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Proliferative Markers in Diagnosis of Thyroid Tumors: A Comparative Study of MIB-1 and Topoisomerase II-α Immunostaining

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Abstract. *Background: The differential diagnosis of well-differentiated tumors of follicular cell origin remains a most problematic task in thyroid pathology. Therefore, various diagnostic/prognostic thyroid markers are currently being studied. The aim of our study was to evaluate the proliferative MIB-1 and topoisomerase II-α markers in both oncocytic and non-oncocytic epithelial thyroid tumors. Materials and Methods: Proliferative activity was analyzed by means of immunohistochemistry in 215 thyroid tumors and the evaluated proliferative indices (PI) were correlated with morphological diagnosis. Comparison of both proliferative markers was made. The results were statistically analyzed using Wilcoxon tests (significance level $p<0.05$) and the Spearman correlation coefficient. Results: Carcinomas generally showed significantly higher PI than adenomas, irrespective of their oncocytic or non-oncocytic features. Moreover, PI in oncocytic adenomas was significantly higher than in non-oncocytic ones. However, PI in both oncocytic and non-oncocytic carcinomas, including papillary microcarcinomas, were similar. The studied proliferative markers correlated with each other. Conclusion: PI can help the differential diagnosis of morphologically difficult cases of thyroid tumors. Oncocytic adenomas have higher malignant potential and should be promptly surgically removed. Both MIB-1 and topoisomerase II-α are recommended for the evaluation of thyroid tumor cell proliferation.*

Thyroid neoplasms represent a broad spectrum of lesions with different histopathological features. Well-differentiated thyroid tumors of follicular cell origin (follicular adenoma and carcinoma, papillocarcinoma) still present diagnostic pitfalls. Specific morphological criteria only (capsular and/or vascular invasion, nuclear characteristics) are crucial points used in the diagnosis of these neoplasms. However, the assessment of such criteria seems to be inconclusive in some cases (1). Futhermore, oncocytic (Hürthle cell, oxyphilic) variants of the above-mentioned thyroid neoplasms are regarded as controversial tumors in terms of their natural history and biological behavior. According to the World Health Organisation, they are variants of the existing thyroid tumor categories of follicular cell origin, although the possibility that oncocytic tumors are distinct neoplastic entities is still under debate (2, 3). Therefore, various other diagnostic/prognostic markers are currently being studied in oncocytic and non-oncocytic thyroid tumors. Particularly, the determination of proliferative activity has become a well-established method in tumor pathology (4-6). However, data on thyroid lesions are only sporadic in the literature.

The aim of our study was to evaluate the proliferative MIB-1 and topoisomerase II-α (topo II-α) markers in epithelial thyroid tumors with regard to morphological changes of neoplastic cells.

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Key Words: Proliferative tumor marker, Ki-67 (MIB-1) antigen, topoisomerase II-α, thyroid tumors.

Materials and Methods

Materials. The studied thyroid neoplasms were retrieved from surgical and consultation files of one author (M.L.). All cases were histologically classified according to the criteria proposed by the World Health Organisation (15) and divided into the following groups: follicular adenoma, follicular carcinoma and papillary carcinoma. Within each category of tumors, oncocytic and non-oncocytic variants were defined: non-oncocytic follicular adenomas (NA), oncocytic follicular adenomas (OA), non-oncocytic follicular carcinomas (FC), oncocytic follicular carcinomas (OFC), non-oncocytic papillary carcinomas (PC) and oncocytic papillary carcinomas (OPC) (Table I) (2).

Immunohistochemistry. Immunohistochemical examination was performed on paraffin sections using the SABC (Novostain ABC, Novocastra, Newcastle-upon-Tyne, UK) immunoperoxidase method with monoclonal antibodies against mitochondrial antigen (clone

Table I. *Clinicopathological characteristics of studied groups of tumors.*

Type of tumor	Number of cases	Sex	
		F	M
Oncocytic follicular adenoma (OA)	69	59	10
Non-oncocytic follicular adenoma (NA)	40	31	9
Oncocytic follicular carcinoma (OFC)	27	19	8
Non-oncocytic follicular carcinoma (FC)	7	5	2
Oncocytic papillocarcinoma (OPC)	24	20	4
Non-oncocytic papillocarcinoma (PC)	48	41	7

Abbreviations: F, female; M, male

113-1; Biogenex; 1:800), antigen Ki-67 (clone MIB-1; DAKO; 1:1000) and topoisomerase II-α (Ki-S1, DAKO, 1:50), according to the manufacturer's instructions. The retrieval of both proliferative antigens (Ki-67 and topo II-α) was performed before immunohistochemistry with 0.1 mol/L citrate, pH 6.0, in a microwave oven for 15 minutes (6). The peroxidase activity was visualized with 3,3-diaminobenzidine-tetrachloride (DAB, Fluka), followed by counterstaining with Gill's hematoxylin. A negative control (by omitting the primary antibody) as well as a positive one (breast carcinoma) were used.

Quantitative analysis. The proliferative index (PI^{MIB-1}, $PI^{topo\ II-\alpha}$) was estimated using an Olympus BX-40 light microscope equipped with a 40x magnification objective and special eye-piece 10x10-mm grid. The percentages of MIB-1- and topo II-α-positive neoplastic epithelial cells were determined by counting at least 1000 tumor cells per slide in randomly selected fields. Positive cells of vascular origin and hematogenous cells were not considered (8, 9). All specimens were assessed independently by two persons.

Statistical analysis. This was performed using the S.A.S. (Statistical Analysis Software, release 8.02) package. The data were analyzed using the Wilcoxon test and Spearman correlation coefficient; values equal to or less than 0.05 were considered as significant.

Results

The studied cases were divided on the basis of the histological examination into six groups. Their basic clinicopathological features are summarized in Table I. One hundred and nine tumors were classified as follicular adenoma; forty of them had oncocytic features. Neoplastic cells of follicular tumors were arranged in solid, trabecular and microfollicular patterns, respectively. The diagnosis of follicular adenoma was based on the presence of a complete thin fibrous capsule (*i.e.* the absence of capsular and vascular invasion). All studied follicular carcinomas (34 cases including 7 non-oncocytic ones) were classified as minimally invasive carcinomas with vascular and capsular penetrance. The diagnosis of conventional papillary carcinoma (41 tumors) was based on the presence of architectural features, such as true papillae and characteristic nuclear changes. Seven cases of non-oncocytic papillo-carcinomas were compatible with a diagnosis of papillary microcarcinoma; their size was maximally 10 mm in diameter. The oncocytic variant of papillocarcinoma, with lymphoid-rich stroma (Warthin-like PC), was represented in 24 cases. This is a less common type of PC and its biological behavior is not fully understood (10, 11). Tumors were considered as oncocytic (Hürthle cell) if more than 75% neoplastic cells had pale, slightly granulated cytoplasm packed by mitochondria, and enlarged polymorphic nuclei with distinct nucleoli (3, 12). Oncocytic differentiation was proven by immunohistochemical positivity of mitochondrial antigen (13).

Assessment of proliferative activity. We investigated the proliferative activity of thyroid tumors by immuno-histochemistry using two different antibodies. MIB-1- and topo II-α-positive nuclei of cycling cells stained dark brown and could be easily distinguished from the negatives ones (Figures 1, 2). The reaction product was observed not only in the epithelial cells, but also in the nuclei of other cells, particularly lymphocytes. PI of both markers (mean, median, standard deviation and range min – max) of the studied groups of tumors are shown in Table II and Figure 3.

Statistical analysis. Both PI between studied groups were compared by Wilcoxon test and *p*-values were established. All results are listed in Table III. Carcinomas had a significantly higher proliferation rate than adenomas (*p*-value <0.0001-1.0098). However, no significant PI differences were found between FC and OA in both the studied markers (MIB-1: *p*-value = 0.1041; topo II-α: *p*-value = 0.0776). Surprisingly, PI^{MIB-1}, $PI^{topo\ II-\alpha}$ were significantly higher in OA than in NA ($p=0.1041$, $p<0.0001$). Concerning the analysis of the proliferative indices MIB-1 and topo II-α of both studied variants of PC (conventional papillocarcinoma and papillary microcarcinoma, respectively), no significant differences in growth activity were confirmed ($p^{MIB-1}=0.0495$, $p^{topo\ II-\alpha}=1.0000$).

Using Spearman correlation coefficient analysis, we determined the correlation of both PI in the studied groups of tumors. All calculated coefficients ($r>0.75725$) with significant levels of *p*-value indicated that both proliferative markers were concordant. Setting NA, OA and NA+OA as control groups, we calculated, at recommended specificities 95% and 97.5%, the cut-offs of PI. The reached sensitivities are summarized in Table IV. All sensitivities were too low to recommend application of corresponding cut-offs in differential diagnosis between oncocytic and non-oncocytic follicular adenomas and carcinomas.

Discussion

Some cases of well-differentiated thyroid tumors of follicular cell origin still remain a matter of controversy (1,

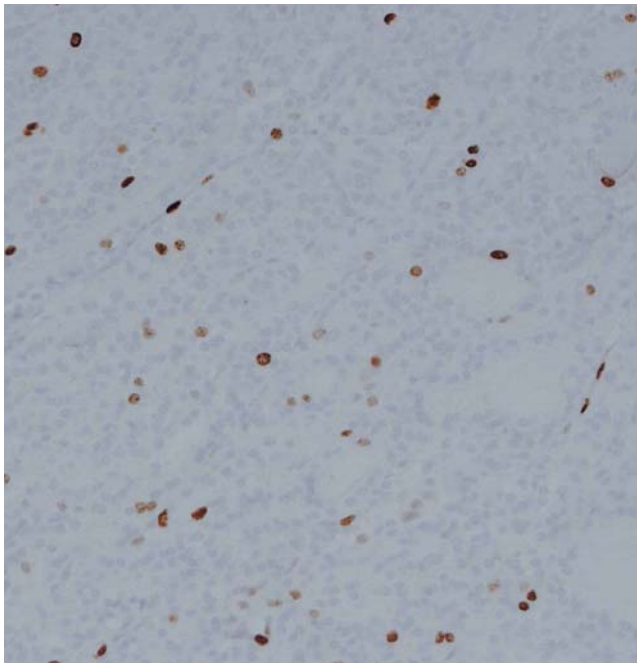

Figure 1. *Nuclear positivity of MIB-1 in non-oncocytic follicular carcinoma (MIB-1 immunohistochemistry, x200).*

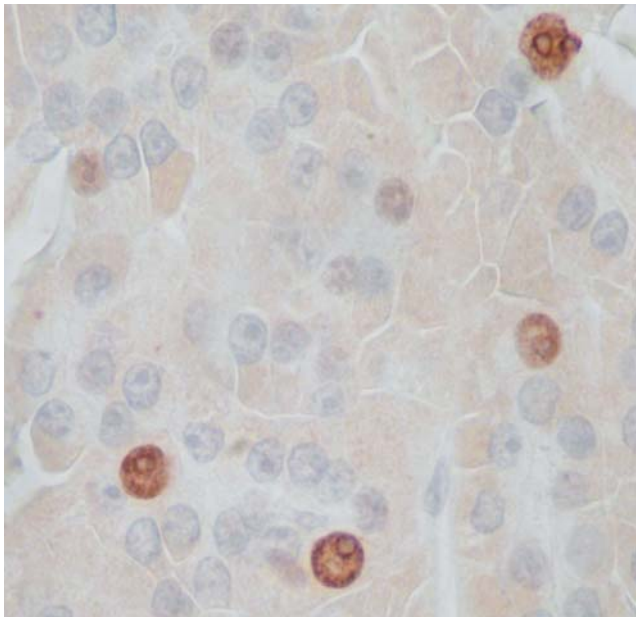

Figure 2. *Topoisomerase II-α nuclear expression in oncocytic follicular adenoma (topoII-α immunohistochemistry, x400).*

Table II. *Proliferative indices of studied tumors.*

Type of tumor	Proliferative index (%) MIB-1/topo II-α			
	Mean	Median	SD	Min-Max
OA	2.6 / 2.6	2.2 / 2.5	1.65 / 1.41	0.4 – 9.3 / 0.3 – 8.9
NA	1.6 / 1.4	1.6 / 1.2	0.94 / 0.69	0.1 – 5.0 / 0.2 – 3.0
OFC	4.9 / 4.3	3.9 / 3.7	3.76 / 2.34	0.4 – 20.3 / 1.1 – 11.0
FC	3.3 / 3.3	3.7 / 3.8	1.04 / 0.99	1.7 – 4.5 / 1.6 – 4.2
OPC	4.0 / 3.3	3.4 / 3.2	2.18 / 1.00	0.9 – 10.7 / 1.9 – 6.3
PC – P	3.6 / 3.4	3.3 / 3.2	2.07 / 1.66	0.2 – 8.1 / 0.8 – 7.0
PC – mp	5.5 / 3.4	5.7 / 3.4	2.28 / 1.73	2.2 – 8.5 / 1.2 – 5.7

Abbreviations: OA, oncocytic follicular adenoma; NA, non-oncocytic follicular adenoma, OFC, oncocytic follicular carcinoma; FC, non-oncocytic follicular carcinoma; OPC, oncocytic papillary carcinoma; PC, non-oncocytic papillocarcinoma; P, conventional papillocarcinoma; mp, papillary microcarcinoma; SD, standard deviation; Min-Max, rate.

12, 14, 15). The diagnosis of distinct types of thyroid tumors (minimally invasive follicular carcinomas, papillary carcinomas) is based on morphological criteria (like capsular and vascular invasion and nuclear characteristics) only. The search for capsular and vascular invasion, which distinguishes follicular carcinoma from adenoma, requires extensive sampling and, in some cases, the use of special staining methods. The nuclear characteristics as well as papillary arrangement of papillary carcinomas are not specific and similar changes have been described in other non-neoplastic thyroid lesions, such as Hashimoto thyroiditis and hyperplastic goiter (16). Moreover, oncocytic variants of the above-mentioned tumors represent about 7% of all epithelial thyroid neoplasms and seem not to be fully understood, particularly with respect to their malignant potential and aggressiveness. According to some published results, oncocytic lesions are supposed to be more aggressive than their non-oncocytic counterparts (3). Determining and predicting the biological behavior of thyroid follicular tumors is, therefore, a difficult problem. The various indices (size of tumor, age of patient, p53, bcl-2) were previously studied, but none of them appeared to be completely satisfactory in discriminating between benign and malignant thyroid lesions (16-19). Studies of the proliferative marker Ki-67 (particularly of its formalin-resistant epitope MIB-1) provide promising results and it is currently being used in routine diagnosis of some tumors (4, 5, 6, 20). However, we found only sporadic studies dealing with the proliferative activity of thyroid tumors in the literature (18, 21-25). These studies were almost all carried out on the proliferative proteins PCNA and Ki-67, while less commonly cyclins and cyclin-dependent kinases, p53, bcl-2 and topo II-α (18, 19, 21-28) were studied. The majority of these studies are inconclusive with regards to the determination of sensitive differential diagnostic and prognostic markers of thyroid neoplasms. Proliferation activity studies discovered statistically significant differences in cell kinetics between thyroid carcinomas and adenomas

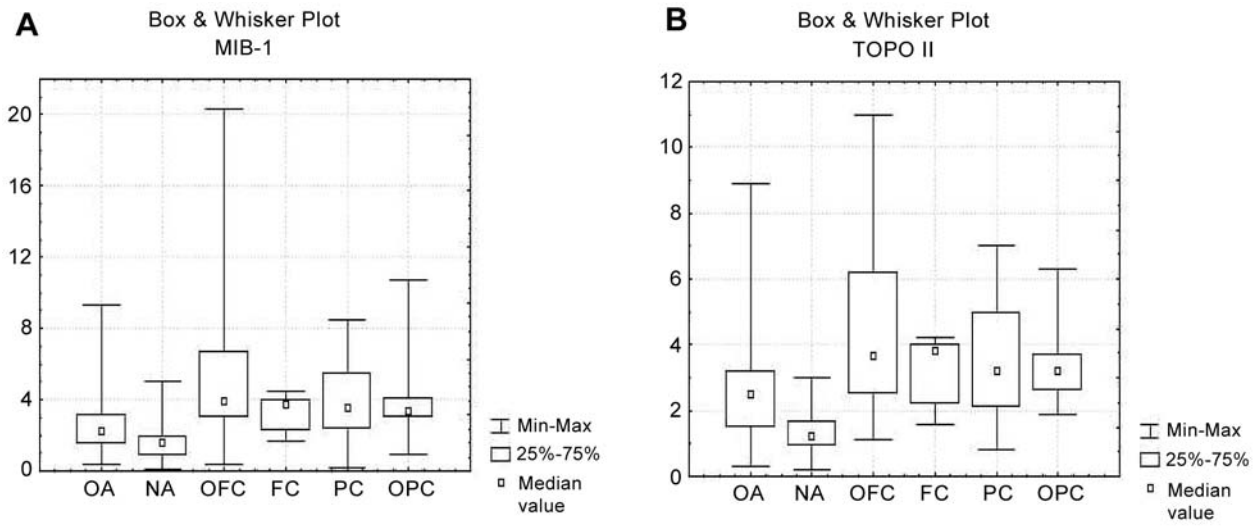

Figure 3. *Distribution of PI^{MIB-1} (A) and $PI^{topo\ II-\alpha}$ (B) in studied groups of tumors.*

(19, 25, 29). However, in all the mentioned studies, oncocytic and non-oncocytic tumors were evaluated separately without comparison of proliferative activity between both these variants of thyroid neoplasms. Tateyama *et al.* only found no difference in proliferative activity between oncocytic and non-oncocytic tumors using PCNA marker (24). To the best of our knowledge, no study has so far evaluated both Ki-67 (MIB-1) and topo II-α in the differential diagnosis of thyroid tumors. Moreover, the complex group of all types of well-differentiated thyroid tumors of follicular cell origin, including both oncocytic and non-oncocytic variants, has not been studied yet.

Both the studied proliferative markers differ from each other with regard to their distribution during the cell cycle. Topo II-α is a nuclear enzyme required for chromatin condensation and chromosomal segregation during mitosis and expressed in the S-, G2- and M- phases only. Ki-67 is a nuclear antigen of unknown function present during the whole cell cycle (excluding G0-phase) (30). Formerly, immunohistochemistry could be performed on fresh unfixed material only due to loss of Ki-67 protein activity after formalin fixation (31). Later, antibodies against the formalin-resistant epitope MIB-1 (of Ki-67 protein) were discovered and paraffin-embedded specimens could be used for studies (32). The distribution of both proliferative markers in the cell cycle is shown in Figure 4. Moreover, topo II-α serves as a target for many anticancer drugs known as topoisomerase II inhibitors (33). Topo II-α has also been suggested as a predictive marker (34, 35). As regards both the evaluated proliferative markers (Ki-67 and topo II-α), their PIs correlated with each other in all studied groups of thyroid tumors ($r>0.83$). The correlation coefficient with NA ($r=0.65851$, $p<0.0001$) and OPC ($r=0.75725$, $p=0.0001$) reached slightly lower but still significant values, which may be explained by the variability of immunoexpression markers. Nevertheless, the MIB-1 indices were always higher than the respective topo II-α indices, because of the differences in their distribution during the cell cycle.

Table III. *Levels of significance (p-value) in proliferative activity between defined groups of tumors.*

Comparison of tumors	Wilcoxon test (*p*-value)	
	MIB-1	topo II-α
FC *versus* OA	0.1041	0.0776
FC *versus* NA	**0.0025**	**0.0008**
FC *versus* OFC	0.2587	0.4943
FC *versus* OPC	0.5892	0.5395
FC *versus* PC	0.4519	0.8647
NA *versus* OA	**0.0003**	**< 0.0001**
NA *versus* OFC	**< 0.0001**	**< 0.0001**
NA *versus* OPC	**< 0.0001**	**< 0.0001**
NA *versus* PC	**< 0.0001**	**< 0.0001**
OA *versus* OFC	**0.0002**	**0.0007**
OA *versus* PC	**0.0006**	**0.0098**
OA *versus* OPC	**0.0009**	**0.0047**
PC *versus* OFC	0.4203	0.1749
PC *versus* OPC	0.8441	0.9570
OPC *versus* OFC	0.3650	0.2786
OA a NA *versus* OFC a FC	**< 0.0001**	**< 0.0001**
OA a NA *versus* OPC a PC	**< 0.0001**	**< 0.0001**
OFC a FC *versus* OPC a PC	0.5931	0.2054

Concerning proliferative activity, we confirmed a significant difference in PI^{MIB-1} and $PI^{topo\ II-\alpha}$ between follicular adenomas and carcinomas, irrespective of their oncocytic or non-oncocytic features. Our findings are in

Table IV. *Sensitivities and threshold (cut-off) of proliferative indices considering specificities 97.5% and 95%, respectively.*

	NA x FC				OA x OFC				NA+OA x FC+OFC			
SP	MIB-1		topo II-a		MIB-1		topo II-a		MIB-1		topo II-a	
	CO	SN	CO	SN	CO	SN	CO	SN	CO	SN	CO	SN
97.5 %	3.7	57.14	3.0	71.43	6.4	25.92	5.8	30.77	6.2	23.53	5.2	24.24
95 %	3.1	57.14	2.7	71.43	6.1	29.63	5.1	30.77	5.1	26.47	4.2	36.36

Abbreviations: SP, specificity; SN, sensitivity; CO, cut-off.

agreement with other reports on thyroid gland tumors (19, 22, 26, 36, 37). By contrast, Rigaud and Bogomelze found no statistical significance in the relationship between proliferative activity and type of thyroid tumors (38).

The other aim of our study was the comparison of PI in oncocytic and non-oncocytic tumors of the thyroid gland. Oncocytic follicular adenomas showed significantly higher PI than non-oncocytic adenomas. Similar results were also obtained earlier by Muller and Hocker, Tateyama *et al.* and Tretiakova *et al.*, but not confirmed by Lee *et al.* (19, 23-25). Based on our study, we suppose the higher risk of malignant transformation of OA in comparison with NA to be due to higher proliferative activity. Therefore, a greater tendency of OA to invade into the capsule and vessels and, thus, transform into the minimally invasive OFC should become, in everyday practice, a reason for prior surgical treatment of oncocytic proliferative neoplasms verified by cytology. However, the study submitted did not manifest any statistically significant differences in the proliferative activity of oncocytic and non-oncocytic carcinomas. On the basis of both these findings and prior references, it can be assumed that an oncocytic appearance does not play any significant role in the biological behavior of thyroid carcinoma of follicular cell origin (in contrast to oncocytic benign tumors) (29). The higher aggressiveness of OFC reported by some authors may apparently be connected with their response to radioiodine treatment (12, 16). It is not known whether PI of conventional papillocarcinomas differ from the proliferative activity of papillary micro-carcinomas. Nevertheless, this conclusion is limited by a small number of microcarcinoma cases included in the study. No threshold of PI usable for solid differential diagnosis of disputed cases of follicular tumors (disputed existence of capsular and/or vascular invasion in case of capsulated follicular nodes) was found. Overlapping of PIs between tumor groups becomes a limiting factor when searching for cut-off values. Considering the low sensitivities of PI, with the chosen specificities of 97.5% and 95%, we could not recommend PI cut-off values for unambiguous differentiation between follicular adenomas and carcinomas. A similar conclusion has also resulted from other studies (37).

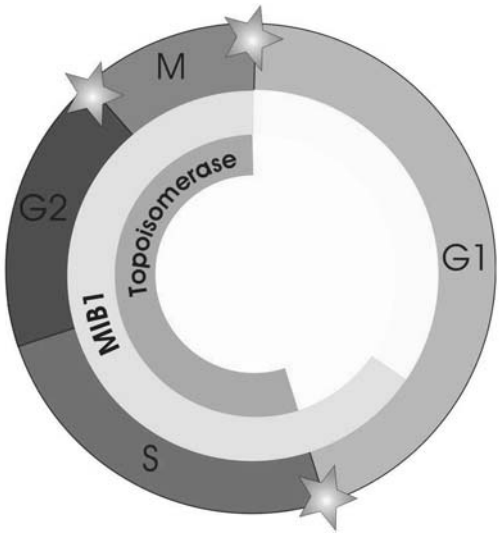

Figure 4. *Distribution of MIB-1 and topo II-α during cell cycle.*

PI in the differential diagnostics of thyroid carcinomas can be used in routine practice as an auxiliary diagnostic and prognostic marker only, and it is necessary to keep looking for other new molecular markers. The proliferative activity of thyroid lesions assessed in accordance with the topo II-α marker correlated with the MIB-1 values of the proliferative index. Topo II-α appears to be another applicable marker for assessment of cell growth.

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