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Original Article

Serum miRNA-21, miRNA-153 and miRNA-22 levels were identified as diagnostic biomarkers of patients with hepatocellular carcinoma: a Chinese population-based study

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Abstract: Background: Hepatocellular carcinoma (HCC) is the most common primary malignant tumor worldwide. The aim of this study is to identify the diagnostic values of serum miRNA-21, miRNA-22 and miRNA-153 of patients with HCC in a large Asian group. Methods: We retrospectively reviewed 65 patients with HCC and 65 patients with hepatic cirrhosis and HBV, respectively. The expression of serum miRNA-21, miRNA-22 and miRNA-153 was detected by real-time PCR. The comparison of diagnostic performance of serum miRNAs and AFP levels was measured using the area under ROC curve (AUC). Results: In the present study, we compared expression levels of miRNA-21, miRNA-153 and miRNA-22 in plasma among HCC, cirrhotic and the HBV groups. We found expression of serum miRNA-21, miRNA-153 and miRNA-22 were significantly higher in patients with HCC compared with cirrhotic and HBV groups. Secondly, we found serum miRNAs had better diagnostic significance for patients compared with AFP levels in all patients. Conclusions: In this study, we found expression of serum miRNA-21, miRNA-153 and miRNA-22 were significantly higher in patients with HCC compared with cirrhotic and HBV groups, and we confirmed the serum miRNA-21, miRNA-153 and miRNA-22 could be defined as novel diagnostic biomarkers for patients with HCC.

Keywords: miRNA-21, miRNA-153, miRNA-22, HCC

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequently diagnosed cancer worldwide and the second most frequent cause of cancer death [1], with the highest incidence in Asian and especially in China [2]. Partial hepatectomy remains the most commonly used curative therapy modality for HCC [3]. Although the prognosis of patients with HCC has been improved recently, the survival outcomes of patients with HCC following surgical resection may vary, as several factors are associated with the prognosis of HCC, including completeness of tumor removal, serum alpha-fetoprotein (AFP) levels, tumor size, tumor multifocality and distant metastases, etc [4, 5]. The poor prognosis of patients with HCC is attributed to the lack of an effective means of early diagnosis. Only 30% to 40% of patients are candidates for potentially curative hepatectomy at the time of diagnosis [6]. Discovery of an effective and reliable tool for early diagnosis of HCC would play a pivotal role in improving the prognosis of patients with HCC.

MicroRNAs (miRNAs) are critical regulators of gene expression that utilize sequence complementarity to bind to and decrease the stability or translation efficiency of target mRNAs [7]. Recent studies have revealed that miRNAs participate in various biological processes such as organogenesis, cellular proliferation and differentiation, apoptosis, innate and adaptive immu-

Table 1. Patient and tumor characteristics (N=195)

Variable	HBV group	Cirrhosis group	HCC group
Case, n	65	65	65
Age	61.5±4.6	61.0±8.6	62.3±6.1
Sex			
Female	22	25	23
Male	43	40	42
HBsAg			
Positive	65	50	48
Negative	0	15	17
HBeAg			
Positive	34	42	45
Negative	31	23	20
Liver cirrhosis			
Yes	0	65	53
No	65	0	12
TBL (μmol/l)	12.5±8.3	15.1±7.3	16.1±8.2
ALB (g/dl)	39.4±6.6	38.9±6.5	37.9±4.6
ALT (U/L)	25.7±14.1	50.4±30.2	79.4±66.5
AFP at diagnosis (ng/ml)			
≤ 400	65	42	40
> 400	0	23	25
Tumor size (cm)			
> 5 cm	_	_	28
≤ 5 cm	_	_	37
Microvascular invasion			
Yes	_	_	20
No	_	_	45
TNM staging			
I	_	_	10
II	_	_	17
III-IV	_	_	38
Metastases			
Yes	_	_	21
No	_	_	44

Abbreviations: TBL: total bilirubin; ALB: albumin; ALT: alanine aminotransferase; AFP: alpha-fetoprotein; HBV: Hepatitis B Virus.

nity, inflammation, and tumorigenesis [8, 9]. MiRNAs are ideal candidates for biomarkers because of their resistance to endogenous RNase and high stability under different storage conditions. Recent studies have shown that human serum miRNAs are aberrantly expressed in many malignancies such as liver [10, 11], colorectal cancer [12], and pancreatic cancer [13].

Several studies had demonstrated that expression of microRNA-21 (miRNA-21) was significantly different in many human cancers compared with the healthy people, and miRNA-21 level was identified as a promising biochemical marker [14-16]. Similarly, miRNA-22 had been found to be ubiquitously expressed in various tissues [17], and previous studies suggested that miRNA-22 functioned in multiple cellular processes such as proliferation, differentiation, apoptosis, senescence, and its deregulation is a hallmark of cancer [18-20]. miRNA-153 was first discovered as one of the several brain-specific miRNAs, based on analysis of expression profile of over one hundred miRNAs in adult organs [21]. Recent evidences have indicated that miRNA-153 was dramatically down-regulated in several cancer cells [22, 23].

However, few studies focused on the diagnostic significance of serum miRNA-21, miRNA-22 and miRNA-153 in patients with HCC. In this study, we aimed to explore the diagnostic values of serum miRNA-21, miRNA-22 and miRNA-153 in patients with HCC compared with HBV and cirrhotic patients. These results of our study shed new light on the identification of new diagnostic and prognostic biomarkers for HCC patients.

Materials and methods

Patients

Bloodsamples from 65 patients with HCC and 65 patients with hepatic cirrhosis were obtained at Department of Hepatobiliary Surgery, Lanzhou General Hospital, Lanzhou Military Area Command, PLA from 1st September 2013 to 30th November 2014 prior to definitive therapy. The tumor type and the grade of cell differentiation were diagnosed based on the criteria of World Health Organization (WHO), whereas the pathological stage of each tumor was determined by the International Union Against Cancer (UICC) TNM classification. Blood samples were also collected from 65 patients with HBV infection and matching ages and genders to the patients group with HCC and cirrhosis. Written consents were obtained from all subjects prior to the recruitment. The study protocol was approved by the Institutional

Review Board of Hospital Ethics Committee. The clinical characteristics of the subjects are listed in **Table 1**.

Total RNA isolation

Total RNA was isolated from 300 μl of serum using the mirVana PARIS Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Briefly, for each sample, total RNA was extracted from 300 μl of serum with 2× denaturing solution, acid-phenol: chloroform, and 100% ethanol. After several washing and centrifugation, the RNA was eluted into 60 μl of preheated (95°C) elution solution. RNA quantity and purity were determined using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA, USA). RNA purity was considered satisfactory with A260/A280 of 1.9-2.1. The RNA samples were stored at -80°C until reverse transcription.

Real-time quantitative PCR

We typically extracted 2 μg to 9 μg of total RNA, and OD260/280 ratios typically ranged from 1.8 to 2.0, indicating high RNA purity. 10 ng of total RNA was used for each miRNA quantification. miRNA detection was performed run on the Eppendorf Mastercycler EP Gradient S (Eppendorf, Germany) using commercial assays (TaqMan microRNA assays; Applied Biosystems, Foster City, CA, USA) for miRNAs. Relative quantification was calculated using $2^{-\Delta\Delta Ct}$, where Ct is cycle threshold. Normalization was performed with universal small nuclear RNA U6 (RNU6B). Each sample was examined in triplicate, and the mean values were calculated. mRNA levels in tumor samples/nontumorous samples of 0.5-fold was defined as under-expression of the gene, whereas a ratio of 2.0-fold was defined as over-expression.

Diagnosis and treatment

After a detailed history and a complete physical examination, the hepatitis B and C serology, liver function test and tumor markers examination which included alpha-fetoprotein (AFP), carbohydrate antigen 19-9 (CA19-9), and carcinoembryonic antigen (CEA) was routinely performed. Other routine investigations were chest X-ray, upper gastrointestinal endoscopy, abdominal ultrasound, contrast-enhanced computerized tomography (CT) and/or magnetic resonance imaging (MRI). A clinical diagnosis of HCC was based on the criteria of the American Association for the Study of Liver Diseases (AASLD) [24].

The type of partial hepatectomy carried out was based on the tumor size, number, location, presence/absence of cirrhosis and estimated volume of future liver remnant. As far as possible, anatomical liver resection was carried out basing on Couinaud's liver segments, sectors and hemilivers.

Histopathological study of the resected specimens was carried out independently by three pathologists who came to a consensus by discussion if there was any discrepancy.

Statistical analysis

Continuous variables were expressed as mean ± SD (standard deviation) and compared using a two-tailed unpaired Student's t test; categorical variables were compared using χ^2 or Fisher analysis. The predictive performance of serum miRNAs were measured using the area under ROC curve (AUC). AUCs were also used to compare serum miRNAs and AFP level using the Hanleyand McNeil method [25]. Statistical analyses were conducted with the SPSS for Windows version 18.0 release (SPSS, Inc., Chicago, IL) and ROC curve analysis were computed using MedCalcV.11.0.3.0 (MedCalc software, Mariakerke, Belgium). A value of $P < 0.05$ was considered significant in all the analysis.

Results

Characteristics of the patients

The characteristics of patients with HCC, cirrhotic and HBV groups enrolled in this study were shown in **Table 1**.

Comparing expression levels of serum miRNA-21, miRNA-153 and miRNA-22 among three groups

Expression of serum miRNA-21 was significantly higher in patients with HCC compared with cirrhotic and HBV groups ($P < 0.001$, **Figure 1A**,

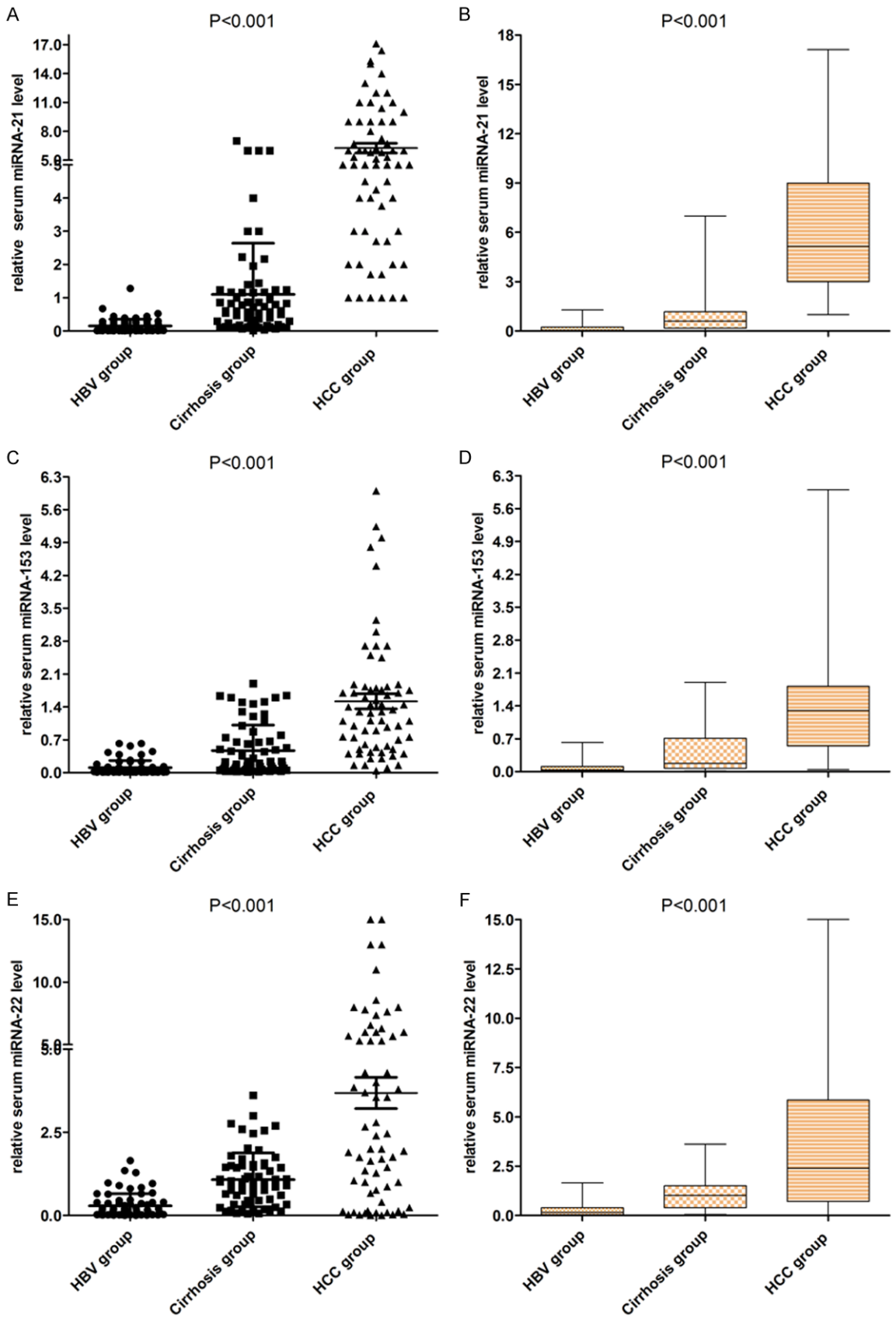
A
P<0.001
relative serum miRNA-21 level
17.0
14.0
11.0
8.0
5.8
4
3
2
1
0
HBV group
Cirrhosis group
HCC group
B
P<0.001
relative serum miRNA-21 level
18
15
12
9
6
3
0
HBV group
Cirrhosis group
HCC group
C
P<0.001
relative serum miRNA-153 level
6.3
5.6
4.9
4.2
3.5
2.8
2.1
1.4
0.7
0.0
HBV group
Cirrhosis group
HCC group
D
P<0.001
relative serum miRNA-153 level
6.3
5.6
4.9
4.2
3.5
2.8
2.1
1.4
0.7
0.0
HBV group
Cirrhosis group
HCC group
E
P<0.001
relative serum miRNA-22 level
15.0
10.0
5.8
2.5
0.0
HBV group
Cirrhosis group
HCC group
F
P<0.001
relative serum miRNA-22 level
15.0
12.5
10.0
7.5
5.0
2.5
0.0
HBV group
Cirrhosis group
HCC group

Figure 1. Serum miRNA-21, miRNA-153 and miRNA-22 were significantly higher in HCC patients. A, B. Serum miRNA-21 levels were significantly higher in patients with HCC compared with cirrhotic and HBV groups ($P < 0.001$); C, D. Serum miRNA-153 levels in were significantly higher in patients with HCC compared with cirrhotic and HBV groups ($P < 0.001$); E, F. Serum miRNA-22 levels in were significantly higher in patients with HCC compared with cirrhotic and HBV groups ($P < 0.001$).

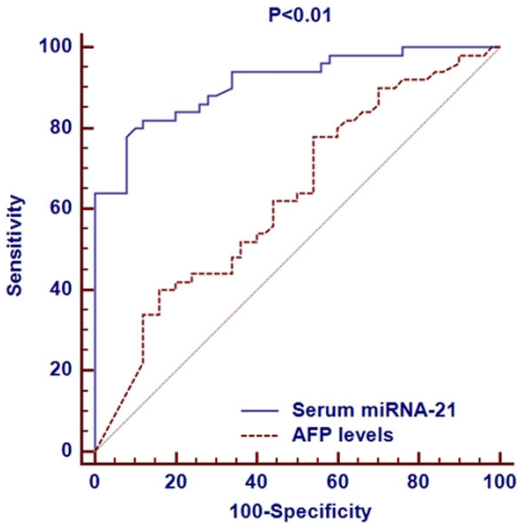

Figure 2. Comparison of diagnostic significance for patients with HCC between serum miRNA-21 and AFP levels.

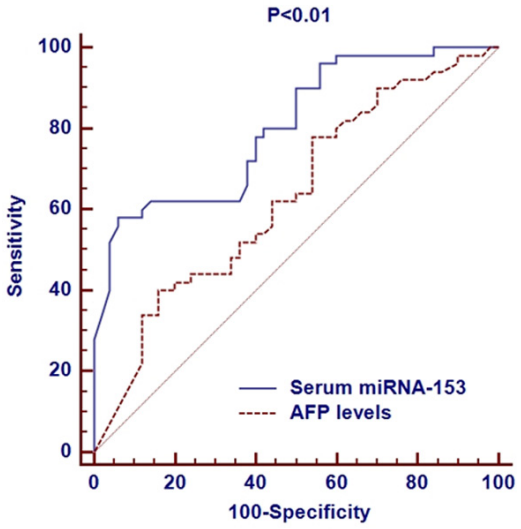

Figure 3. Comparison of diagnostic significance for patients with HCC between serum miRNA-153 and AFP levels.

1B). Expression of serum miRNA-153 was significantly higher in patients with HCC compared with cirrhotic and HBV groups (P < 0.001, **Figure 1C**, **1D**). Expression of serum miRNA-22 was significantly higher in patients with HCC compared with cirrhotic and HBV groups (P < 0.001, **Figure 1E**, **1F**).

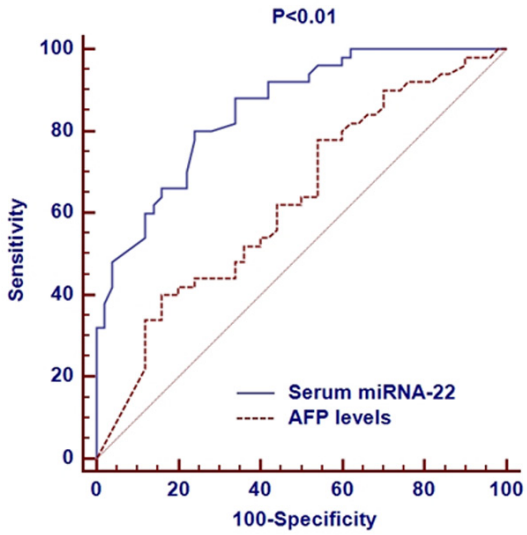

Figure 4. Comparison of diagnostic significance for patients with HCC between serum miRNA-22 and AFP levels.

Comparison of diagnostic significance for patients with HCC between serum miRNAs and AFP levels among different groups

Among the three groups, we performed ROC curves to compare the diagnosis values of different miRNAs with AFP levels. Serum miRNA-21 was significantly more accurate in diagnosing HCC than the AFP level. The AUC of serum miRNA-21 was 0.862 (95% CI: 0.775 to 0.896), which was larger than that of AFP level (0.654, 95% CI: 0.587 to 0.736, **Figure 2**); Serum miRNA-153 was significantly more accurate in diagnosing HCC than the AFP level. The AUC of serum miRNA-153 was 0.802 (95% CI: 0.711 to 0.875), which was larger that of AFP level (0.654, 95% CI: 0.587 to 0.736, **Figure 3**); Serum miRNA-22 was significantly more accurate in diagnosing HCC than the AFP level. The AUC of serum miRNA-22 was 0.832 (95% CI: 0.726 to 0.903), which was larger than that of AFP level (0.654, 95% CI: 0.587 to 0.736, **Figure 4**).

Discussion

The outcomes for patients with HCC have improved markedly over the last 30 years due to the presence of various therapeutic modalities and advances in surgical treatment [26]. Many Asian studies, however, included a high proportion of patients with advanced disease, including those categorized as TNM III or IV, and patients with primary refractory disease and metastatic disease [27]. Early diagnosis could be "a sense of urgency" for improving the prognosis and reducing the burden of patients with HCC.

Many studies showed that miRNAs could be used as diagnostic indicator and prognostic factor in various cancers. Increasing evidence suggested that unique serum miRNAs expression signatures might serve as new noninvasive biomarkers for cancer diagnosis including HCC [28]. Previous studies showed miRNA-21 increased in several types of cancers, such as breast cancer, colon cancer and lung cancer [29-31]. Hu et al found that miRNA-21 were expressed at higher levels in the laryngeal squamous cell carcinoma samples compared to the normal samples; furthermore, they indicated that patients with high miRNA-21 expression in tumor tissues had poorer prognosis compared to patients with lower miRNA-21 expression [32]. With respect to miRNA-153, mechanistic investigations indicated that miRNA-153 promoted invasiveness indirectly by inducing matrix metalloprotease enzyme 9 (MMP9) production and miRNA-153 played an important role in promoting proliferation of human prostate cancer cells and presents a novel mechanism of miRNA-mediated direct suppression of *PTEN* expression in prostate cancer [33]. While miRNA-22 was also an important factor associated with cancer pathogenesis, evolutionary clustering suggested that miRNA-22 was highly conserved in vertebrate evolution, indicating its functional importance in vertebrate species. It had been deduced from the statistical analysis of 3'-UTR in transcriptome that miRNA-22 is involved in the regulation of many target genes [34]. Meanwhile, miRNA-22 was found to be associated with both diagnosis and prognosis of various cancers [35]. In the present study, we chose to evaluate the diagnostic significance of three distinctly differentially expressed miRNA-21, miRNA-153 and miRNA-22. Firstly, we compared expression levels of miRNA-21, miRNA-153 and miRNA-22 in plasma among HCC, cirrhotic and the HBV groups. We found expression of serum miRNA-21, miRNA-153 and miRNA-22 were significantly higher in patients with HCC compared with cirrhotic and HBV groups. Secondly, we found serum miRNAs had better diagnostic significance for patients compared with AFP levels in all patients. This study proposed new direction of diagnostic biomarker research in patients with HCC in the future.

However, there are limitations of this study: (1) the sample size is too small in this study, and further larger sample study is needed to confirm the present experimental results. (2) whether the three miRNAs have the optimal specificity and sensitivity for liver cancer diagnosis also needs future confirmation.

In conclusion, we found expression of serum miRNA-21, miRNA-153 and miRNA-22 were significantly higher in patients with HCC compared with cirrhotic and HBV groups, and we confirmed the serum miRNA-21, miRNA-153 and miRNA-22 could be defined as novel diagnostic biomarkers for patients with HCC.

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Disclosure of conflict of interest

None.

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