BD ProExTMC immunostaining in extramammary Paget's disease and perineal melanoma

Ann E Walts and Shikha Bose

Department of Pathology and Laboratory Medicine, Cedars-Sinai Medical Center, Los Angeles, CA, USA

The differential diagnosis of perineal biopsies can include squamous intraepithelial lesions, extramammary Paget's disease, and melanoma. Less frequently two of these lesions coexist. BD ProExTMC is a recently developed immunoassay that targets expression of two genes shown to be associated with cervical cancer. Immunostaining for ProExTMC has been validated in cervical cytology and positive staining has also been shown to be strongly associated with human papilloma virus (HPV)-induced cervical and anal intraepithelial neoplasia in biopsies. We observed positive staining for ProExTMC in Paget cells in all of 26 cases of Paget's disease irrespective of tissue site (extramammary, mammary) and in melanoma cells in all of 12 cases of primary perineal melanoma with immunostaining in >50% of malignant cells in 73% of Paget disease cases and 43% of perineal melanoma cases. Positive staining was heterogeneous and exclusively nuclear in all cases. *In situ* hybridization was negative for low-risk and high-risk HPV subtypes in all Paget and melanoma cases that were tested. Currently neither of these lesions is known to be HPV related although according to the literature the possibility of a role for HPV in melanoma is still unsettled. Relevant literature is reviewed.

Modern Pathology (2009) 22, 246–251; doi:10.1038/modpathol.2008.171; published online 17 October 2008

Keywords: BD ProExTMC; human papilloma virus; extramammary Paget's disease; melanoma; anorectal; vulvar

BD ProExTMC is a recently developed immunoassay that targets the expression of minichromosome maintenance protein 2 (MCM2) and topoisomerase II- α (TOP2A), two genes shown by microarray analysis to be associated with cervical carcinoma. ProExTMC has been validated in cervical cytology¹ and Kelly *et al*² have suggested that ProExTMC may be useful in the diagnosis of high-grade squamous intraepithelial lesions in cervical cytology. Anal epithelium, similar to cervical epithelium, has a proclivity for human papilloma virus (HPV) infection and high-risk serotypes of HPV have been shown to be involved in the pathogenesis of cervical and anal squamous carcinomas and their precursor lesions. We have recently shown that ProExTMC staining is strongly associated with HPV-induced cervical and anal squamous intraepithelial lesions.^{3,4} In selected cases, the differential diagnosis of squamous intraepithelial lesion in the

perineum can include extramammary Paget's disease and melanoma. Infrequently two of these lesions occur together. We encountered an anal biopsy that exhibited positive staining for ProExTMC in Paget cells and in the coexistent anal intraepithelial neoplasia (AIN). Hence, this study was designed to assess immunostaining for ProExTMC in extramammary Paget's disease and perineal melanoma.

Materials and methods

After IRB approval, 26 cases of Paget's disease (7 perianal, 11 vulvar, 8 mammary) and 12 cases of primary perineal melanoma (1 vaginal, 7 vulvar, 4 anorectal) were retrieved from our files. In most cases, one or more additional stains (CEA, cytokeratin 7, mucicarmine, S100, HMB45, melan A) had been performed before sign-out for confirmation of diagnoses. Patients ranged in age from 40 to 87 years. Thirty-five (92% of the Paget's and 92% of the melanomas) of the lesions occurred in women. Three of the lesions (two perianal Paget's, one anal melanoma) occurred in men. Invasive tumor was present in eight (31%) of the Paget and ten (83%) of the melanoma cases. (*Note:* invasive tumor was

Correspondence: Dr AE Walts, MD, Department of Pathology and Laboratory Medicine, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Los Angeles, CA 90048, USA.

E-mail: walts@cshs.org

This study was presented in part as a poster at the USCAP annual meeting, Denver, CO, USA, March 2008.

Received 28 April 2008; revised and accepted 16 September 2008; published online 17 October 2008

present in only seven of the eight Paget blocks immunostained.)

After slides were reviewed and diagnoses confirmed (AW, SB), serial sections of a representative formalin fixed paraffin embedded block from each case and appropriate positive and negative controls were immunostained for ProEx™C utilizing the ABC immunoperoxidase method and antibodies directed against ProEx™C (clone MCM2 26H6.19, MCM2 27C5.6, TOP2A SWT3D1 from TriPath Imaging Inc., Burlington, NC, USA). Sections of an HPV-positive cervical biopsy served as the positive control whereas sections of the cervical biopsy run substituting mouse IgG for ProEx™C served as the negative control. The ProEx™C antibody and its detection solutions are available as a kit and the staining protocol applied was as per the manufacturer's recommendation. Immunostained slides were reviewed by two pathologists (AW, SB) and the percentage of Paget cells with positive nuclear staining was recorded as <25, 25–50, 50–75, and >75%. Intensity of positive staining was recorded as 1+ to 3+.

In situ hybridization for low-risk and high-risk HPV subtypes was performed on fifteen of the extramammary Paget's disease (seven perianal and eight vulvar) and four of the anorectal melanomas utilizing INFORM® HPV II Family 6 Probe (cocktail of HPV 6 and 11) and the INFORM® HPV III Family 16 Probe (B; cocktail of HPV subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, and 66) from Ventana Medical Systems Inc., Tucson, AZ, USA. *In situ* hybridization was performed as per the manufacturer's recommendations and preparations were evaluated for the presence of low-risk and high-risk HPV DNA. In four of these cases, PCR for HPV types 16 and 18 was also performed utilizing HPV16F96 (5'-GGTCGGTGGACCGGTCGATG-3') and HPV16R96 (5'-GCAATGTAGGTGTATCTCCA-3') as primers for HPV 16 and HPV18F115 (5'-CCTTGGACGTAAATTT TTGG-3') and HPV18R115 (5'-CACGCACACGCTTG GCAGGT-3') as primers for HPV 18 as previously described.⁵

Results

Immunostaining for ProEx™C in Paget's Disease

Positive nuclear staining for ProEx™C was present in Paget cells in 100% of the cases irrespective of tissue site (Table 1; Figures 1–3). Staining was heteroge-

neous and exclusively nuclear with 2–3+ intensity in each case. In 19 (73%) cases (100% anal, 45% vulvar, 88% mammary), 50% or more of the Paget cells stained positive. In some cases, variable numbers of benign squamous cells in the basal layers also stained positive. When present in the section associated invasive adenocarcinomas (two anal, three vulvar, two mammary) and ductal carcinoma-*in situ* also stained positive for ProEx™C to approximately the same extent as the Paget cells. *In situ* hybridization was negative for low-risk and high-risk HPV subtypes in all of the extramammary Paget's disease cases that were tested (seven anal and five vulvar each with positive staining for ProEx™C in 50% or more Paget cells; three vulvar each with positive staining for ProEx™C in 25–50% Paget cells) including one anal Paget's with PCR positive for HPV 16 and ProEx™C positivity in >75% of Paget cells (Figures 1–3).

Immunostaining for ProEx™C in Perineal Melanoma

Positive nuclear staining for ProEx™C was present in melanoma cells in 100% of the cases irrespective of tissue site (Table 2; Figure 4). Staining was heterogeneous and exclusively nuclear. The percentage of melanoma cells that stained varied between cases and from microscopic field to field within some cases. In five (42%) of the perineal melanomas (100% anorectal, 14% vulvar), 50% or more of the melanoma cells in at least one tumor field in the section stained positive. In one polypoid/nodular anorectal melanoma, the percentage of melanoma cells that stained positive for ProEx™C ranged from <10 to >90% and intensity of staining ranged from 1+ to 3+. As in the Paget cases, variable numbers of benign squamous cells in the basal layers also stained positive. Neither the extent nor the intensity of staining for ProEx™C correlated with the presence of or depth of invasive tumor. *In situ* hybridization was negative for low-risk and high-risk HPV subtypes in all of the perineal melanomas that were tested (three anorectal and one vulvar each with positive staining for ProEx™C in 50% or more melanoma cells and one anorectal in which <10 to >90% of melanoma cells stained positive for ProEx™C depending on the microscopic field selected) (Table 2; Figure 4).

Discussion

The incidence of anal squamous carcinoma and its precursor dysplastic lesions is increasing.^{6,7} Although anal cytology is useful for screening individuals at high risk for HPV-associated AIN, biopsy remains the gold standard for diagnosis and treatment. In selected cases, the differential diagnosis of HPV-induced squamous intraepithelial lesions in the perineum (AIN, VIN, VAIN) can include extramammary Paget's disease (an intrae-

Table 1 ProEx™C immunostaining in Paget's disease (N=26)

% Paget cells ProEx™C+	Anal	Vulvar	Mammary
<25	0	2	1
25–50	0	4	0
50–75	5	3	3
>75	2	2	4

Figure 1 Mammary Paget's disease with underlying invasive ductal carcinoma showing positive ProEx™C staining. (a, c) Hematoxylin and eosin, (b, d) ProEx™C; (a, b) original magnification $\times 10$, (c, d) original magnification $\times 20$.

epithelial proliferation of glandular-type cells)⁸ and/or superficial spreading melanoma (an intraepithelial proliferation of atypical melanocytic cells). Infrequently, these lesions can coexist.^{8–11}

ProEx™C is a recently developed assay that has been shown to be useful in the diagnosis of HPV-associated squamous intraepithelial lesions involving anogenital epithelium but has not yet been widely utilized. Strong nuclear staining for ProEx™C was seen in $>50\%$ of lesional cells in 48% of HPV-associated low-grade cervical intraepithelial neoplasia, 48% of low-grade AIN, 92% of high-grade CIN, and 70% of high-grade AIN.^{3,4} Positive staining in $>50\%$ of cells correlated strongly with presence of HPV DNA as determined by *in situ* hybridization in both cervical and anal lesions. In the current study, we observed immunoreactivity for ProEx™C in all cases of extramammary Paget's disease and perineal malignant melanoma with strong immunostaining in $>50\%$ of malignant cells in most (73%) cases of Paget's disease and 43% of perineal melanoma cases, findings not previously reported. Although 50% of our cases of AIN and CIN showed HPV by *in situ* hybridization, no HPV was detected by *in situ* hybridization in the cases of Paget's disease and

melanoma. *In situ* hybridization, although a specific test, is known to lack sensitivity. Currently neither of these lesions is known to be HPV related, but according to the literature the possibility of a role for HPV in melanoma is still unsettled.

In situ hybridization studies for low-risk and high-risk HPV DNA have uniformly failed to detect the presence of HPV in extramammary Paget's disease, although viral DNA has been identified in the coexistent HPV-associated lesions (low-risk HPV DNA in the condylomatous component of a scrotal Paget's disease, high-risk HPV DNA in coexistent Bowen's disease of the scrotum, and high-risk HPV DNA in coexistent squamous carcinoma of the vulva.^{8,9,12–14} PCR studies for HPV have also been reported as negative in all mammary and extramammary Paget's disease (20/20 and 14/14 cases, respectively).^{15,16} Although most of our cases of anal Paget's disease were positive for ProEx™C, HPV type 16 was detected by PCR in one case where 75% of Paget cells stained positive. This case did not show histologic changes diagnostic of HPV nor was HPV DNA detected by *in situ* hybridization. Thus, it appears that the immunostaining for ProEx™C observed in Paget's disease might be unrelated to HPV.

Figure 2 Anal Paget's disease showing positive ProEx™C staining. (a) Hematoxylin and eosin, (b) ProEx™C; (a) original magnification $\times 10$, (b) original magnification $\times 20$.

Figure 3 Vulvar Paget's disease showing positive ProEx™C staining. (a) Hematoxylin and eosin, (b) ProEx™C; (a, b) original magnification $\times 20$.

The etiology of malignant melanoma is unknown. The possibility of HPV as an etiologic agent or as a cofactor with or without a role in tumor promotion has been queried for cutaneous as well as anogenital melanoma. Although a few studies have reported the presence of HPV type 16 in melanoma,^{17–20} most studies have reported the presence of epidermodysplasia verruciformis/cutaneous HPV DNA. In 1986, Scheurlen *et al*²¹ reported the presence of a specific DNA sequence corresponding to HPV subtype 38 in a superficial spreading melanoma of an immunosuppressed patient. HPV 38 has been associated with some cases of epidermodysplasia verruciformis and is not currently considered a genital mucosal HPV.¹⁷ Takamiyagi *et al*¹⁸ reported HPV 16 DNA in a malignant melanoma that had developed as a result of rapid coalescence of papular and nodular pigmented tumors on the leg of a 37-year-old woman and Roussaki-Schulze *et al*¹⁹ reported HPV type 16 DNA in two of 20 cutaneous melanomas. In a study of cutaneous melanomas, Dreau *et al*²² found that the HPV-positive melanomas grew better in tissue culture and were associated with recurrences and

clinically aggressive tumors whereas the HPV-negative melanomas failed to grow in tissue culture and the corresponding patients remained alive and free of tumor recurrence. They suggested that HPV might have a role as a cofactor in the development of melanoma and might 'modulate a more aggressive phenotype' in HPV-containing melanoma. Rohwedder *et al*¹⁷ reported DNA for multiple HPV subtypes including HPV 16 in two vulvar melanomas and surrounding skin (one with lichen sclerosus and one with pigmented vulvar squamous papillomatosis) and suggested that the presence of DNA of multiple HPV types and of integrated HPV DNA in skin associated with vulvar melanomas might indicate a direct or indirect role for HPV in the development of melanoma. In a subsequent study,²³ they also identified HPV DNA in six of nine vulvovaginal melanomas. Only nongenital-mucosal HPV subtypes were identified; type specific probes for HPV 16 were negative. Dahlgren *et al*²⁴ evaluated 35 primary melanomas in non-sun exposed areas utilizing a protocol that they describe as highly sensitive for identification of DNA sequences from

Figure 4 Anal melanoma showing positive ProEx™C staining. (a) Hematoxylin and eosin, (b) ProEx™C; (a, b) original magnification $\times 20$.

Table 2 ProEx™C immunostaining in perineal melanoma (N=12)

% Melanoma cells ProEx™C+	Anal	Vulvar	Vaginal
< 25	0	4	1
25–50	0	2	0
50–75	2	1	0
> 75	1	0	0
<10–>90 ^a	1	0	0

^aIn one melanoma, the percentage of lesional cells that stained positive for ProEx™C varied from <10 to >90% depending upon the microscopic field selected for evaluation.

36 HPV subtypes including HPV 16 and concluded that HPV is rarely detected in primary melanomas of non-sun exposed areas. Using a PCR-enzyme-linked immunosorbent assay, La Placa *et al*²⁵ detected mucosal high-risk HPV DNA in 27% of 51 primary melanomas and in 24% of acquired dysplastic melanocytic nevi, which they considered as precursor lesions for melanoma. Utilizing a combination of fluorescent *in situ* hybridization and

chemiluminescent immunohistochemistry, Ambretti *et al*²⁶ recently reported colocalization of high-risk HPV (HPV 16 and 18) nucleic acids and HMB45 (a melanoma marker) in melanoma cells, which they interpreted as supportive of a possible active role for HPV in malignant melanoma. The plethora of HPV subtypes (currently more than 100), cross-reactivity for HPV antigens, and limited specificity and sensitivity of various testing modalities have no doubt contributed to the complexity of this issue. Thus at this time, the role of HPV in melanoma remains controversial. ProEx™C staining in melanoma cells could be indirect evidence in support of a role for HPV or could represent nonspecific staining similar to that observed in extramammary Paget's disease.

Successful application of an immunostain requires awareness of its staining spectrum. Our findings suggest that immunostaining for ProEx™C has a limited role for the diagnosis of squamous intraepithelial lesions when extramammary Paget's disease and/or melanoma are in the differential diagnosis. TOP2A (a topoisomerase) and MCM2 (a helicase) are both involved in DNA replication during the G₁ to S-phase of the cell cycle. High-risk HPV infection provides one but not the only mechanism that can result in aberrant S-phase induction (premature and prolonged entry into the S-phase) manifesting as overexpression of these proteins. Hence, ProEx™C positivity is not equivalent to the presence of high-risk HPV.

Acknowledgement

Support: BD ProEx™C antibody was provided free of cost for this study by BD Diagnostics—TriPath Imaging.

Disclosure/Conflict of interest

No relevant financial or other disclosures or conflicts of interest.

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