

Micrometastasis and Expression of nm23 Messenger RNA of Lymph Nodes from Lung Cancer and the Postoperative Clinical Outcome

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Background: Based on the metastatic route in lymph nodes from lung cancer, we investigated micrometastasis in the dissected lymph nodes by genetic analysis of keratin 19 and nm23-H1 (the expression of a tumor-metastatic suppressor gene) and evaluated the postoperative outcomes.

Methods: Ten patients operated with lung cancer were 4 males and 6 females, who were stage IA; 2, stage IB; 3, stage IIA; 2, stage IIB; 1, and stage IIIA; 2, respectively. After total RNA extraction from the dissected lymph nodes, the expression of nm23-H1 and keratin 19 messenger ribonucleic acid (mRNA) were analyzed with reverse-transcribed polymerase chain reaction (RT-PCR).

Results: The confirmation of micrometastasis in lymph nodes was realized in seven of 10 cases (70%), in their 5-year follow-up term. In three patients there was recurrence (43%, 3/7), and the one of them had died from the mediastinal recurrence. On the expression of nm23-H1 mRNA in lymph nodes, there was no significant difference between the pathologically lymph-node metastasis positive group and the negative one, and between the group with a tumor size over 30 mm and the group with a tumor size under 30 mm, respectively. The expression ratio of nm23-H1 gene was significantly expressed in the group with micrometastasis in lymph nodes (47%, 9/19) as compared to those without micrometastasis (10%, 1/10) ($p < 0.05$). On the all-positive expression of nm23-H1 in the examined lymph nodes ($n=4$), no patient had recurrence (0%, 0/4). However, in the rest of the six patients without the all-positive expression of nm23-H1 in those lymph nodes ($n=6$), four patients had recurrence (67%, 4/6). There was no significance between the recurrent ratio in the all-positive expression of nm23-H1 suggesting lower incidence as compared to that in patients without all-positive expression of nm23-H1.

Conclusion: A detection of micrometastasis in lymph nodes could be a useful tool to identify the subpopulation of patients who might have a higher risk of recurrence and distant metastases. The nm23-H1 gene might be involved in a suppression role for micrometastasis in lymph nodes through the lymphatic route in lung cancer. (*Ann Thorac Cardiovasc Surg* 2004; 10: 152–9)

Key words: nm23 messenger ribonucleic acid (mRNA), lung cancer, lymph nodes, micrometastasis, postoperative course

Introduction

The presence of lymph node metastasis is an important prognostic factor in primary lung cancer. Even in patients

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with stage I of non-small-cell lung cancer (NSCLC), without an apparent metastasis in the regional lymph nodes, recurrent disease might develop within an early interval after the operation. The 5-year survivals after the operation estimate recurrence in about 64% to 75% of patients.^{1,2} This suggests that the pathologic stage as determined by conventional hematoxylin-eosin (HE) staining for specimens from lymph nodes may be underestimated and thus the occult metastasis stage may remain undetected. An accurate assessment of the presence or absence of tumor cells in the regional lymph nodes is therefore

Table 1. Clinicopathological characteristics of the patients

Case	Age/gender	TNM-stage	Pathology*	Operation**	Postoperative therapy	
1	66/M	T2N0M0	IB	Ad	R-UML	None
2	62/F	T1N1M0	IIA	Ad	R-LL	Oral tegafur
3	63/M	T3N2M0	IIIA	Adsq	R-UL	Radiotherapy
4	56/M	T2N0M0	IB	Sq	L-P	Oral tegafur
5	67/F	T1N1M0	IIA	Ad	R-LL	Oral tegafur
6	64/F	T3N2M0	IIIA	Sm	L-LL	Chemotherapy (cisplatin, etoposide)
7	54/F	T2N1M0	IIB	Ad	R-MLL	Oral tegafur
8	72/F	T2N0M0	IB	Ad	R-UL	None
9	64/M	T1N0M0	IA	Ad	L-LL	None
10	66/F	T1N0M0	IA	Ad	R-LL	None

* Ad: adenocarcinoma, adsq: adenosquamous carcinoma, sq: squamous cell carcinoma, sm: small cell carcinoma.

** R-: right, L-: left, UML: upper-middle lobectomy, LL: lower lobectomy, UL: upper lobectomy, P: pneumonectomy, MLL: middle-lower lobectomy.

critical for making an accurate prognosis for patients with lung cancer.³⁻⁵⁾

It has recently been recognized that the nm23 gene and its product are closely related to the metastatic potential of some tumor cells.^{6,7)} Low levels of nm23 RNA and the corresponding protein have been reported to reflect high metastatic potential in both experimental animal tumors and human breast cancer.⁶⁻¹¹⁾ However, from the different studies examining colon carcinoma or neuroblastoma, an increased nm23-H1 gene expression was found to be associated with advanced stages of the disease.^{12,13)} Therefore, the significance of nm23 expression in human cancers may be different according to the various organs in which the tumor might be developed. Few studies have been performed on lung carcinoma, and they were all done on NSCLC. Clinical studies on nm23 expression in NSCLC have demonstrated conflicting results, however; some studies suggested that nm23 was associated with antimetastatic potential,¹⁴⁻¹⁶⁾ and others did not.^{17,18)} Some studies suggested that nm23 expression was correlated with tumor progression.¹⁹⁾

Studies of the messenger ribonucleic acid (mRNA) level of the nm23 gene in lymph nodes of patients with lung cancer have not yet been carried out. Whether or not the expression of nm23 mRNA suppresses the early micrometastatic stage in lymph nodal route has not yet been elucidated at genetic levels except for immunohistochemical analysis of the nm23-gene product. We report on the expression of nm23-H1 and keratin-19 gene in resected lymph nodes and evaluate relationships of nm23 mRNA expression and micrometastasis in lymph nodes as a metastatic route, and the observation of the postoperative clinical

course, that is, recurrence and metastasis for the postoperative 5-year interval.

Patients and Methods

Patients

Ten surgically obtained specimens from the patients with lung cancer, were studied, with their consent at our department in Miyazaki Medical College Hospital from June 1997 to February 1998. They underwent a lobectomy combined with a formal mediastinal and hilar node dissection, or pneumonectomy. The patient characteristics are summarized in Table 1. Pathological stage was determined according to the tumor-node-metastasis (TNM) classification, the clinicopathological stages found among these cases were follows: stage IA, 2 tumors; stage IB, 3; stage IIA, 2; stage IIB, 1; stage IIIA; 2. Histological type was defined due to the classification by the World Health Organization. After surgery, the postoperative recurrent disease was investigated by chest roentgenograms, chest computed tomography (CT), whole-body bone scanning data, and serum tumor marker of carcinoembryonic antigen (CEA) and CYFRA 21-1 (cytokeratin fragment 19). They were followed up for five years at our outpatient clinic until September 2002, and if treatment for recurrence or metastasis was necessary, postoperative comprehensive therapy was performed at our clinic. The postoperative clinical observation was conducted by outpatient medical records.

Samples

Resected primary lung cancer and lymph nodes were immediately frozen in liquid nitrogen and stored in a deep

freezer at -80°C until usage. Half of the dissected lymph node was used for conventional pathological diagnosis of metastasis with HE staining, and the other half was immediately frozen for further genetic analysis as above. Surgically resected tissues obtained were 10 of the primary lung cancer tissues and 28 of the dissected regional lymph nodes. Regarding the criteria of the selection of those dissected mediastinal and hilar nodes, considering the skip metastasis, at least two nodes from the first group (#10 or #11) and the second one (#3 or #7) were genetically analyzed.

Genetic analysis

Total RNA was purified from each tissue verified from 0.1 to 0.5 g of the frozen homogenized specimen under liquid nitrogen. Total RNA from tumor and lymph nodes was extracted by the guanidinium thiocyanate method followed by ISOGEN instruction method (Nippon Gene Co., Ltd., Tokyo, Japan). The SuperScriptTM Preamplification system for First Strand cDNA Synthesis (LIFE Technologies Co., Tokyo, Japan) was used to generate complementary deoxyribonucleic acid (cDNA) of the target gene. The target cDNA was amplified directly with TaKaRa Taq (TaKaRa Shuzo Co., Ltd., Kyoto, Japan) by the reverse-transcribed polymerase chain reaction (RT-PCR). PCR was performed with a DNA Thermal Cycler (TaKaRa Thermal Cycler MP, TP-3000, TaKaRa Shuzo Co., Ltd.). The following primers were synthesized with a DNA synthesizer (Applied Biosystems, Model 394). DNA sequence of the target primer is listed as below: sense of nm23-H1 is 5'-CGCAGTTCAAACTAAGCAGCAGCTGG-3', antisense of nm23-H1 is 5'-AGATCCAGTTCTGAGCACAGCTCG-3', (483 bp),²⁰⁾ sense of keratin 19 is 5'-AGGTGGATTCGCTCCGGGCA-3', antisense of keratin 19 is 5'-ATCTTCCTGTCCCTCGAGCA-3' for keratin 19 mRNA, (460 bp).²¹⁾ The primer for β -actin (838 bp) was purchased from BD Bioscience Clontech Co. (Tokyo, Japan); sense of β -actin is 5'-ATCTGGCACCACACTTCTACAATGAGGCTGCG-3', antisense of β -actin is 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3'.²²⁾ The PCR conditions followed were: nm23-H1: 95°C , 30 sec, 55°C , 30 sec, 72°C , 30 sec, 32 cycles, and 72°C for 7 min and 4°C , soaked; keratin 19: 94°C , 60 sec, 60°C , 60 sec, 72°C , 60 sec, 50 cycles, and 72°C for 7 min and 4°C , soaked; β -actin: 94°C , 45 sec, 60°C , 45 sec, 72°C , 2 min, 35 cycles, and 72°C for 7 min and 4°C , soaked. RT-PCR amplification of nm23-H1, keratin 19, and β -actin was applied in 1.8% Agarose gel (Wako Pure Chemical

Industries, Ltd., Osaka, Japan), and electrophoresed by 150 V. After staining with ethidium bromide for 10 min, the gel was photographed under UV illumination.

Statistical analysis

The χ^2 test and Fisher's exact methods were used for comparison of the incidence among groups. All p values less than 0.05 were considered statically significant.

Results

Agarose gel electrophoresis of nm23-H1 and keratin 19 products

To rule out RNA degradation, RT-PCR amplification of β -actin mRNA was performed which resulted in the presence of a 838 bp fragment in every sample, guaranteeing the integrity of the total RNA used for RT-PCR. Four representative patterns of results are shown in Fig. 1 (the other is not shown). The expression of nm23-H1 product with RT-PCR, a DNA product band of 483 bp fragment of nm23-H1 and a 460 bp fragment of keratin 19 product were determined by agarose gel electrophoresis. Case 1 was stage IB, and pathologically metastasis-negative in lymph nodes but micrometastasis-positive in the first and the second groups of lymph nodes by a genetic analysis. The pathological stage IIB might have been revised to be stage IIIA. The expression of nm23-H1 mRNA was not recognized in the second group of lymph nodes (#3). Fourteen months postoperatively, the patient had mediastinal recurrence and died at postoperative 19 months. Case 2 was stage IIA, and pathologically metastasis-positive in the first group of lymph nodes and pathological negative in the second ones, however, there was no indication of micrometastasis in lymph nodes by genetic analysis. The expression of nm23-H1 was recognized in the first group of lymph nodes (#3) but not in the second ones. Five years postoperatively, small multiple metastases in the bilateral lungs have been found and chemotherapy has been continued. Case 6 was stage IIIA, and pathologically metastasis-positive in the second group of lymph nodes. The expressions of nm23-H1 mRNA were recognized in both first and second groups of lymph nodes. Postoperative adjuvant chemotherapy had been carried out and the patient is alive to date without any recurrence. Case 9 was stage IA, and pathological metastasis-negative in lymph nodes but micrometastasis-positive in the first group of lymph nodes. The expressions of nm23-H1 mRNA were recognized in both the first and second group of lymph nodes. The patient is alive without any recur-

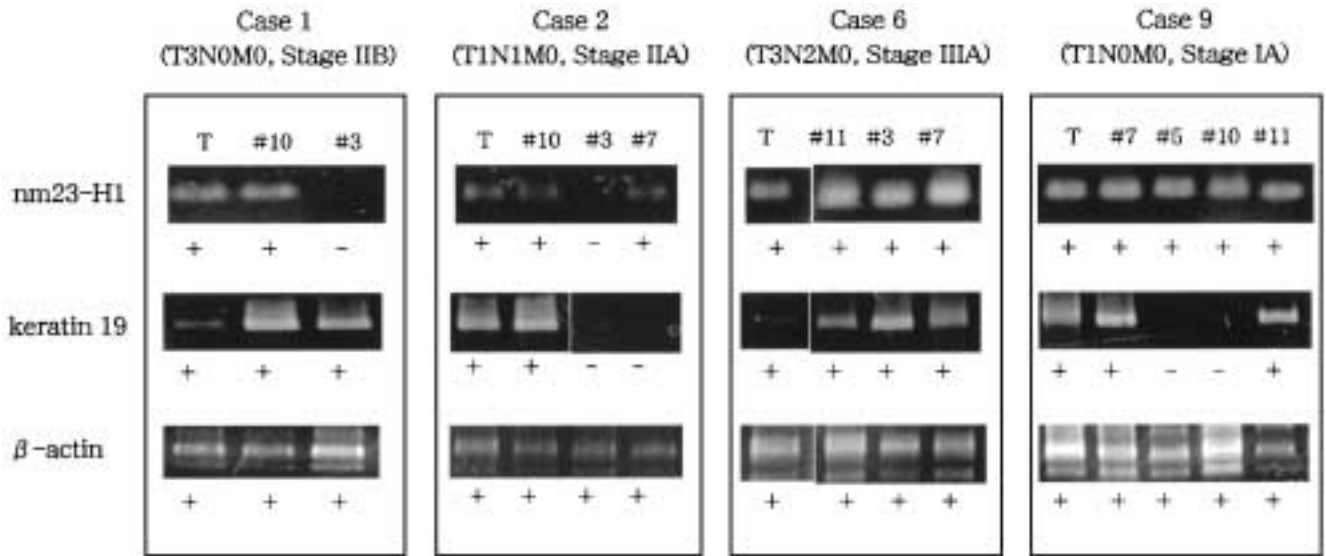


Fig. 1. Representative RT-PCR of nm23-H1, keratin 19, and β -actin. Amplified fragments were electrophoresed on 1.8% agarose gels and stained with ethidium bromide. T: tumor

rence. From these clinical courses, even though from a preliminary consideration, in the cases with micrometastasis-positive by a genetic analysis and/or with no expressions of nm23-H1 mRNA in lymph nodes, we have found unfavorable outcomes such as early recurrence and a poor prognosis in the postoperative term.

Group classification by clinicopathological characteristics of the patients

We divided these cases into two groups as follows: LN(+) (lymph node metastasis was confirmed pathologically positive) versus LN(-) (lymph node metastasis was also negative); size>30 group (tumor size is larger than 30 mm in diameter) versus size≤30 (tumor size is smaller than 30 mm in diameter); micrometa(+) (micrometastasis-positive, pathological metastasis was negative but the expression of keratin 19 mRNA in lymph nodes was positive) versus micrometa(-) (micrometastasis-negative, patho-

logical metastasis was negative and the expression of keratin 19 mRNA in lymph nodes was negative).

The expression of nm23-H1 mRNA

In primary lung cancer, the expression of nm23-H1 mRNA was 100%. In lymph nodes, the results of the expression of nm23-H1 mRNA between the LN(+) group versus the LN(-) group, and those between size>30 group versus size≤30 one were summarized in Table 2. There were no significant differences among those groups and the expression ratio was similarly observed between these two groups. Micrometastasis in lymph nodes was observed in seven of the 10 cases (70%). On the expression of nm23-H1 between the micrometa(+) group versus micrometa(-) group, there was a significant difference between these groups (p<0.05), the expression ratio was highly observed in micrometa(+) group (47%, 9/19) compared with that in micrometa(-) group (10%, 1/10).

Table 2. Expression of nm23-H1 mRNA in lymph nodes

		nm23-H1		p value
		Positive	Negative	
Lymph nodes metastasis	-	7	7	N.S.
	+	12	3	
Size (mm)	>30	8	6	N.S.
	≤30	10	5	
Micrometastasis	+	10	9	<0.05
	-	9	1	

Table 3. The postoperative clinical outcomes

Case	Lymph nodes metastasis	Size	Micrometastasis	nm23-H1 expression*	Recurrence	Survival	Disease-free term	Comments
1	-	>30	+	-	+	Dead (19 M)	13 M	Recurrence of mediastinum
2	+	≤30	-	-	+	Alive	5 Y	Chemotherapy for multiple lung metastasis
3	+	>30	+	-	-	Alive	5 Y	Operation for pharyngeal cancer
4	-	>30	+	-	-	Alive	4 Y 11 M	
5	+	≤30	-	+	-	Alive	4 Y 11 M	
6	+	>30	-	+	-	Alive	5 Y 0 M	Chemotherapy
7	+	>30	+	-	+	Alive	2 Y 9 M	Radiotherapy and chemotherapy
8	-	≤30	+	+	-	Alive	4 Y 7 M	
9	-	≤30	+	+	-	Alive	4 Y 10 M	
10	-	≤30	+	-	+	Alive	4 Y 8 M	Partial resection of lung metastasis, chemotherapy

nm23-H1 expression*

+: all positive for nm23-H1 expression in the examined lymph nodes

-: without all positive for nm23-H1 expression in the examined lymph nodes

The postoperative clinical course and the expression of nm23-H1 mRNA

The postoperative clinical course was summarized in Table 3. As a postoperative adjuvant therapy, chemotherapy, or radiotherapy was administered for two patients of stage IIIA, oral tegafur (300 mg) have been prescribed for four cases of stages IIA and IIB, the other four cases of stages IA and IB have been under follow-up. In the 5-year follow-up, one patient had died of mediastinal recurrence after the postoperative 13-month period and nine are still alive, and five patients had been treated again with operation, chemotherapy, and radiotherapy. However, the other four patients have been healthy without any recurrence. Micrometastasis in lymph nodes was observed in seven of the 10 cases (70%, 7/10). Three patients have had recurrence in the micrometa(+) group (n=7, 3/7, 43%). There were four patients with all-positive expression of nm23-H1 in the examined lymph nodes, who did not have recurrence (n=4, 0/4, 0%), however, there were six patients without all-positive expression of nm23-H1 in the examined lymph nodes amongst these recurrence has not been observed in four patients (n=6, 4/6, 67%). There was no significant difference in those groups, however, the recurrent ratio in the all-positive expression of nm23-H1 in lymph nodes was observed to be lower compared to that in patients without all positive for nm23-H1.

Discussion

A tumor metastasis suppressor gene, nm23, encodes a human Mr 17,000 nuclear and cytoplasmic protein containing 152 amino acids.²³⁾ nm23 was originally identi-

fied as the antimetastatic gene,⁶⁾ and its product, nucleotide diphosphate kinase, is a ubiquitous enzyme that catalyzes the adenosine triphosphate-dependent synthesis of ribo- and deoxyribonucleoside triphosphates from the corresponding diphosphates via a phosphoenzyme intermediate.

By immunohistochemical detection of nm23 protein in pulmonary adenocarcinoma, any correlation was not revealed between the expression of nm23 and metastatic potentials,¹⁷⁾ and an independent prognostic factor was suggested not to be found.²⁴⁾ In comparison with this point of view, in the report from another group on pulmonary adenocarcinoma with immunohistochemical assessment, nm23 protein expression in pulmonary tumors was shown to correlate inversely with advancing pathologic stage and the degree of metastasis in the regional lymph nodes (p<0.05).¹⁴⁾ In lung adenocarcinoma of Clara cell type, high levels of nm23 expression were associated with tumor progression (pathologic stage, positive lymph node status, and poorer prognosis) (p<0.05); however, there was no correlation with other cell types.¹⁹⁾ Our group reported a positive relationship between expression of nm23-H1 gene product and extent of metastasis in mediastinal lymph nodes from lung cancer patients, and they concluded that normal germinal center cells suggested that nm23-H1 gene product expression did not play a specific biological role in suppressing tumor or metastasis in lung cancer.¹⁸⁾ In immunohistochemical analysis of nm23-H1 in stage I NSCLC, the level of nm23-H1 protein was a more useful indicator than the T-status or histological type for the prediction of distant metastases and was a helpful tool to identify the subpopulation of patients with early stage lung cancer who have a high risk of distant me-

tastases and might benefit from postoperative adjuvant chemotherapy.¹⁵⁾

In recent reports, the 5-year survival rates of nm23-positive and nm23-negative patients in resected pathologic-stage I, NSCLC, were reported to be 79.7% and 57.8%, respectively, suggesting a significantly poorer prognosis in nm23-negative patients ($p=0.013$) by a multivariate analysis.²⁵⁾ From this point, the expression of nm23 mRNA in lymph nodes is of our interest, and there have not been any reports concerning the expression of nm23 mRNA in surgically resected lymph nodes, which was focusing on the metastatic route in lung cancer via lymphatic flow. In fact, histologically metastasis-negative cases could be revised to be metastasis-positive cases followed by genetic diagnosis by detecting of keratin 19 mRNA in the dissected lymph nodes.²¹⁾ In immunohistochemical detection of micrometastasis in regional lymph nodes of patients with stage I NSCLC,²⁶⁾ cytokeratin-positive cells was reported to be observed in 27% of 56 patients and 2.1% of 1,024 lymph nodes and that recurrence occurred more frequently in patients with micrometastasis than without micrometastasis ($p=0.053$). Concerning the patients with lung cancer who were diagnosed as N0, using a conventional pathologic diagnosis, a wide spectrum of percentage of nodal microdissemination of cytokeratin-positive cells has been reported as 10.4-70%.^{16,27-30)} The development of sensitive immunohistochemical techniques and specific monoclonal antibodies, against keratin, cytokeratin, and BerEp4, has enhanced our capacity to detect small clusters of tumor cells in breast and lung cancer or even single tumor cells in the lymph nodes suspected to be negative by the examination of HE-stained slides.^{27,31-33)} In the present genetic analysis, seven of 10 cases (70%) were observed and micrometastasis was highly suspected.

The expression of nm23-H1 mRNA in the micrometa(+) group (with micrometastasis) was observed to be higher compared with that in the micrometa(-) group (without micrometastasis). No recurrence has been recognized in the patients with all-positive expression of nm23-H1 in lymph nodes (0/4, 0%) compared to that in patients without all positive for nm23-H1 (4/6, 67%). Even though there was no significance, the nm23-H1 gene might play a tumor-suppression role in lymph nodes. Patients with no micrometastasis even though, there is positive expression of nm23-H1 mRNA in lymph nodes with micrometastasis might have less incidence of distant metastasis. On the other hand, the patients with a positive micrometastasis and/or no expression of nm23-H1

mRNA in lymph nodes might have a metastatic potential. These genetic analyses for the dissected lymph nodes should be a useful tool for the high-risk group of metastatic potentials. To obtain a better postoperative follow-up, in these suspicious cases, a postoperative adjuvant therapy should be managed under a strict investigation of recurrence and metastasis. No correlation with micrometastases in lymph nodes and prognosis was reported,³⁰⁾ on the other hand, it was more frequently reported that micrometastasis-positive cases had a much worse prognosis than micrometastasis-negative ones.^{28,29,33)}

From the present preliminary clinical observation, we conclude that a genetic diagnosis compared to an immunohistochemical detection is much more sensitive but can not be routinely performed because of its complicated detection technique, however, we representatively analyzed key lymph nodes of #10, #7, and #3. As we did not analyze all of the dissected nodes, this was a limitation of this study. We might explain a suppressive role of nm23 with keratin 19 mRNA in the lymph nodes but we might not do it without keratin 19 mRNA. The expression of nm23 might play a suppressive role of micrometastasis in lymph nodes, even though there were no cancer cells in the primary step of lymph nodal metastasis. The immunohistochemical analysis on the expression of keratin 19 could be easily performed but we obtained limited information because of our results from only a cut-surface of the resected lymph nodal tissue and we could not show the full nodal metastatic state. On the other hand, the genetic analysis of keratin 19 in lymph node should be much more sensitive because of its mRNA detection of the very tiny micrometastasis in the resected whole nodes. Both genetic and immunohistochemical analyses should be performed to make up for complement. These results suggest the inquiry for postoperative adjuvant treatment (chemotherapy and/or radiotherapy), for a better postoperative follow-up and an early finding of recurrence. In the future, a further genetic study of micrometastasis and expression of nm23-H1 in lymph nodes would be elucidated on a malignancy of tumor-metastatic progression in the lymphatic route in lung cancer cases.

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