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ORIGINAL ARTICLE

Expression of Glut-1 in Malignant Melanoma and Melanocytic Nevi: an Immunohistochemical Study of 400 Cases

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Abstract The glucose transporter-1 (Glut-1) is a cell membrane glycoprotein involved in glucose uptake. An increased expression of Glut-1 is an important cell adaptation mechanism against hypoxia. An upregulation of Glut-1 can be found in several types of malignant tumors, which are able to reprogram their metabolism from oxidative phosphorylation to aerobic glycolysis (Warburg effect). However, the data regarding melanocytic lesions is equivocal. We performed comprehensive immunohistochemical analysis of the Glut-1 expression in 225 malignant melanomas (MM) and 175 benign nevi. Only the membranous expression of Glut-1 was regarded as positive. The expression of Glut-1 (the cut-off for positivity was determined as H-score 15) was found in 69/225 malignant melanomas. The number of positive cases and the H-score of Glut-1 increased where there was a higher Breslow thickness ($p < 0.00001$) when comparing pT1- pT4 MM groups. All benign nevi were classified as negative. In conclusion, the membranous expression of Glut-1 is a common feature of a malignant melanoma but this type of expression is very rare in benign melanocytic nevi. Our results suggest that the membranous expression of Glut-1 can be used as a surrogate marker in the assessing of the biological nature of benign and malignant melanocytic lesions. However, despite its high specificity, the sensitivity of this marker is relatively low. Moreover, due to the fact that the increased expression of Glut-1 correlates with a shorter survival period (10-year disease free survival, recurrence free survival and metastasis free survival and MFS), it can be used as a prognostically adverse factor.

Keywords Glut-1 · Malignant melanoma · Melanocytic nevus · Immunohistochemistry · Follow-up

Background

The uptake of glucose is in the majority of mammalian cells mediated by members of the Glut (SLC2A) family of membrane transport proteins. This family includes 14 Glut proteins, however, only Glut1–4 are well established with known substrates [1]. An increased expression of Glut-1 can be found in several malignant tumors and seems to be an adverse prognostic factor associated with a higher tumor grade, higher stage and lymphovascular space/lymph node involvement [2–5]. In non-tumor tissue, the expression of Glut-1 can be detected, for example, in erythrocytes, perineurial cells, trophoblastic cells, reactive germinal centers, squamous epithelium, renal tubules and some endothelial cells. An expression of Glut-1 can also be found in some benign tumors (e.g. perineuriomas, infantile hemangiomas), but is relatively rare and usually weak [4, 6]. Only a few studies analyzed the expression of Glut-1 in melanocytic lesions and the results of these studies are equivocal [7–9]. The goals of the present study were (i) to analyze the expression of Glut-1 in the, thus far, biggest published sample set of 225 malignant melanomas and 175 melanocytic nevi; (ii) to find a potential use of Glut-1 expression in differential diagnostics between benign and

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malignant melanocytic lesions; and (iii) to assess the prognostic significance of Glut-1 expression in malignant melanoma.

Material and Methods

Formalin-fixed paraffin-embedded (FFPE) tissue blocks were obtained from the archive files processed at the Institute of Pathology and the Department of Dermatology and Venereology, First Faculty of Medicine, Charles University and General University Hospital in Prague. A review of the hematoxylin and eosin-stained slides was performed in all cases. In total, 400 FFPE specimens were selected for immunohistochemical analysis (Table 1), comprising of 225 malignant melanomas which were divided according to TNM classification into 4 groups (pT1 ≤ 1 mm, pT2 > 1–2 mm, pT3 > 2–4 mm, and pT4 > 4 mm) and 175 benign melanocytic nevi (89 of compound type, 83 of intradermal type and 3 of other type – junctional, blue, combined type). Other types of melanocytic lesions, including dysplastic nevi and equivocal lesions with uncertain malignant potential, were not included in the study.

The MMs were divided according to their location to the following groups: head and neck (35 cases; one case was conjunctival melanoma, two cases were localized at the neck, two cases were localized at the auricle), upper extremity including the shoulder (42 cases), lower extremity including the hip (37 cases), and trunk (111 cases). The mean age of patients with malignant melanoma was 63 years (median 65; range 15 to 93 years), and the mean age of patients with benign nevi was 36 years (median 35; range 5 to 79 years). In compliance with the Helsinki Declaration, the project has been approved by the Ethics Committee of the General University Hospital in Prague.

Immunohistochemical Analysis

Immunohistochemical analysis was performed manually using the avidin-biotin complex method with antibody against the Glut-1 (polyclonal, 1:100; Cell Marque, Rocklin, CA). Antigen retrieval was performed, including pretreatment in 0.01 M citrate buffer (pH 9.0), for 40 min in a water bath at 98 °C. Only membranous positivity was evaluated. The expression of Glut-1 was double-blindly evaluated by two pathologists. Ambiguous cases were evaluated by a third independent pathologist. The immunohistochemical results of membranous staining were assessed semi-quantitatively by an H-score method as described previously [10]. The H-score combines the percentage of positive cells and staining intensity level (weak 1+, moderate 2+, strong 3+). The score for each sample was calculated using the following formula: [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)]. The total score ranges from 0 to 300. Positive and negative internal controls were assessed for all the evaluated cases. Staining of erythrocytes, perineurium and squamous cells of the epidermis was used to serve as a positive control, while the staining of other structures such as connective tissue, adipose tissue and smooth muscles were used as a negative control.

Statistical Analyses

The software STATISTICA 10 (StatSoft, Tulsa, OK) was used to perform a chi-square test in order to compare the different groups of malignant melanomas and nevi with clinicopathological variables and immunohistochemical results. Cox's Proportional Hazard Method was used to test univariable and multivariable statistical effects of H-score of Glut-1 on the patient survival. The sample set was divided into three groups according to the calculated H-score of Glut-1 (<15 – no expression, 15–50 – weak expression of Glut-1, >50 – moderate to strong expression of Glut-1; threshold of 50 was set to obtain reasonable number of cases in each group). Time-to-event analysis was performed with a total of 3 outcomes, disease-specific survival (DSS), local recurrence-free survival (RFS) and distant metastasis-free survival (MFS). The date of diagnosis was the date of primary sample accession in the pathology database. The Kaplan-Meier method was used to compose the survival curves. All performed tests were two-sided and a *P*-value of less than 0.05 was considered as significant.

Table 1 Characteristics of study groups and summary of the H-score of Glut-1 membranous expression

Group	Number of cases (mean age)	Gender Male/Female	H-score (mean)	H-score range
Nevi	175 (36)	50/125	0.34	0–11
Malignant melanoma	225 (63)	134/91		
pT1 – Breslow ≤1 mm	101 (62)	58/43	5.32	0–150
pT2 – Breslow >1–2 mm	47 (57)	25/22	21.4	0–135
pT3 – Breslow >2–4 mm	44 (63)	29/15	17.36	0–85
pT4 – Breslow >4 mm	33 (73)	22/11	65.7	0–200

Results

Immunohistochemical Findings

The immunohistochemical findings are summarized in Table 1. Representative images of the Glut-1 expression in melanocytic lesions are shown in Fig. 1.

The cut-off for positivity of membranous Glut-1 expression was set at H-score 15, where is the highest sensitivity together with a high specificity (Table 2).

A membranous expression of Glut-1 was found in 69/225 (30.7%) cases of malignant melanoma (MM), the more pronounced of these expressions correlated with a growing Breslow thickness ($p < 0{,}00001$). A positivity of Glut-1 (Table 1) was observed in 10/101 pT1 MM cases (10%; median H-score of positive cases was 30.5; mean 47), 17/47 pT2 MM cases (36%; median H-score of positive cases was 48; mean 53.2), 16/44 pT3 MM cases (36.4%; median H-score of positive cases was 34.5; mean 41.5), and 26/33 pT4 MM cases (78.8%; median H-score of positive cases was 67.5; mean 82.9). In some cases of MM (mainly in MMs with Breslow >4 mm), there was an apparent zonation of Glut-1 expression with the intensity of positivity increasing with distance from stroma (either neovascularized tumor stroma or from invasive margin stroma) (Fig. 1b, c). In other cases, however, the expression was heterogeneous without any apparent relation with distance from tumor stroma or areas of necrosis. In the group of melanocytic nevi, all 175 cases showed negative membranous staining (the Glut-1 expression of median H-score 2 was detected in 24/175; 13.7%).

In our study there was no significant relationship between the expression of Glut-1 and other clinicopathological variables including gender, age and tumor location ($p > 0.05$, data not shown).

The multivariate analysis model included, besides the H-score of Glut-1, a number of other clinicopathological co-variables: Breslow thickness, pT classification, location, age, and sex. We confirmed the Breslow thickness and location to be the strongest prognostic markers. The H-score of Glut-1 served as a statistically significant marker only in the univariate analysis, which showed that increased membranous expression of Glut-1 correlated with a decreased 10-year DSS ($p = 0.00734$; 24 patients died of MM), 10-year RFS ($p = 0.00003$; 36 patients developed local recurrence), and MFS ($p = 0.00050$; 41 patients developed distant metastases) (Fig. 2).

Discussion

Malignant melanoma is quite a common tumor with increased incidence in especially the Caucasian population [11, 12]. Despite recent progress in molecular targeted therapy and immunotherapy, the prognosis of advanced stages is still serious [13].

The histological diagnosis of malignant melanoma is usually straightforward. However, in certain cases there can be ambiguous features and in these cases distinguishing between benign and malignant melanocytic lesions is difficult. Ancillary methods able to help us in this differential diagnostics would be beneficial. Melanomas are commonly associated with some specific genetic aberrations including copy number increases of RREB1 (6p25), CCND1 (11q13) and MYC (8q24) gene regions as well as loss of MYB (6q23) and CDKN2A (9p21). Some studies suggest fluorescence in

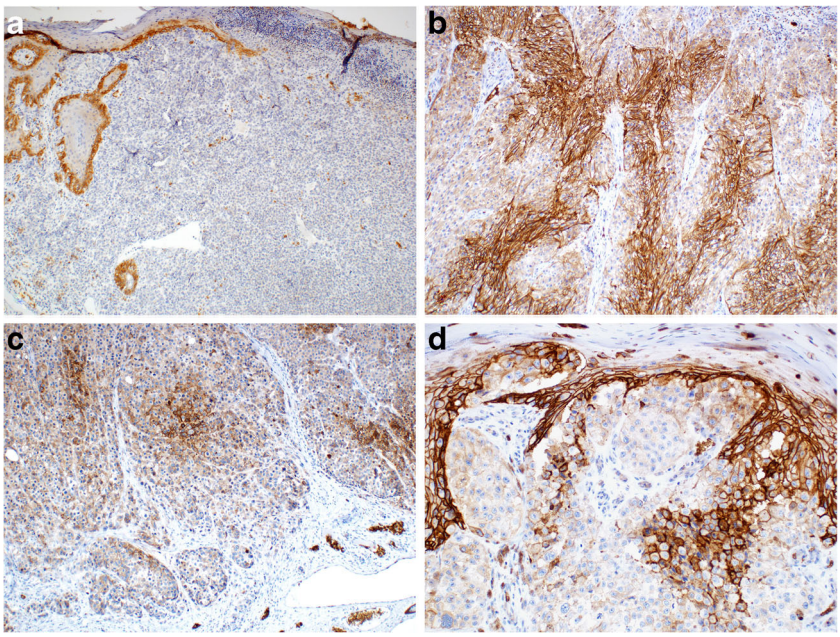

Fig. 1 Representative examples of immunohistochemical assessment of Glut-1 protein expression. **a** – complete negativity of Glut-1 expression in melanoma cells (positivity in epidermis and erythrocytes as an internal control); **b, c** – strong membranous positivity in melanoma cells (note zonation of Glut-1 expression dependent on the distance from tumor stroma); **d** – weak membranous positivity in some melanoma cells (strong positivity in epidermal cells can be observed). Magnification 100x (**a-c**), 200x (**d**)

Table 2 Discriminatory thresholds for negativity and positivity (nevi vs MM)

H-score	Nevi			MM			Sensitivity	Specificity
Cut-off	total	positive Glut-1 expression (false +)	negative Glut-1 expression (true -)	total	positive Glut-1 expression(true +)	negative Glut-1 expression(false -)		
50	175	0	175	225	32	193	14.22% CI (9.94–19.48%)	100% CI (97.9–100%)
15	175	0	175	225	65	160	28.89% CI (23.06–35.29%)	100% CI (97.91–100%)
10	175	1	174	225	73	152	32.4% CI (26.37–38.99%)	99.4% CI (96.86–99.4%)

situ hybridization (FISH) as a helpful method in distinguishing between malignant melanomas and benign melanocytic lesions [14–16]. However, this method is expensive, time-consuming and requires specific equipment which

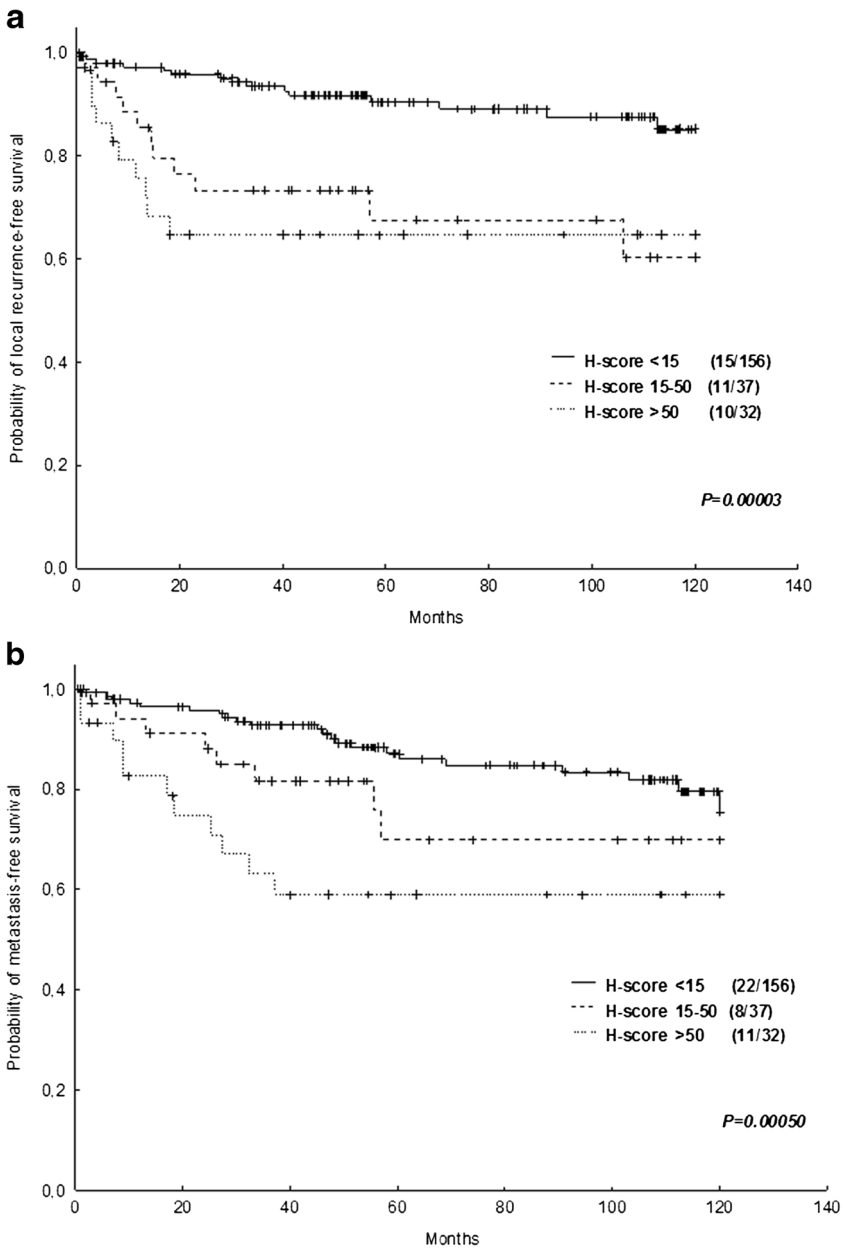

Fig. 2 Higher Glut-1 membranous expression is correlated with a higher risk of local recurrence (recurrence free survival) (**a**) and distant recurrence (metastasis free survival) (**b**)

is not available in every laboratory. On the contrary, immunohistochemistry is a cheap method routinely used in most laboratories. Unfortunately, despite the fact that several immunohistochemical markers have been suggested as valuable in differential diagnostics of equivocal melanocytic lesions, their practical significance is limited. The final diagnosis in dubious cases is made by clinicopathological correlation considering several characteristics including histological features, symmetry and size of a lesion, nuclear atypia, mitoses, inflammatory infiltrate, clinical appearance, dermoscopic features, age, family history, etc.

Immunohistochemical markers, which have been evaluated in malignant melanomas for prognostic purposes, include expressions of HIF-1α, Glut-1, p53, bcl-2 and MIB-1 [17–25]. The expression of HIF-1α is increased in the advanced stages of malignant melanoma and according to some previous studies this protein contributes to a melanoma's progression and/or metastasis. However, the results are equivocal as other studies have not detected a correlation between HIF-1α expression and any clinicopathological variables [23].

The expression of Glut-1 is influenced by hypoxia. An important role in this process is played by HIF-1α. In one study, the authors found a significant inverse correlation between Glut-1 expression and the presence of microvessels [22]. Some studies have also shown a positive correlation between increasing proliferative index Ki67 and tumor thickness, mitotic count, vertical growth, vascular invasion and metastatic potential [26]. However, the prognostic significance of the Ki67 proliferative index in malignant melanoma is controversial in literature [19–21].

Some other markers, including bcl-2 and p53, have been studied as prognostic and also predictive markers. According to some studies, a high expression of bcl-2 in tumor cells is an adverse prognostic marker in intermediate-thickness primary melanoma and high levels of p53 expression are associated with an unfavorable prognosis [24, 25]. Bcl-2 has been also studied as a potential therapeutic target with promising results, but it has yet to be confirmed by additional clinical studies [17, 18].

One of the characteristic features of malignant tumors is their ability to reprogramme glucose metabolism from the oxidative phosphorylation pathway to glucose aerobic glycolysis (Warburg's effect), which represents a selective growth advantage [27, 28]. However, the process of aerobic glycolysis, when compared to oxidative phosphorylation, results in a significantly lower production of ATP molecules (2 ATPs vs 32 ATPs) [29]. Nevertheless, tumor cells are able to compensate for this energy deficit by upregulating glucose transporters expression, especially Glut-1, which allows for an increased glucose uptake. The mechanisms of these processes are various and include mitochondrial damage, stabilization of hypoxia-inducible factor-1α (HIF-1α), inactivation of tumor suppressors such as p53 and PTEN, and upregulation of several oncogenes of the PI3K/Akt/mTOR signaling pathway [30, 31]. The resulting impact of these changes is complex and includes not only the upregulation of glucose transporters, but also the resistance to apoptosis and promotion of further proliferation. The PI3K/Akt/mTOR pathway plays a major role in glucose metabolism regulation and inhibition of this pathway represents a possible therapeutic approach in several malignant tumors. However, the benefit of such treatment in malignant melanoma is equivocal [32]. Another therapeutic approach includes competitive inhibition of glucose uptake and utilization with the use of a glucose analogue 2-deoxy-D-glucose (2DG), or targeting the Glut-1 metabolic pathway by small molecule inhibitors [33, 34].

In our study, we focused on the assessing of Glut-1 expression with respect to its significance in differential diagnostics between benign and malignant melanocytic lesions and prognostic significance. An increased expression of Glut-1 was described in several malignant tumors, including lung, breast, pancreatic, esophageal, colorectal, uterine and head and neck cancer [5, 8, 35–39]. However, the expression of Glut-1 in malignant melanomas and benign melanocytic lesions has been analyzed in only a few studies [7, 8] and the results are equivocal. Some studies failed to detect any positivity of Glut-1 in both benign and malignant melanocytic lesions [4, 8, 40]. In another study, the authors found an expression of Glut-1 in all (24) melanocytic nevi and Spitz nevi, but only in 9/20 malignant melanomas [7]. Based on these results, the authors suggest that downregulation of Glut-1 can be used as a marker able to discriminate between malignant melanomas and melanocytic nevi. However, other studies have found that Glut-1 is expressed in a significant amount of malignant melanomas ranging from 12% to 89.5% of positive cases [22, 23, 41]. Moreover, in one study Glut-1 expression was found in all four melanoma cell lines tested [42]. Higher levels of Glut-1 were detected in advanced melanomas. In some studies, there was correlation between Glut-1 expression and shortened overall or disease free survival [22, 23]. In addition, one study demonstrated a negative prognostic significance of Glut-1 expression in the lung melanoma metastasis for a post-metastasectomy survivor [43].

In our study, using a cut-off for positivity of H-score 15, we have found membranous expression of Glut-1 in 30.7% of malignant melanomas but all benign melanocytic nevi were negative. We determined this cut-off value to be the most discriminative (with respect to sensitivity and specificity), based on the comparison between benign nevi and malignant melanomas (Table 2). Despite the fact that this expression is relatively low (15% of cells showing weak membranous expression or

5% of cells showing strong membranous expression), the assessment at threshold of 15 is not problematic. The selfsame or lower positivity thresholds are also used for example in the routine practice when evaluating the expression of PD-L1 or hormonal receptors. The pattern of Glut-1 expression was irregular and heterogeneous, and in most cases we were not able to find any association with vascularization or relation between the Glut-1 expression and distance either from the invasive margin or tumor stroma. However, in some advanced melanomas, the expression of Glut-1 showed apparent zonation with increased intensity in areas more distant from the tumor stroma. This effect has been mentioned in the literature in malignant melanomas and in some other tumors as well [22]. This staining heterogeneity is probably given by the increased expression of Glut-1 in areas of ischaemia, and in some studies Glut-1 expression is regarded as a marker of intralesional regions of hypoxia [44]. However, Glut-1 expression can also reflect the metabolic state of tumor cells (aerobic glycolysis) linked to malignant transformation. In these cases, hypoxia-inducible gene expression is independent of hypoxia and can be caused by lactate and pyruvate (the end product of glycolysis) produced by tumor cells. These substances cause accumulation of HIF-1α and enhance expression of several HIF-1α activated genes, including Glut-1 [45].

The reasons of such equivocal results regarding Glut-1 expression in melanocytic nevi and malignant melanomas are unclear. Nevertheless, we suppose that this could be due to several factors. Firstly, there are several antibodies used in reported studies which can cover different epitopes of the protein Glut-1. Secondly, different cut-offs for positivity can influence the results as well. For example, in one study which reported complete negativity in 67 cases of malignant melanoma the cut-off for positivity was 10% of cells [8]. In this study, tissue microarrays were used and this can impinge on the results as well, because of limited sampling of the tumor area and intralesional heterogeneity of Glut-1 expression. Thirdly, an important factor is the staining pattern of Glut-1 expression. Some studies reported only cytoplasmic expression, others only membranous or both membranous and cytoplasmic expressions [7, 23, 41]. However, Glut-1 is a membrane transport protein and in our study we focused only on its membranous expression. Weak cytoplasmic expression can be seen in both benign and malignant lesions and we consider this as a non-specific staining pattern which should not be assessed.

Univariate Cox regression model analysis showed that membraneous expression of Glut-1 is negatively associated with the length of 5-year (data not shown) and 10-year survival. The prognostic significance is diminished in the multivariate analysis, which includes stronger prognostic markers (i.e. the strongest prognostic marker of MM – Breslow score, and location).

Conclusions

In conclusion, we performed a comprehensive analysis of Glut-1 expression in the largest series of malignant melanomas and benign melanocytic nevi to date. Based on our results, we suppose that a membranous (but not cytoplasmic) expression of Glut-1 can be used as a surrogate marker in differential diagnostics of benign and malignant melanocytic lesions. However, when considering a routine use of this marker in differential diagnostics, further studies on equivocal cases are needed. Moreover, we should be aware of the fact that despite the high specificity, the sensitivity of this marker seems to be relatively low. According to our results the expression of Glut-1 is a prognostically adverse factor, but only in the univariate analysis and its practical use is limited compared to other standard prognostic factors.

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Compliance with Ethical Standards In compliance with the Helsinki Declaration, the project has been approved by Ethics Committee of the General University Hospital, Prague (reference number č.j. 56/15 Grant VES 2016 AZV 1. LF UK).

Competing Interests The authors declare that they have no competing interests.

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