

## Aberrant promoter methylation of EDNRB in lung cancer in Taiwan

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**Abstract.** Hypermethylation has been shown in the promoter region of the endothelin receptor B (EDNRB) gene in several human tumors, but its role in lung cancer formation is unclear. In this study, genomic DNA from lung cancer patients was subjected to methylation-specific PCR to determine the methylation status of the EDNRB gene in lung cancer. Aberrant methylation of the EDNRB gene was detected in 32.9% (26 of 79) lung cancer patients. Promoter hypermethylation of EDNRB was found to significantly differ with histological type but was not correlated to other clinicopathological characteristics. Decreased mRNA transcripts were correlated to aberrant methylation. Treatment with 5-aza-deoxycytidine reversed the methylation status and re-expression of the EDNRB gene in the H1355 human lung cancer cell line. Our results suggest that inactivation of the EDNRB gene through epigenetic alteration is highly prevalent in lung cancer in Taiwan.

### Introduction

Lung cancer is one of the leading causes of cancer death worldwide. In Taiwan, the annual mortality rate of lung cancer is approximately 20% of the total cancer-related mortality rate. In the United States, the equivalent annual mortality rate is approximately 30%, exceeding the number

of deaths from breast, prostate and colon cancer combined (1). Most of the patients who die are at the late stages of the disease when they are diagnosed. However, some patients who are diagnosed at an early stage and undergo curative resection still die of cancer due to early recurrence and metastasis (2). Due to diagnostic difficulties and low survival rates, lung cancer is a serious health problem.

Several known putative tumor suppressor genes (TSGs) have been identified as involved in the pathogenesis of lung cancer and are frequently inactivated by methylation (3). Frequent loss of retinoic acid receptor  $\beta$ -2 expression mediated by methylation was found in non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (4). The genes for several cell surface glycoproteins, including E-cadherin and H-cadherin, were also reported to be hypermethylated in lung cancer (5,6). Other genes, such as the cyclin-dependent protein kinase inhibitors p16INK4a, death-associated protein kinase and adenomatous polyposis coli genes, are also frequently silenced by promoter hypermethylation (7-9). Using a highly sensitive PCR method to detect the methylation of DNA sequences, Palmisano *et al* have reported that the methylation of p16INK4a and/or O<sup>6</sup>-methylguanine-DNA-methyltransferase can be detected in DNA from sputum in patients with squamous cell lung carcinoma prior to clinical diagnosis (10). Furthermore, patients with methylation of TSGs have a shorter overall survival than patients whose tumors were not methylated (3,11,12).

The endothelin receptor type B (EDNRB) gene has been localized to the chromosome 13q22 region and encodes a G-protein coupled receptor (13). Mutation of EDNRB is associated with Hirschsprung's disease, a common congenital malformation characterized by megacolon and abnormal skin pigmentation (13,14). Recently, evidence has been shown that the 5' flanking region of EDNRB contains numerous CpG dinucleotide repeats, and the methylation of these CpG sites can regulate gene expression (15). Moreover, using the arbitrarily primed PCR (AP-PCR) technique, it has been found that the 5' region of the EDNRB gene is hypermethylated

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in cancer compared to normal white blood cells (WBC) (16). Pao *et al* demonstrated that the EDNRB gene is unmethylated in normal bladder and prostate tissue, whereas it is frequently hypermethylated in tumors derived from these tissues (16). After treatment with 5-aza-2'-deoxycytidine (5-Aza-CdR), expression of EDNRB is induced by DNA demethylation in T24 cancer cells (16). Silencing of EDNRB gene expression mediated through promoter hypermethylation has also been identified in nasopharyngeal and prostate carcinomas and melanoma (15,17,18). The high frequency of promoter hypermethylation suggests that downregulation of the EDNRB gene may be involved in human tumorigenesis. Smith *et al* have reported that lower EDNRB expression was found in four primary small cell lung carcinomas compared to normal bronchial epithelium (19); however, the methylation status and expression levels of EDNRB remain to be elucidated in lung cancer in Taiwan. Here, we report that aberrant promoter hypermethylation of EDNRB was found in lung cancer and correlated with the histological type. In addition, the treatment of the human lung cancer cell line, H1355, with demethylation agent restored the expression of EDNRB. These results suggested that downregulation of EDNRB expression through promoter hypermethylation was highly prevalent in lung cancer in Taiwan.

## Materials and methods

**Sample collection and cell culture.** Tumor tissue and corresponding non-malignant tissue were obtained following surgical resection of 79 paired lung cancer samples (34 adenocarcinomas and 45 squamous carcinomas) in the Department of Surgery of Chang Hua Christian Hospital. All samples were frozen in liquid nitrogen immediately after surgical removal and stored at  $-70^{\circ}\text{C}$  until use.

The human H1355 cell line was a kind gift from Dr J.L. Ko (Institute of Medical and Molecular Toxicology, Chung Shan Medical University). H1355 cells were cultured in RPMI-1640 supplemented with 5% FBS and incubated in a 5%  $\text{CO}_2$  incubator.

**Genomic DNA purification and bisulfite modification.** Genomic DNA (1  $\mu\text{g}$ ) was subjected to bisulfite modification as previously described (20). Briefly, DNA (1  $\mu\text{g}$ ) was denatured with NaOH and modified with 3 M sodium bisulfite. Modified DNA was purified using the Gene-Spin™ 1-4-3 DNA extraction kit according to the manufacturer's instructions (Protech Technology, Taiwan) and eluted into 50  $\mu\text{l}$  of water. Modification was stopped by NaOH treatment (final concentration 0.3 M) for 5 min at room temperature, and followed by ethanol precipitation. DNA was resuspended in water and used immediately or stored at  $-20^{\circ}\text{C}$ . The EDNRB promoter methylation status was determined by methylation-specific PCR (MS-PCR) as described (17). Bisulfite-modified DNA was amplified by specific primers for unmethylated and methylated EDNRB, respectively. The primer sequences and annealing temperatures were as described (17). The PCR conditions were as follows: 5 min at  $95^{\circ}\text{C}$ ; 35 cycles of denaturing at  $94^{\circ}\text{C}$  for 30 sec, annealing at  $55^{\circ}\text{C}$  for 30 sec, and extension at  $72^{\circ}\text{C}$  for 30 sec; and a final extension of 10 min at  $72^{\circ}\text{C}$ . A negative control (water instead of DNA) was included with each amplification. The PCR products were

separated on 3% agarose gel, stained with ethidium bromide, and visualized under UV illumination. To determine the methylation sites, the PCR products were purified and sub-cloned into pGEMT-easy vector and sequenced. Individual clones containing insert from unmethylated and methylated samples were sequenced using an Applied Biosystems PRISM dye terminator cycle sequencer.

**RT-PCR amplification of EDNRB from lung cancer patients.** Total RNA derived from 20 paired samples was isolated using TRIzol reagent according to the manufacturer's recommendations. Ten  $\mu\text{g}$  of total RNA was converted into cDNA using MMLV-reverse transcriptase (Superscript II, Gibco-BRL, Gaithersburg, MD, USA) in 50  $\mu\text{l}$  of reaction buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM  $\text{MgCl}_2$ , 0.1 M DTT, 0.5 mM dNTP mix, and 0.5  $\mu\text{g}$  of oligo-dT primer. The expression of the three isoforms of the EDNRB gene (EDNRB  $\Delta 1$ ,  $\Delta 2$  and  $\Delta 3$ ) in SCC samples was examined by RT-PCR using primer set as: sense 5'-CATCAAGCTGCTGGCAGAGG-3' and anti-sense 5'-GATTCGCA GATAACTTTTGTAG-3'. The PCR reaction was performed as follows: hot start at  $94^{\circ}\text{C}$  for 5 min; 35 cycles of  $94^{\circ}\text{C}$  for 30 sec,  $52^{\circ}\text{C}$  for 30 sec, and  $72^{\circ}\text{C}$  for 30 sec; and a final extension at  $72^{\circ}\text{C}$  for 10 min. A fragment of GAPDH was amplified as an internal control.

**5-aza-2'-deoxycytidine treatment.** The human lung cancer cell line, H1355, was treated with 0.5, 1 and 5  $\mu\text{M}$  of 5-aza-2'-deoxycytidine. The culture medium and drugs were changed every 24 h. After 4-day exposure, genomic DNA derived from these cells was subjected to sodium bisulfite modification and MS-PCR as described above.

**Western blot analysis of EDNRB protein expression.** The cell lysates from 5-aza-2'-deoxycytidine-treated H1355 cells were obtained and the concentration was measured using a Bradford protein assay kit (Bio-Rad, USA). Fifty  $\mu\text{g}$  of protein was separated by 10% polyacrylamide gel and electrotransferred to nitrocellulose membrane. The membrane was blocked by PBS containing 0.5% non-fat milk for 1 h at room temperature. Afterwards, the membrane was incubated with goat anti-human-EDNRB antibody (Santa Cruz Biotechnology, USA; 1:500 dilution) at room temperature for 1 h. The membrane was washed with PBS containing 0.1% Tween-20, followed by reacting with HRP-conjugated bovine anti-goat IgG antibody (Santa Cruz Biotechnology; 1:5000 dilution). The reactive signal was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, UK).  $\beta$ -actin expression was used as the internal control.

**Statistical analysis.** The relationships of EDNRB hypermethylation with different characteristics, including gender, smoking, and clinicopathological parameters such as histological type, tumor stage, and tumor differentiation were calculated by Chi-square test. A p-value  $<0.05$  was considered as a significant difference.

## Results

**Methylation status of the EDNRB gene in lung cancer patients.** To investigate the methylation status of the EDNRB gene in

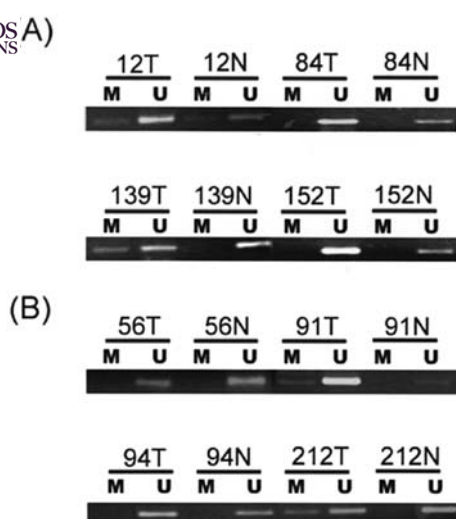


Figure 1. Methylation-specific PCR analysis of the EDNRB promoter of lung cancer. The methylation status of (A) adenocarcinoma and (B) squamous cell carcinoma was analyzed by specific primer for methylated DNA (M) or unmethylated DNA (U). N, non-malignant; T, tumor.

adenocarcinomas and squamous carcinomas of the lung, we first performed MS-PCR assay of 79 paired adenocarcinomas and squamous cell carcinomas (SCC). The specimens were obtained from 64 men and 15 women patients with lung cancer. The 79 lung cancer samples were divided into 38 stage I lesions, 12 stage II lesions, 26 stage III lesions, and 3 stage IV lesions. As shown in Fig. 1A, aberrant hypermethylation was found in cases 12, 139, 91, and 212. Promoter hypermethylation of EDNRB was detected in 26/79 lung carcinomas (7 in adenocarcinoma and 19 in SCC) and in 13 paired non-malignant parts (3 in adenocarcinoma and 10 in SCC). A significantly higher aberrant methylation frequency was found in tumors compared to non-malignant tissue (32.9% vs 16.5%,  $p=0.016$ ).

**Sequence analysis of bisulfite-modified DNA.** To ensure the successful modification of the DNA by bisulfite treatment and to determine a more detailed map of methylation sites, the PCR products derived from methylated and unmethylated fragments were subcloned and positive colonies were subjected to sequence analysis. The 150-bp PCR fragment spanning from nucleotide 550 to 700 in the promoter region of EDNRB (accession no. D13612) contains 11 CpG sites. Cytosine residues within the unmethylated fragment were all converted to thymine. Sequencing analysis from five methylated colonies showed a heterogeneous methylation pattern of cytosine in the 11 CpG islands of the promoter region with positions 654 and 673 as the less methylated sites (Fig. 2).

**Downregulation of EDNRB expression in lung cancer by methylation.** It is accepted that promoter hypermethylation can trigger silencing of gene expression (21). To determine the expression level of the EDNRB gene, we have performed RT-PCR in primary NSCLC with and without methylation. As shown in Fig. 3, the EDNRB mRNA expression level was decreased to approximately 50% in tumors with promoter hypermethylation compared to non-tumor tissue (cases 12,

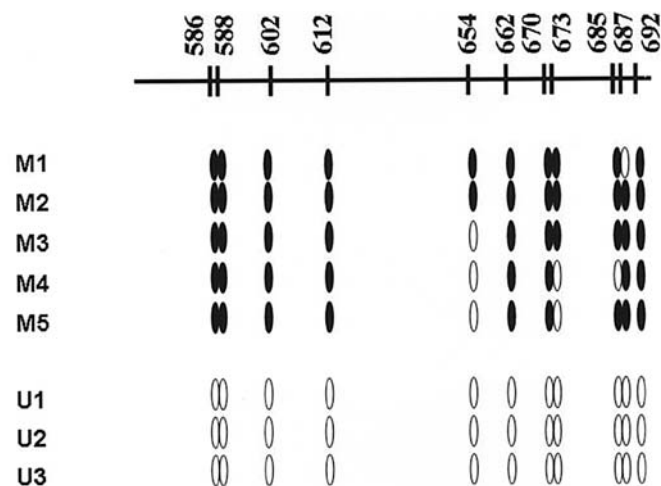


Figure 2. DNA sequencing analysis of methylation-specific PCR products of the EDNRB promoter region derived from case 212. MS-PCR products were subcloned into pGEMT-easy plasmid and transformed into JM109 cells. Five clones from methylated product and three clones from unmethylated product were sequenced. The top shows the promoter sequence of EDNRB (accession no. D13162) from 550 to 700, containing 11 CpG islands marked by number. Open circle, unmethylated site; closed circle, methylated site.

139, 91, and 212). Intriguingly, the expression of EDNRB was higher in the tumors of cases 56 and 94.

**Demethylation and re-expression of the EDNRB gene by 5-aza-deoxycytidine treatment.** Considering that silencing of EDNRB expression in lung cancer patients was mediated through promoter hypermethylation, it is reasonable to accept that treatment with a demethylation agent will reverse the methylation status of EDNRB and re-express EDNRB. We examined the methylation of CpG islands in the EDNRB gene of the human lung cancer cell line, H1355, after treatment with 5-aza-dC. H1355 cells were treated with various concentration of 5-aza-dC for 4 days and subjected to MS-PCR. Decreased signals showing methylated EDNRB were reversed to unmethylated from H1355 cells after, but not before, treatment with 1 and 5  $\mu\text{M}$  of 5-aza-dC (Fig. 4A). Furthermore, using Western blot analysis, we detected re-expression of the EDNRB gene in H1355 cells treated with 0.5, 1 and 5  $\mu\text{M}$  of 5-aza-dC (Fig. 4B).

**Clinicopathological correlations with EDNRB promoter methylation.** The association between aberrant methylation and the clinicopathological characteristics of patients and tumors is summarized in Table I. Aberrant methylation of EDNRB was found in 35.7% of tumors from patients aged <60 years, and 32.3% in those of >60 years. The frequency of EDNRB hypermethylation was 20.6% (7 of 34) in adenocarcinomas and 42.2% (19 of 35) in squamous cell carcinomas. Methylation of the EDNRB gene was significantly different in SCC histology compared to adenocarcinomas ( $p<0.05$ ). The frequency of EDNRB methylation tended to increase in relation to the clinical stage in SCC (31.6% in stage I; 44.4% in stage II; 52.9% in stage III/IV), although this difference did not reach statistical significance ( $p>0.05$ ). Promoter hypermethylation was also significantly different in stage-III/IV but not in stage-I/II SCCs compared to adenocarcinomas

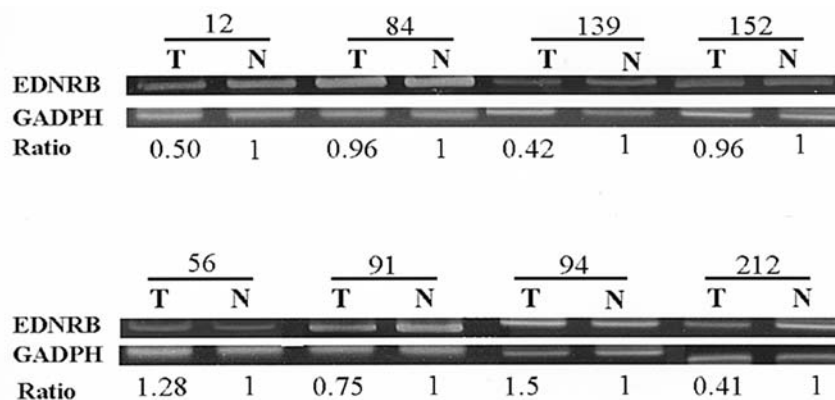


Figure 3. Expression pattern of EDNRB in lung cancer patients. Reverse transcription-PCR analysis was performed to determine the EDNRB mRNA expression in lung cancer patients. N, non-malignant; T, tumor. Represented samples are shown. The GADPH gene was used as control for RNA quality and loading. Ratio, mRNA level of EDNRB divided by GADPH in tumor tissue and compared with non-tumor tissue.

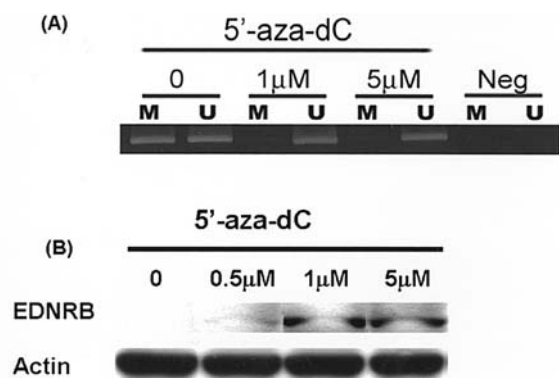


Figure 4. Demethylation and re-expression of EDNRB in lung cancer cell line by 5'-aza-dC. Lung cancer cell line, H1355, was treated with 0.5 µM, 1 µM or 5 µM 5'-aza-dC for 4 days. (A) Methylation status of these cells was detected by MS-PCR. U and M, PCR products amplified by unmethylated and methylated gene-specific primers. (B) Fifty µg total cell lysates were subjected to Western blot analysis. Strong re-expression of EDNRB was found in 5'-aza-dC-treated cell lysates. All samples were normalized by β-actin.

(52.9% vs. 16.7%;  $p < 0.05$ ). There was no association between hypermethylation of EDNRB and gender, smoking status, or tumor differentiation.

To explore whether the methylation status of the EDNRB gene was significantly associated with the survival of patients, the 5-year survival rates of the 79 patients were determined. Overall, the patients survived for 0.2-83.3 months, with a mean of 25.8 months. The follow-up data revealed no significant association between survival and the methylation status of the EDNRB gene (mean 26.5 months for unmethylated and 24.4 months for methylated,  $p > 0.05$ ).

**Discussion**

Lung cancer is one of the most prevalent cancers and is the leading cause of cancer-related death in the world (22). Epigenetic alternation of several tumor suppressor genes by prompter hypermethylation is frequently detected in lung cancer and is thought to be an important factor in the pathogenesis of lung cancer (23,24). In this study, we first demonstrated aberrant promoter methylation of the EDNRB

Table I. Correlation between methylation status and clinicopathological features of lung cancer patients.

	Methylation status of EDNRB		p-value
	Methylated (%)	Unmethylated (%)	
<b>Histological type</b>			
Adeno	7/34 (20.6)	27/34 (79.4)	0.04 <sup>a</sup>
SCC	19/45 (42.2)	26/45 (57.8)	
<b>Gender</b>			
Female	4/15 (26.7)	11/15 (73.3)	0.76
Male	22/64 (36.4)	42/64 (63.6)	
<b>Stage</b>			
Adeno I	2/19 (10.5)	17/19 (79.5)	0.05 <sup>b</sup>
Adeno II	3/3 (100)	0/0 (0)	
Adeno III/IV	2/12 (16.7)	10/12 (83.3)	
SCC I	6/19 (31.6)	13/19 (69.4)	
SCC II	4/9 (44.4)	5/9 (55.6)	
SCC III/IV	9/17 (52.9)	8/17 (47.1)	
<b>Differentiation</b>			
Well	1/4 (25)	3/4 (75)	0.65
Moderate	22/61 (36.1)	39/61 (63.9)	
Poor	3/14 (21.4)	11/14 (78.6)	
<b>Smoker</b>			
No	15/51 (29.4)	36/51 (70.6)	0.37
Yes	11/38 (39.3)	27/38 (60.7)	
<b>Survival (years)</b>			
<5	26/73 (35.6)	47/73 (64.4)	0.17
≥5	0 (0)	6/6 (100)	

Adeno, adenocarcinoma; SCC, squamous cell carcinoma. <sup>a</sup> $p = 0.04$  between Adeno and SCC by Chi-square test. <sup>b</sup> $p = 0.05$  between Adeno III/IV and SCC III/IV by Chi-square test.

gene in lung cancer patients in Taiwan. Hypermethylation of the EDNRB gene promoter was found in 32.9% of primary lung tumor samples and correlated with histological type



SPANDIDOS PUBLICATIONS SCC than in adenocarcinoma). Reduced EDNRB transcripts were found in patient samples containing aberrant methylation but not in unmethylated samples. Demethylation agents, such as 5-aza-2'-doxycytidine, can reverse the methylation of EDNRB in the human lung cancer cell line, H1355.

Hypermethylation of the EDNRB gene was associated with the histological type of lung cancer. A higher methylation frequency was observed in SCCs than in adenocarcinomas. However, our results reveal that different histological types of lung cancer display a distinct methylation status. In agreement with our finding, the prevalence of p16 hypermethylation was higher in squamous cell carcinomas than in adenocarcinomas (25). This result may reflect the distinct activity of *de novo* methyltransferases in different cell types (26). Moreover, a significant difference in the frequency of methylation between adenocarcinoma and SCC was found at advanced stages (52.9% in stage III/IV SCCs and 16.7% in stage III/IV adenocarcinomas;  $p < 0.05$ ) but not early stages. Our results imply that hypermethylation of the EDNRB gene may be a histologically specific event and be involved in advanced SCC tumorigenesis. However, the methylation status of the EDNRB gene did not correlate with gender, age, clinical stage, or differentiation.

Based on our data, a trend in the frequency of EDNRB gene methylation was detected in SCC in relation to clinical stage, but it did not reach statistical significance ( $p > 0.05$ ). The correlation between EDNRB gene methylation and disease stage is controversial. By performing MS-PCR using primers to amplify the -9 to -139 region of the EDNRB promoter, Jeronimo *et al* evaluated the methylation status of the EDNRB gene and found methylation in both normal and tumor tissue in prostate tissue specimens (18). Similarly, EDNRB gene methylation in medulloblastoma was found to reflect a normal level of tissue-specific methylation rather than a tumor-related event (27). Yegnasubramanian *et al* demonstrated that promoter hypermethylation of the EDNRB gene correlated with the grade and stage of primary prostate cancer (28). In contrast, Woodson *et al* showed that methylation of the EDNRB gene correlated with the stage of disease but not the tumor grade (29). These discrepant results may arise from the use of different primer sets and the detection of different CpG sites.

It has been demonstrated that downregulation of the EDNRB gene is found in advanced prostate cancer (30). Using a suppression subtraction hybridization technique, Smith *et al* reported that downregulation of EDNRB gene expression was detected in metastatic uveal melanomas and correlated with death due to metastases (19). In this study, we also detected reduced EDNRB expression in promoter methylated lung cancers; however, no significant difference in the 5-year survival rate was seen between methylated and unmethylated groups. The data indicated that the aberrant methylation of EDNRB may not be an independent prognostic factor for lung cancer.

In conclusion, we have found a high incidence of hypermethylation of the EDNRB gene in NSCLC. The frequency of aberrant methylation was significantly higher in SCCs than adenocarcinomas, suggesting that this methylation may be a suitable biomarker to distinguish SCCs from adeno-

carcinoma of the lung. Our results strongly suggest that hypermethylation of the EDNRB gene promoter leading to the downregulation of EDNRB expression may be a critical event in lung cancer tumorigenesis and may play a role in tumor-related death. However, more studies are needed to elucidate the functional consequences of EDNRB downregulation in lung cancer.

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