

Higher expression level of tyrosine kinase-like orphan receptor 2 and Wnt member 5a in papillary thyroid carcinoma is associated with poor prognosis

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Abstract. The tyrosine kinase-like orphan receptor 2 (ROR2) has a wnt-mediated, pro-tumorigenic role in certain types of cancer. The present study was designed to assess the protein expression level of ROR2 and its putative ligand Wnt member 5a (Wnt5a), as well as the association with clinicopathological features in papillary thyroid carcinoma (PTC). A total of 58 patients were recruited, resulting in 58 human PTC tissue samples and their paired adjacent noncancerous tissue samples being obtained. The protein expression levels of ROR2 and Wnt5a were evaluated by immunohistochemistry and western blotting, and messenger RNA expression levels were determined by reverse transcription-quantitative polymerase chain reaction. ROR2 and Wnt5a protein and mRNA expression were significantly overexpressed in PTC tissues ($P < 0.05$). The present study also revealed that ROR2 and Wnt5a were significantly associated with tumor stage and lymph node metastasis ($P < 0.05$). There was a positive association between ROR2 and Wnt5a expression levels ($r = 0.857$; $P = 0.007$). In conclusion, ROR2 and Wnt5a may act as tumor suppressor genes in the development of PTC; overexpression of ROR2 and Wnt5a in PTC may be important for tumorigenesis and tumor development.

Introduction

Thyroid cancer is the most common type of endocrine tumor; since 2000, overall thyroid cancer incidence rates have increased by ~8% per year (1). Papillary thyroid carcinoma (PTC) is the major histological type and accounts for ~80% of all thyroid types of cancer (2,3). Highly effective for

the treatment of PTC involves surgery and radioactive iodine; however, the traditional therapies are ineffective against advanced radioactive iodine-resistant PTC. The majority of patients with PTC demonstrate excellent clinical outcomes; however, distant metastasis of PTC can be fatal (4).

Tyrosine kinase receptors represent targets of great interest for cancer therapy. The tyrosine kinase-like orphan receptor 2 (ROR2), is also a Wnt ligand receptor. ROR2 has been revealed to specifically interact with Wnt5a. Aberrant activation of Wnt signaling is involved in the development of various types of tumors (4,5). In renal cell carcinoma, a high expression level of ROR2 demonstrated a significant association with higher clinical stage, nuclear grade and tumor stage (5). Recent data suggested that the Wnt signaling pathway also altered PTC with RET/PTC mutations (6). To the best of our knowledge, the present study revealed for the first time that ROR2 and Wnt5a expression levels may also be altered in PTC tissues compared with normal tissues.

The present study evaluated the protein expression levels of ROR2 and Wnt5a in human PTC and adjacent normal tissues, investigated the changes and clinical significance of ROR2 in PTC and its association with Wnt5a expression.

Materials and methods

Patients and samples. The present study utilized 58 human PTC tissues and paired adjacent noncancerous tissues excised from patients with histologically confirmed PTC between January 2014 and July 2015 at the Department of Oncological Surgery, Central Hospital of Cangzhou (Cangzhou, China). The tissue samples were frozen immediately in liquid nitrogen on removal from the patients. The present study was approved by the Ethics Committee of the Central Hospital of Cangzhou. Written informed consent was obtained from all patients prior to enrollment in the present study.

Immunohistochemistry analysis. The rabbit anti-human ROR2 polyclonal antibody (cat. no. SC-98486; 1:1,000) and the rabbit anti-human β -actin polyclonal antibodies (cat. no. SC-130656; 1:1,000) were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). The rabbit anti-human

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Wnt5a polyclonal antibody (cat. no. 55184-1-AP; 1:1,000) was purchased from Abcam (Cambridge, UK). The tissues were fixed with 4% paraformaldehyde for 12 h and then embedded in paraffin and sliced into 4- μ m sections; these sections were then dewaxed in xylene for 3 min three times, 100% ethanol for 2 min three times, 95% ethanol for 2 min, 80% ethanol for 2 min, 70% ethanol for 2 min and PBS for 5 min. Endogenous peroxidase activity was blocked by soaking slides in 3% H₂O₂ for 15 min at room temperature (RT). The slides were then agitated and excess PBS removed. All tumor sections were circled with a PAP pen. A total of 75 μ l blocking buffer (Shanghai Xin Le Biological Technology Co., Ltd, Shanghai, China) was added to each section immediately. The slides were then incubated for 1 h to overnight at RT in a humidified chamber. The appropriate primary antibody was applied overnight at 4°C. PBS-incubated slides were used as a negative control. Subsequent to washing in PBS three times, the tissue sections were incubated with biotin-conjugated secondary antibody (Goat anti-rabbit antibody; cat. no. ab6720; Abcam; 1:1,000) at room temperature for 1 h. Subsequently, the tissue sections were visualized using 0.05% diaminobenzidine in PBS for 5 min at 37°C. The tissue sections were counterstained with hematoxylin (10%) at room temperature for 1 min, dehydrated using graded ethanol (70, 80 and 100%) and sealed and covered with glass coverslips. All slides were processed by the same pathologist.

Two independent pathologists reviewed all histological tissue sections and evaluated the immunohistochemistry staining results according to the criteria of the World Health Organization (7). The pathologists randomly observed 10 high power fields of view with 100 cells in each view using a light microscope at magnification, x400 (Olympus Corporation, Tokyo, Japan). Comprehensive evaluation was performed according to the intensity and percentage of the stained tumor cells. Staining intensity was graded according to the following criteria: 0, no staining; 1, yellow; 2, deep yellow; and 3, brown. The proportion of positive tumor cells was scored as follows: 0, no positive tumor cells; 1, <10% positive tumor cells; 2, 10-50% positive tumor cells; 3, 51-80% positive tumor cells; and 4, >80% positive tumor cells. The immunohistochemical expression level was based on the total scores. Total score = points of staining intensity + points of percentage of positive cells. The tissue samples were classified into two groups, as follows: Negative expression, 0-1 points; and positive expression, 2-6 points.

Western blot analysis. Proteins were extracted from nitrogen-frozen tissue fragments of tissue samples. The tissues were homogenized in 1 ml radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) and subsequently with protease inhibitor cocktail (Beyotime Institute of Biotechnology) at 0°C for 20 min, followed by incubation for 20 min on ice and centrifugation at 12,000 x g for 15 min at 4°C. Following collection of the supernatant fluid, the sample was boiled for 15 min then stored at -80°C. Proteins (50 μ g/well) were separated by 8% SDS-PAGE. The absorbance of proteins at A562 nm was measured by a microplate reader. The protein concentration was calculated from the standard curve. Following electrophoresis, proteins were transferred to polyvinylidene

Table I. Analysis of ROR2 expression level in PTC and normal tissues.

Group	Cases, n	ROR2 expression, n		χ^2	P-value
		Negative	Positive		
PTC	58	22	36	5.829	0.016
Normal	58	35	23		

ROR2, tyrosine kinase-like orphan receptor 2; PTC, papillary thyroid carcinoma.

Table II. Analysis of Wnt5a expression level in PTC and normal tissues.

Group	Cases, n	Wnt5a expression, n		χ^2	P-value
		Negative	Positive		
PTC	58	19	39	15.211	<0.001
Normal	58	40	18		

Wnt5a, Wnt member 5a; PTC, papillary thyroid carcinoma.

difluoride membranes and blocked for 1 h with 5% fat-free milk at room temperature. The membranes were incubated with primary antibodies, anti-ROR2 (SC-98486; 1:1,000) and anti-Wnt5a (55184-1-AP; 1:1,000), overnight at 4°C. β -actin antibody (SC-130656; 1:1,000) was used as the loading control. Subsequently, the membranes were incubated with secondary antibody (horseradish peroxidase-conjugated antibodies) at room temperature for 1 h (goat anti-rabbit antibody; ab6721; Abcam, 1:1,000). Following three washes with TBS for 15 min at room temperature, the membranes were treated with a chemiluminescence detection kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Protein bands were quantified using densitometry (Gel-Doc Gel-It2 310 Imaging Analysis System, Bio-Rad Laboratories, Hercules, CA, USA).

RNA extraction and cDNA synthesis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Total RNA isolation and cDNA synthesis were performed as previously described (8). Total RNA of PTC tissues and paired adjacent noncancerous tissues were isolated using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), according to manufacturer's instructions. RNA quality and concentration were assessed on the NanoDrop 1000 (Thermo Fisher Scientific, Inc.). A total of 1 μ g RNA was reverse transcribed using the First-Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) with Oligo(dT) primer. PCR primers used were as follows: ROR2 forward, 5'-GGCAGAACCCATCCT CGTG-3' and reverse, 5'-CGACTGCGAATCCAGGACC-3'; Wnt5a forward, 5'-ACCACATGCAGTACATCGGAG-3' and reverse, 5'-GAGGTGTTATCCACAGTGCTG-3'; and GAPDH forward, 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse,

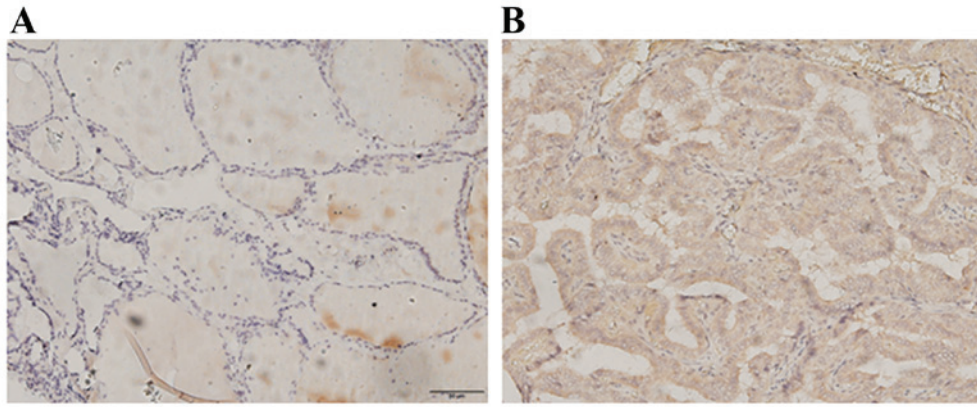


Figure 1. The expression level of tyrosine kinase-like orphan receptor 2 in (A) normal thyroid tissues and (B) papillary thyroid carcinoma tissues by immunohistochemistry (magnification, x200).

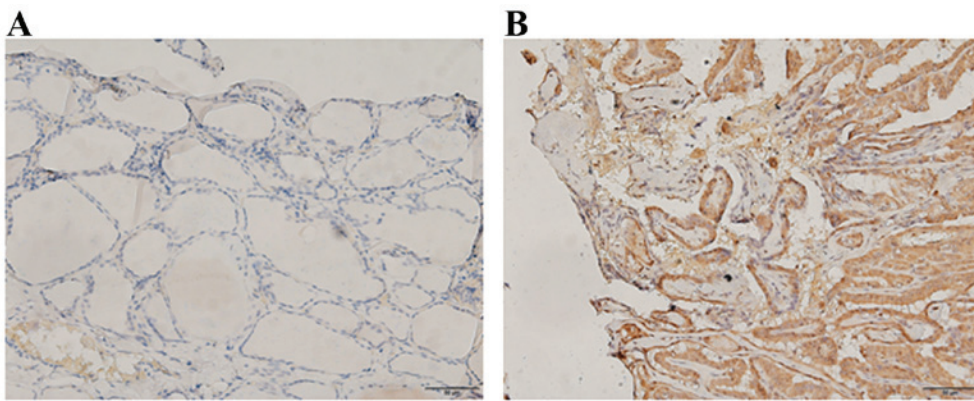


Figure 2. The expression level of Wnt member 5a in (A) normal thyroid tissues and (B) papillary thyroid carcinoma tissues by immunohistochemistry (magnification, x200).

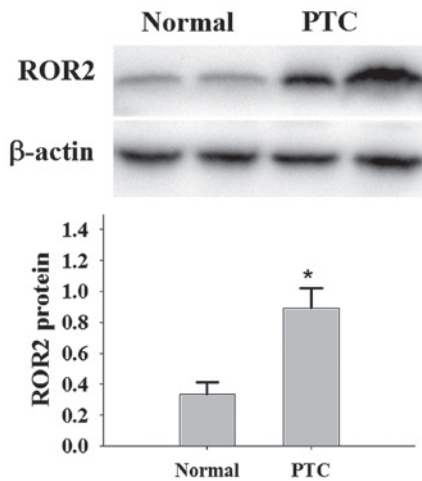


Figure 3. The expression level of ROR2 in PTC tissues and the corresponding normal tissues. A relatively high ROR2 protein expression level was revealed in PTC tissues compared with in normal thyroid tissues (* $P < 0.05$). ROR2, tyrosine kinase-like orphan receptor 2; PTC, papillary thyroid carcinoma.

5'-GAAGATGGTGATGGGATTTC-3'. The PCR reaction was performed for 40 cycles of a 3-step program: 95°C for 15 sec, annealing temperature at 60°C for 15 sec, 72°C for 1 min. mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method (9).

Statistical analysis. Statistical analysis was performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). The expression level of mRNA was represented as fold change using the $2^{-\Delta\Delta Cq}$ method. The χ^2 test was performed to determine the association between protein ROR2 expression level and clinicopathological features. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ROR2 protein and Wnt5a protein expression is increased in PTC. The patients in this study comprised 43 females and 15 males with an age range of 16-57 years. The present study selected the most representative PTC and adjacent normal tissues. Immunohistochemistry demonstrated that 36 patients exhibited positive expression of the ROR2 protein ($P < 0.05$; Fig. 1; Table I) and 39 patients exhibited positive expression of the Wnt5a protein ($P < 0.05$; Fig. 2; Table II). Western blot analysis demonstrated that the protein expression levels of ROR2 and Wnt5a in PTC were significantly increased compared with the expression in normal thyroid tissue samples (Figs. 3 and 4).

ROR2 gene and Wnt5a gene expression is increased in PTC. ROR2 and Wnt5a mRNA expression levels were significantly

Table III. Association between ROR2 expression level and clinicopathological characteristics in patients with PTC.

Clinicopathological characteristics	Cases, n	ROR2 expression, n		χ^2	P-value
		Negative	Positive		
Age				0.648	0.421
<45 years	20	9	11		
≥ 45 years	38	13	25		
Sex				2.039	0.153
Male	15	8	7		
Female	43	14	29		
Tumor Stage				6.408	0.007
I, II	40	20	20		
III, IV	18	2	16		
Tumor size				0.741	0.389
<1 cm	41	17	24		
≥ 1 cm	17	5	12		
Lymph node involvement				5.084	0.028
Yes	24	5	19		
No	34	17	17		
Multifocal				0.056	0.814
Yes	20	8	12		
No	38	14	24		

ROR2, tyrosine kinase-like orphan receptor 2; PTC, papillary thyroid carcinoma.

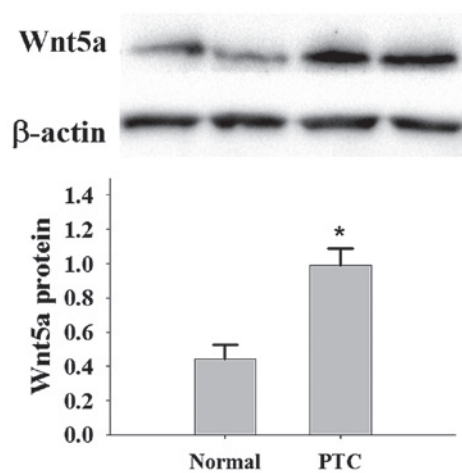


Figure 4. The expression levels of Wnt5a in PTC tissues and its counterpart normal tissues. Relatively high Wnt5a protein expression levels were revealed in PTC tissues compared with normal thyroid tissues (* $P < 0.05$). Wnt5a, Wnt member 5a; PTC, papillary thyroid carcinoma.

increased in PTC samples compared with in normal thyroid tissue samples (Figs. 5 and 6).

ROR2 and Wnt5a expression with pathological features. The expression level of ROR2 was associated with tumor stage and lymph node metastasis in PTC; however, there was no association with age, sex, tumor size and the number of tumors (Table III). Wnt5a was also associated with tumor stage and lymph node metastasis; no significant differences were

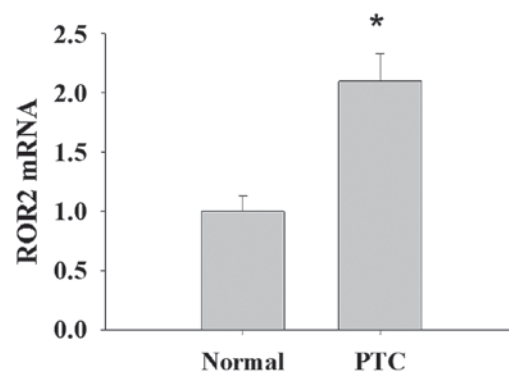


Figure 5. The expression levels of ROR2 mRNA in PTC tissues and its counterpart normal tissues were detected by reverse transcription-quantitative polymerase chain reaction (* $P < 0.05$). ROR2, tyrosine kinase-like orphan receptor 2; PTC, papillary thyroid carcinoma.

identified between Wnt5a expression levels and other clinicopathological findings, including age, sex, tumor size and the number of tumors (Table IV).

Correlation between ROR2 and Wnt5a in PTC. There were significant positive associations between ROR2 and Wnt5a (Table V).

Discussion

Thyroid cancer incidence increased, on average, 3.6% per year during 1974-2013, this was primarily associated

Table IV. Association between Wnt5a expression level and clinicopathological characteristics in patients with PTC.

Clinicopathological characteristics	Cases, n	Wnt5a expression, n		χ^2	P-value
		Negative	Positive		
Age				0.727	0.394
<45 years	20	8	12		
≥45 years	38	11	27		
Sex				0.482	0.488
Male	15	6	9		
Female	43	13	30		
Tumor Stage				7.069	0.003
I, II	40	18	22		
III, IV	18	1	17		
Tumor size				0.070	0.791
<1 cm	41	13	28		
≥1 cm	17	6	11		
Lymph node involvement				3.647	0.046
Yes	24	4	20		
No	34	15	19		
Multifocal				0.070	0.792
Yes	20	7	13		
No	38	12	26		

Wnt5a, Wnt member 5a; PTC, papillary thyroid carcinoma.

Table V. Association between ROR2 and Wnt5a expression levels in PTC.

Protein	r-value	P-value
ROR2		
Wnt5a	0.857	0.007

Wnt5a, Wnt member 5a; PTC, papillary thyroid carcinoma; ROR2, tyrosine kinase-like orphan receptor 2.

with increases in PTC of 4.4% per year (10). The main treatment for PTC involves total or subtotal thyroidectomy, radioactive iodine and thyroid hormone inhibitory therapy; however, the traditional therapies are ineffective against advanced radioactive iodine-resistant PTC (11). The majority of patients with PTC have a favorable prognosis and asymptomatic long-term survival; however, invasion and metastasis is also one of the major causes of mortality in patients with PTC (12). The invasion and metastasis of PTC is an interactive effect mediated by diverse genes and factors (13,14).

A previous study revealed that ROR2 has a role in cell migration and invasion (15,16). ROR2 is a transmembrane protein and acts as a Wnt ligand receptor that participates in Wnt signaling (17). A previous study suggested that ROR2 mediates Wnt5a signaling in a variety of tumor types,

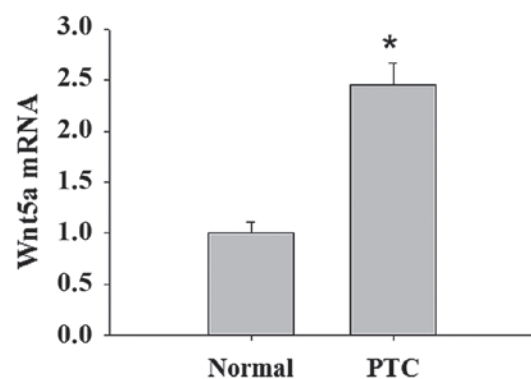


Figure 6. The expression levels of Wnt5a mRNA in PTC tissues and corresponding normal tissues were detected by reverse transcription-quantitative polymerase chain reaction (*P<0.05). Wnt5a, Wnt member 5a; PTC, papillary thyroid carcinoma.

including in human metastatic melanoma, leiomyosarcoma and gastrointestinal stromal tumors, renal cell carcinoma and osteosarcoma (8,18-21). Although it is accepted that altered Wnt signaling is a late event in thyroid cell transformation that affects anaplastic thyroid tumors, previous data suggested that it is also altered in PTC with RET/PTC mutations (22). The present study inferred that ROR2 and Wnt5a may also have an important role in PTC.

The present study demonstrated that ROR2 and Wnt5a protein translation and gene transcription were upregulated in PTC tissues in comparison with the matched adjacent

normal thyroid tissues. ROR2 is a member of a family of proteins known as receptor protein kinases, which have a key role in cell growth, differentiation and cell movement. Wnt5a binds to its receptor ROR2 and activates a serine/threonine-specific protein kinase, CamKII, which negatively regulates the canonical Wnt/ β -catenin signaling via the MAPK signaling pathway. An association was revealed between ROR2 expression level and tumor stage as well as lymph nodes metastasis. Wnt5a protein expression level was observed to be increased more significantly in patients with advanced stage and lymph node metastases; however, the role of the Wnt5a/ROR2 signaling pathway in cancer remains unknown (23). Wnt5a was demonstrated to signal via ROR2 to induce cellular migration and invasion in murine fibroblast NIH3T3 cells (24). Wright *et al* (21) demonstrated that ROR2 promotes tumor growth potential in renal cell carcinoma. McDonald and Silver (23) revealed that in cancer cells, the oncogenic potential of ROR2 may be conferred by Wnt5a via the promotion of cancer cell invasion. In the present study, compared with in the normal thyroid tissues, the expression level of ROR2 and Wnt5a in PTC tissues was increased markedly; therefore, ROR2 and Wnt5a may be involved in the progress of PTC or may become additional tumor markers for the diagnosis of PTC. However, it remains unclear how the Wnt5a/ROR2 signaling pathway acts in PTC.

The present study investigated the clinical significance of ROR2 and Wnt5a in patients with PTC for the first time and the result demonstrated that ROR2 and Wnt5a were significantly upregulated in PTC tissues compared with in the matched adjacent normal thyroid tissues. High ROR2 and Wnt5a expression levels were associated with tumor stage and lymph node metastases. Furthermore, there was a significant positive association between ROR2 and Wnt5a expression levels. These results indicated the cancer-promoting activity of the Wnt5a/ROR2 signaling pathway in PTC tissues. Rasmussen *et al* (6) revealed that in patients with renal cell carcinoma a high expression level of ROR2 was associated with a significantly lower overall survival rate, cancer specific survival and recurrence free survival compared with renal cell carcinoma patients with low expression levels of ROR2; these findings suggested that ROR2 may be considered as a negative prognostic biomarker and potential therapeutic target in this type of cancer. A study investigating cervical cancer indicated that ROR2 was significantly associated with cancer progression and poor prognosis (25). Lara *et al* (26) demonstrated that epigenetic alteration of ROR2 has a Wnt-mediated, pro-tumorigenic role in colon cancer. In brief, ROR2 with Wnt5a may have an important role in the infiltration and metastasis of PTC.

In summary, compared with normal thyroid tissues, the present study revealed a high expression level of ROR2 and Wnt5a in PTC tissues. Furthermore, the expression levels of ROR2 and Wnt5a were associated with tumor stage and lymph node metastasis. There was a significant positive association between ROR2 and Wnt5a expression levels. The results of the present study indicated that the Wnt5a/ROR2 signaling pathway may have a critical role in driving cell proliferation and migration. The expression level of ROR2 and Wnt5a mRNA significantly increased with tumor

progression, which suggested that the ROR2 and Wnt5a genes have potential as targets for cancer gene therapy. In conclusion, these results indicated that ROR2 and Wnt5a may be promising biomarkers and potential therapeutic targets for PTC in the future.

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