Low expression of citron kinase is associated with poor patient outcomes in hepatocellular carcinoma

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Background: Citron kinase (CIT) is a protein related to cytokinesis and is an important abscission regulator. However, the relationship between CIT and hepatocellular carcinoma (HCC) is unclear. The aim of this study was to investigate the expression CIT in HCC tissues, and explore the connection between this expression and clinicopathological characteristics of HCC.

Methods: Immunohistochemistry staining on 235 HCC tissues and 96 non-tumorous liver tissues controls was performed to examine the CIT protein expression. We then analyzed the correlation between protein expression and clinicopathological parameters via χ^2 tests, and we performed overall survival analyses via the Kaplan-Meier survival approach. Based on the online Oncomine Expression Array and UALCAN databases, we more broadly compared CIT mRNA expression between normal and HCC tissues. Finally, we compared CIT mRNA expression in these databases to protein expression in our study and explored potential sources for any observe differences.

Results: Compared to normal tissues, CIT expression was significantly lower in HCC tissues. Low CIT expression was found to be related to gender, tumor size, Edmondson Grade, Microvascular invasion, serum AFP levels and poor overall survival. Based on the online databases, CIT mRNA expression was found to be high in HCC tissues and decreased in normal tissues. We hypothesize that this unexpected result is due to a negative feedback loop whereby low protein CIT levels mediate increased CIT mRNA levels.

Conclusions: Lower CIT protein levels are associated with a poorer prognosis in HCC patients, and lower CIT protein levels may mediate a negative feedback loop leading to increased CIT mRNA levels.

Keywords: Citron kinase (CIT); hepatocellular carcinoma (HCC); immunohistochemistry

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common tumor type and the third leading cause of death related to cancer worldwide (1). Up to half of all HCC cases occur in China, and there were a total of 782,500 new liver cancer cases and 745,500 deaths worldwide in 2012, with 84.6% of liver cancer incidence and 86.3% of liver cancer deaths in the WHO Western Pacific region (WPRO) occurring

in China (1-3). HCC is one of the most common forms of cancer in China and thus underscoring the urgent need for novel therapeutic treatment strategies for this deadly disease.

Citron kinase (CIT) is typed as an AGC (cAMP-dependent, cGMP-dependent and protein kinase C) protein kinase, and it is influenced by second messengers, including lipids such as PKC and cyclic AMP (4). The target proteins directly phosphorylated by CIT remain to be identified, although it is known to interact with proteins including KIF14 and TUBB3 (5,6). Known roles for CIT include a relationship with neurogenic cytokinesis, and this gene is known to be mutated in primary microcephaly, suggesting a key role for this gene in central nervous system development (7). A single nucleotide polymorphism in CIT has been found to be significantly related to schizophrenia, and the interaction between polymorphisms in CIT, NDEL1, and DISC1 is known to influence schizophrenia risk (8). CIK can also induce HIV-1 virion production by promoting Gag ubiquitination as well as improving viral release via the multivesicular bodies pathway (9). With respect to cancer, CIT appears to be related with the time to progression of ovarian cancer, and is also associated with therapeutic outcomes (10). CIT has been found to be overexpressed in human colon cancer tissues, wherein it may accelerate cancer cell growth via influencing the p53 signaling pathway (11). CIT protein is also overexpressed in breast cancer tissues, where it is associated with more aggressive forms of this disease (12). CIT depletion can mediate a failure of cytokinesis that may therefore valuable therapeutic efficacy as an anti-cancer strategy in cervical, breast, and colorectal cancer, and potentially in additional cancers (13).

Given the high incidence of HCC and the myriad roles of CIT, we were interested in exploring the role of CIT in HCC. We therefore investigated the expression of CIT in 235 HCC tissues and 96 non-tumorous liver tissues by immunohistochemistry staining to examine the correlations between CIT expression and clinicopathological parameters, as well as to compare this expression with overall patient survival rate. We found that CIT expression was associated with gender, tumor size, Edmondson Grade, Microvascular invasion, serum AFP level, and poor overall survival. We further reviewed available datasets in the Oncomine and UALCAN Expression Array databases to assess CIT mRNA expression. Unexpectedly, we found CIT mRNA expression to be higher in HCC tissues relative to normal liver tissue controls, leading us to speculate regarding the potential reasons for this discrepancy.

Methods

Patients and tissue samples

All the human tissues were acquired from HCC patients at Zhejiang Provincial People's Hospital (Hangzhou, China). This research was approved by the Ethics Committee of Zhejiang Provincial People's Hospital (Hangzhou, China). All the patients provided written informed consent.

A total of 235 paraffin-embedded HCC tissue samples and 96 non-tumorous liver tissue samples were acquired from April 2008 to September 2014. This patient cohort consisted 191 males and 44 females. Survival time was calculated based on the time between the date of surgery and the end of follow-up or date of death. All the tissues were used for a tissue microarray (TMA) analysis constructed by Shanghai Biochip Co., Ltd (Shanghai, China).

Immunohistochemical staining and evaluation

According to the manufacturer's instructions, immunohistochemical staining was conducted using the Histostain-Plus IHC Kit (cat.no.856143; Invetrogen, USA). We heated 5 µm sections from the TMAs at 70 ℃ for 2 hours, after which they were de-paraffinized, rehydrated, and boiled in TE buffer for 3 min to retrieve antigen. Next, the sections were blocked with 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity, and they were then incubated with 10% goat non-immune serum for 20 min to decrease background non-specific staining. After that, samples were probed with a rabbit anti-CIT polyclonal antibody (1:400; lot. no. GR48652-1; ab110897; Abcam, Cambridge, UK) at 4 ℃ overnight, followed by incubation with a biotin-labeled secondary antibody for 20 min at room temperature, and then with HRP-conjugated streptavidin for 20 min at room temperature. A DAB Kit (ZSGB-BIO, Beijing, China) was additionally used to enhance color development. Finally, the sections were counterstained with hematoxylin, dehydrated, cleared, and mounted.

Two independent pathologists reviewed and scored all samples based on the strength of staining and on the percent of positively stained cells. A four-tiered scoring system was used as followed: for staining, 0 = negative, 1 = weak, 2 = moderate, and 3 = strong; for cell staining positivity: 0 for no cell stained, 1 for 1–25% of cells stained, 2 for 26–50% of cell stained, 3 for more than 50% of cells stained. As all sections tended to exhibit >50% staining in both HCC tissues and non-tumorous liver tissues, scores for

Table 1 Expression of Citron kinase (CIT) in hepatocellular carcinoma (HCC) and non-cancerous liver tissues

Samples	CIT expression		P
	Low	High	
Non-cancerous liver tissues	2	94	<0.001
HCC tissues	61	174	

the percent of positively stained cells were 3 for all samples. Scores for strength and percent of positively stained cells were multiplied together to yield an overall score. Scores <6 was indicative of low CIT expression, while scores ≥6 were indicative of high CIT expression.

Oncomine and UALCAN database analyses

A comprehensive analysis of extant datasets in the Oncomine Expression Array database (www.oncomine.org) was conducted to compare the mRNA expression of CIT between HCC and normal tissues, using the following search items: 'CIT', 'mRNA', 'Cancer *vs.* Normal Analysis' and 'Hepatocellular Carcinoma'. We identified five relevant datasets including Chen Liver (14), Roessler Liver, Roessler Liver 2 (15), Mas Liver (16), and Wurmbach Liver (17). An additional analysis based on the online UALCAN database analysis (ualcan.path.uab.edu) was also conducted as above, unsing the search items: 'Analysis', 'CIT', 'Liver Hepatocellular Carcinoma'.

Statistical analysis

Statistical analysis was fulfilled by using SPSS v13.0 (SPSS Inc., Chicago, IL). Chi-squared tests were used to assess the statistical significance of the relationship between CIT protein expression and clinicopathological parameters. We additionally used the Kaplan-Meier method to estimate survival curves, and the log-rank test was used for calculating differences between these curves. P<0.05 was the threshold of statistical significance.

Results

Expression of CIT in HCC and adjacent non-cancerous tissues

Immunostaining for CIT was evident in the cytoplasm and absent in the nuclei of both non-cancerous liver and HCC tissues. CIT was highly expressed in 94 of the 96 (97.91%)

samples of adjacent non-cancerous liver tissues (*Table 1*). CIT expression was clearly decreased in HCC tissues, with low expression of CIT in 174 of the 235 (74.04%) HCC tissues (*Figures 1,2*). These decrease in CIT expression was significant (P<0.001).

Relationship between CIT expression and clinicopathologic parameters

The relation between the expression of CIT and clinical variables was explored, and the results are shown in *Table 2*. CIT expression was significantly associated with gender, tumor size, Edmondson Grade, microvascular invasion, and AFP. The expression of CIT was significantly decreased in tumor of females, large tumors, tumors with a high Edmondson Grade, tumors with evident microvascular invasion or those with a high AFP level. There was no significant relationship between CIT expression and other assessed clinicopathologic parameters. These parameters and lower CIT expression are thus associated with poorer patient outcomes.

Survival analysis

The 5-year cumulative survival rate of patients with low CIT expression was 33.3%, while that of patients with high CIT expression was 68.9% based on a Kaplan-Meier survival analysis. Patients with low CIT expression had a mean survival time of 26.706±7.563 months, which was significantly shorter than that of patients in the high CIT expression group (45.860±4.449, P<0.001). These data indicated that low expression of CIT is related with poor overall survival (*Figure 3*).

Analysis of CIT expression according to Oncomine and UALCAN databases

We next explored available datasets in the Oncomine database to compare CIT mRNA expression in HCC with normal tissues. What we found is that CIT mRNA
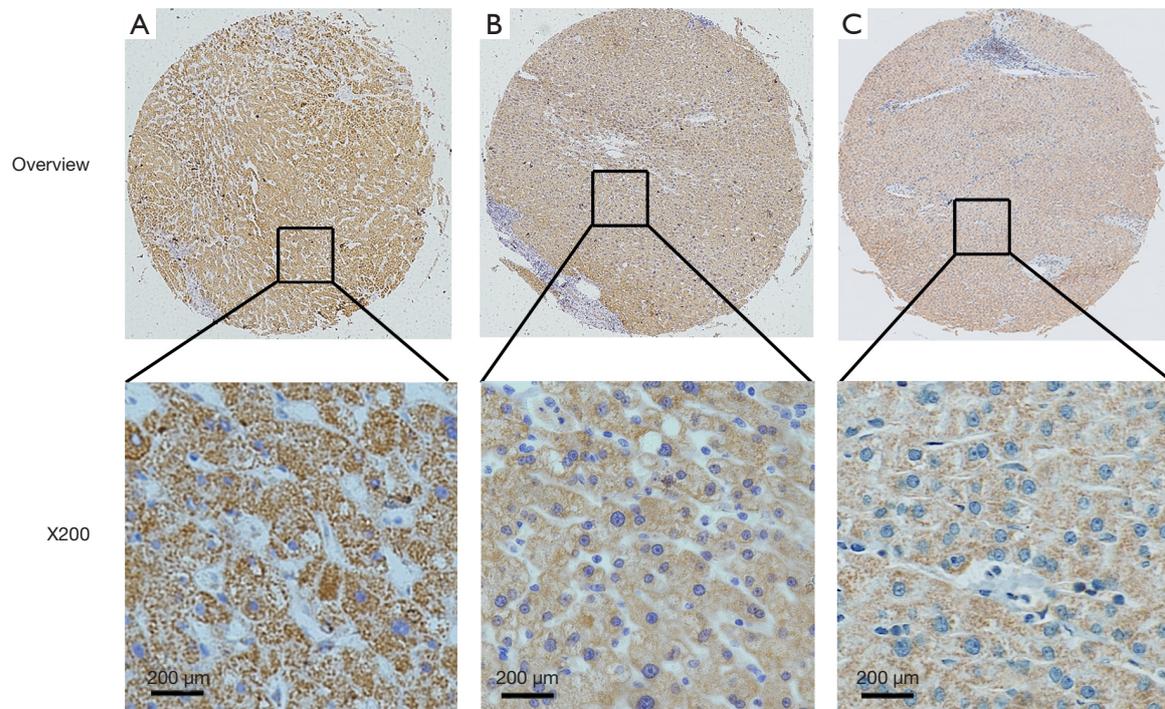
Figure 1 Immunohistochemical staining of Citron Kinase in non-cancerous liver tissues. (A) Cirrhosis tissues; (B) hyperplastic tissues; (C) inflammatory tissues.
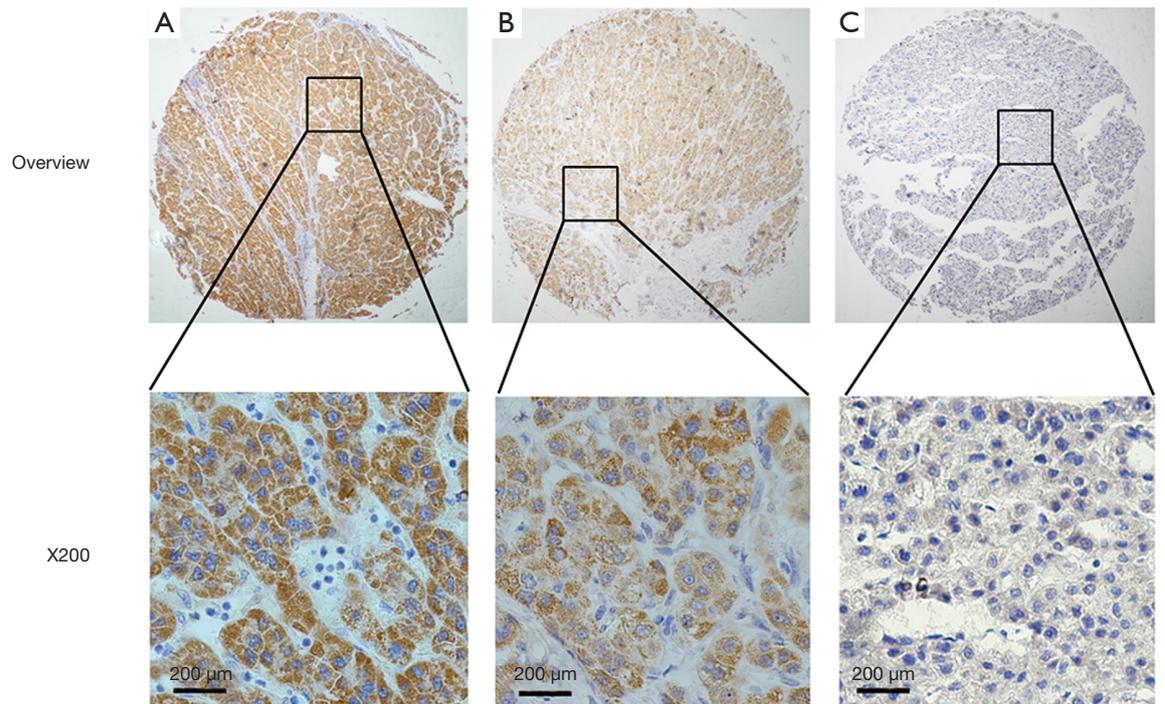
Figure 2 Immunohistochemical staining of Citron Kinase in hepatocellular carcinoma tissues. (A) Strong expression; (B) moderate expression; (C) negative expression.

Table 2 Relationship between CIT expression and clinicopathologic parameters

Clinical parameters	All cases	CIT		P value
		Low	High	
Age (years)				0.240
<55	93	28	65	
≥55	142	33	109	
Gender				0.033
Male	191	44	147	
Female	44	17	27	
Size				0.017
<5	111	21	90	
≥5	119	39	80	
Tumour number				0.726
Single	193	51	142	
Multiple	42	10	32	
Edmondson grade				0.011
I+II	151	31	120	
III	84	30	54	
Metastasis				0.180
M0	212	52	160	
M1	18	7	11	
Microvascular invasion				0.015
Absence	85	17	68	
Presence	99	36	63	
HBs antigen				0.123
Negative	46	16	30	
Positive	186	44	142	
AFP				0.003
<50	96	10	86	
≥50	82	23	59	
Status				<0.001
Alive	75	13	62	
Dead	54	26	28	

expression was higher in HCC tissues relative to normal controls (*Figure 4*, all P<0.05, except P=0.980 between HCC and Mas Liver). Similarly, we found CIT mRNA expression to be higher in HCC tissues compared with

normal tissues in the UALCAN Expression Array database (*Figure 5*, P<0.05). Survival analyses of these HCC patients showed that the patients with high CIT mRNA expression had significantly shorter survival times as compared with

patients in the low CIT expression group based on the UALCAN database. These data thus demonstrate that high CIT mRNA expression was related to with poorer overall survival (*Figure 6*, P<0.05). These data highlight a discrepancy between these mRNA data and our protein level data, leading us to speculate that low CIT protein levels may mediate a feedback loop that results in a compensatory over-expression of the CIT mRNA.

Discussion

HCC is among the most common tumors in the world, and there is thus an urgent need to better understand and develop novel treatments for this disease. CIT is a multi-functional protein that is most highly expressed in fetal liver, with levels gradually decreasing after birth. Src and

CIT are also downstream effectors of the Eph-induced signal transduction cascade, and Eph kinase activity control abscission (18). Indeed, CIT is known to be tightly linked with cytokinesis, a process which it controls using its coiled-coil domain, mediating the transition from constriction to abscission (19). Low level of TUBB3 in mitotic cells can be detrimental to their cytokinesis, and CIT can control TUBB3 phosphorylation to stabilize mid-body microtubules and cytokinesis (6). Two-pore channel 1 and $p27^{Kip1}$ (p27) also interact with CIT to regulate cytokinesis (20,21). We had found no evidence for CIT to play a role in other mitotic events besides cytokinesis, until a recent research reported that it is required for the orientation of the mitotic spindle during metaphase (22). Aurora B, ASPM and CIT control mid-body architecture and spindle positioning during cytokinesis, and are necessary to maintain proliferating cell division (22,23). Specific tyrosine residues in CIT are known to be phosphorylated, and it has been demonstrated that such tyrosine phosphorylation of CIT impairs cytokinesis (19). CIT is an important abscission regulator, using active RhoA and anillin to promote mid-body stability (24). In our research, we found CIT to be expressed at lower levels in HCC tissues and at higher levels in normal tissues. This result suggests that CIT may function as a tumor suppressor in HCC, and may thus be an ideal therapeutic target in this disease. We thus conclude that lower CIT levels are associated with poorer patient outcomes in HCC.

Interestingly, while we observed a decrease in CIT levels in HCC samples at the protein level, we observed the opposite finding when assessing CIT mRNA levels in normal and HCC tissues using the Oncomine and
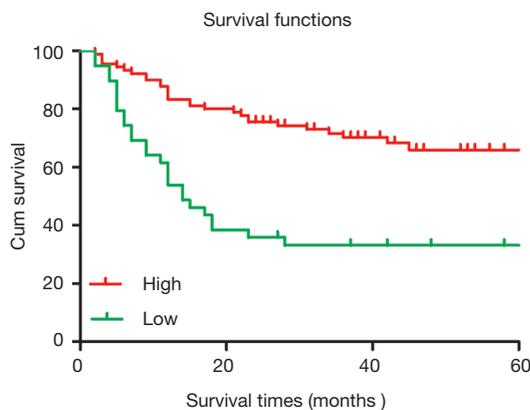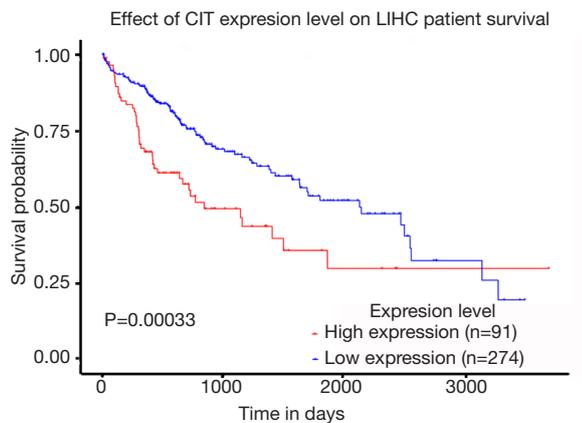

Figure 3 Kaplan-Meier survival curves of the hepatocellular carcinoma patients with high or low citron kinase expression.
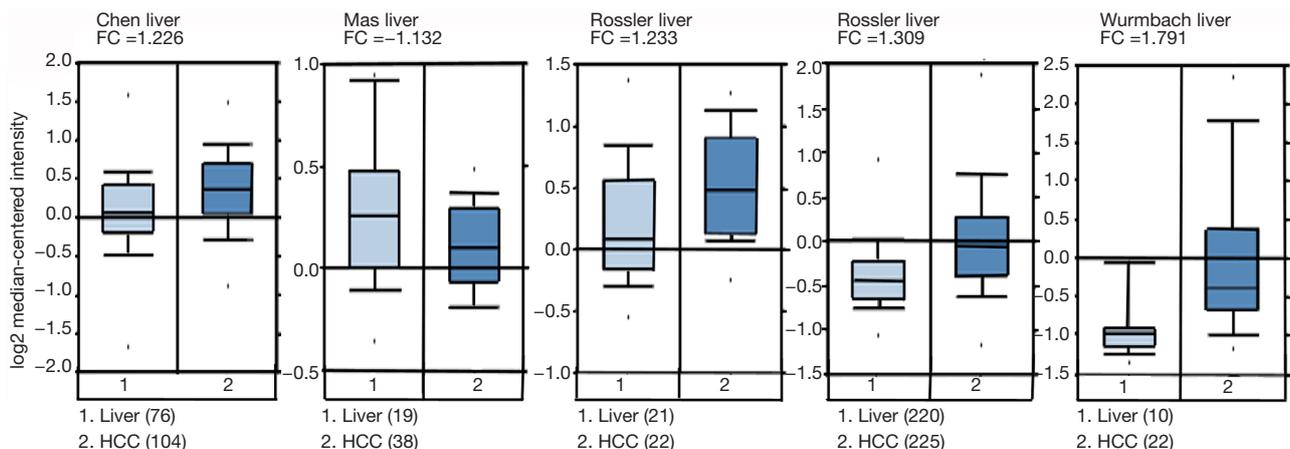
Figure 4 Expression of citron kinase in normal liver and hepatocellular carcinoma tissues according to Oncomine database.

Expresion of CIT in LIHC based on sample types
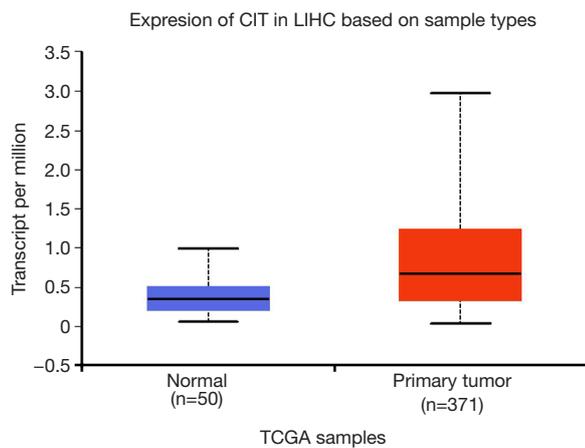
Figure 5 Expression of citron kinase in normal liver and hepatocellular carcinoma tissues based on UALCAN database.

Figure 6 Survival curves of the hepatocellular carcinoma patients with high or low citron kinase expression based on UALCAN database.

UALCAN databases. Indeed, these results suggested that higher CIT expression was associated with poorer HCC prognosis, and a published article has also detected elevated CIT mRNA levels in HCC (25). These results are thus at odds with our protein level finding, which may be due to a difference in the readout being examined. Indeed, it may be that the decrease CIT protein levels which we observed in the present study initiated a feedback loop, resulting in increasing CIT mRNA expression in order to compensate for the decrease protein levels within cells, although further research will be needed to verify this finding.

In conclusion, we found that low CIT protein expression is associated with poorer outcomes in HCC patients. Low

CIT expression was associated with gender, tumor size, Edmondson Grade, microvascular invasion, serum AFP level, and poor overall survival. Differences in the specific readout being examined may explain the discrepancies in the data between the present study and published databases.

Acknowledgments

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2020.03.58). All authors reports grants from Zhejiang Province Bureau of Health, during the conduct of the study.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The research was allowed by Review Board of Hospital Ethics Committee, and the informed consent from every patient was obtained before we collected the data. Written informed consent was obtained from the patient to publish this paper.

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