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Epithelial-mesenchymal transition-like events in vulvar cancer and its relation with HPV

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Background: Epithelial-to-mesenchymal transition (EMT) still remains an obscure event in vulvar squamous cell carcinoma (VSCC).

Methods: Immunohistochemistry (IHC) expression of E-cadherin, β-catenin, Snail, Slug, Twist and Vimentin was analysed in 87 VSCC, controlled for human papillomavirus (HPV) positivity, considering tumour front and central tumour as different morphological categories from the same tumour.

Results: Lower β-catenin and higher Vimentin expression was associated with invasive front when compared with the central tumour ($P=0.013$ and $P\leqslant0.001$, respectively). Higher expression of E-cadherin in central tumour was significantly related to absence of vascular and perineural invasion, lower invasion depth and ⩽2 lymph node involvement. Loss of β-catenin and high Slug, Snail and Twist expression was associated with HPV-negative tumours. Moreover, β-catenin lower expression associated with gain in Slug expression predicts a subgroup with worst outcome ($P=0.001$). Lower expression of β-catenin in both central tumour and invasive front correlated with lower overall survival ($P=0.021$ and $P=0.011$, respectively). Also, multivariate analysis showed that lower β-catenin expression was independently associated with poorer outcome ($P=0.044$).

Conclusion: Human papillomavirus-related tumours show better prognosis and outcome; besides, they do not progress through EMT phenomenon. Immunohistochemical analysis of β-catenin in invasive tumour front is a key issue for establishing prognosis of vulva cancer.

The incidence of vulvar cancer has risen steadily at 20% over the last 40 years (Lanneau *et al*, 2009) and represents nowadays the fourth most common type of gynaecological cancer, with an incidence of two to three per 100 000 women yearly (van de Nieuwenhof *et al*, 2011). Vulvar squamous cell carcinoma (VSCC) comprises 70% of all cases of vulvar cancer and is primarily a disease of postmenopausal women with a mean age at diagnosis of ~70 years. Vulvar squamous cell carcinoma is usually diagnosed in a stage still amenable to potentially curative treatments, including surgery and/or radiation therapy with or without chemotherapy. However, several patients present metastatic disease at diagnosis, among those, 30–50% will relapse (De Melo Maia *et al*, 2012; Santeufemia *et al*, 2012). Local or regional recurrences and distant metastasis have a critical role in progress of VSCC and the mechanism underlying their occurrence remains poorly understood.

During metastatic progression, important early events are loss of cell–cell adhesion detachment of tumour cells from the primary site (Cooke *et al*, 2012) and hypoxia that induces epithelial-to-mesenchymal transition (EMT) of cells specifically by activation of master regulators of EMT, Twist and Slug (Sun *et al*, 2009), which are suggested to have an essential role in promoting metastasis (Cardiff, 2005).

E-cadherin-mediated cell adhesion requires intracellular attachment to the actin cytoskeleton by interaction with catenins, such as β-catenin, that may also act as an oncoprotein, becoming one of the key downstream effectors in the Wnt–Akt signalling pathway. In normal epithelial cells, β-catenin is localised at the cell

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membrane. The unbound β-catenin is degraded by the ubiquitin-proteasome system that involves the glycogen synthase kinase 3 beta. Stabilisation of cytoplasmic β-catenin by aberrant activation of Wnt signalling leads to its accumulation, complexed with lymphoid enhancer factor/Tcf (LEF/Tcf) transcription factors and transactivation of LEF/Tcf target genes. Activation of these genes leads to cell proliferation and inhibition of apoptosis (Bhangu *et al*, 2012). Some studies reported that reduced assembly of membranous β-catenin induces upregulation of Slug and the mesenchymal marker, Vimentin, key markers for EMT characterisation (Peinado *et al*, 2007; Heuberger and Birchmeier, 2010).

There are few studies assessing the impact of EMT markers on the progression of VSCC, and it remains largely unknown whether these markers have significance in the prognosis of this type of cancer. Therefore, considering VSCC morphological heterogeneity, evaluating the expression of EMT-related protein set in the tumour invasion may bring a novel perspective in establishing prognosis of patients with vulvar cancer, and also in the understanding of VSCC tumour biology. In this retrospective study, we evaluated the expression of E-cadherin, β-catenin, Snail, Slug, Twist and Vimentin in VSCC at the invasive front and central tumour, and associated their expression with clinical data collected from patient's medical records and the presence or not of human papillomavirus (HPV) infection.

MATERIALS AND METHODS

Sample selection. Eighty-seven cases of VSCC diagnosed between 1979 and 2006 were selected from the Anatomic Pathology Department of AC Camargo Cancer Center, Brazil. *In situ* carcinoma and patients subjected to preoperative chemotherapy and radiotherapy were excluded. Original H&E slides were reviewed by experienced pathologist (MMAS) in order to confirm diagnosis and select the most suitable paraffin-embedded tissue for further studies in whole sections. Clinical and follow-up information were obtained up to 5 years after diagnosis for all patients.

HPV DNA detection and genotyping

DNA extraction. Total DNA was extracted from up to eight tissue sections (10-μm-thick). Sections were pretreated with 350 μl ATL lysis buffer from the DNA FFPE kit (Qiagen, Valencia, CA, USA), added directly to the paraffin sections, followed by incubation of the tightly closed tubes at 120 °C for 20 min. All procedures followed the supplier's specification. DNA was then quantified at NanoDrop ND-1000 spectrophotometer (Wilmington, DE, USA) and analysed on 1% agarose gel.

HPV genotyping. Human papillomavirus detection and typing was performed using the linear array HPV-genotyping test (Roche Molecular Systems; Branchburg, NJ, USA). The assay is based on L1 consensus PCR with PGMY primers yielding a 450-bp amplicon, with type-specific hybridisation to detect 37 individual types (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72 73, 81, 82, 83, 84, 89 and IS39). In addition, the assay includes β-globin primers (150-bp amplicon) detected on the HPV typing strip as positive control for amplifiable sample DNA. Linear array HPV-genotyping strips were manually interpreted using the HPV reference guide provided. The products of hybridisation were detected by a colour reaction with alkaline phosphatase–streptavidin conjugate and substrate (5-bromo-4-chloro-3-indolyl phosphate and nitrobluetetrazolium), which results in a purple precipitate. Hybridisation results were visually assessed by comparison with the standard grid.

Immunohistochemistry. Immunohistochemistry (IHC) was performed in whole sections (4-μm-thick) of formalin-fixed paraffin-embedded using a Ventana Benchmark XT automated stainer (Ventana Medical Systems; Tucson, AZ, USA). Table 1 presents the primary antibodies used in this study. All IHC-stained slides were digitised and analysed using a whole-slide imaging system (ScanScope XT; Aperio Technologies; San Diego, CA, USA; Table 3). All slides were scanned at 0.25 μm per pixel resolution, and the images saved in the password-protected database (Spectrum version 10.2.2.2314) provided by Aperio via web-accessible server.

Scoring of IHC expression. All sections were blindly analysed and the expression pattern was evaluated in a quantitative manner, whereby the levels of expression are represented by the percentage of positive cells and the intensity of immunostaining (HSCORE = $\Sigma(i \times Pi)$, where Pi = percentage of positive pixels, varied from 0 to 100% and pixel staining intensity i = 0, 1, 2 or 3), with a ranking between 0 and 300 (Alvarenga *et al*, 2013).

Two areas of each tumour – central tumour and invasion front – were morphologically selected and assessed for expression of each protein. Concerning central tumour, it was considered as the largest extension tumour area, and at least three fields of each section were randomly selected and analysed. Invasion front, defined as single cells or clusters of up to five cells detached from the main tumour mass that broadly infiltrate the adjacent stroma (Paterson *et al*, 2013), was evaluated in 10 different randomly selected fields.

The levels of immunoreactivity for all markers were established as numerical variables. Membrane β-catenin expression was assigned as negative (0) or positive (1) by the software.

Clinicopathological parameters of the patients. The clinical data obtained from the medical records were: age and 5-year follow-up. Pathological data, such as lymph node involvement, pathological stage (FIGO staging), inflammatory infiltration, presence of vascular and perineural invasion, associated lesions (differentiated and high-grade VIN, lichen sclerosus and squamous hyperplasia) and invasion depth were evaluated.

Statistics analysis. The Statistical Package for the Social Sciences (SPSS, Upper Saddle River, NJ, USA) 17.0 software was used. The association between β-catenin and clinicopathological variables was analysed with the χ^2-test for categorical data and Fisher's exact test was used for small numbers. Comparison of the protein expression between the central tumour and invasion front was performed using Wilcoxon signed-rank test. Mann–Whitney test

Table 1. Protein, clones, supplier and dilution of the primary antibodies used in the immunohistochemical assays

Antibody	Clone	Supplier	Dilution
E-cadherin	36/E-cadherin	BD Biosciences, Franklin Lakes, NJ, USA	1/2000
β-catenin	17C2	NeoMarkers Biotechnology, Fremont, CA, USA	1/50
Snai1	A242	Bioworld Technology, Minneapolis, MN, USA	1/200
Slug	C1967	Cell Signaling, Beverly, MA, USA	1/100
Twist2	3C8	Abnova, Jhongli, Taiwan	1/25
Vimentin	V9	Dako, Carpinteria, CA, USA	1/1500

Table 2. Clinicopathological data and HPV infection

Characteristic Clinicopathological	Value*N* (%)	*N*/total HPV positive (%)	*P*-value
Age (years), median (range)	68.75 (46-90)	–	–
46–49 years old	30 (34.5)	11/34 (32.3)	0.067
⩾50 years old	57 (65.5)	23/34 (67.7)	
Histologic types			
Grade 1 SCC	27 (31)	9/34 (26.5)	—
Grade 2 SCC	46 (52.9)	13/34 (38.2)	—
Grade 3 SCC	8 (9.2)	5/34 (14.7)	—
Basaloid Ca	4 (4.6)	4/34 (11.8)	—
Verrucous Ca	2 (2.3)	0	—
Inflammatory infiltrate			
No	79 (90.8)	16/57 (28.1)	0.951
Yes	8 (9.2)	29/40 (72.5)	
Vascular/perineural invasion			
No	73 (84)	47/56 (83.9)	0.445
Yes	14 (13)	4/38 (10.5)	
Lymph node involvement			
⩽2	80 (92)	28/30 (93.3)	1.000
>2	2 (2.3)	2/23 (8.7)	
No	5 (5.7)		
Associated lesions			
VIN	25 (29)	8/22 (36.4)	0.683
Lichen sclerosus and squamous hyperplasia	8 (9)	4/10 (40)	
No	54 (62)		
Invasion depth			
Superficial and mid dermis	37 (42.5)	32/59 (54.2)	0.570
Deep dermis and subcutis	50 (57.5)	16/40 (40)	
FIGO stage			
I and II	56 (64.4)	30/55 (54.5)	0.179
III and IV	31 (35.6)	12/38 (31.6)	

Abbreviations: ⩽2 = two or less lymph node involvement; >2 = more than two lymph node involvement; FIGO = International Federation of Gynecology and Obstetrics; HPV = human papillomavirus; VIN = vulvar intraepithelial neoplasia (differentiated and high grade). χ^2-test ($N = 87$).

was employed for interpretation of the association between protein expression and clinical parameters, and $P \leqslant 0.05$ was considered statistically significant for all tests. Kaplan–Meier curves were generated for protein expression in overall survival. The log-rank test was applied to test the significance of differences between stratified survival functions. Cox proportional-hazards regression analysis was performed to test the statistical independence and significance between pathological, molecular and clinical variables.

RESULTS

Sample characteristics and HPV Infection. Clinicopathological data of all patients are provided in Table 2. Local recurrence was observed in 25 out of 87 (28.7%) patients. Human papillomavirus infection was detected in 34 out of 87 (39.1%) cases. The most frequently detected high-risk HPV types were HPV16 (35.3%), followed by HPV33 (8.9%), HPV18 and HPV35 (both 5.9%). Human papillomavirus coinfection was found in eight cases (23.5%; Table 3). There was no association between HPV infection and vascular and perineural invasion, lymph node involvement, FIGO stage, invasion depth and presence of associated lesion (Table 2). However, HPV-positive cases showed better overall survival than the HPV-negative ones (Figure 1).

Table 3. HPV type in patients with vulvar carcinoma

HPV type	*N* (%)
Positive cases	34 (39.1)
Type HPV	
HPV 16	12 (35.3)
HPV 18	2 (5.9)
HPV 33	3 (8.9)
HPV 35	2 (5.9)
HPV 45	1 (2.9)
HPV 53	4 (11.8)
HPV 71	2 (5.9)
Coinfection	
HPV 16 + HPV 18 + HPV 33	1 (2.9)
HPV 16 + HPV 33	2 (5.9)
HPV 16 + HPV 33 + HPV 35	1 (2.9)
HPV 16 + HPV 33 + HPV 84	1 (2.9)
HPV 18 + HPV 33 + HPV 35	1 (2.9)
HPV 31 + HPV 33 + HPV 82	1 (2.9)
HPV 42 + HPV 54	1 (2.9)

Abbreviation: HPV = human papillomavirus.

E-cadherin expression. Immunoexpression for E-cadherin was considered as membrane staining; it was positive at the central tumour and invasive front of 47 (54%) and 45 (51.7%) cases, respectively, (Figure 2A and D). There was no difference of E-cadherin expression between central tumour and invasive front ($P=0.692$; Figure 2G). Lower expression of E-cadherin at the central tumour was significantly related to vascular and perineural invasion, invasion depth and $\geqslant 2$ lymph node involvement (Table 4A). Similarly, lower expression of E-cadherin at the tumour invasive front was related with vascular and perineural invasion, and FIGO stage III/IV (Table 4B). On the other hand, there was no relation between E-cadherin expression and HPV infection (Table 5), inflammatory infiltrate, associated diseases, and overall and disease-free survival (Supplementary Table S1).

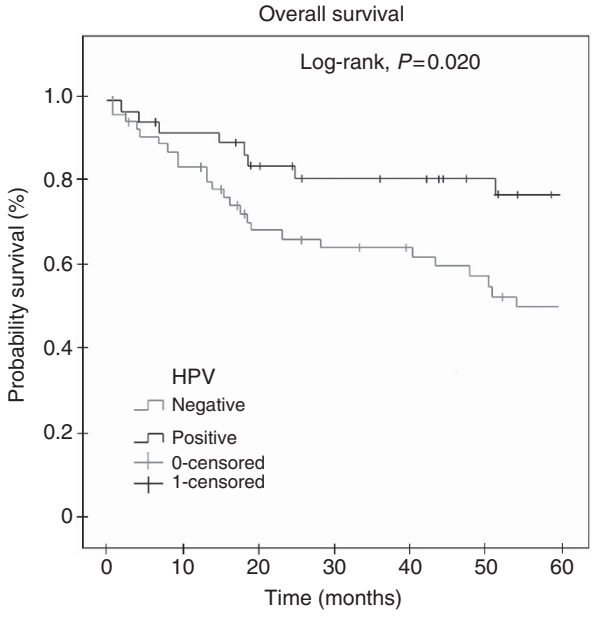

Figure 1. Overall survival curve of patients according to HPV infection. Shorter overall survival was observed for HPV-negative cases ($P=0.020$). Kaplan–Meier method, log-rank test.

***β*-catenin expression.** Immunoexpression for *β*-catenin was specifically well localised in cell membrane, and positive in central tumour and invasion front in 50 out of 87 (57.5%) and 40 out of 87 (46%), respectively, (Figures 2B and E). *β*-catenin was significantly less expressed at the invasive front when compared with central tumour ($P=0.013$) (Figure 2H). Nuclear *β*-catenin expression was not detected at all. There was no relation between *β*-catenin positivity and vascular and perineural invasion, invasion depth, lymph node involvement, associated lesions and inflammatory infiltration (Table 6). In contrast, loss of *β*-catenin expression at the central tumour and invasive front was significantly associated with FIGO stages III/IV cases ($P=0.016$ and $P=0.023$; Table 6) and absence of HPV infection ($P=0.001$ and $P=0.000$; Table 7).

Patients with lower expression of *β*-catenin in both central tumour and invasive front presented lower overall survival ($P=0.021$ and $P=0.011$, respectively; Figure 3A and B). Concerning multivariate analysis, lower expression of *β*-catenin in invasive front was independently associated with lower overall survival ($P=0.044$; Table 8).

Vimentin expression. Vimentin expression presented as specific, sharp and well-localised cytoplasmic immunostaining pattern, being positive in 45 out of 87 cases (51.7%) at the central tumour and 44 out of 87 cases (50.6%) at the invasive front (Figure 2C and F). There was a significant difference in Vimentin expression between invasive front and central tumour ($P<0.001$; Figure 2I). Vimentin positivity was also significantly associated with invasion depth ($P=0.017$; Table 4A). Conversely, there was no association between Vimentin and vascular and perineural invasion, lymph node involvement, inflammatory infiltration, associated lesions, overall, disease-free survival (Supplementary Table S1 and Table 4B) and HPV infection (Table 5).

Slug expression. Slug expression presented as specific, sharp and well-localised nuclear staining pattern, with variable staining intensity degree along the same tumour. Slug expression was observed at the central tumour and invasive front in 42 out of 87 cases (48.2%) and 44 out of 87 cases (50.6%), respectively,

Table 4A. E-cadherin, Snail, Twist and Vimentin expression and clinicopathological data in central tumour

Protein expression	Variables	Category	Quartile1	Median	Quartile 3	*P*-value
E-cadherin	Vascular/perineural invasion	No	110.70	150.713	172.44	0.010[a]
		Yes	37.40	89.47	144.50	
	Invasion depth	SMD	117.97	157.17	175.96	0.001[a]
		DDS	60.90	117.00	161.66	
	Lymph node involvement	⩽2	94.47	139.67	169.37	0.044[a]
		>2	28.308	56.06	72.620	
Snail	Vascular/perineural invasion	No	1.58	6.31	28.0	0.031[a]
		Yes	0.64	1.74	7.37	
	Invasion depth	SMD	1.73	8.26	37.00	0.029[a]
		DDS	0.73	2.17	7.54	
Twist 2	Associated lesions	VIN	30.32	76.55	117.26	0.014[a]
		LH	0	17.60	46.72	
Vimentin	Invasion depth	SMD	21.88	35.59	52.42	0.017[a]
		DDS	13.97	24.19	38.85	

Abbreviations: ⩽2 = two or less lymph node involvement; >2 = more than two lymph node involvement; DDS = deep dermis and subcutis; LH = lichen sclerosus and squamous hyperplasia; SMD = superficial and mind dermis, VIN = vulvar intraepithelial neoplasia (differentiated and high grade). Mann–Whitney test ($N=87$).
[a]Statistically significant, $P\leqslant 0.05$.

Table 4B. E-cadherin, Slug, Snail, Twist2 and Vimentin expression and clinicopathological factors at the invasive front

Protein expression	Variables	Category	Quartile 1	Median	Quartile 3	*P*-value
E-cadherin	Vascular/perineural invasion	No	109.92	147.06	173.2	0.044[a]
		Yes	49.53	138.96	148.41	
	FIGO stage	I–II	121.44	149.91	174.86	0.009[a]
		III–IV	73.108	130.86	146.80	
	Invasion depth	SMD	101.30	145.17	166.72	0.481
		DDS	116.27	141.73	161.05	
	Lymph node involvement	⩽2	99.65	133.12	162.22	0.747
		>2	76.399	141.76	148.54	
	Associated lesions	VIN	81.49	144.41	165.09	0.887
		LH	108.33	135.98	168.33	
	Inflammatory infiltrate	No	126.87	146.00	170.05	0.236
		Yes	91.97	140.78	157.72	
Slug	Vascular/perineural invasion	No	1,204	4.69	18.24	0.991
		Yes	1.185	2.95	20.97	
	Invasion depth	SMD	2.049	5.42	23.88	0.176
		DDS	0.619	2.76	13.23	
	Lymph node involvement	⩽2	0.766	5.18	15.54	0.747
		>2	1.352	1.50	12.48	
	Associated lesions	VIN	2.43	4.37	23.66	0.029[a]
		LH	0.61	0.97	2.33	
	FIGO stage	I–II	1.134	2.91	13.54	0.100
		III–IV	1.695	4.32	34.36	
	Inflammatory infiltrate	No	1.06	2.79	14.69	0.236
		Yes	1.32	4.83	21.15	
Snail	Vascular/perineural invasion	No	0.98	6.41	25.80	0.148
		Yes	0.19	2.28	10.97	
	Invasion depth	SMD	0.57	6.41	29.72	0.511
		DDS	0.60	4.02	12.58	
	Lymph Node Involvement	⩽2	1.01	5.89	33.89	0.081
		>2	0	0	1.14	
	Associated lesions	VIN	4.04	7.37	21.44	0.625
		LH	0.54	5.31	19.09	
	FIGO stage	I–II	0.59	4.83	15.62	0.171
		III–IV	0.63	5.33	33.92	
	Inflammatory infiltrate	No	0.39	5.07	23.92	0.510
		Yes	0.68	5.26	22.62	
Twist	Vascular/perineural invasion	No	0.28	5.77	19.88	0.258
		Yes	0.34	3.11	9.10	
	Invasion depth	SMD	0.57	6.43	19.38	0.487
		DDS	0.10	3.58	9.35	
	Lymph Node Involvement	⩽2	0.07	6.21	20.32	0.428
		>2	1.65	3.31	5.01	
	Associated lesions	VIN	53.74	77.11	145.22	0.052
		LH	0	18.87	74.97	
	FIGO stage	I–II	0.05	5.10	9.68	0.856
		III–IV	0.58	5.00	20.32	
	Inflammatory infiltrate	No	0.05	2.93	9.25	0.219
		Yes	0.67	6.17	18.54	
Vimentin	Vascular/perineural invasion	No	40.52	68.14	105.91	0.287
		Yes	45.07	54.96	72.48	

Table 4B. (Continued)

Protein expression	Variables	Category	Quartile 1	Median	Quartile 3	*P*-value
	Invasion depth	SMD	45.83	68.86	102.43	0.148
		DDS	40.30	54.96	74.76	
	Lymph node involvement	⩽2	40.54	58.94	79.89	0.617
		>2	42.32	54.96	67.37	
	Associated lesions	VIN	32.63	58.64	82.48	0.569
		LH	51.74	67.20	74.09	
	FIGO stage	I–II	44.02	62.32	75.53	0.725
		III–IV	58.36	57.07	106.00	
	Inflammatory infiltrate	No	43.48	66.91	75.98	0.676
		Yes	42.36	57.99	102.81	

Abbreviations: ⩽2 = two or less lymph node involvement; >2 = more than two lymph node involvement; C = cytoplasmic; DDS = deep dermis and subcutis; FIGO = International Federation of Gynecology and Obstetrics; LH = lichen sclerosus and squamous hyperplasia; N = nuclear; SMD = superficial and mind dermis; VIN = vulvar intraepithelial neoplasia (differentiated and high grade). Mann–Whitney test ($N = 87$).
[a]Statistically significant, $P \leqslant 0.05$.

Table 5. E-cadherin, Slug, Snail, Twist and Vimentin expression according to the HPV infection

Protein expression (immunopositivity)	Region	HPV infection	Quartile 1	Median	Quartile 3	*P*-value
E-cadherin	Central tumour	No	97.95	115.18	168.92	0.721
		Yes	73.451	131.49	175.50	
	Invasive front	No	109.924	141.74	162.36	0.862
		Yes	88.476	145.22	158.95	
Slug	Central tumour	No	2.127	8.09	31.95	0.018[a]
		Yes	0.690	2.11	8.66	
	Invasive front	No	1.952	9.80	37.98	0.004[a]
		Yes	0.905	2.33	9.64	
Snail	Central tumour	No	1.74	5.96	28.52	0.068
		Yes	0	1.96	15.50	
	Invasive front	No	0.98	8.10	29.41	0.018[a]
		Yes	0.18	2.54	8.70	
Twist	Central tumour	No	36.08	67.90	110.80	0.007[a]
		Yes	1.13	35.29	69.47	
	Invasive front	No	0.88	6.67	20.33	0.009[a]
		Yes	0	2.10	8.43	
Vimentin	Central tumour	No	16.03	30.04	48.02	0.486
		Yes	22.25	33.23	44.67	
	Invasive front	No	44.54	64.24	103.00	0.389
		Yes	42.00	59.74	76.00	

Abbreviation: HPV = human papillomavirus. Mann–Whitney test ($N = 87$).
[a]Statistically significant, $P \leqslant 0.05$.

(Figure 4A and D). There was no difference of Slug expression at the invasive front when compared with the central tumour ($P = 0.059$; Figure 4G). Also, Slug expression was not associated with vascular and perineural invasion, lymph node involvement, FIGO stage, invasion depth, inflammatory infiltration, overall and disease-free survival (Supplementary Table S1 and Table 4B). Slug positivity was significantly associated with associated lesions, specially differentiated and high-grade VIN ($P = 0.029$; Table 4B). Higher expression of Slug at the central tumour and invasive front was also most frequently observed in negative HPV cases ($P = 0.018$ and $P = 0.004$, respectively; Table 5).

Snail and Twist2 expression. Snail and Twist2 expression presented as sharp, well-localised nuclear and cytoplasmic staining. Snail was positive in both central tumour and invasive front in 44 out of 87 cases (50.6%; Figure 4B and E). Twist2 was positive at the central tumour and invasive front in 42 out of 87 (48.3%) and 44 out of 87 cases (50.6%; Figure 4C and Figure 4F). There was no difference in Snail expression at invasive front when compared with central tumour ($P = 0.813$; Figure 4H). Snail expression in central tumour was related to vascular and perineural invasion, invasion depth and associated lesions ($P = 0.031$, $P = 0.029$ and $P = 0.018$; Table 4A); on the other hand, Snail expression at the

Table 6. β-catenin^{low} and β-catenin^{low}/Slug^{high} expression *vs* clinicopathological data

			Protein expression		Protein expression	
Variables	**Category**	**Region**	**β-catenin^{low} N/total (%)**	***P*-value**	**β-catenin^{low}/Slug^{high} N/total (%)**	***P*-value**
Vascular/perineural invasion	No	IF	34/65 (52.3)	0.293	16/65 (24.6)	0.293
	Yes		13/19 (68.4)		7/19 (36.8)	
	No	CT	28/65 (43.1)	0.740	17/65(26.2)	0.364
	Yes		9/19 (47.4)		7/19(36.8)	
Invasion depth	SMD	IF	25/50 (50.0)	0.701	14/50 (28.0)	0.701
	DDS		22/37 (59.5)		9/37 (24.3)	
	SMD	CT	20/50(40.0)	0.579	12/50 (24.0)	0.384
	DDS		17/37(45.9)		12/37 (32.4)	
Lymph node involvement	$\leqslant 2$	IF	23/42 (54.8)	0.870	12/42 (28.6)	1.000
	>2		2/3 (66.7)		1/3 (33.3)	
	$\leqslant 2$	CT	21/42 (50.0)	1.000	12/42 (28.6)	1.000
	>2		2/3 (66.7)		1/3(33.3)	
Associated lesions	VIN	IF	4/10 (40.0)	0.446	8/27 (78.5)	0.637
	LH		12/23 (52.2)		2/5 (38.9)	
	VIN	CT	3/10 (30)	0.438	6/22 (41.7)	0.473
	LH		8/23 (34.8)		4/10/20 (75)	
FIGO stage	I–II	IF	23/52 (44.2)	0.023[a]	9/52 (17.3)	0.023[a]
	III–IV		21/30 (70.0)		12/30 (40.0)	
	I–II	CT	17/52 (32.7)	0.016[a]	10/52 (19.2)	0.041[a]
	III–IV		18/30 (60.0)		12/30 (40.0)	
Inflammatory infiltrate	No	IF	16/28 (57.1)	0.466	6/28 (21.4)	0.466
	Yes		31/59 (52.5)		17/59 (28.8)	
	No	CT	11/28 (39.3)	0.673	6/28 (21.4)	0.376
	Yes		26/59 (44.1)		18/59 (30.5)	

Abbreviations: $\leqslant 2$ = two or less lymph node involvement; >2 = more than two lymph node involvement; CT = central tumour; DDS = deep dermis and subcutis; FIGO = International Federation of Gynecology and Obstetrics; IF = invasive front; LH = lichen sclerosus and squamous hyperplasia; SMD = superficial and mind dermis; VIN = vulvar intraepithelial neoplasia (differentiated and high grade). χ^2- and Fisher's exact test ($N = 87$).
[a]Statistically significant, $P \leqslant 0.05$.

invasive front was related to negative HPV cases ($P = 0.018$; Table 5). There was also no difference in Twist2 expression at the invasive front when compared with the central tumour ($P = 0.113$; Figure 4I). However, Twist2 expression at the invasive front was associated with negative HPV cases ($P = 0.009$ and $P = 0.005$; Table 5). Besides, Twist2 expression was also related to the presence of vulvar intraepithelial neoplasia differentiated ($P = 0.014$; Table 4B). However, no association between Snail or Twist2 with lymph node involvement, inflammatory infiltration, FIGO stage, overall and disease-free survival was observed (Supplementary Table S1 and Table 4B).

Loss of expression of β-catenin together with high expression of Slug. Cases that expressed β-catenin^{low}/Slug^{high} at the invasive front were related with FIGO stages III and IV ($P = 0.023$) and negativity for HPV ($P = 0.000$; Table 7). This pattern of expression (β-catenin^{low}/Slug^{high}) was observed at the central tumour and invasive front in 24 out of 87 (27.6%) and 21 out of 87 cases (24.1%; Figure 5), and these patients had the poorest overall survival ($P = 0.001$; Figure 3C and D).

DISCUSSION

Alterations in cell adhesion are among the hallmark characteristics of a malignant epithelial tumour, including irregularities in expression and distribution of adhesion molecules, which is accompanied by degradation of extracellular matrix, and gain of mesenchymal cytoskeletal proteins such as N-cadherin, Vimentin, fibronectin and smooth muscle alfa-actin (Peinado *et al*, 2007; Thiery *et al*, 2009). Therefore, we investigated the expression of E-cadherin, β-catenin, Vimentin and the EMT markers Snail, Slug and Twist2 in a large series of 87 cases of vulvar carcinoma. Also, concerning different morphological subpopulations of tumour cells, we evaluated the immunoexpression of these proteins in both central tumour and invasive front, in order to determine whether the expression of these molecules induce EMT-like, the acquisition of migratory and invasive behaviour and whether these events are related with HPV infection.

Loss of E-cadherin expression leads to reduced cell adhesiveness and detachment of tumour cells from each other, resulting in increased invasive potential of the neoplastic cells. The results of the present study reveal that there was statistical association between lower E-cadherin expression and vascular and perineural invasion, FIGO stages III and IV, deeper invasion and $\geqslant 2$ lymph node involvement (Table 4A and B). E-cadherin expression was not significantly reduced at the invasive front compared with the central tumour. Also, no association was found between decrease of E-cadherin expression, inflammatory infiltration and HPV infection (Table 5). Carico *et al* (2012) demonstrated that higher expression of E-cadherin was associated with specific subtypes of laryngeal cancer, such as *in situ* carcinoma, in which a strong and

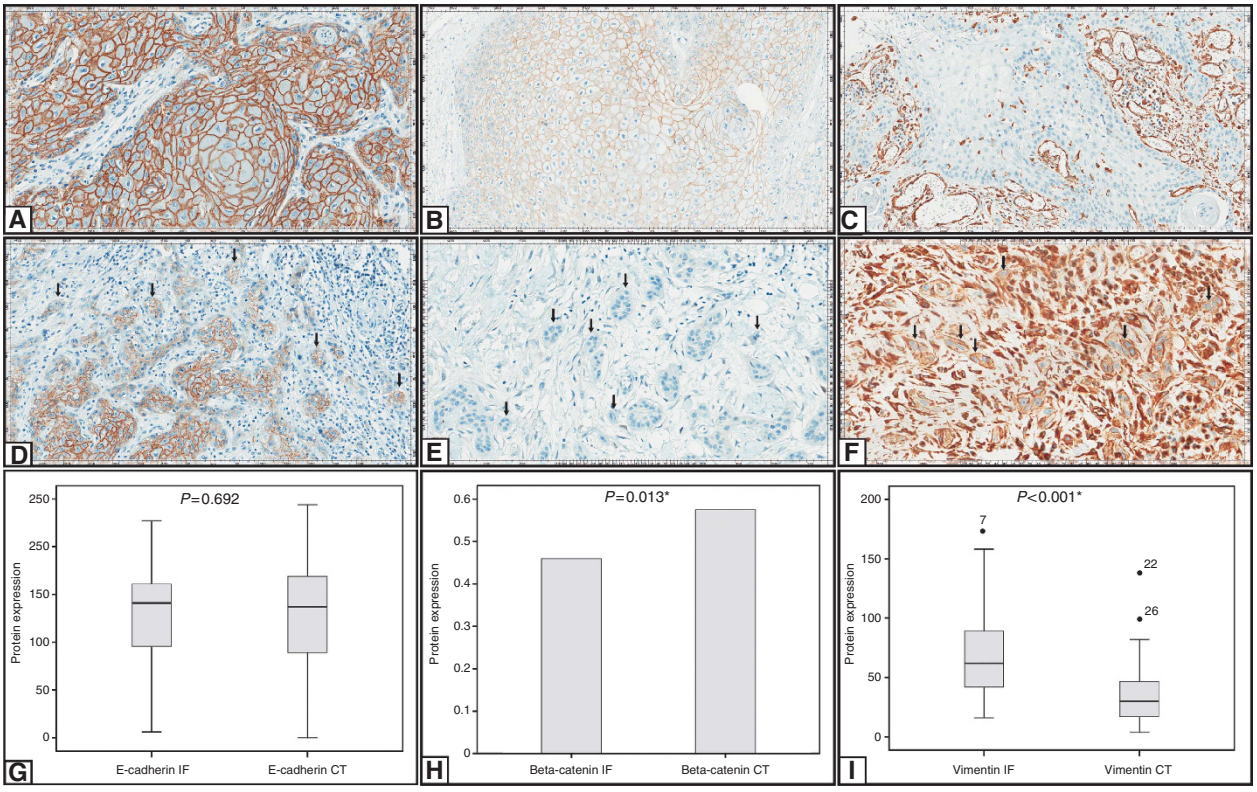

Figure 2. Immunohistochemical staining for E-cadherin, β-catenin and Vimentin is shown at central tumour and invasive front. Positive immunostaining for E-cadherin at central tumour (**A**) and invasive front (**D**); positive immunostaining for β-catenin at central tumour (**B**) and negative immunostaining for β-catenin at invasive front (**E**); negative immunostaining for Vimentin at central tumour (**C**) and positive immunostaining for vimentin at invasive front (**F**). Comparison between the expression of E-cadherin (**G**), β-catenin (**H**) and Vimentin (**I**) in central tumour and invasive front. All images were captured at ×200 magnification. *Statistical significance, $P \leqslant 0.05$.

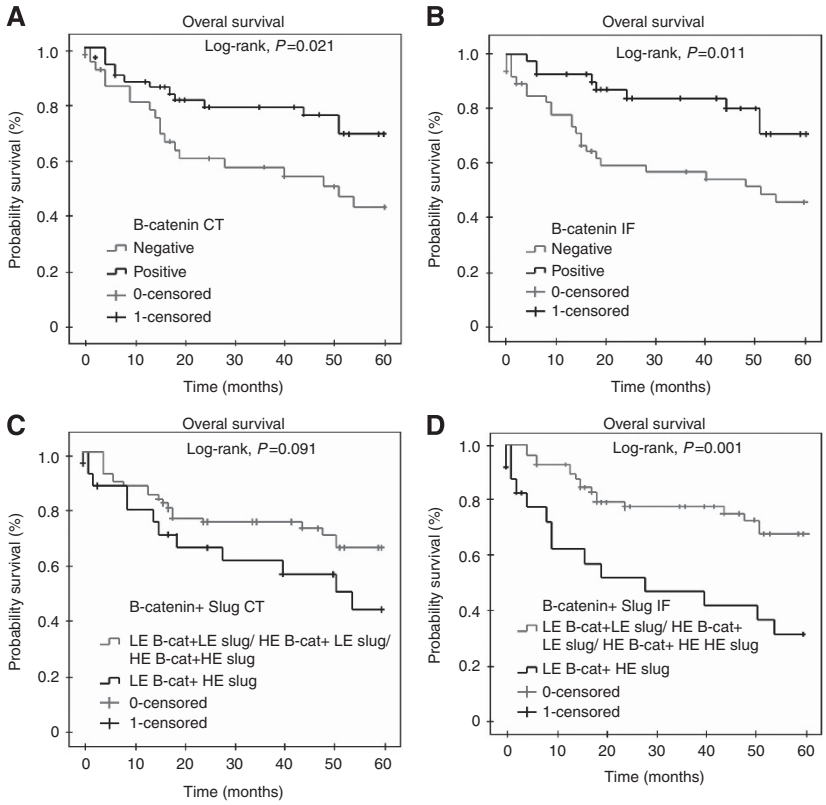

Figure 3. Overall survival analysis of 87 cases of VSCC. Shorter overall survival was observed for patients whose tumours lacked β-catenin expression at the central tumour ($P = 0.021$; **A**) and invasive front ($P = 0.011$; **B**). Overall survival curve of patients whose tumours expressed low β-catenin and high Slug at center tumour (**C**) and invasion front ($P = 0.001$; **D**). Kaplan–Meier method, log-rank test.

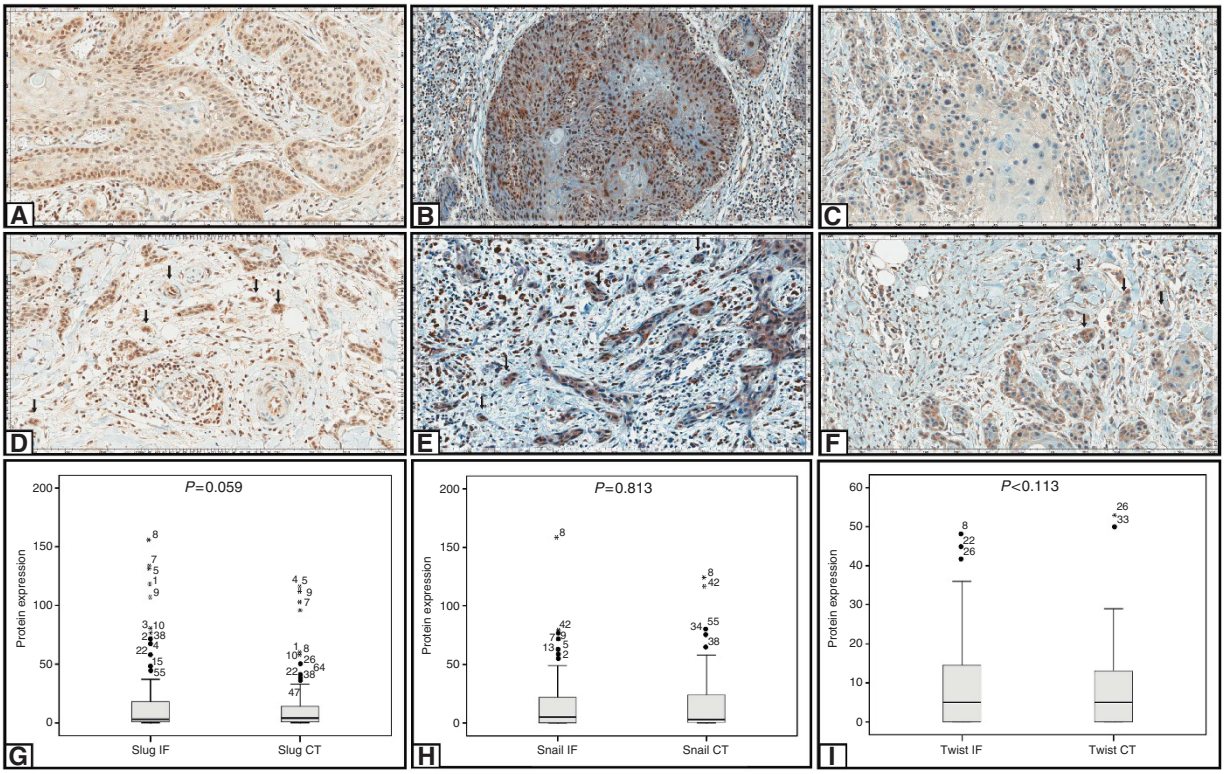

Figure 4. Immunohistochemical staining for Slug, Snail and Twist is shown at central tumour and invasive front. Positive immunostaining for Slug at central tumour (**A**) and invasive front (**D**); positive immunostaining for Snail at central tumour (**B**) and invasive front (**E**); positive immunostaining for Twist at central tumour (**C**) and invasive front (**F**). Comparison between the expression of Slug (**G**), Snail (**H**) and Twist (**I**) in central tumour and invasive front. Images **A**, **B**, **D**, **E** and **F** were captured at $\times 200$ magnification. Image **C** was captured at $\times 400$ magnification. *Statistical significance, $P \leqslant 0.05$.

Table 7. β-catenin and β-catenin/Slug expression *vs* HPV infection

Protein expression	Region	Category	HPV positive *N*/total (%)	*P*-value
β-catenin^{low}	IF	No	37/53 (69.8)	0.000[a]
		Yes	10/34(29.4)	
	CT	No	30/53 (56.6)	0.001[a]
		Yes	7/34 (20.6)	
β-catenin^{low}/slug^{high}	IF	No	21/53 (39.6)	0.000[a]
		Yes	2/34 (5.9)	
	CT	No	21/53 (39.6)	0.002[a]
		Yes	3/34 (8.8)	

Abbreviation: HPV = human papillomavirus. χ^2-test ($N = 87$).
[a]Statistically significant, $P \leqslant 0.05$.

Table 8. Correlation between β-catenin expression and clinical data of patients with vulvar carcinoma

Variables	*N*	*P*-value	Hazard ratio for survival	95.0% CI
Vascular and perineural invasion	19	0.204	1.650	0.762–3.569
FIGO stages	30	0.577	1.264	0.555–2.881
HPV	53	0.367	1.493	0.625–3.564
β-catenin central tumour	37	0.928	1.047	0.386–2.841
β-catenin invasion front	47	0.044[a]	2.131	1.022–5.234

Abbreviations: CI = confidence interval; FIGO = International Federation of Gynecology and Obstetrics; HPV = human papillomavirus. multivariate analysis ($N = 87$)
[a]Statistically significant, $P \leqslant 0.05$.

diffuse immunostaining pattern was observed. Furthermore, the majority of previous studies indicated that loss of E-cadherin expression has a close correlation with metastasis (Sawada *et al*, 2008; von Burstin *et al*, 2009; Tang *et al*, 2011). In the light of ours and other results, it is suggestive that E-cadherin expression may be lower in primary tumours that can potentially progress to lymph node or distant metastasis.

Association between E-cadherin and β-catenin is known to be essential for proper anchorage to the cytoskeleton and is necessary for E-cadherin-binding function (Thiery *et al*, 2009). Our results have demonstrated that the loss of β-catenin expression strongly characterises the transition from cells at the central tumour to invasive nests or isolated tumour cells in elderly patients with VSCC having no HPV infection (Table 7). Similar findings have been recorded in cervical squamous carcinoma, in which there was reduced β-catenin immunoexpression in many of infiltrative tumour cells, particularly at the tumour–stromal interface (Koay *et al*, 2012). In the present study, loss of β-catenin expression was statistically associated with FIGO stages III and IV, and Cox regression model also demonstrated that loss β-catenin expression was an independent factor associated with poor survival (Table 8). Previous studies demonstrated that β-catenin is an important marker of invasion for cervical neoplasms, and therefore can be useful in the diagnostic setting (Stewart *et al*, 2011). Our results allow us to confirm these findings and also to suggest that the assessment of β-catenin expression by IHC at the invasive tumour front may represent an important tool in establishing prognosis of patients with vulvar cancer.

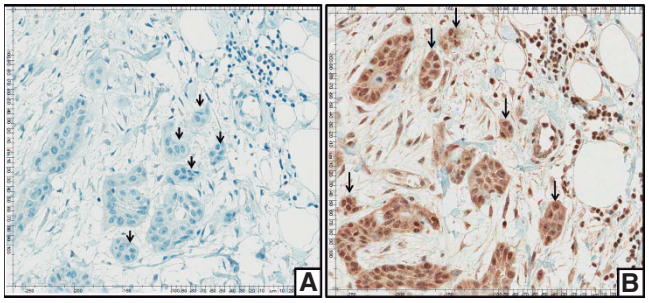

Figure 5. Immunohistochemical staining for Slug and β-catenin. Negative immunostaining for β-catenin at invasive front (**A**). Positive immunostaining for Slug at invasive front (**B**).

In our series, loss of β-catenin expression was associated with a significant increase in the expression of Slug and Vimentin at the invasive front when compared with central tumour. Loss of expression of β-catenin and gain of Slug could be a key feature of EMT-like events, and therefore the current findings could suggest that EMT-like has an important role in invasion of VSCC. Kojc *et al* (2009) showed that upregulation of transcription repressors Snail, Slug and Twist on both mRNA and protein levels presumably results in transient EMT, which has been postulated to be responsible for tumour progression and metastasising (Yuen *et al*, 2007). Interestingly, our results show strong association between high expression of Slug, Snail and Twist2 at the invasive front with negative HPV cases (Table 5). Furthermore, the strongest association was between β-catenin^{low}/Slug^{high} tumours with HPV-negative, late stage and patients with poorest overall survival. Other data of our group are consistent with the fact that vulvar tumours associated with HPV infection, which has been shown to be an independent predictor of better survival, have a better prognosis compared with tumours with p53 mutations (Kumar *et al*, 2009; De Melo Maia *et al*, 2013). Furthermore, our results are in agreement with these studies, as our HPV-positive cases showed better overall survival than those of HPV-negative ones; however, the multivariate analysis did not show an independent prognostic significance in these cases.

These observations led us to hypothesise that those alterations in β-catenin and Slug expression may characterise a potentially more aggressive behaviour of the tumour cells in the tumour front, with increased risk of deeper invasion and metastasis. These results, together with other results from our group, drive us to conclude that HPV-positive tumours do not show evidences of EMT-like events, with usually better prognosis (De Melo Maia *et al*, 2012; Lavorato-Rocha *et al* 2013). On the other hand, the HPV-negative tumours develop EMT-like and, therefore increased capability of invasion and progression, leading to worse prognosis and poorer outcome.

The metastatic process of epithelial tumours is characterised by alteration in E-cadherin and β-catenin expression, and seems to occur at different time points, depending on the patient's HPV status. Al Moustafa *et al* (2004) showed that overexpression of E6()E7 in combination with ErbB-2 activation downregulates E-cadherin and membranous β-catenin expression. This is accompanied by nuclear translocation of β-catenin with subsequent upregulation of oncoproteins responsible for tumour progression (Al Moustafa *et al*, 2004). In our series, besides loss of β-catenin being related with negativity for HPV infection (Table 7), no nuclear β-catenin was detected at all, suggesting that other pathways apart from HPV-related Wnt activation may be related with EMT-like events in vulvar cancer.

Vimentin is another upregulated protein in aggressive tumour phenotypes related to EMT phenomenon (Brabletz *et al*, 2005; Otsuki *et al*, 2011). Vimentin is ubiquitously expressed by cells of mesenchymal origin including fibroblasts, endothelial cells, smooth muscle cells and leucocytes (Mor-Vaknin *et al*, 2003). Our results indicated that the invasive front expressed higher Vimentin than that of central tumour. Furthermore, Vimentin immunopositivity was also significantly associated with invasion depth and associated lesions (lichen sclerosus and squamous hyperplasia; Table 3A). De Araujo *et al* (1993) demonstrated that the expression of the Vimentin was most marked in oral squamous cell carcinoma with high tumour grade. Also, significant correlation between Vimentin expression and aggressive tumour features, including lymph node involvement, was recently shown by immunophenotypic analysis of the head and neck squamous cell carcinoma (Mandal *et al*, 2008; Dal Vechio *et al*, 2011). Our results did not indicate significant association between Vimentin expression and HPV infection.

In conclusion, E-cadherin, β-catenin, Slug, Snail, Twist2 and Vimentin expression are important features for the characterisation of EMT-like events, known to be related to invasive and metastatic phenotype in many tumours, including vulvar cancer. According to our results, loss of E-cadherin expression is observed in advanced stages of tumour invasion. β-catenin represents an important biomarker for establishing prognosis in vulvar cancer, as its loss of expression is related to poorer outcome, even in the multivariate analysis. Furthermore, β-catenin lower expression associated with gain in Slug expression characterises a subgroup of EMT-related HPV-negative tumours with the worst outcome, increased invasiveness and progression. Human papillomavirus-positive tumours did not exhibit EMT-like events, and had better prognosis. In the clinical setting, IHC-comparative assessment of β-catenin expression between invasive front and central tumour may represent an additional tool for establishing prognosis of vulvar cancer.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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