The Significance of Immunohistochemical Expression of Sphingosine Kinase-1 and Galectin-3 in Differentiation Between Follicular Adenoma, Non-invasive Follicular Tumor with Papillary Nuclear Features and Follicular Variant Papillary Thyroid Carcinoma

Elkabsh MM[1], Sherkawy FA[2], Yousef M[3], Fadel SAM[1]

[1] Pathology Department, Faculty of Medicine, Assiut University, Assiut, Egypt.
[2] Pathology Department, Faculty of Medicine, South Valley University, Qena, Egypt.
[3] General surgery Department, Qena University Hospital, South Valley University, Qena, Egypt.

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Abstract:

Background and Study Aims: This study aims to evaluate the frequency of sphingosine kinase-1 (SPHK-1) and galectin 3 (Gal-3) immunohistochemical (IHC) expression in noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP), follicular adenoma (FA), and follicular variant of papillary thyroid carcinoma (FVPTC), as well as the relationship between SPHK-1 and Gal 3 IHC expression in these thyroid lesions.

Patients and Methods: 56 individuals underwent IHC analysis to evaluate the expression of the SPHK-1 and Gal-3 proteins.

Results: The findings showed a significant correlation between FVPTC and elevated expression of SPHK-1 and Gal-3 ($p < 0.001$ for both markers). Gal-3 expression did not vary statistically significantly between FA and NIFTP patients ($p = 0.271$). The difference in the expression of SPHK-1 in FA and NIFTP patients was statistically significant ($p < 0.001$).

Conclusions: Our results showed that both SPHK-1 and Gal-3 expression have diagnostic value for FVPTC. As an innovative strategy, only SPHK-1 could be used as a diagnostic factor for NIFTP.

Keywords: Sphingosine kinase 1, Galectin-3, Immunohistochemistry (IHC), Follicular adenoma (FA), Non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP), Follicular variant of papillary thyroid carcinoma (FVPTC), Thyroid neoplasms, Thyroid tumor biomarkers

Authors Information:
Mai M Elkabsh
Pathology Department, Faculty of Medicine, Assiut University, Assiut, Egypt.
email: Maielkabsh939@aun.edu.eg

Fatma-Elzhraa Ahmed Sherkawy
Pathology Department, Faculty of Medicine, South Valley University, Qena, Egypt.
email: fatmaelzhraa@med.svu.edu.eg

Mohamed Yousef A
General surgery Department, Qena University Hospital, South Valley University, Qena, Egypt.
email: Myousef76@med.svu.edu.eg

Sabah Ahmed Mohamed Fadel
Pathology Department, Faculty of Medicine, Assiut University, Assiut, Egypt.
email: sabah_fadel77@yahoo.com

Corresponding Author:
Mai M Elkabsh
Pathology Department, Faculty of Medicine, Assiut University, Assiut, Egypt.
email: Maielkabsh939@aun.edu.eg

Introduction:

Thyroid tumors make up around 90% of all endocrine tumors and 3.1% of all malignancies worldwide, making them the most prevalent neoplasms within the endocrine system [1]. In 2020, over 586,000 new cases were recorded annually [2]. In the globe, it ranks as the ninth most prevalent cancer [3]. In Egypt, it ranks sixth among women and seventeenth among men, and it is responsible for around 1.5% of all cancers and 30% of endocrine malignancies [4].

Additionally, Qian et al. [5] noted that thyroid cancer in children is becoming more common in several nations, including the US. Risk factors include radiation exposure, excess iodine, lymphocytic thyroiditis, and other environmental contaminants [6].

The most prevalent thyroid cancer is papillary thyroid carcinoma (PTC). The classic, follicular, tall cell, hobnail, cribriform morular, columnar cell, and diffuse sclerosing variants are among the histological variations included in this category [7]. Within these,

PTC with follicular variant (FVPTC) has a broad morphological spectrum of epicentrifugal, microfollicular, and macrofollicular with diffuse growth. Capillary is either encapsulated or infiltrative and may mimic other follicular neoplasms [8].

Two primary subtypes of FVPTC have been further subclassified as [9] encapsulated and infiltrative. Nikiforov suggested in 2016 that non-invasive encapsulated FVPTC should be reclassified as noninvasive follicular thyroid neoplasm with papillary-like nuclear characteristics (NIFTP) [10].

Specific morphological and diagnostic criteria of NIFTP have been defined, including [1] encapsulation, follicular growth pattern, lack of true papillae and psammoma bodies, less than 30% solid/trabecular/insular architecture; nuclear grade of 2–3; lack of tumor necrosis, low mitotic activity [9]; and [2] absence of capsular and vascular invasion, in contrast to invasive encapsulated FVPTC.

Inhibition of apoptosis and promotion of cell proliferation and angiogenesis are central to sphingosine kinases' role in cancer biology [11]. Humanoids have only been identified with two isoenzymes, sphingosine kinase 1 (SPHK1) and sphingosine kinase 2 (SPHK2) [12]. SPHK1 is essential for growth, proliferation, survival, and apoptosis mediated by Sphingosine 1 phosphate (S1P) [13]. In particular, SPHK1 has been linked to cancer initiation, progressions, metastasis, and neovascularization.

Galectin 3 (Gal-3) is mainly cytoplasmic but can also be found in the perinuclear membrane, the nucleus, and extracellularly. Its physiological and pathological effects include cell proliferation, apoptosis, adhesion, inflammation, transformation, tumor progression, and metastasis [7].

This study's purpose was to assess the immunohistochemical expression of Gal-3 and SPHK-1 in FA, NIFTP, and FVPTC patients and determine the correlation between Gal-3 and SPHK-1 expression in these thyroid lesions.

Patients and Methods:

Case Selection

A retrospective analysis was carried out on 56 formalin-fixed paraffin-embedded (FFPE) tissue blocks of FA, NIFTP, and FVPTC that were retrieved from the archives of the South Valley University Hospital and Assiut University Hospital Surgical Pathology Laboratories and diagnosed between January 2018 and December 2021.

Hematoxylin and eosin (H&E) staining was performed on paraffin-embedded blocks that had been cut to a thickness of 3 to 5 um and placed onto regular glass slides. Thus, the H&E-stained slides were first microscopically examined. Tumors were graded using the World Health Organization (WHO) grading criteria.

IHC

Deparaffinization and Blocking

FFPE tissue sections, each measuring a micrometer in thickness, were mounted to coated slides. After 30 minutes of xylene dewaxing, the sections were rehydrated in ethanol-to-alcohol-graded solutions, culminating in distilled water. Hydrogen peroxide blocked endogenous peroxidase activity, incubated for about 10 to 15 min, and then applied V block at 22 degrees C.

We incubated tissue sections with a mouse monoclonal SPHK-1 antibody (Abcam, clone: OTI1A6) at 1:100 dilutions and Gal-3 antibody (Quartett , clone 1-GA079-02 H-5) at a 1:100 dilution for one hour at room temperature. Then, we washed with phosphate-buffered saline (PBS). Following these steps, 10 minutes of biotinylated goat anti-poly valent secondary antibody was applied, and then cells were washed twice with PBS. For 5-10 minutes, a chromogen of DAB was used. To ensure robust and consistent tissue clearing, tissue clearing and mounting were carried out, and then Mayer's hematoxylin counterstaining was performed.

Positive Controls

SPHK1 expression was evaluated in colonic adenocarcinoma sections, which served as a positive control for those assays and Gal-3 expression was assessed in sections from papillary thyroid carcinoma.

Negative Controls

Tissue sections from the same cases were similarly stained using the same immunohistochemical protocol, with the primary antibody omitted as negative controls.

Examination Methods

As is done for all slides, evaluation of both SPHK1 and Gal-3 immunoreactivity was performed on all slides by two experienced pathologists independently. At Low magnification (×4 and ×10) immunostained sections of each case were used to identify positively stained cells, and subsequent high magnification (×40 and ×100) of five randomly selected fields was used to analyze staining intensity.

Evaluation of SPHK1 and Gal-3 Immunohistochemical Expression

Semi-quantitative analysis was done for the expression levels of SPHK1 and Gal-3. Positively stained cells were evaluated as follows: 0 (less than 5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), or 4 (more than 75%). Secondly, a four-point grading system assessed this staining intensity: 0 for negative, 1 for weak, 2 for moderate, and 3 for strong. Consequently, the IRS for every example was determined by multiplying the intensity score by the percentage score. The IRS values were then categorized into a 'low' and a 'high' expression level based on the median score (median = 6), and the median cut point served as the cutoff for classification for statistical analysis. [17]

Statistical analysis:

Data were collected, coded, reviewed, and entered into the Statistical Package for the Social Sciences (IBM SPSS version 27). The frequencies and percentages were summarized for categorical variables, and for numerical variables, means and standard

deviations were reported. The Kolmogorov–Smirnov and Shapiro–Wilk tests were used to test the normality of continuous data. Using the independent samples t-test for continuous variables and the chi-square test for categorical variables, we assessed the relationships between SPHK-1 and Galectin-3 expression and clinicopathologic features. The impact of histopathological classifications on the SPHK-1 and Galectin-3 IRS in more than two groups was compared using one-way analysis of variance (ANOVA); the SPHK-1 and Gal-3 IRS were compared between two groups using the independent samples t-test. Using Pearson's correlation coefficient, we investigated the connection between the expression levels of SPHK-1 and Gal-3. For statistical significance, a p-value of less than 0.05 was considered.

Results:

Clinicopathologic Parameters of Cases of the Study

Table 1 lists the clinicopathological features of 56 thyroid neoplasm patients enrolled in the current study. These patients included follicular adenoma, follicular variant of papillary thyroid carcinoma, and non-invasive follicular thyroid neoplasm with NIFTP.

Sphingosine kinase-1

• *Immunohistochemical expression of Sphingosine kinase-1 in thyroid FVPTC, NIFTP and FA, (fig.1).*

SPHK-1 was expressed in thyroid tumor cells and specifically stained cytoplasmically. As shown in Fig. 1C, there was a statistically significant association between cases of FA and FVPTC and SPHK-1 expression (p-value < 0.001). In particular, high SPHK-1 expression was specific for FVPTC cases; however, no FA cases presented this expression (Table 2).Also, There was statistically significant association in SPHK-1 expression between cases of FA and NIFTP (p-value =< 0.001), where 70% of NIFTP cases had high SPHK-1 compared to 0% in adenoma cases. (Table 3).

Immunohistochemical expression of Galectin-3 in thyroid FVPTC, NIFTP and FA (Fig. 2):

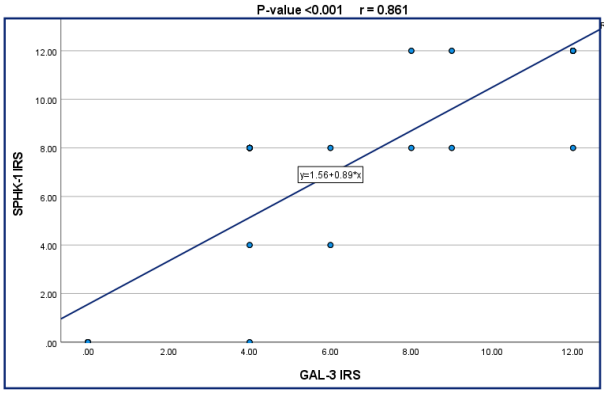
Expression of GAL-3 was identified in thyroid tumor cells, and the staining pattern was cytoplasmic. In the expression of Gal-3, we observed a statistically significant association between FA and FVPTC (p-value <0.001). In particular, 91.67% of FVPTC cases displayed high Gal-3 expression, but none of the FA cases exhibited elevated Gal-3 expression (Table 4).No statistical significant association in expression of Gal-3 between cases of FA and NIFTP was detected (p-value = 0.271) (Table 5).

The Relationship between Gal-3 and SPHK-1 expression in FA, NIFTP, FVPTC

As regard the relation between Gal-3 and SPHK-1 IHC staining results, there was statistically significant association between overexpression of Gal-3 and SPHK-1 IRS (p-value <0.001). (Table 6).

Correlation between Gal-3 and SPHK-1 immunohistochemical expression:

The results indicated a significant positive association between the expression of both Gal-3 and SPHK-1, where the Pearson correlation coefficient was 0.861 and the p-value of < 0.001 (Graph 1).



Graph (1): Correlation between Gal-3 index and SPHK-1 index

Discussion:

Non-invasive follicular thyroid neoplasm with papillary-like features (NIFTP) has a favorable prognosis regardless of tumor size and can be treated similarly to benign thyroid tumors, as suggested in clinical practice guidelines [14].

Our study aimed to determine the degree of staining of Gal-3 and SPHK-1 proteins on fifty-six FFPE tissue blocks originating from FA, NIFTP, and FVPTC. These samples were obtained from the specimens stored in the archives of the Surgical Pathology Laboratories of both South Valley University Hospital and Assiut University Hospital from 2019 to December 2021.

There was a relatively high level of a significant relationship between FA and FVPTC in terms of the changes in the expression of Gal-3 (p-value < 0.001), with 91.67% of FVPTC cases having a high intensity of Gal-3. In comparison, 0% of FA cases had the same. No significant relationship between the FA and the NIFTP cases could be established (p = 0.327). In line with this observation, Haugen et al. [15] also found that the level of Gal-3 was raised in invasive FVPTC compared to NIFTP with a significance value of < 0.001 and benign nodule with a significance value of < 0.001. Moreover, when comparing NIFTP with benign lesions, it was found statistically at p = 0.064 significance level. This is in contrast to the findings by Cho et al. [16], whereby they established that invasive FVPTC had higher HBME-1, CK19, Gal-3, and CD56 than encapsulated FVPTC.

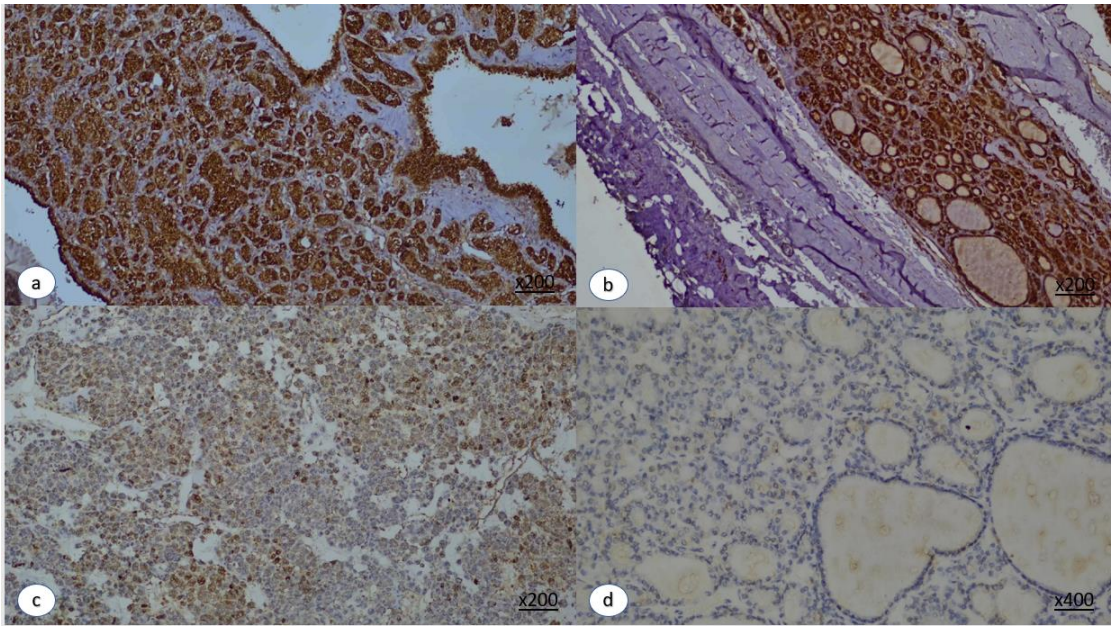


Fig.1: SPHK1IRS expression:
Fig.1.a. Strong SPHK1 expression in FVPTC (x200).
Fig.1.b. Strong SPHK1 expression in NIFTP (x200).
Fig.1.c. Moderate SPHK1 expression in NIFTP (x200).
Fig.1.d. Negative SPHK1 expression in FA (x400).

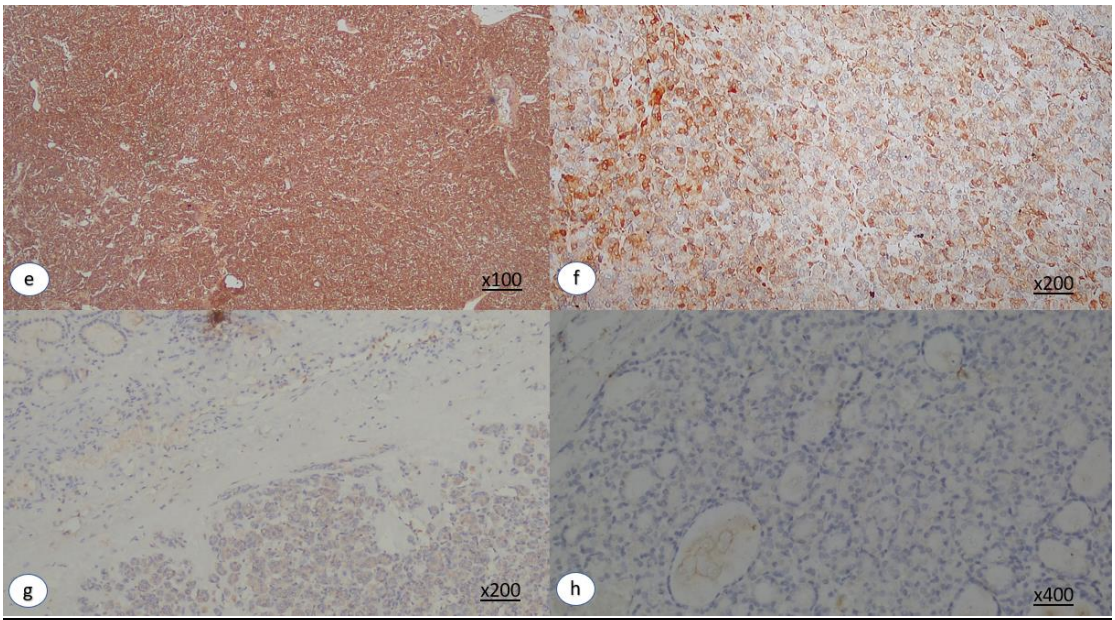


Fig.2: Gal-3 IRS expression:
Fig.2.e: Strong Gal-3 IHC expression in PTC (x100).
Fig.2.f: Moderate Gal-3 IHC expression in PTC (x200).
Fig.2.g: Mild Gal-3 IHC expression in NIFTP (x200).
Fig.2.h: Negative Gal-3 IHC expression in FA (x400).

Table (1): Clinicopathological parameters.

Parameters		Frequency	Percentage %
Age (years)	≤ 40	40	71.4%
	>40	16	28.6%
	Mean ± SD	34.89 ± 10.196	
	Median (range)	34.50 (18-64)	
Gender	Male	2	3.6 %
	Female	54	96.4%
Histopathology	Follicular adenoma	12	21.4 %
	FVPTC	24	42.9 %
	NIFTP	20	35.7%
T staging of FVPTC	T1	8	33.33%
	T2	6	25%
	T3	6	25%
	T4	4	16.67%
Extra-thyroid extension of FVPTC	Positive	10	41.66%

Table (2): SPHK-1 expression in cases of FVPTC vs FA.

		Histopathology		P value
		FA(n=12)	FVPTC (n=24)	
		Number (%)	Number (%)	
SPHK-1 expression	Low	12 (100%)	0 (0%)	<0.001*
	High	0 (0%)	24 (100%)	

*Chi-square test

Table (3): SPHK-1 expression in cases of NIFTP vs FA.

		Histopathology		P value
		FA (n=12)	NIFTP (n=20)	
		Number (%)	Number (%)	
SPHK-1 expression	Low	12 (100%)	6 (30%)	<0.001*
	High	0 (0%)	14 (70%)	

*Chi-square test

Table (4): Gal-3 expression in cases of FVPTC vs FA.

		Histopathology		P value
		FA (n=12)	FVPTC (n=24)	
		Number (%)	Number (%)	
Gal-3 expression	Low	12 (100%)	2 (8.33%)	<0.001*
	High	0 (0%)	22 (91.67%)	

*Chi-square test

Table (5): Gal-3 expression in cases of NIFTP vs FA.

		Histopathology		P value
		FA(n=12)	NIFTP (n=20)	
		Number (%)	Number (%)	
Gal-3 expression	Low	12 (100%)	16 (80%)	0.271
	High	0 (0%)	4 (20%)	

*Chi-square test

Table (6): The Relationship among Gal-3 and SPHK-1 IHC staining results in FA, NIFTP, and FVPTC

SPHK-1 IRS	Gal-3 IRS				P value
	Low expression (n= 30)		High expression (n=26)		
	No.	%	No.	%	
Low expression (18)	16	53.33%	2	7.7%	<0.001*
High expression (38)	14	46.67%	24	92.3%	

*Chi-square test

These results differ from those stated by Feilchenfeldt et al. [17], who detected Gal-3 expression in all PTC and even in certain benign diseases, desolate hyperplastic nodule (1/8) and FA (1/5). This may be because the level of Gal-3 RNA can vary from 0.03 to 2.75 Amol/µg in normal and benign thyroid tissues. Moreover, such positive findings in benign lesions can pose challenges when cytological control is lacking.

However, in the present study, it was possible to observe a positive statistical correlation between the immunohistochemical expression of SPHK-1 and FA and FVPTC ($p < 0.001$). Regarding SPHK-1, all FVPTC cases have high markings, while no FA cases have high markings. This is consistent with the study by Do et al [18], who established higher sphingosine kinase-1 levels in papillary thyroid carcinoma tissue than in nodular goiter tissue ($p < 0.001$) or normal thyroid tissue ($p < 0.001$). Our findings are similar to the study conducted by Sheng Lau et al [19], where they established overexpression of SPHK-1 in thirteen cancers of the cancers sampled, including the invasive breast carcinoma, head and neck squamous cell carcinoma, liver hepatocellular carcinoma, lung squamous cell carcinoma and thyroid carcinoma with p values of < 0.001, = 0.002 and < 0. 001, < 0.001 and < 0.001 respectively.

These findings can be explained based on the function of SPHK-1, a proto-oncogene synthesizing sphingosine-1-phosphate S1P liberated by human ABC transporter. S1P binds to other phosphosphingolipid partners within the interstitial fluid surrounding the tumor microenvironment; hence, the protein plays a vital role in signal transduction and modules of cancer cell communication, proliferation, invasion, and metastasis. Moreover, S1P is implicated in various aspects such as anti-apoptosis, cell invasion, epithelial-mesenchymal transition, angiogenesis, and resistance to chemotherapy [20], [21].

Surprisingly, our study has demonstrated that the SPHK-1 expression difference is statistically significant between FA and NIFTP (Chi-square value = 137.76, p-value < 0.001). More particularly, high SPHK-1 expression was noted in 70% of NIFTP cases as opposed to no adenoma cases. In this regard, because SPHK-1 transduces S1P, which is known to have pro-survival and anti-apoptotic effects in cancer cells by increasing their proliferation, this isoform functions as a cancer-related kinase involved in the most essential features of cancer. It is widely accepted as a crucial factor in oncogenesis [21].

It was also identified that there is a positive correlation between the IHC staining scores of Gal-3 and SPHK-1. The correlation between high IHC nuclear expression of both factors and the corresponding IHC scores was significant at $p < 0.001$. Also, a significant association was revealed between the immunohistochemical staining of Gal-3 and SPHK-1 in the present cases ($p < 0.001$, $r = 0.861$). This was further supported by the fact that most FVPTC cases (91.67%) exhibited high expression of GAL-3, and all the FVPTC patients displayed high expression of SPHK-1. Galectin-3 binds to other survival-related proteins like B-cell lymphoma protein 2 (Bcl-2) and GTP-bound active Ras-K (K-Ras) proteins involved in differentiation, transformation, and angiogenic events. In the same context, it has been observed that high expression levels of SPHK-1 promote Ras-mediated neoplastic transformation and, thus, the formation of large and more angiogenic tumors resistant to treatments and poor prognosis [22].

Conclusion:

SPHK-1 and Gal-3 are highly expressed in follicular variant of papillary thyroid carcinoma patients, so both of them have diagnostic value in infiltrative FVPTC. Only SPHK-1 has a diagnostic role in NIFTP. Both Gal-3 and SPHK-1 are correlated together.

Ethical approval:
SUV-MED-PAT005-2-21-12-289

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