2b, shows the histological features of the penile condylomas which revealed

167 hyperplasia and low-grade dysplasia with many HPV72-FISH-positive nuclei, while FISH analysis

168 for HPV16, 18, 51, and 61 genomes was negative as was p16^{INK4a} staining (data not shown). The

169 MCM7 signal was only being apparent in the upper epithelial layers.[7,31]

170   A different staining pattern was visualized in the cutaneous lesions. As shown in Figure 3,

171 the epithelium of the flat wart-like lesions displayed the unequivocal histological features associated

172 with HPV infection by cutaneous genotypes. In these areas, co-immunostaining of HPV24-DNA by

173 FISH and E4 by immunofluorescence revealed the presence of many cells exhibiting intense

174 HPV24 DNA-positive nuclei and cytoplasmic E4 staining. In contrast, viral genome amplification

175 was no longer detected in the central dysplastic area by FISH, while cytoplasmic E4 expression was

176 still present in the more superficial layers. FISH analysis for HPV8 was negative, as it was also for

177 HPV51 and 72 (data not shown). MCM7 expression was increased in the lesion in comparison with

178 the adjacent normal epithelium, and was well evident in the basal layers, extending into the

179 suprabasal layers in the productive areas and to a higher extent in the dysplastic central area.

180 p16^{INK4a} staining was negative throughout the entire lesion (data not shown). Expression of the

181 major coat protein L1 occurred in a subset of E4-positive cells in the upper epithelial layers in the

182 areas displaying FISH-positive nuclei, while it showed an aberrant cytoplasmic expression in the

183 mid-superficial layers in the central dysplastic area.

184

185 **DISCUSSION**

186   The present study describes a case of primary T-cell immunodeficiency with a remarkable

187 and specific susceptibility to HPV infections, who does not carry any of the genetic mutations

188 currently associated with EV,[21] and WHIM syndrome.[25] He has a T-cell defect characterized by

189 abnormally low numbers of naïve T cells (affecting both the CD4+ and CD8+ compartments), very

190 likely due to a developmental defect of T lymphocytes, and high numbers of memory T cells

191 presenting an exhausted phenotype that probably results from chronic viral infection. Despite some

192 commonalities with PIDs harboring mutations in *RHOH* and *MST1* genes, sequence analysis of

193 these genes did not reveal any causative mutation.[26,27] The lack of primary lymphedema also

194 excludes any correlation with WILD syndrome.[33] The patient suffered some recurrent bacterial

195 infections during his childhood; but since his teenage years, he has not had any major health

196 problems other than those resulting from the HPV infection. Another interesting feature of this

197 patient is that his susceptibility to HPV infection involves both cutaneous and genital sites. This is

198 very different from the situation in EV patients where susceptibility is considered to be restricted to

199 the β genus, as genital lesions caused by α genus have never been reported in this setting.[11,14,34]

200 Characterization of the HPV infection pattern by both PCR and immunohistochemistry (for

201 the viral proteins E4 and L1) in a number of lesional and non-lesional body sites revealed that the

202 patient's genital lesions were exclusively caused by α-genotypes (high-risk type HPV51 in the anal

203 condylomas and low-risk type HPV72 in the penile condylomas); the opposite was true for the skin

204 lesions, which were infected by β-genotypes only. Two β-genotypes were found, namely HPV8 and

205 24; of which, HPV24 was always the predominant type in terms of viral loads and the only one

206 found in productive areas of infection. These HPV24-induced skin lesions provide a good example

207 of the cytopathic effect caused by the β-genotypes, which display unique features compared with

208 those reported for the α-types: the lesions are characterized by enlarged cells with prominent blue-

209 grey pallor, perinuclear halos, and cytoplasmic granuli. Visualization of the E4 viral protein was

210 also confirmed as an invaluable marker for the detection of areas of productive infection for the β-

211 genotypes, as its expression consistently overlaps with areas of viral genome amplification as

212 detected by FISH.[35] Consistent with the data reported for cervical lesions caused by high-risk

213 genotypes (e.g. HPV16 and 18),[35,36] stimulation of cell cycle entry was very apparent in the basal

214 and above layers in the HPV51-positive condyloma (high-risk α-type), with many cells being driven

215 through mitosis. In the lesion caused by HPV72 (low-risk α-type), the stimulation of cell cycle entry

216 in the basal layers was much less obvious, and the MCM7 signal indicating cell cycle re-entry (but

217 not cell division) was only apparent in cells of the mid-epithelial layers where viral genome

218 amplification was shown to take place. The lower ability of low-risk HPV types to drive cell

219 proliferation is currently correlated with a lower incidence in neoplasia.[7] Consistent with this

220 finding, $p16^{INK4a}$ overexpression was only observed in the anal condylomas caused by HPV51.

221 In the HPV24-positive lesions, cytoplasmic E4 expression was constantly found in the areas

222 displaying clear-cut cytopathic effects that coincided with viral genome amplification and

223 expression of the late structural protein L1 in dying superficial cells, as has been reported for many

224 Papillomaviruses.[37] Of interest, in these productive areas, MCM7 expression was very strong and

225 always present in the basal and some of the above layers, indicating that cells were stimulated to

226 entry the cell cycle. In addition, a dysplastic area was found where MCM7 expression extended

227 throughout the epithelium in the absence of detectable viral genome amplification, but with E4

228 expression maintained in the superficial layers. This MCM7 staining pattern was closer to that of

229 the high-risk α−genotypes rather than low-risk types, indicating that β-HPV replication drives the

230 cells above the basal layer to enter the cell cycle in order to facilitate the amplification of its

231 genome.[31] This observed stimulation of basal cell proliferation may contribute, in association with

232 other transforming agents, such as UVB irradiation, to the transformation process.[11,12,14]

233 Although the patient was very young (26 years), he had already developed high-grade

234 dysplasia in some genital condylomas and also showed areas of early stage dysplasia in the skin

235 lesions caused by the β-genotype HPV24. These findings prompt us to propose the following

236 affirmations: i) symptomatic β-HPV infection of the skin is not restricted to patients harboring

237 EVER gene deficiencies, which are thought to be compromised at the keratinocyte level; ii) β-HPV

238 susceptibility is primarily associated with loss of immunosurveillance, rather than with alteration of

239 the infected keratinocytes, as demonstrated in this patient and all the other reported PIDs without

240 EVER genes mutations; iii) the patient's inability to clear HPV infections has led to the

241 uncontrolled replication of a few genotypes from both the α and β genera with a clear-cut tropism;

242   iii) both genera are causing proliferative lesions with a high probability of progressing to invasive

243   cancer. It is indeed very likely that he will develop skin cancer with a more aggressive phenotype in

244   the future, as can be envisaged from the dysplastic area already found in a skin wart-like lesion and

245   the clinical picture of his forehead.

246   Overall, our findings provide further compelling evidence that in the immunocompromised

247   host, regardless of his EVER gene genetic status, persistence of high rate replication of β-genotypes

248   causes skin proliferative lesions with a documented risk of progression to skin cancer.

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364

365   **FIGURE LEGENDS**

366

367   **Figure 1. Photographs of affected skin areas in the study patient.** The top picture shows the flat,

368   reddish papular lesions (wart-like) on the back. The inset is a higher magnification of these papular

369   lesions; the bottom picture shows the forehead with many red, flat-topped, small papular lesions.

370

371   **Figure 2. Distribution of the viral L1 protein, HPV DNA, and cellular markers (MCM7 and**

372   **p16^{INK4a}) in biopsies from anal (a) and penile (b) condylomas.** (**a**) The top pictures show a

373   biopsy tissue section stained using H&E corresponding to areas of low-grade (left column) and

374   high-grade dysplasia (right column). The panels in the second row display the same section stained

375   for HPV51 DNA using FISH (red) to visualize the cells in which viral genome amplification was

376   occurring. In the third row, a serial section was stained for the cellular proliferation marker MCM7

377   (red). The image of the fourth row left column shows a serial section stained with antibodies to the

378   late capsid protein L1 (green). The white dotted line indicates the basal layer. All sections were

379   counterstained with DAPI (blue) to visualize cell nuclei. The lower left picture and the image in

380   fourth row right column show a serial section stained for the cellular protein p16^{INK4a} by

381   immunoenzymatic staining. The bottom right picture presents a photograph of the anal condylomas.

382   (**b**) The top picture shows the H&E staining pattern in a biopsy section of the penile condylomas. In

383   the lower panel, the same section was stained for HPV72 DNA using FISH to detect viral genome

384   amplification (red). A serial section was double stained with antibodies to the cellular proliferation

385   marker MCM7 (red), third image from the top, and the late capsid protein L1 (fourth image from

386   the top) (green). The white dotted line indicates the basal layer. All sections were counterstained

387   with DAPI (blue) to visualize cell nuclei. The bottom picture shows a photograph of the penile

388   condylomas. Scale Bar = 100 μm.

389

390 **Figure 3. Distribution of viral proteins E4 and L1, HPV DNA, and MCM7 (marker of cell**

391 **proliferation) in biopsies from a papular wart-like lesion of the neck shown in Figure 1.** The

392 top pictures show H&E staining in biopsy sections. The panels below in the left hand column

393 correspond to the region indicated by the red rectangle in the H&E image, showing a dysplastic

394 area; while panels in the central column correspond to the region indicated by the red square,

395 showing a productive area with the classical β-HPV-induced cytopathic effects. The right hand

396 column shows the edge of the lesion at its interface with the normal epithelium. In the upper panels,

397 sections first stained with H&E were then double stained for the early viral protein E4 expression

398 (green) and viral genome amplification by HPV24 DNA-FISH (red). The central panels show serial

399 sections double stained with antibodies to the late viral capsid protein L1 (red) and E4 (green). The

400 lower panels show serial sections immunostained for the cell proliferation marker MCM7. All

401 sections were counterstained with DAPI (blue) to visualize cell nuclei. The white dotted line

402 indicates the basal layer. Scale bar = 50 μm.

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Abaiations

HPV, Human Papillomavirus; PID, primary immunodeficiency;EV, Epidermodysplasia Verruciformis; WHIM, warts hypogammaglobulinemia infections and myelokathexis; PBMC, peripheral blood mononuclear cells; RTE, recent thymic emigrant; FFPE, formalin-fixed paraffin embedded; MCM, minichromosome maintenance protein; FISH, Fluorescent *in situ* hybridization; HSIL, high grade squamous intraepithelial lesion; WILD, Warts, depressed cell-mediated Immunity, primary Lymphedema, and anogenital Dysplasia.
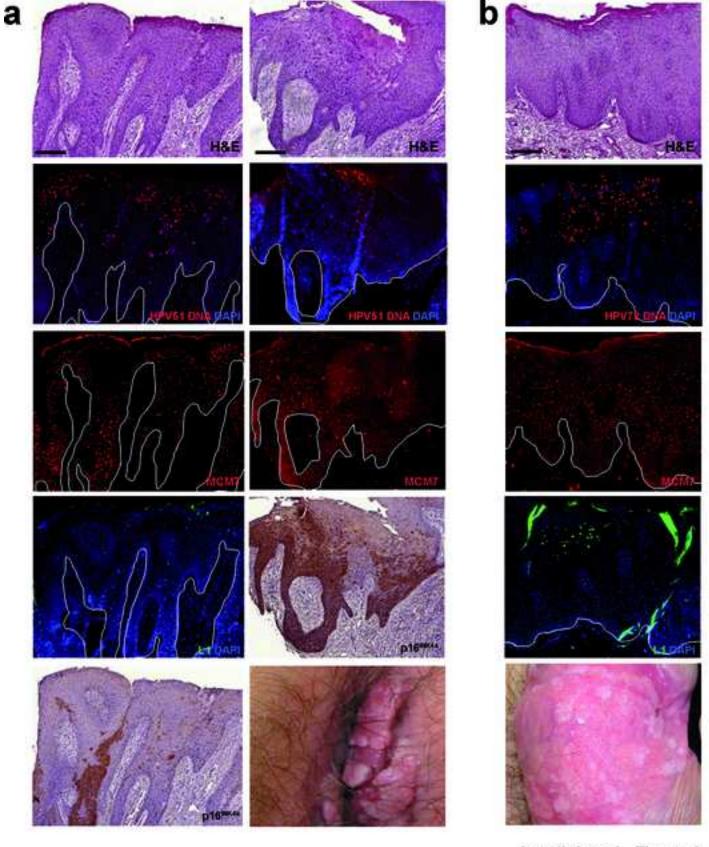
ACKNOWLEDGMENTS

Table 1. Human Papillomavirus DNA genotyping in swabs and hair bulbs from different body sites

Samples	alpha HPV types (copies/cell)	beta HPV types (copies/cell)
Hair bulbs		
Eyebrows	**18** (**<**0.1), **51** (<0.1), **61** (<0.1)	**8** (16), **24** (273)
inguinal hair	**16** (<0.1), **51** (<0.1), **61** (<0.1)	**8** (0.2), **24** (19)
Swabs		
forehead (macular lesion)	**51** (<0.1)	**8** (1×10^3), **24** (6×10^3)
arm (normal skin)	**51** (1)	**8** (<0.1), **24** (1×10^5)
anal region (condyloma)	**51** (228), **61** (2), **72** (60)	**8** (<0.1), **24** (80)
buttock (normal skin)	**51** (<0.1), **61** (<0.1)	**8** (<0.1), **24** (2×10^3)
penis (condyloma)	**51** (<0.1), **72** (4)	**8** (<0.1), **24** (8)
genital region (normal skin)	**51** (<0.1)	**8** (<0.1), **24** (2×10^3)

Figure 1
Click here to download high resolution image
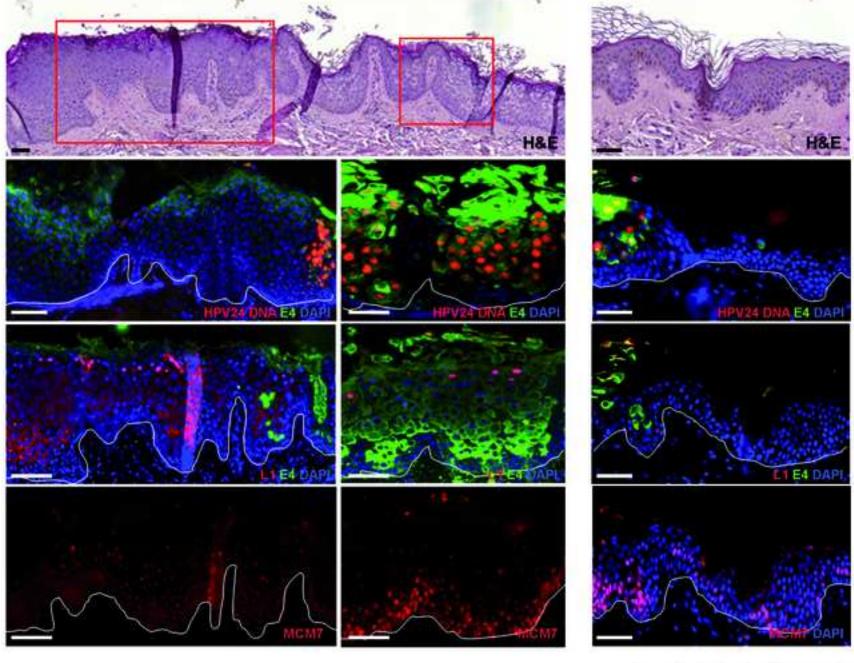

Landini et al., Figure 1

Landini et al,. Figure 2

Figure 3
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Landini et al., Figure 3