Molecular Therapy
Nucleic Acids
Original Article

Expression Analysis of *MALAT1*, *GAS5*, *SRA*, and *NEAT1* lncRNAs in Breast Cancer Tissues from Young Women and Women over 45 Years of Age

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Breast cancer, as the most common cancer in women worldwide, represents about 30% of all cancers affecting women. Long non-coding RNAs (lncRNAs) have been implicated in the regulation of several biological processes, and their dysregulation in cancer has well been documented. To investigate possible age-dependent variations in expression profiles of lncRNAs, we evaluated the expression levels of four lncRNAs, i.e., *MALAT1*, *GAS5*, *SRA*, and *NEAT1*, in breast cancer (BC) samples obtained from younger (<45 years) and older (>45 years) women. Tumor samples (n = 23) and 15 normal tissues were collected from BC patients. All tumor and normal samples were morphologically confirmed by a pathologist. RNA was extracted from the tissues and cDNAs were then synthesized. The lncRNA expression levels were evaluated by qRT-PCR. The changes in the expression levels were determined using the ΔΔCt method. Compared to normal tissues, BC tissues from both age groups (women under 45 years of age and women above 45 years of age) showed upregulation of *MALAT1* (p = 0.003 and p = 0.0002), *SRA* (p = 0.005 and p = 0.0002), and *NEAT1* (p = 0.010 and p = 0.0002) and downregulation of *GAS5* (p = 0.0002 and p = 0.0005). Additionally, our analysis showed significant and direct correlation between the age and the expression levels of three of the four lncRNAs studied in this work. All four lncRNAs were overexpressed in both MDA-MB-231 and MCF7 cell lines (p = 0.1000). Our data show that *MALAT1*, *GAS5*, *SRA*, and *NEAT1* lncRNAs are dysregulated in BC samples. However, except for *MALAT1*, the expression levels of all of these lncRNAs were significantly lower in cancers developed in younger cases, where poorer prognosis is suggested. Of note, *GAS5* reduced expression has been documented to correlate with tumor progression.

INTRODUCTION

Breast cancer (BC), as the primary cause of death by cancer in women, is the most common malignant tumor worldwide.[1] BC is estimated to count up to 29% of all new cancers detected in women.[2] On average, women in Iran are diagnosed with BC at the age of 45, while this figure is at least ten years higher in other countries.[3] The etiology of BC is complex and multifactorial. It is generally considered that genetic, environmental, and reproductive factors all contribute to the development of BC.[4] The complex association between circadian rhythm (CR) disruption and BC development can be used as an example of this etiologic complexity. Disruption in CR has been correlated with increased risk of BC development.[5] Interestingly, several epigenetic changes and genomic polymorphisms in genes controlling the CR have been shown to be significantly associated with BC development.[6,7] Further, CR disruption has been correlated with decreased telomere length, where short telomere length itself is associated with BC development.[8]

Long non-coding RNAs (lncRNAs) are a category of non-coding RNAs, generally longer than 200 bp, which are transcribed from the genome with various regulatory or unknown functions. It has been demonstrated that lncRNAs are indispensable in normal cell and tissue development and differentiation as well as in the initiation and progression of various pathogenic conditions, including cancer.[9] In this regard, dysregulated expression of lncRNAs is observed in BC as well as in many other malignancies.[10] A better understanding of the molecular biology of cancer, including that related to the function and behavior of lncRNAs, can well be helpful in early detection as well as in designing targeted therapy for this multifaceted disorder.[9]

Steroid Receptor RNA (*SRA*) was first reported in 1999 as a functional ncRNA able to co-activate steroid nuclear receptors.[11] It has also been

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Table 1. Characteristics of the Cancer Patients and Specimens Used in This Study

	Frequency	Valid Percent	Cumulative Percent
Age			
≤45	15	65.2	65.2
>45	8	34.8	100
Histologic Grade			
Well differentiated	1	6.7	6.7
Moderately differentiated	10	66.7	73.3
Poorly differentiated	4	26.7	100
Tumor Side			
Left	7	46.7	46.7
Right	8	53.3	100
Prevascular Invasion			
Negative	4	26.7	26.7
Positive	10	66.7	93.3
Other	1	6.7	100
Preneural Invasion			
Negative	3	20	20
Positive	12	80	100
Lymph Node Involvement Status			
Free	6	40	40
Involved	9	60	100
Total	15	100	
Staging (Clinical)			
I	8	53.3	53.3
II	7	46.7	100
Total	15	100	

shown that overexpression of *SRA* RNAs increases transcriptional transactivation of steroid receptors. *SRA* is an lncRNA with multiple isoform variants. The predominant *SRA* transcripts in normal tissue are approximately 0.7–0.9 kb long, while less abundant but larger transcripts (1.3–1.5 kb) have also been identified.[12] In addition to functioning as co-regulators for steroid and non-steroid nuclear receptors, *SRA*s also contribute to the action of several other transcription factors;[13] plus, the *SRA1* locus codes for protein-coding transcripts as well.[14]

Growth arrest-specific 5 (*GAS5*) is an lncRNA 650 bases in length expressed at high levels during growth arrest. This lncRNA was originally isolated from NIH 3T3 cell line using subtraction hybridization in 1998.[15] The human *GAS5* has been classified as a member of the 59-terminal oligopyrimidine tract (59 TOP) gene family, and this gene is also the host of multiple small nucleolar RNAs within its 11 introns.[16] At least four splice variants have so far been detected for *GAS5*,[17] and it has been shown that this gene can modulate cellular response to various apoptotic stimuli, including those triggered by a range of chemotherapeutic drugs, a notion that may suggest for a mechanism linking dysregulated *GAS5* expression in cancer with prognosis.[18]

Nuclear Enriched Abundant Transcript 1 (*NEAT1*) is a nuclear lncRNA that is a necessary scaffolding factor for the formation of nuclear paraspeckles.[19] Paraspeckles are nuclear bodies comprised of a *NEAT1* backbone that interacts with other core paraspeckle proteins, including polypyrimidine tract-binding protein-associated splicing factor (PSF), 54-kDa nuclear RNA-binding protein (p54nrb), and polymerase suppressor protein 1 (PSP1).[20] These proteins along with other paraspeckle proteins sequester certain mRNA transcripts at the paraspeckle and mediate post-transcriptional splicing.[21] Paraspeckle-independent functions of *NEAT1* are mostly uncharacterized. However, similar to *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1) (see below), *NEAT1* has also been shown to bind epigenetically active chromatin, and it may be an important activator of gene transcription.[22]

MALAT1 is a highly conserved lncRNA that was first identified as an upregulated lncRNA in lung cancer with a high tendency to metastasize.[23] *MALAT1* transcript is highly abundant in mammalian cells, and the primary transcript is processed into two smaller RNAs: a long 6.7-kb transcript that localizes to the nuclear speckles[24] and a tRNA-like small RNA (61 nt) that localizes to the cytoplasm.[25] *MALAT1* has been involved in the regulation of pre-mRNA alternative splicing, and its knockdown results in cell-cycle arrest.[26] *MALAT1* is also necessary for E2F target gene activation by repositioning E2F from polycomb bodies to transcriptionally active nuclear sites in a serum-dependent manner.[27] Recently, two genome-wide association studies have indicated that *MALAT1*, along with *NEAT1*, binds to the transcription start sites (TSSs) and to the gene bodies of those genes being actively transcribed.[22] Overexpression of *MALAT1* has been found to be associated with poor prognosis and shorter survival time in early-stage lung cancer.[28]

In this study we evaluated the expression levels of four lncRNAs, i.e., *MALAT1*, *GAS5*, *SRA*, and *NEAT1*, in BC samples from women under and above 45 years of age, using qRT-PCR technique. Our data indicate age-related differences in the expression levels of some of these lncRNAs in BC tissues.

RESULTS

Patients' General and Clinical Information

Table 1 summarizes the available patients' demographic and clinical data.

Expression of lncRNAs in MDA-MB-231 and MCF-7 Cell Lines

qPCR analysis on RNA samples from cancer cell lines showed that all of four lncRNAs, *i.e.*, *MALAT1*, *GAS5*, *SRA*, and *NEAT1*, were overexpressed in both MDA-MB-231 and MCF-7 BC cell lines compared to MCF-10A control cell line (Figure 1).

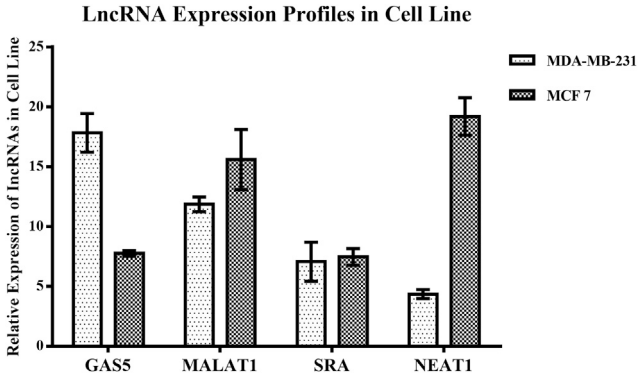

Figure 1. Relative Expression of lncRNAs in MDA-MB-231 and MCF-7 Cell Lines Compared to that in the MCF-10A Cell Line

Expression levels of the lncRNA were evaluated by qPCR and compared to that in the MCF-10A control cell line by the ΔΔCt method. The numbers on the y axis show fold changes and the star on the bars indicates significant ($p < 0.05$) change. Error bars show ± SE.

lncRNA Expression Profiles in BC Samples

The transcriptional status of *MALAT1*, *GAS5*, *SRA*, and *NEAT1* lncRNAs in BC samples from women aged under 45 and over 45 years (BC < 45 and BC > 45) was evaluated using qRT-PCR.

Compared to the average expression in normal tissues, *MALAT1* ($p = 0.003$ and $p = 0.0002$), *SRA* ($p = 0.005$ and $p = 0.0002$), and *NEAT1* ($p = 0.010$ and $p = 0.0002$) were upregulated in both age groups while *GAS5* ($p = 0.0002$ and $p = 0.0005$) was downregulated in all samples tested (Figure 2).

While *MALAT1* showed a rather similar overexpression pattern in both age groups, *NEAT1*, *SRA*, and *GAS5* showed lower expression levels in BC < 45 compared to BC > 45 (Figures 2 and 3). Table 2 also provides an overview on the correlations between the lncRNA expression levels and age, tumor size, and number of the lymph nodes involved.

DISCUSSION

lncRNAs are recently attracting attention for their documented roles in the molecular pathobiology of various cancers.[29] BC happens in women of different ages, but that affecting younger women is of poorer prognosis.[30] The current study evaluated the expression levels of four lncRNAs, *i.e.*, *MALAT1*, *SRA*, *GAS5*, and *NEAT1*, in the BC tissues from young women and women over 45 years of age to verify possible age effect(s) on the status of these lncRNA, which could be used to explain the age-dependent pathobiology.

Our expression analyses showed that *MALAT1*, *SRA*, and *NEAT1* were upregulated while *GAS5* was downregulated in both age groups (Figures 2 and 3). However, except for *MALAT1*, the extents of changes were considerably different in these two age groups so that tumor tissues from younger patients showed lower expression levels for these lncRNAs (Figures 2 and 3). The expression level differences were even more pronounced when the samples were grouped into patients under 35 years of age and older cases (Figure 4).

As mentioned earlier, there are documented indications of poorer prognosis for BC in young women (below 40 years of age) compared to that happening in older age.[31–33] Interestingly, it has already been

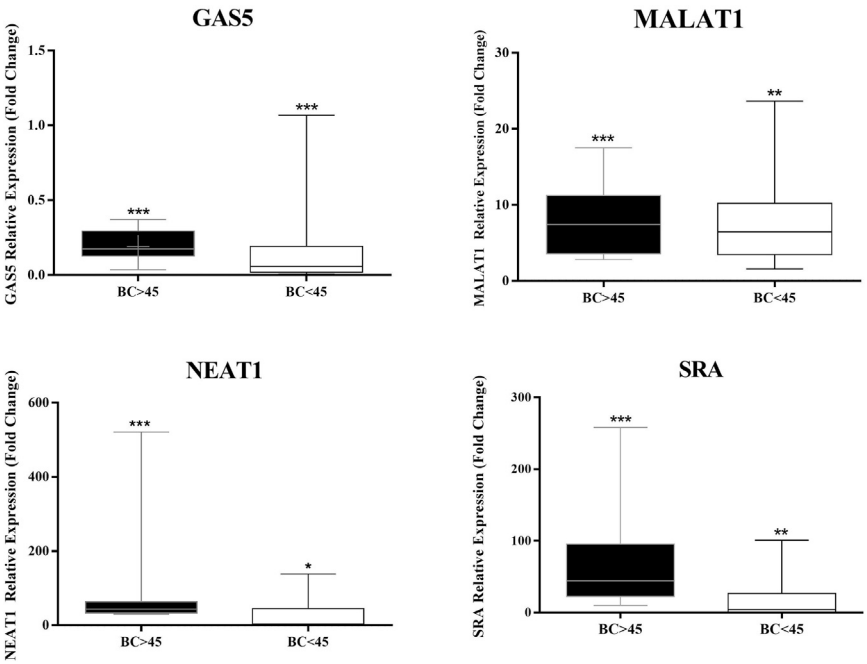

Figure 2. Expression Levels of lncRNAs in Samples from Women over 45 Years of Age (BC > 45) and in Those from Younger Patients (BC < 45) Compared to Unaffected Tissues.

qRT-PCR was used to quantify the expression levels of *GAS5*, *MALAT1*, *NEAT1*, and *SRA* lncRNAs in breast cancer. *significant at the 0.05 level; **significant at the 0.001 level; ***significant at the 0.0001 level. Error bars show the minimum and maximum variables.

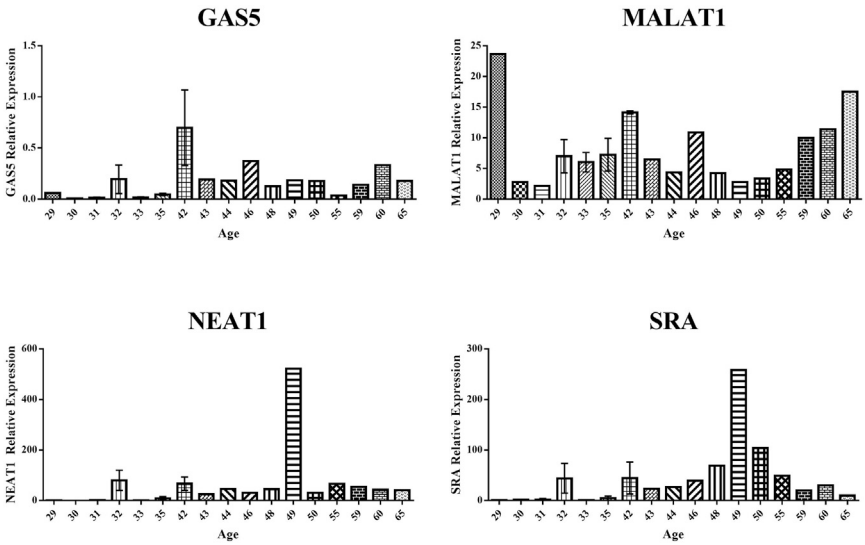

Figure 3. Normalized Expression of Each of the Four lncRNAs Studied in This Work per Sample

The x axis numbers are the ages of the patients from whom the samples were obtained. The y axis gives the normalized expression levels. Error bars show ± SE.

shown that *GAS5* has a tumor-suppressive role by controlling mammalian cell apoptosis, and its downregulation is thought to contribute to tumor formation.[34] Further, *GAS5* low expression levels correlate with a poor prognosis in head and neck squamous cell carcinoma.[35] Therefore, our finding of significant *GAS5* downregulation in tumor samples from young BC patients compared to those from older patients may explain the molecular mechanism causing a poorer prognosis of BC in this age group.

Conclusions

Based on previous research works and the data presented in our analyses, dysregulation of lncRNA expression is a major component in the tumorigenesis process. However, the expression pattern of these RNAs differs in different tumors and even in the same tumors developed in individuals at different ages, a notion that highlights the importance of considering the variable pathobiology of tumors when therapeutic approaches are going to be undertaken. In this line our work specifically showed that at least three of the four lncRNAs evaluated in this study show different levels of expression in BCs developed in younger women compared to those happening in older women. Specifically, backed by previous publications, the differences in the expression of one of these differentially expressed lncRNAs, *i.e.*, *GAS5*, can well explain the worse outcome of BC in younger patients at molecular levels. These findings can also be useful for defining future therapeutic regimens.

MATERIALS AND METHODS

Breast Sample Collection

Eight BC samples from women over the age of 45 and 15 BC samples from women under 45, plus 15 normal breast epithelial tissues, were received from patients referred to Shiraz General Hospital. Samples were obtained from patients undergoing hysterectomy without preoperative chemotherapy or radiotherapy, and they were histologically evaluated for type and grade (Table 1). All samples were transferred to RNAlater immediately after resection and stored at −20°C until used for RNA extraction. Informed consents were obtained, and the

Table 2. Correlation Coefficients between lncRNAs and Age, Tumor Size, and Number of Involved Lymph Nodes

		GAS5 lncRNA Expression	MALAT1 lncRNA Expression	SRA lncRNA Expression	NEAT1 lncRNA Expression	Age	Tumor Size	Number of Involved Lymph Nodes
GAS5 lncRNA expression	correlation coefficient	1.000	0.305	0.671**	0.678**	0.531**	0.042	−0.165
	significance (2-tailed)		0.157	0.000	0.000	0.009	0.882	0.556
	n	23	23	23	23	23	15	15
MALAT1 lncRNA expression	correlation coefficient	0.305	1.000	−0.197	−0.060	0.223	0.297	0.215
	significance (2-tailed)	0.157		0.368	0.785	0.307	0.283	0.442
	n	23	23	23	23	23	15	15
SRA lncRNA expression	correlation coefficient	0.671**	−0.197	1.000	0.777**	0.545**	−0.078	−0.167
	significance (2-tailed)	0.000	0.368		0.000	0.007	0.782	0.552
	n	23	23	23	23	23	15	15
NEAT1 lncRNA expression	correlation coefficient	0.678**	−0.060	0.777**	1.000	0.538**	−0.322	−0.046
	significance (2-tailed)	0.000	0.785	0.000		0.008	0.242	0.872
	n	23	23	23	23	23	15	15

**Correlation is significant at the 0.01 level (2-tailed).

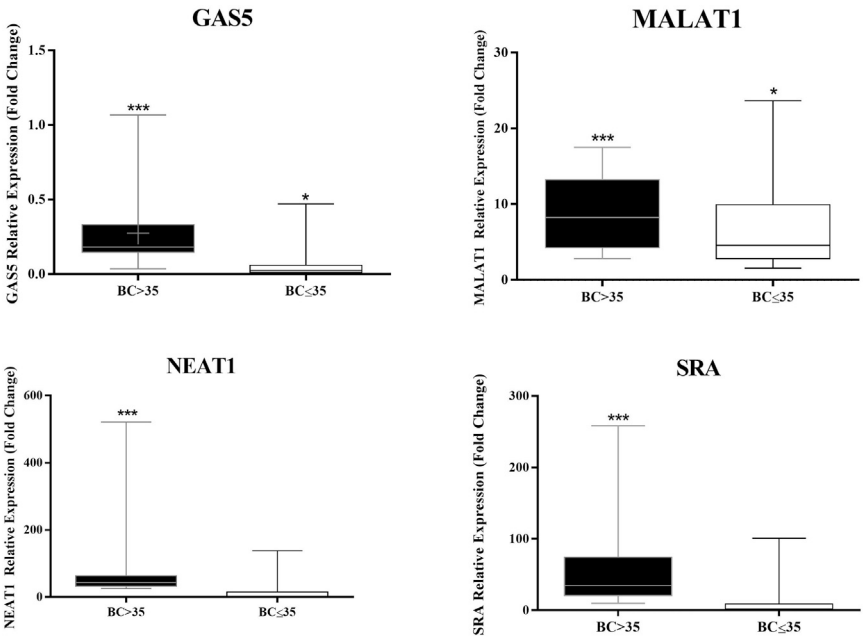

Figure 4. Expression Levels of lncRNAs in Samples from Women over 35 Years of Age (BC > 35) and in Those from Younger Patients (BC < 35) Compared to Unaffected Tissues

qRT-PCR was used to quantify the expression levels of *GAS5*, *MALAT1*, *NEAT1*, and *SRA* lncRNAs in breast cancer. *significant at the 0.05 level; **significant at the 0.001 level; ***significant at the 0.0001 level. Error bars show the minimum and maximum variables.

experimental procedure was approved by the Human Studies Committee of Islamic Azad University, Shahrekord Branch, Shahrekord, Iran (approval 17621105).

Cell Culture

The MDA-MB-231 (ATCC HTB-26), MCF-7 (ATCC HTB22), and MCF-10A (ATCC CRL-10317) cell lines were cultured and maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with L-Glutamine (Sigma-Aldrich, St. Louis, MO, USA), 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a cell culture incubator at 37°C, 5% CO_2, and 95% humidity.

RNA Extraction and cDNA Synthesis

Up to 100 mg tissue per extraction was homogenized in liquid nitrogen using a pestle and a mortar. Total RNA was extracted from tissue samples using the RNX-Plus solution (SinaClon, Iran), according to the manufacturer's instructions. The purity and concentration of the extracted RNA were determined by Thermo Scientific NanoDrop 1000 Spectrophotometer (Thermo Scientific, Germany), and the RNA integrity was confirmed by gel electrophoresis. The RNA extraction step was followed by a *DNase*I treatment (EN0521, Fermentas, Germany); 1 μg RNA was then used for cDNA synthesis using random hexamers as primers and Prime Script-RT kit (Takara, Japan).

Real-Time qPCR

Real-time qPCR was carried out using lncRNA-specific primers (Table 3) and SYBR Premix Ex Taq II kit (Takara, Japan), according to the manufacturer's instruction. Rotor gene 6000 Corbett detection system was used for amplification. The thermal cycling condition was set as follows: an initial activation step for 5 min at 95°C, followed by 40 cycles of 95°C for 15 s and 65°C for 1 min. No template controls (NTCs) were also included in each run. The best primer

Table 3. Sequences and Optimized Concentration of Primers Used in This Study

Primers	Primer Sequences (5′ to 3′)	Primer Concentrations (nM)		Ta (°C)	Product Size (bp)
		Forward	Reverse		
NEAT1	TGGCTAGCTCAGGGCTTCAG	300	300	63	101
	TCTCCTTGCCAAGCTTCCTTC				
SRA	CCTATTTGCACTGTATCACCC	300	600	57	114
	CCCCAATCTCAGTAATCTGG				
MALAT1	GAAGGAAGGAGCGCTAACGA	300	300	62	197
	TACCAACCACTCGCTTTCCC				
GAS5	CACACAGGCATTAGACAGA	300	900	53	187
	GCTCCACACAGTGTAGTCA				
PUM1	CCAGCAGGTAATTAATGAGA	900	900	53	165
	GATAAGGCAAATACCTGTCC				

Ta, annealing temperature.

concentrations were identified by performing a series of experiments with varying primer combinations (Table 3). To verify reaction efficiencies, for each primer set, standard curves were prepared using data from serially diluted samples. Melting curve analyses were also performed for each primer set. Besides, PCR products were electrophoresed on 2% agarose gel to verify the product sizes. The *PUM1* housekeeping gene was used as a normalizer, and the MCF-10A cell line was used as the control group for the cancer cell lines. Relative expressions were calculated using the $2^{-\Delta\Delta Ct}$ method.[36] The qPCR assays were performed in triplicate, and the data are presented as the mean ± SEM where applicable.

Statistical Analysis

qPCR data were analyzed using unpaired t test and Mann-Whitney tests, and the Spearman test was used to examine the effect of age using GraphPad Prism version 7.00 (GraphPad, La Jolla, CA, USA). The level of statistical significance was set at $p < 0.05$.

AUTHOR CONTRIBUTIONS

Conceptualization, M.M.G.S. and M.K.G.; Methodology, experiments, and analyses, A.A. F.S.S., S.O., and E.M.M.; Software, M.K.G. and Z.F.; Writing the Manuscript, A.A., M.M.G.S., and Z.F.; Supervision, M.M.G.S. and M.K.G.

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