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Enhanced expression of non coding miR 92a expression is implicated in the development of lung cancer

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs/miRs) are small noncoding RNAs that primarily function in RNA silencing and gene regulation at the posttranscriptional level in animals, plants and certain viruses. They have been reported to play a vital role in development and progression of diseases such as cancer. The present study was undertaken to establish a correlation between a specific miRNA in this cluster, termed miR92a, and its association with non-small cell lung cancer (NSCLC).

MATERIALS AND METHODS: Total RNA was isolated from the plasma using an RNeasy mini kit and cDNA was synthesized by TaqMan MicroRNA Reverse Transcription kit. The expression of miR-92a was quantified by quantitative Real-time PCR (RT-PCR). All transfections were performed using Lipofectamine 2000. Digoxigenin (DIG)labeled locked nucleic acid (LNA) modified probes for miR92a and negative control oligonucleotides were used for *in vitro* hybridization following manufacturers protocol. Digoxigenin (DIG)labeled locked nucleic acid (LNA)modified probes for miR92a and negative control oligonucleotides (miRCURY LNA MicroRNA Detection Probes; Exiqon, Vedbaek, Denmark) were used. Cell proliferation was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assay.

RESULTS: It was observed that miR92a is highly expressed in NSCLC compared with adjacent tissue samples and plasma from healthy donors. Additionally, the proliferation of three NSCLCderived cell lines, SPCA1, A549 and H2170, was enhanced by miR92a and inhibited by a complementary antimiR92a oligonucleotide sequence. Although the underlying mechanisms of reduced cellular proliferation in the presence of miR92a antagomirs cannot be explained from the current results.

CONCLUSIONS: The upregulation of miR92a expression, in cells and plasma, is manifested during the development of NSCLC.

Key Words:
Lung cancer, miR-92a, Cell proliferation, Expression.

Introduction

Lung cancer is one of the leading causes of cancer-related mortality worldwide. About 8590% of cases of lung cancer are categorized as non-small cell lung cancer (NSCLC), with the majority of patients diagnosed with advanced cancer[1]. The association between microRNAs (miRNAs/miRs) and cancer is well established, with several studies suggesting that miRNAs have a role in cancer initiation and progression[2,3]. Previous studies[4,5] have indicated that mutations or dysregulation of miRNAs correlate with various types of human cancer. Furthermore, careful analysis of miRNAs expression signatures provides valuable information on the developmental origin of tumors and, thus, they are indispensible tools for the diagnosis and treatment of cancer[4,5]. Although conventional methods for the detection of cancer in its early stages have shown promise, miRNA expression profiling may complement these conventional methods. They also exhibit potential to develop into a novel class of effective biomarkers with high sensitivities.

MiR1792 is a polycistronic miRNA cluster that encodes six miRNAs, termed miR17, miR18a, miR19a, miR20a, miR19b1 and miR921, grouped within an 800bp region of human chromosome 13[6]. A number of independent researches[7,8] revealed that the miR1792 cluster can act as oncogenes. Furthermore, expression profiling studies[9] revealed that these miRNAs are overexpressed in cancer of the lung, thyroid, breast, colon, pancreas, stomach, prostate and liver. It is clear that the miR1792 cluster regulates different cellular targets for tumorigenesis in different organs; however, the role and contribution of individual miRNAs in this cluster have not been fully characterized. One such critical individual miRNA is miRNA92a. Several papers[10-12] indicate that aberrant expression of miR92a is implicated in angiogenesis, embryonic development and tumo-

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rigenesis. However, the specific role of miR92a in human NSCLC development remains unclear. The present study aimed to investigate whether miR92a is overexpressed in patients with NSCLC and quantify the relative expression of miR92a with respect to healthy individuals. In addition, the correlation between miR92a expression and the proliferation of human NSCLC cell lines, SPCA1, A549 and H2170, was determined. The present study implicated miR92a in human NSCLC development. Furthermore, the potential of miR92a in blood serum was identified as a viable biomarker for the diagnosis of human NSCLC.

Materials and Methods

Plasma Collection and RNA Isolation

Whole blood samples were collected from healthy individuals and patients with NSCLC, and tumor and adjacent nontumorous tissues were obtained from the patients with NSCLC (n=20) at Qianfoshan Hospital Affiliated to Shandong University and the Fourth People's Hospital of Jinan (Jinan, China) (Table I). The present study was approved by the Institutional Review Board of The Fourth People's Hospital of Jinan and written informed consent was obtained from all subjects. Blood samples of the patients were collected twice 1 day prior to surgery and 1 week after the operation, and were properly stored. The whole blood was separated into plasma and cellular fractions by centrifugation at 2,500 x g for 15 min. Total RNA was isolated from the plasma using an RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's instructions. The RNA sample was suspended in 20 µl nuclease free water. Using this protocol, 500 ng RNA were obtained from 1 ml plasma

Quantitative Real-time Polymerase Chain Reaction (RTqPCR) of Mature miRNAs

The miRNA was quantified using TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instruction, with minor modifications. Briefly, 20 ng total RNA were reverse transcribed by TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). RT reaction mixtures (20 µl) were prepared with 10X RT buffer, 0.2 µl dNTPs (100 mM), 0.2 µl RNase inhibitor (20 U/µl), 1 µl reverse transcriptase (50 U/µl), 1 µl of each of miRNAspecific stemloop primer and 10 µl input RNA. The mixture was incubated at 16°C and 42°C for 30 min each, followed by 85°C for 5 min. Subsequently, RTqPCR was performed using 20µl reaction mixtures containing PCR primers and probes. The reactions were initially incubated at 95°C for 2 min, followed by 55 cycles of 95°C for 15 s and 58°C for 1 min. All data were analyzed using models and algorithms implemented using the Mx3005P qRT-PCR system with MxPro software (Stratagene, San Diego, CA, USA).

In situ Hybridization of miR92a

Digoxigenin (DIG)labeled locked nucleic acid (LNA)modified probes for miR92a and negative control oligonucleotides (miRCURY LNA MicroRNA detection probes; Exiqon, Vedbaek, Denmark) were used. The probe sequences were as follows; 5'ACA GGC CGG GAC AAG TGC AATA3' for miR92a; 5'GTG TCC ACG TTC ATA GCC CCA3' for scrambled oligonucleotides (negative control). *In situ* hybridization of paraffin embedded tissue sections was performed using the DISCOVERY RiboMap kit (Ventana Medical Systems, Inc., Tucson, AZ, USA) on an automated *in situ* hybridization instrument (DISCOVERY XT; Ventana Medical Systems, Inc, Tucson, AZ, USA). *In situ* hybridization steps were performed according to the manufacturer's instructions. Staining was evaluated and graded as follows: negative for < 5% staining of tumor cells; positive for > 5% staining of tumor cells.

Cell Culture, Transfection and in vitro Proliferation Assays

Three NSCLC cell lines, SPCA1, A549 and H2170, were cultured in Dulbecco's modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). The miR92a oligonucleotide (5'UUG CAC UUG UCC CGG CCU G3') was purchased from Dhamacon Inc. (Lafayette, CO, USA). The scrambled oligonucleotide sequences (5'UAU UGC ACU UGU CCC GGC CUG UCC CGG-CC3' and 5'AUU GCA CUU GUC CCG GCC UTT3') are a mix of two different frames of the miR92a sequence. The antagomir oligonucleotide sequence was obtained from Exiqon and used for miR92a knockdown. All transfections were performed using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The effects of miR92a and antimiR92a on the proliferation of SPCA1, A549 and H2170 cells were evaluated 48 or 72 h after transfection using the Vybrant MTT

Table I. Summary of clinical details of patients with non-small cell lung cancer.

Patient ID	Age (years)	Gender	Differentiation	Stage	miR-92a expression
1	55	Male	Moderate	I	+
2	68	Male	Moderate	II	+
3	56	Male	Moderate	III	+
4	64	Male	Moderate	IV-A	+
5	68	Female	High	II	+
6	70	Male	High	I	+
7	57	Female	Moderate	III	+
8	56	Male	Poor	I	+
9	64	Male	Moderate	II	+
10	66	Male	Moderate	II	+
11	65	Male	High	IV-A	+
12	49	Female	Poor	III	+
13	67	Male	Moderate	III	+
14	78	Male	High	II	+
15	72	Male	Moderate	II	+
16	64	Female	High	III	+
17	48	Male	Poor	III	+
18	52	Female	Moderate	IV-A	+
19	54	Male	Moderate	III	+
20	59	Female	High	IV-A	+

Cell Proliferation Assay kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Following the addition of MTT reagents, the cells were incubated at 37°C for 4 h. The absorbance of the formazan produced as a result of MTT reduction was taken at 450 nm on a spectrophotometer.

Statistical Analysis

All experiments were carried out in triplicates and expressed as mean ± SD. $p < 0.05$ was considered to indicate a statistically significant difference. One way ANOVA followed by Tukey's test was used for statistical analysis.

Results

MiR92a is Overexpressed in NSCLC Plasma and Cells

Although much have been learned about the miR1792 polycistronic miRNA cluster in cancer development, the present study independently evaluated whether miR92a is overexpressed in NSCLC using *in situ* hybridization with DIGlabeled LNAmodified probes. Examination of 20 NSCLC tissues revealed that miR92a was strongly expressed in 17/20 cases (Table I; Figure 1). By contrast, miR92a expression was not detected in normal noncancerous cells (data not shown). Furthermore, miR92a levels in NSCLC tissue sections (n=10) were quantified with respect to the adjacent nontumorous sections (n=10) by RTqPCR and higher levels of miR92a expression were observed in NSCLC sections compared with their noncancerous counterparts (Figure 2).

MiR92a is Responsible for Increased Proliferation Rates in SPCA1, A549 and H2170 Cell Lines

Next, we aimed to derive the correlation between miR92a expression lines, SPCA1, A549 and H2170. To answer this question, the aforementioned cell lines were transfected with scramble, miR92a or antagomir oligonucleotides. The effect of transfection on miR92a expression was confirmed and quantified by RTqPCR (Figure 3). Following transfection with miR92a, all the cell lines exhibited an increased proliferation rate compared with the scramble control cells (Figure 3). In addition, the effect of antagomirs on these cells lines was analyzed. Antagomirs are engineered oligonucleotides that are perfectly complementary to the specific miRNA; thus, transfection with antagomirs eventually arrests the action of the specific miRNA. When cells were transfected with the antagomir against miR92a, the cell proliferation rate was reduced with respect to the scramble control oligonucleotide (Figure 3).

MiR92a is a Potential Biomarker for NSCLC

To investigate the predictive value of miR92a as a viable blood serum biomarker, the expression

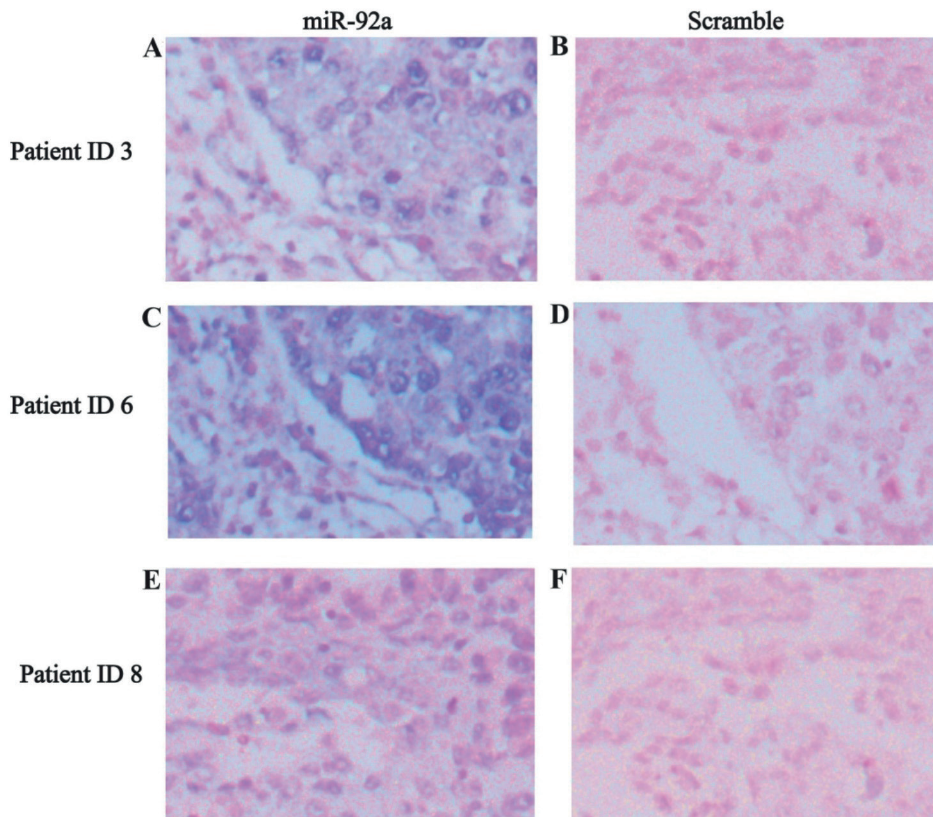

Figure 1. Representative miR 92a expression in non small cell lung cancer (NSCLC) tissues from patient IDs (A and B) 3, (C and D) 6 and (E and F) 8. In situ hybridization (ISH) was performed using locked nucleic acid modified probes for miR 92a and scramble miRNA (negative control). ISH miR 92a positive signals stain blue, while positive nuclei stain red. (A and C) Strong and (B and D) negative miR 92a staining for patient ID 3 and 6 samples. (E) Weak and (F) negative miR 92a staining for patient ID 8 sample. miR/miRNA, microRNA.

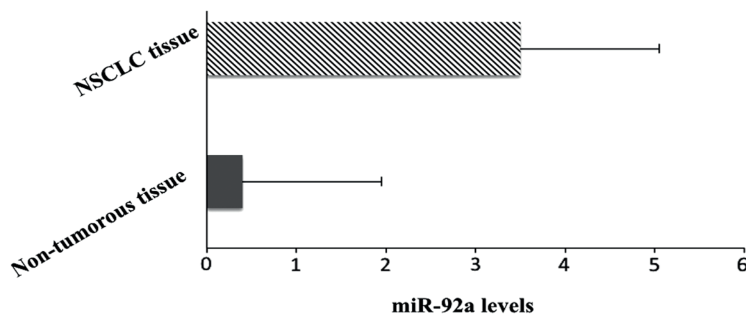

Figure 2. Quantification of miR 92a expression in NSCLC tissue samples. Relative miR 92a levels in NSCLC and adjacent non tumorous tissues were analyzed by quantitative RT-PCR. Error bars indicate standard deviation. NSCLC, non small cell lung carcinoma; miR, microRNA ($p < 0.05$)

levels of miR92a in serum samples of NSCLC patients were measured with respect to healthy individuals. Several previous researches have shown that the implementation of miR638 as a standard improved the precision of miRNA serum expression data[13,14]. Therefore, the present work analyzed the miR92a to miR638 expression level ratios in plasma samples from healthy individuals (n=10) and patients with NSCLC (n=10) by RTqPCR (Figure 4). The analysis revealed a decreased ratio of miR92a to miR638 in the NSCLC plasma samples compared with those from healthy individuals, thus, indicating miR92a as a potential biomarker for NSCLC prognosis.

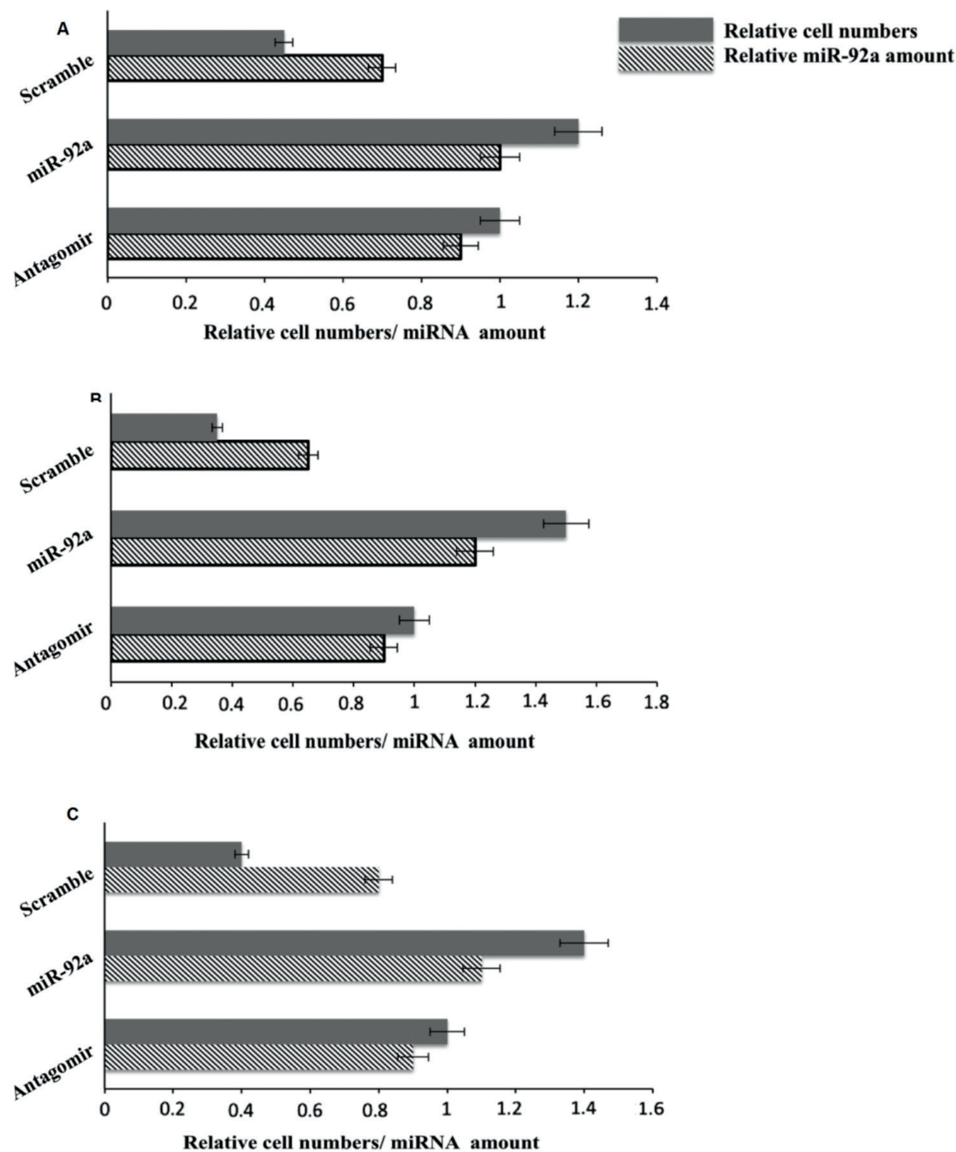

Figure 3. MiR 92a upregulation results in increased proliferation of SPC A1, A549 and H2170 NSCLC cell lines. Relative cell numbers/miR 92a expression in ***(A)*** SPC A1, ***(B)*** A549 and ***(C)*** H2170 cells transfected with scrambled control oligonucleotide, synthetic miR 92a or anti miR 92a antagomir. Cell numbers were determined by MTT assays while the miR 92a expression was determined by quantitative RT-PCR. Error bars indicate standard deviationù; miR/miRNA, microRNA ($p < 0.05$).

Discussion

Lung cancer is one of the leading causes of cancer-related mortality worldwide. Specifically, amongst all lung cancer types, NSCLC accounts for the majority of lung cancer cases. However, little progression has been made in methods for the prognosis and diagnosis of NSCLC, despite the plethora of information regarding this disease type[15]. Thus, it is important to provide novel insights into the underlying mechanisms of NSCLC development in order to identify unique molecular signatures, and exploit this information for the development of novel prognostic and therapeutic strategies. The previous decade has witnessed a surge in the identification and implementation of miRNAs that exhibit potential to act as diagnostic and prognostic biomarkers in cancer[16,17]. It has previously been shown that the expression of miRNA cluster miR1792 is markedly increased in small cell lung cancer[18]. Additionally, a previous study investigated the involvement of miRNA in lung carcinogenesis using a microarray analysis, and identified unique profiles that could discrimi-

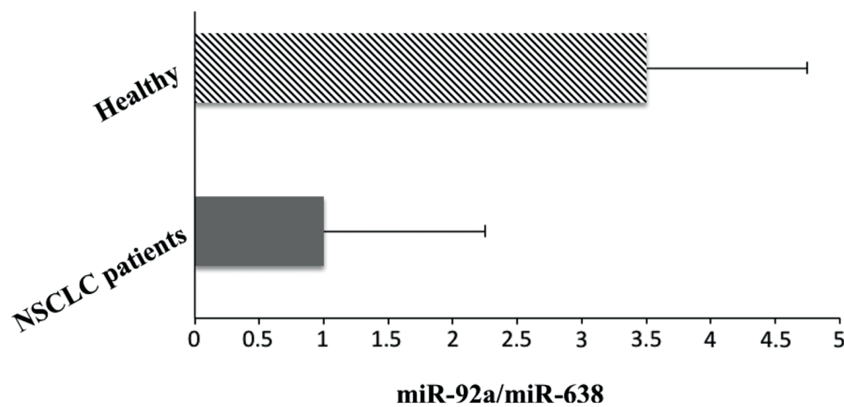

Figure 4. Comparison of miR92a expression levels in plasmas from healthy individuals and patients with NSCLC. The ratio of miR92a to miR638 was quantified by performing quantitative RT-PCR. Error bars indicate standard deviation. NSCLC, nonsmall cell lung carcinoma; miR, microRNA ($p < 0.05$).

nate lung cancer from noncancerous lung tissues, thus, providing a correlation between miRNA molecular profiles, lung adenocarcinoma and patient survival[19].

We aimed to investigate the potential of miR92a, from the miR1792 cluster, as a biomarker candidate for NSCLC. It was observed that miR92a is overexpressed in NSCLC compared with adjacent nontumorous tissue. Furthermore, a correlation was identified between miR92a expression and the proliferation of human NSCLC cell lines; increased proliferation was observed in the NSCLC cell lines, SPCA1, A549 and H2170, upon transfection with miR92a. The results were further validated by transfection of the NSCLC cell lines with an antagomir for miR92a, resulting in a reduction in the cell proliferation rate in all the cell lines. The underlying mechanism by which the antagomirs reduce cellular proliferation requires investigation. Similar studies have been conducted by several independent groups and, recently, it was reported that miR92a is highly expressed in breast tumor tissue compared with matched normal breast tissues, suggesting that miR92a may potentially act as an oncogene[20]. Furthermore, quantification of circulating miR92a has shown potential as a novel breast cancer biomarker[21].

Considering the significance of miRNAs in cancer and their promising potential as prognostic biomarkers, as well as molecular targets for the development of novel cancer therapeutics, we investigated the predictive value of miR92a in blood serum as a viable biomarker for NSCLC. In comparison to another stable miRNA, miR638, the current analysis determined that quantification of miR92a/miR638 level in plasma could prove a potential biomarker for NSCLC. The current study identified that the ratio of miR92a to miR638 in plasma samples from patients with NSCLC were decreased compared with those from healthy individuals. Similar investigations were conducted in plasma samples from patients with acute leukemia and liver cancer[13]. The analyses revealed that using miR638 as the standard and quantifying the ratio of differentially expressed miRNAs with respect to miR638 allowed these studies to identify valuable diagnostic markers for acute leukemia and hepatocelluar carcinoma. Thus, miR92a/miR638 levels in human plasma may act as a gold standard in the prognosis of NSCLC.

Conclusions

We showed on overexpression of miR92a in NSCLC cell lines and tissue samples. Furthermore, quantifying the levels of miR92a with respect to miR638 in the blood may serve as a potential biomarker for patients with human NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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