P16 protein expression in primary cutaneous melanoma with positive and negative lymph node biopsies: Particular aspects of a study performed at the Hospital de Clinicas de Porto Alegre, Brazil

JAC Fauri MD¹, F Ricardi MD², ES Diehl MD³, A Cartell MD⁴, R Furian MD⁵, L Bakos MD⁶, MI Edelweiss MD⁷

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BACKGROUND: Cutaneous melanoma dermal invasion, identified through measurement of maximum tumour thickness and sentinel lymph node (SLN) biopsy, is important to establish melanoma prognosis and progression. P16 protein expression has been shown to be a predictive factor for melanoma evolution and prognosis.

OBJECTIVE: To investigate p16 protein expression in cutaneous melanomas with and without SLN metastasis.

PATIENTS AND METHODS: Sixty-seven paraffin-embedded cutaneous melanoma specimens of patients who had undergone SLN investigation were evaluated from 1995 to 2007. SLN biopsy was negative for metastasis in 34 of these patients (controls); in the remaining 33 patients, SLN biopsy was positive (cases). The expression of p16 protein in the primary tumour was measured using an immunohistochemical assay. The samples were classified according to their nuclear expression.

RESULTS: P16 nuclear expression was absent in 14 cases and in 15 controls; $P=0.812$. There was no statistically significant difference in p16 nuclear expression between cases and controls.

CONCLUSIONS: The present study does not support the findings of other studies that suggest p16 protein expression is important in the prognosis of cutaneous melanoma.

Key Words: *Cutaneous melanoma; Immunohistochemistry; Metastasis; p16; Sentinel lymph node*

The increase in the incidence of cutaneous melanomas from 3% to 7% among Caucasians (1) is a major health concern worldwide. The identification of markers that may accurately predict the chances of a cutaneous melanoma in producing metastases has been the focus of several studies (2-6). Discoveries in the fields of molecular biology and oncology have provided explanations regarding several gene associations relative to tumour development.

The sentinel lymph node (SLN) biopsy in cutaneous melanomas, described by Morton et al (7), changed surgical approaches in centres that specialized in the treatment of these tumours worldwide. The use of a specific and sensitive histological marker might be helpful in the selection of patients undergoing SLN investigation.

Chana et al (8) found high p16 protein expression in melanocytic nevi and lower expression levels in dysplastic melanocytic nevi. Progressively, lower levels were found in primary and metastatic cutaneous melanomas. The same authors (9) studied acral melanomas (lentiginous subtype) and investigated the biological, molecular and

L'expression de la protéine p16 dans des mélanomes cutanés primaires dont les biopsies des ganglions lymphatiques sont positives ou négatives : des aspects précis d'une étude effectuée à l'Hospital de Clinicas de Porto Alegre, au Brésil

HISTORIQUE : Il est important de déterminer l'invasion dermique d'un mélanome cutané, dépisté par la mesure de l'épaisseur maximale de la tumeur et de la biopsie d'un ganglion sentinelle (GS), pour établir le pronostic et l'évolution du mélanome. Il est démontré que l'expression de la protéine p16 est un facteur prédictif de l'évolution et du pronostic du mélanome.

OBJECTIF : Explorer l'expression de la protéine p16 dans les mélanomes cutanés comportant ou non des métastases dans les GS.

PATIENTS ET MÉTHODOLOGIE : De 1995 à 2007, les chercheurs ont évalué 67 échantillons de mélanome cutané en paraffine provenant de patients qui avaient subi une exploration du GS. La biopsie du GS était négative aux métastase chez 34 patients (témoins), et chez les 33 autres, elle était positive (cas). Les chercheurs ont mesuré l'expression de la protéine p16 dans la tumeur primaire au moyen d'un titrage immunohistochimique. Ils ont classé les échantillons d'après leur expression nucléaire.

RÉSULTATS : Il n'y avait pas d'expression nucléaire de la protéine p16 dans 14 cas et chez 15 témoins; $P=0,812$. On ne constatait aucune différence statistiquement significative dans l'expression nucléaire de la protéine p16 des cas et des témoins.

CONCLUSIONS : La présente étude n'étaye pas les résultats d'autres études selon lesquelles la protéine p16 est importante dans le pronostic du mélanome cutané.

cellular basis of these tumours. They suggested that the loss of p16 protein expression is not only associated with the early transformation of benign lesions, but also with disease progression at its invasive stage.

The *p16^{INK4a}* gene was identified as the target of mutations in several types of tumours other than cutaneous melanomas because it encodes the 16 kDa protein that inhibits the cyclin-dependent kinase enzymes CDK4 and CDK (6). These kinases promote the phosphorylation of Rb protein (retinoblastoma) at the G₁ checkpoint of the cell cycle. The hypophosphorylated form of the Rb protein prevents progression to the S phase (or synthesis) of the cell cycle because it sequesters a family of transcription factors, such as E2F, that are necessary for DNA replication and progression of the cell cycle. In the absence of p16 protein, Rb protein undergoes phosphorylation by CDK4 and CDK6; these transcription factors are released and lead to DNA replication and consequent tumour proliferation (9).

We hypothesized that weak or absent p16 protein expression was associated with poor prognosis in cutaneous malignant melanoma.

¹Department of Surgery, Programa de pós graduação em Ciências Cirúrgicas, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul;

²Department of Pathology, Fundação Faculdade Federal de Ciências Médicas de Porto Alegre; ³Department of Clinical Medicine; ⁴Department of Pathology, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul and Hospital de Clínicas de Porto Alegre; ⁵Department of Pathology, Fundação Faculdade Federal de Ciências Médicas de Porto Alegre and Hospital Santa Casa de Misericórdia de Porto Alegre;

⁶Department of Dermatology Service, Faculdade de Medicina; ⁷Department of Pathology, Universidade Federal do Rio Grande do Sul and Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil

Correspondence: Dr JAC Fauri, Av. Maryland, 1367 ap. 902, 90440-191 Porto Alegre, Brazil. Telephone and fax 55-51-3332-0339, e-mail fauri47@gmail.com

TABLE 1
Semiquantitative scale of nuclear staining, according to
Schneider-Stock et al (12)

Nuclear expression	Percentage of stained nuclei	Point scale
Absent	0 to 5	0
Weak	6 to 20	1
Moderate	21 to 50	2
Strong	More than 50	3

In the present study, we verified immunohistochemical p16 protein expression in primary cutaneous melanomas from biopsies prepared for histological analysis. Samples were obtained from patients who later underwent SLN biopsy according to the protocol of our Division of Oncologic Surgery at the Hospital de Clínicas de Porto Alegre (HCPA), Universidade Federal do Rio Grande do Sul (Porto Alegre, Brazil). p16 protein expression was correlated with different histological levels of melanoma progression.

PATIENTS AND METHODS

The present case-control observational study was conducted at the HCPA and at the Faculdade de Medicina da Universidade Federal do Rio Grande do Sul. The objective of the study was to investigate the immunohistochemical expression of p16 protein in primary cutaneous melanomas. The outcome measure assessed was the presence or absence of SLN metastasis.

The independent variables were sex, age, location of primary tumour, histological type, Breslow thickness and Clark's level. Patients with a diagnosis of cutaneous melanoma who were surgically treated in the services listed above and who underwent SLN biopsy were included. Exclusion criteria included the following: previous chemotherapy; history or presence of other concomitant malignant tumours, except skin tumours; immunosuppression; absence or scarcity of material from primary lesion in paraffin block; incomplete patient records and distant metastasis of melanoma.

Between 1995 and 2007, 316 cases of primary cutaneous melanoma were selected from the HCPA and Santa Casa de Misericórdia de Porto Alegre anatomical pathology records. Of these, 163 cases (52%) referred only to the primary lesion, without mention of the SLN investigation, and were excluded from the study. In this group, there were 21 *in situ* melanomas, 10 punch samples, two amelanotic melanomas, one multiple melanoma, one sample with insufficient material and one unclassified melanoma. From the remaining 153 cases (48%), 12 referred only to the SLN biopsy, 16 medical records were incomplete, one was a mucosal melanoma, one was recurrent and 56 were metastatic melanomas. The other 67 (21%) paraffin-embedded specimens of cutaneous melanomas were from patients with complete medical records who underwent SLN investigation during clinical evaluation. SLN biopsies were negative for metastasis in 34 patients (controls), and positive in 33 (cases).

Immunohistochemistry

Representative 3 µm thick biopsy sections of the lesion, fixed in formalin and paraffin embedded, were evaluated with the immunohistochemical technique used in the Experimental Research Center of the HCPA as described by Mills (10) in 1992 and Santos et al (11) in 1999.

The technique includes the following steps: deparaffinization, rehydration, antigen retrieval, endogenous peroxidase inactivation and blocking of nonspecific reactions. The primary antibody – monoclonal mouse antihuman immunoglobulin G p16^{INK4a} (Dako Canada Inc) – at 1:50 dilution was incubated for 12 h overnight at 4°C, followed by application of the streptavidin-biotin-peroxidase complex (LSAB kit [Dako Canada Inc]), developed with diaminobenzidine tetrahydrochloride (DAB kit [Dako Canada Inc]) and counterstained with Giemsa.

For detection of p16 protein in the present study, a stained slide with a high-degree intraepithelial lesion of the uterine cervix was used as a positive control, and a slide without the primary antibody was used as a negative control. Non-neoplastic stromal cells, with nuclear reactivity and lymphocytes, were used as an internal positive control.

Giemsa staining was used to neutralize and differentiate actual staining from the immunohistochemical technique (brownish because of the DAB kit) with the melanotic brownish colour of the lesion, which became markedly bluish after counterstaining.

Two pathologists blinded to the histological and clinical data read and classified the intensity of p16 oncoprotein on the slides. The final results were obtained through consensus between the two pathologists.

p16^{INK4a} interpretation

The intensity of nuclear expression of p16 protein was classified into four levels: absent, weak, moderate and strong. After evaluation of 10 high-powered fields, each field was defined as having a 400× magnification, as suggested by Schneider-Stock et al (12). A section was classified as immunohistochemically positive for p16 protein when the nuclei of the tumour cells were stained, with or without cytoplasmic expression, and was graded using a semiquantitative 0- to 3-point scale: 0 = absent (0% to 5% stained nuclei); 1 = weak (6% to 20% stained nuclei); 2 = moderate (21% to 50% stained nuclei); and 3 = strong (more than 50% stained nuclei) (Table 1).

Figure 1 shows images of nuclear expression reactions in study cases, and their grades on the 0- to 3-point scale.

Histopathological analysis

The morphological analysis of slides was made by two pathologists. The pathological results of cases and controls were classified and analyzed according to several subtypes of cutaneous melanoma based on the histogenetic classification described by Clark (13): acral, superficial spreading, nodular and lentigo maligna melanoma. The histological criteria of lesion progression – Clark's level (13) and Breslow thickness (14) – were also reviewed.

All cases were evaluated clinically and histologically for the presence or absence of metastasis in the SLN by reviewing results of histological examinations.

Statistical analysis

SPSS for Windows, version 13.0 (IBM Corporation, USA) was used for data analysis. Descriptive analysis was expressed as absolute and relative frequencies. Pearson's χ^2 test, Fisher's exact test and Student's *t* test were used to compare demographic, clinical and histological data between cases and controls. The level of significance was set at 0.05.

Sample calculation: Considering that the mean ($\pm$ SD) expression of p16 has been found to be approximately 1.5 ± 1 in positive SLN biopsy samples, and 2.3 ± 1 in negative samples, a sample size of 60 was calculated to be necessary to achieve 80% power with a 95% CI.

Maximum lesion microthickness according to Breslow thickness (14) was correlated with greater or lower expression of the p16 protein using the Pearson's correlation coefficient (*r*). Likewise, histological types and tumour locations were compared with levels of expression.

Ethical issues

Permission to use biological material in the present study was granted by the ethics committees of the HCPA, protocol no. 06/072, and of Santa Casa de Misericórdia de Porto Alegre, according to the consolidated opinion issued under no. 316/07. The material was collected from patients whose biopsies were performed by the surgical and dermatological team of the two hospitals, and stored in paraffin blocks in the two surgical pathology departments.

RESULTS

Thirty-three cases with SLN metastasis and 34 controls without SLN metastasis were studied. Thirteen (39%) patients were female, and 20 (61%) were male. Twenty-five (73%) of the controls were female patients, and nine (27%) were male ($P=0.010$). The mean age of the patients with positive SLN biopsies (cases) was 54 ± 14 years, and the mean age of the patients with negative SLN biopsies (controls) was 53 ± 13 years ($P=0.662$). The most frequent anatomical tumour site was the back: 10 (30%) cases and eight (26%) controls ($P=0.156$) (Table 2).

Figure 1 A Strong expression: Digital image of cutaneous melanoma with strong (yellow arrow) and moderate (red arrow) nuclear expression of p16 protein. See weak cytoplasmic staining – not evaluated in the study. B Moderate expression: Digital image of cutaneous melanoma showing moderate expression of p16 protein (yellow arrows) in some nuclei. C Weak expression: Digital image of cutaneous melanoma with weak nuclear expression of p16 protein. Note areas with nuclei that are not intensely stained (yellow arrows). D No expression: Digital image of cutaneous melanoma with no nuclear expression of p16 protein. Blue areas show melanin reaction (yellow arrows) and Giemsa counterstaining (p16 immunohistochemical staining, $\times 400$ original magnification)

The predominant histological tumour subtype was superficial spreading melanoma: 22 (67%) cases and 25 (78%) controls, followed by the acral subtype: six (18.2%) cases and four (11.8%) controls. The nodular subtype was present in four (12.1%) cases and two (5.9%) controls, and lentigo maligna melanoma in one (2.9%) control. Unclassified melanomas were present in one (3%) case and in two (5.9%) controls; $P=0.653$ (Fisher's exact test).

The classification according to Breslow thickness showed that most tumours had a thickness ranging from 1.01 mm to 4.00 mm: 20 (61%) cases and 21 (62%) controls ($P=0.003$). Most tumours were classified as Clark's level III or IV. Clarke's level III: four (12%) cases and 10 (29%) controls; Clark's level IV: 23 (70%) cases and 23 (68%) control; $P=0.080$ (Table 3).

The analysis of p16 protein expression intensity (12) revealed that it was absent in 14 (42%) cases and 15 (44%) controls; it was weak in three (9.1%) cases and in three (8.8%) controls (OR 1.07; 95% CI 0.14 to 8.34); moderate in three (9.1%) cases and one (2.9%) control (OR of 3.21; 95% CI 0.24 to 90.82); and strong in 13 (39%) cases and in 15 (44%) controls (OR of 0.93; 95% CI 0.29 to 2.99) ($P=0.812$) (Table 4).

A comparison of p16 protein expression (mean percentage of stained nuclei) with the histological tumour type in the cases revealed the following values: acral melanoma $35 \pm 0.40\%$; superficial spreading melanoma $37 \pm 0.39\%$ and nodular melanoma $35 \pm 0.34\%$ (Figure 2).

A comparison of p16 protein expression (mean percentage of stained nuclei) with the histological tumour type in the controls

revealed the following values: acral melanoma $43 \pm 0.38\%$, superficial spreading melanoma $35 \pm 0.40\%$, nodular melanoma $45 \pm 0.49\%$ and lentigo maligna melanoma 50% (Figure 2).

A comparison of maximum tumour thickness (Breslow thickness) with the percentage of stained nuclei in cases (expression) did not reveal any correlation between Breslow thickness and positive p16 protein results (percentage of stained nuclei); $r=0.029$, $P=0.873$ (Figure 3).

A comparison of maximum tumour thickness (Breslow thickness) with the percentage of stained nuclei in controls (expression) did not reveal any correlation between Breslow thickness and positive p16 protein results (percentage of stained nuclei); $r=0.146$, $P=0.410$ (Figure 4).

DISCUSSION

In the present study, we verified the expression of p16 protein in primary cutaneous melanomas with and without SLN metastasis.

Several studies of cutaneous melanoma analyzed the use of p16 protein (2-6). Some important findings were reported by Straume et al (5) who conducted a study with 202 cases of cutaneous melanomas in the vertical growth phase, and found that 77% of the metastatic cases (lymph nodes or distant) did not express p16. Bachmann et al (6) also found an association between the loss of p16 protein expression in cutaneous melanomas with tumour cell proliferation and poor prognosis. Zhang and Rosdahl (15) studied the loss of p16 protein expression using the Western blot technique and correlated it with primary and metastatic cutaneous melanomas. They showed that p16 protein was

TABLE 2
Demographic characteristics of the study population with cutaneous melanoma (n=67)

Variable	Case (n=33)	Control (n=34)	P
Sex, n (%)			0.010*
Female	13 (39.4)	25 (73.5)	
Male	20 (60.6)	9 (26.5)	
Age, mean $\pm$ SD	54 $\pm$ 14	53 $\pm$ 13	0.662†
Site, n (%)			0.156‡
Back	10 (30.3)	8 (25.8)	
Foot	7 (21.2)	4 (12.9)	
Lower extremity, except foot	5 (15.2)	8 (25.8)	
Scalp/neck	3 (9.1)	0 (0)	
Thorax	3 (9.1)	0 (0)	
Upper extremities	2 (6.1)	7 (22.6)	
Face	2 (6.1)	2 (6.5)	
Abdomen	1 (3)	2 (6.5)	

* χ^2 test; †Student's t test; ‡Fisher's exact test

TABLE 3
Tumour thickness (Breslow) and Clark's levels of cutaneous melanomas in cases and controls

Variable	Case (n=33)	Control (n=34)	P
Breslow thickness, mm			0.003*
Up to 0.75	1 (3)	5 (14.7)	
0.76 to 1.00	2 (6.1)	7 (20.6)	
1.01 to 4.00	20 (60.6)	21 (61.8)	
4.01 or greater	10 (30.3)	1 (2.9)	
Clark's level			0.080*
2	1 (3)	0 (0)	
3	4 (12.1)	10 (29.4)	
4	23 (69.7)	23 (67.6)	
5	5 (15.2)	1 (2.9)	

Data presented as n (%). *Fisher's exact test

TABLE 4
P16 expression intensity in cases and controls (16)

Variable	Intensity	Case (n=33)	Control (n=34)	P	OR (95%CI)
Expression	Absent	14 (42.4)	15 (44.1)	0.812*	1
	Weak	3 (9.1)	3 (8.8)		1.07 (0.14–8.34)
	Moderate	3 (9.1)	1 (2.9)		3.21 (0.24–90.82)
	Strong	13 (39.4)	15 (44.1)		0.93 (0.29–2.99)

Data presented as n (%). *Fisher's exact test

strongly expressed in two cell lines of primary melanomas and weakly expressed in one cell line of metastatic melanomas. Fearfield et al (16) found that the expression of p16 protein was lower in recurrent thin melanomas than in tumours without metastases.

Sparrow et al (17) analyzed the immunohistochemical expression of 110 benign and malignant melanocytic lesions. The expression of p16 protein was different in cutaneous melanocytic lesions, and loss of expression was associated with the invasive stage of tumour growth. p16 protein expression was found in 89% of benign melanocytic lesions, in 92% of in situ melanomas, in 50% of primary invasive melanomas and in 64% of metastatic melanomas. Alonso et al (2) studied 39 different antibodies and, after analyzing 165 samples of cutaneous melanomas from 88 patients, they also found a lower expression of p16 protein in 89% of the cases with vertical growth and in 71% of metastatic cases, whereas p16 protein expression in benign lesions was 100% (nine of nine cases). They also reported that the best predictors of survival were p16 protein and B-cell lymphoma-6 protein, and that the risk of death was eight times greater when there was loss of p16 protein expression or when B-cell lymphoma-6 protein was positive.

In our study, demographic data regarding sex, age and location were similar to those reported in the literature and by the Brazilian

Figure 2) Mean percentage of stained nuclei according to histological tumour type in cases and controls

Figure 3) Distribution of stained nuclei (%) in relation to tumour thickness in cases

Figure 4) Distribution of stained nuclei (%) in relation to tumour thickness in controls

Cancer Institute. The analysis of histological types revealed that the superficial spreading type was the most frequent, as expected, in the State of Rio Grande do Sul (1,2,18). The difference in maximum tumour thickness (Breslow) between cases and controls was statistically significant ($P=0.003$), which showed that this morphological marker is an important predictive factor of tumour aggressiveness (19). The differences in Clark's levels, however, were not statistically significant between cases and controls ($P=0.080$).

Although SLN biopsy is a standard procedure in institutions that treat cutaneous melanomas, research results often do not correspond to the exact clinical condition of the patient. Rossi et al (20) examined 96 consecutive patients with positive SLN biopsy who underwent complete lymphadenectomy, and found 20 patients (21%) with positive non-SLNs, which may be clinically significant.

In June 2008, Karim et al (21) examined 74 SLN biopsies from 33 patients and published a study that reported false-negative results in SLN biopsies, which may have resulted from failures in nuclear medicine, surgery or pathology. They reviewed lymphoscintigraphies, surgery data and histological findings, and aggregated multimarkers using real-time quantitative reverse transcription-polymerase chain reaction and antimony (a residue of technetium 99m) concentration measurements as markers of the 'true' status of the SLN obtained with the use of inductively coupled plasma mass spectroscopy, and found that false-negative results may represent 25% of the cases in long-term follow-up. Yet, Morton et al (7) confirmed the existence of a learning curve for the surgical technique to evaluate SLNs. According to their results, only after 55 surgeries does the surgeon begin to master the use of this technique.

Our study used samples from two institutions where different surgeons with different levels of expertise performed SLN biopsies. Our results differed greatly from those found in the literature. Technical problems may have resulted from the fact that cases and controls were

not adequately studied because the SLNs removed might have yielded false-negative results. There was statistical, but not clinical, significance in sex differences ($P=0.01$) because cases and controls were enrolled consecutively. This is believed to have occurred by chance due to the selection of paraffin blocks. Only the differences in Breslow thickness ($P=0.003$) were statistically and clinically significant between cases and controls, and had a prognostic value. The results of immunohistochemical expression of p16 protein in our study sample, contrary to studies in the international literature (2-6,8,15,22), did not show statistically significant differences between cases and controls. The lack of standard procedures to collect material from the primary lesion, as well as to determine the maximum time between this procedure and the SLN biopsy, may explain the inconsistencies. Incisional or punch biopsies sent to the pathology services, together with the refusal by some patients to undergo SLN biopsy, may have complicated the collection of a larger number of surgical specimens for correct staging, thus limiting the identification of the prognostic factor under study – the absence of nuclear p16 protein expression – in a larger number of cases.

P16 protein should be regarded as an important tool in the prognosis of cutaneous melanoma. Together with Breslow thickness, it may provide a more accurate prediction of SLN metastases, but this was not confirmed in our study. Yet, weak or absent p16 protein expression related to more aggressive or metastatic melanomas may reveal those with hematic dissemination regardless of lymph node status for micrometastasis (positive or negative). For an adequate evaluation, services should follow standard procedures to treat patients with cutaneous melanoma to improve not only the SLN biopsy techniques, but also the collection of primary lesion samples, and to establish a time frame for SLN biopsy after the diagnosis of the primary tumour.

The correct histological description of specimens is extremely important and was one of the best described items provided by the pathology services where the material for the present study was obtained. The description included all the items suggested by the Brazilian Melanoma Group such as the following: histological type of melanoma; growth phase (radial or vertical); Clark's level; depth

according to Breslow thickness; mitotic index; inflammatory lymphocyte infiltrate; peritumoral inflammatory infiltrate; intratumoral inflammatory infiltrate; lymphatic and blood vessel invasion; perineural invasion; ulceration; regression; microscopic satellitosis and surgical margins. Histological examination of SLN was designed to investigate metastasis, micrometastases (diameter less than 2 mm) or groups of cells circulating in the lymph node, and should be regarded as a special protocol. SLN should be fully examined in serial sections according to the methods reported in the literature (23).

The photographic standardization of lesions, the existence of a database common to all the services where all protocol items are entered, the development of molecular methods of investigation by means of more complex techniques, such as tissue microarray, will provide conditions for more accurate studies and responses to patient diagnosis, prognosis, treatment and follow-up. Therefore, the epidemiological profile of patients with cutaneous melanoma should be better outlined so that those patients will, as a result, be treated and followed up according to established standards used by international institutions.

Finally, the present study did not find a statistically significant difference in the loss of p16 protein expression between cutaneous melanomas both with or without SLN metastasis. The standardization of protocols in different institutions and of the care provided to patients with cutaneous melanoma, associated with complementary studies of p16 protein or other tumour markers and proteomics studies, may help to outline a prognostic molecular profile for primary cutaneous melanomas, which may eventually support surgical and clinical decisions.

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