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Editorial Board Member of *World Journal of Hepatology*, Manuel Luis Rodríguez-Perálvarez, MD, PhD, Consultant Hepatologist and Associate Professor of Medicine, Department of Hepatology and Liver Transplantation, Reina Sofía University Hospital, Córdoba 14014, Spain. ropeml@hotmail.com

AIMS AND SCOPE

The primary aim of *World Journal of Hepatology* (*WJH*, *World J Hepatol*) is to provide scholars and readers from various fields of hepatology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

   WJH mainly publishes articles reporting research results and findings obtained in the field of hepatology and covering a wide range of topics including chronic cholestatic liver diseases, cirrhosis and its complications, clinical alcoholic liver disease, drug induced liver disease autoimmune, fatty liver disease, genetic and pediatric liver diseases, hepatocellular carcinoma, hepatic stellate cells and fibrosis, liver immunology, liver regeneration, hepatic surgery, liver transplantation, biliary tract pathophysiology, non-invasive markers of liver fibrosis, viral hepatitis.

INDEXING/ABSTRACTING

The *WJH* is now abstracted and indexed in PubMed, PubMed Central, Emerging Sources Citation Index (Web of Science), Scopus, China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database. The 2021 edition of Journal Citation Reports® cites the 2020 Journal Citation Indicator (JCI) for *WJH* as 0.61. The *WJH*'s CiteScore for 2020 is 5.6 and Scopus CiteScore rank 2020: Hepatology is 24/62.

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Observational Study

New stem cell autophagy surrogate diagnostic biomarkers in early-stage hepatocellular carcinoma in Egypt: A pilot study

Tarek Yosef, Wesam Ahmed Ibrahim, Marwa Matboli, Amina Ahmed Swilam, Sarah El-Nakeep

ORCID number: Tarek Yosef 0000-0002-0003-7548; Wesam Ahmed Ibrahim 0000-0003-1813-5248; Marwa Matboli 0000-0002-6852-3954; Amina Ahmed Swilam 0000-0002-1464-1269; Sarah El-Nakeep 0000-0003-2830-5052.

Author contributions: Yosef T supervised the conduction of the study; Ibrahim WA and El-Nakeep S followed the clinical collection of data and the availability of patients; El-Nakeep S and Matboli M formulated the research question and its applicability and wrote the final draft; Matboli M conducted the laboratory analysis; Swilam AA collected the data from the patients; all authors revised and accepted the final submitted manuscript.

Institutional review board statement: The Internal Medicine Department, Faculty of Medicine, Ain Shams University, approved this study's protocol in 2016 for ethics of conducting the study and in accordance with the ethical standards of the Declaration of Helsinki. Informed consent was obtained from each participant. Both the patients and controls were randomly selected.

Informed consent statement: Informed consent was obtained from each participant. Both the

Tarek Yosef, Wesam Ahmed Ibrahim, Sarah El-Nakeep, Gastroenterology and Hepatology Unit, Department of Internal Medicine, Faculty of Medicine, Ain Shams University, Cairo 11591, Egypt

Marwa Matboli, Department of Biochemistry, Faculty of Medicine, Ain Shams University, Cairo 11591, Egypt

Amina Ahmed Swilam, Department of Internal Medicine, Health Affair Directorate, Ministry of Health and Population, Cairo 11591, Egypt

Corresponding author: Sarah El-Nakeep, MD, Associate Professor, Gastroenterology and Hepatology Unit, Department of Internal Medicine, Faculty of Medicine, Ain Shams University, Ramsees Street, Cairo 11591, Egypt. sarahnakeep@gmail.com

Abstract

BACKGROUND

Stem cell autophagy disruption is responsible for the development of hepatocellular carcinoma (HCC). Many non-coding RNAs are linked to the activation and inhibition of certain genes. The *SQSTM1* gene controls stem cell autophagy as shown in previous studies. The upregulation of *SQSTM1* is associated with the inhibition of autophagy in cancerous stem cells in patients with HCC.

AIM

To determine whether serum microRNA, hsa-miR-519d, is linked to *SQSTM1* gene and whether they could be used as diagnostic biomarkers for early-stage HCC.

METHODS

In silico analysis was performed to determine the most correlated genes of autophagy with microRNAs. *SQSTM1* and hsa-miR-519d were validated through this pilot clinical study. This study included 50 Egyptian participants, who were classified into three subgroups: Group 1 included 34 patients with early-stage HCC, Group 2 included 11 patients with chronic liver disease, and Group 3 (control) included 5 healthy subjects. All patients were subjected to full laboratory investigations, including viral markers and alpha-fetoprotein (AFP), abdominal ultrasound, and clinical assessment with the Child–Pugh score calculation. Besides, the patients with HCC underwent triphasic computed tomography with contrast to diagnose and determine the tumor site, size, and number. Quantitative

real-time polymerase chain reaction was used to assess hsa-miR-519d-3p and *SQSTM1* in the serum of all the study participants.

RESULTS

Hsa-miR-519d-3p was significantly upregulated in patients with HCC compared with those with chronic liver disease and healthy subjects with an area under the curve (AUC) of 0.939, with cutoff value 8.34, sensitivity of 91.2%, and specificity of 81.8%. *SQSTM1* was upregulated with an AUC of 0.995, with cutoff value 7.89, sensitivity of 97.1%, and specificity of 100%. AFP significantly increased in patients with HCC with an AUC of 0.794, with cutoff value 7.30 ng/mL, sensitivity of 76.5%, and specificity of 72.7%.

CONCLUSION

This study is the first to show a direct relation between *SQSTM1* and hsa-miR-519d-3p; they are both upregulated in HCC. Thus, they could be used as surrogate diagnostic markers for stem cell autophagy disturbance in early-stage HCC.

Key Words: Autophagy; Hepatocellular carcinoma; miRNA; miR-519d; Stem cell; *SQSTM1*

Core Tip: Hepatocellular carcinoma (HCC) is the most common primary liver cancer. HCC is associated with poor prognosis due to difficult discovery at an early stage. The molecular pathophysiology behind HCC is not yet fully understood. Autophagy is one of the important affected pathways in HCC pathogenesis. In this study we used *in silico* analysis to determine a new molecular pathway and find the underlying background controlling genetic and epigenetic pathways. We found that autophagy-controlling gene *SQSTM1* is related to hsa-miR-519d-3p. Also, we found that their use as early detecting biomarkers for HCC diagnosis are more efficient than the currently used alpha-fetoprotein.

INTRODUCTION

The scientists who discovered the mechanism of autophagy were awarded a Nobel Prize, and autophagy subsequently became a topic of great scientific interest for researchers. Autophagy is defined as cellular "self-eating," where lysosomal degradation of cellular elements occurs[1-3]. This process has three types: Chaperone-mediated autophagy, microautophagy, and macroautophagy. Autophagy is considered a "dynamic cellular recycling"[4] and provides cancerous cell preservation through the production of amino acids from degraded proteins[5]. The activation of autophagy increases resistance to cisplatin and sorafenib in patients with hepato-cellular carcinoma (HCC); this could be reversed upon deactivation[6].

The discovery of "epigenetic–genetic" links is an important area of research. Studies on the regulation of targeted genes by microRNAs (miRNAs) must answer two questions: The mechanism of regulation and the effect of dysfunction on specific cancerous molecular pathways[7].

MiR-519d dysregulation is not only linked to the initiation and progression of many cancers as breast[8], skin[9], and gastrointestinal cancers[10,11] but also associated with obesity[12].

SQSTM1, also known as p62 protein, is a multifunctional protein responsible for various stress-induced cellular functions, including apoptosis and autophagy; its coding gene is the *SQSTM1* gene located on chromosome 5[13]. The impairment of

autophagy causes the accumulation of p62 proteins in the hepatoma cells of mice[14]. Meanwhile, its upregulation significantly contributes to the resistance of hepatoma cells to sorafenib[15]. *SQSTM1* was initially believed to only control several cellular metabolic pathways, including the mechanistic target of rapamycin, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and mitogen-activated protein kinase signaling pathways, but later was also linked to the control of selective autophagy[16].

Here, in this study, we used *in silico* analysis to search for a new link among epigenetic–genetic biomarkers to identify and detect their relationship with early-stage HCC. We found significant *in silico* data relation between hsa-miR-519d-3p and *SQSTM1* and their link to HCC pathophysiology. We clinically validated the data by examining serum samples to assess their ability to be used in the diagnosis of HCC.

MATERIALS AND METHODS

This was a cross-sectional study conducted on randomly selected 50 Egyptian participants from outpatient clinics and inpatients attending the Gastroenterology and Hepatology Unit of the Internal Medicine Department at Ainshams University Hospitals, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

The participants were divided into three groups

Group 1: Consisted of 34 patients with HCC that were diagnosed according to the American Association for the Study of Liver Diseases practice guidelines and staged according to the Barcelona Clinic Liver Cancer as stages A to D[17].

Group 2: Consisted of 11 patients with chronic liver disease.

Group 3: Consisted of 5 healthy subjects (control), who were enlisted during routine checkups and as volunteers.

Inclusion criteria for the study

Age more than 18 years.

The ability to provide informed consent.

Proven diagnosis of HCC according to the American Association for the Study of Liver Diseases practice guidelines for group 1[17].

Exclusion criteria for the study

Patients actively undergoing chemotherapy or radiation therapy for HCC.

Patients with other malignancies or treated within the last 5 years.

The Internal Medicine Department, Faculty of Medicine, Ain Shams University, approved this study's protocol in 2016 for ethics of conducting the study and in accordance with the ethical standards of the Declaration of Helsinki. Informed consent was obtained from each participant. Both the patients and controls were randomly selected. This study was not funded.

Data of samples

The following parameters were documented for the participants: Full personal history and thorough clinical examination.

Laboratory investigations included the following: (1) Liver function: Serum albumin, prothrombin time and international normalized ratio, and total and direct bilirubin; (2) Liver enzymes: Serum aspartate transaminase, alanine transaminase, alpha-fetoprotein (AFP), hepatitis C virus antibody, and hepatitis B virus surface antigen (HBsAg); and (3) Abdominal ultrasound: Tumor size, the number of nodules, local spread, lymph node metastasis, cirrhosis, and the presence of ascites.

Triphasic spiral contrast-enhanced computed tomography in the HCC group.

Biomarker identification and bioinformatics analysis

Bioinformatics analysis was performed to retrieve biomarkers relevant to HCC based on previous microarray studies. This step included the following.

Biomarker retrieval and verification: In this concern, we used the public databases, including miRDB, miRTargetLink Human, GeneCards, and Human Protein Atlas, to choose a set of miRNAs and its targeted messenger RNA (mRNA) that is related to HCC. According to the data retrieved, we chose the microRNA-519d, hsa-miR-519d-

3p, and the targeted mRNA, *SQSTM1*, as a point to be studied in relation to HCC. *In silico* analysis is shown in detail in Figure 1.

Sample collection: Blood was collected from all participants in a plain test tube. These blood samples were left at room temperature for a minimum of 30 min to allow complete blood clotting.

The clotted blood samples were centrifuged for 20 min.

The serum was carefully separated and transferred to 1.5 mL aliquots and stored at 80 °C until assayed.

An identifier was used to label each serum sample to protect the confidentiality of the participants.

Laboratory work

Extraction of total RNA: An miRNEasy RNA isolation kit (Qiagen, Hilden, Germany) was used to extract total RNA from the serum samples according to the manufacturer's instructions. The RNA concentration and integrity were assessed using an Ultraspec 1000 UV/visible spectrophotometer (Amersham Pharmacia Biotech, Cambridge, United Kingdom). Then, 72 µL diethyl pyrocarbonate–water was added to 3 µL RNA solution (dilution 1:25). The sample was read at 260 nm for RNA detection and 280 nm for protein detection using a spectrophotometer. Next, 40 µg RNA/mL is equivalent to 1 absorbance, so the concentration of RNA in a sample (µg/mL) = sample absorbance at 260 nm × 40/1 × dilution factor (25). The samples were considered to have high RNA quality if the RNA–protein ratio (260:280 ratio) was more than 1.8–2.

Reverse transcription-polymerase chain reaction: The extracted total RNA underwent reverse transcription into cDNA using a miScript II RT Kit (Qiagen) according to the manufacturer's protocol using a Hybaid thermal cycler (Thermo Electron, Waltham, MA, United States).

Quantitative reverse transcription-polymerase chain reaction: RNA levels were examined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to ensure sensitive and specific RNA detection and quantification with high amplification efficacy. All PCR primers were obtained from Qiagen. All steps followed the manufacturer's suggested protocol.

Quantitative PCR for the detection of *SQSTM1* mRNA: The relative expression of *SQSTM1* mRNA was assessed using a QuantiTect SYBR Green PCR Kit (Qiagen) on a Rotor-Gene real-time PCR detection system (Qiagen) with specific primers provided by Qiagen. Beta-actin (ACTB) was used as a housekeeping gene.

The QuantiTect SYBR Green PCR Kit provides accurate real-time quantification of cDNA targets in an easy-to-handle format. The kit can be used in real-time two-step RT-PCR of RNA targets following reverse transcription with the fluorescent dye SYBR Green I in the master mix, which enables the analysis of many targets without having to synthesize target-specific labeled probes. It uses the SYBR Green I dye to detect PCR products by binding to double-stranded DNA formed during the PCR. It binds to each new copy of double-stranded DNA generated during each PCR cycle. The result is an increase in fluorescence intensity proportional to the number of double-stranded PCR products produced.

High specificity and sensitivity in PCR are achieved using the hot-start enzyme HotStarTaq DNA Polymerase together with a specialized PCR buffer. In addition, the buffer contains ROX dye, allowing fluorescence normalization on certain cyclers. The kit has been optimized for use with any real-time cycler, including Rotor-Gene® cyclers. A melting point analysis was performed to monitor the homogeneity and specificity of the quantitative PCR (qPCR) products.

qPCR for the detection of hsa-miR-519d-3p: A relative miRNA expression level for hsa-miR-519d-3p was analyzed by mixing the total cDNA with the reagent provided in the miScript SYBR Green PCR Kit (Qiagen) according to the manufacturer's suggested protocol, in addition to the manufacturer-provided miScript universal primer. RNU-6 was used as a housekeeping gene.

For detecting mature miRNA, cDNA prepared in a reverse transcription reaction using miScript HiSpec Buffer or miScript HiFlex Buffer serves as the template for real-time PCR analysis using a miRNA-specific miScript Primer Assay (forward primer) and the miScript SYBR Green PCR Kit, which contains the miScript Universal Primer (reverse primer) and QuantiTect SYBR Green PCR Master Mix.
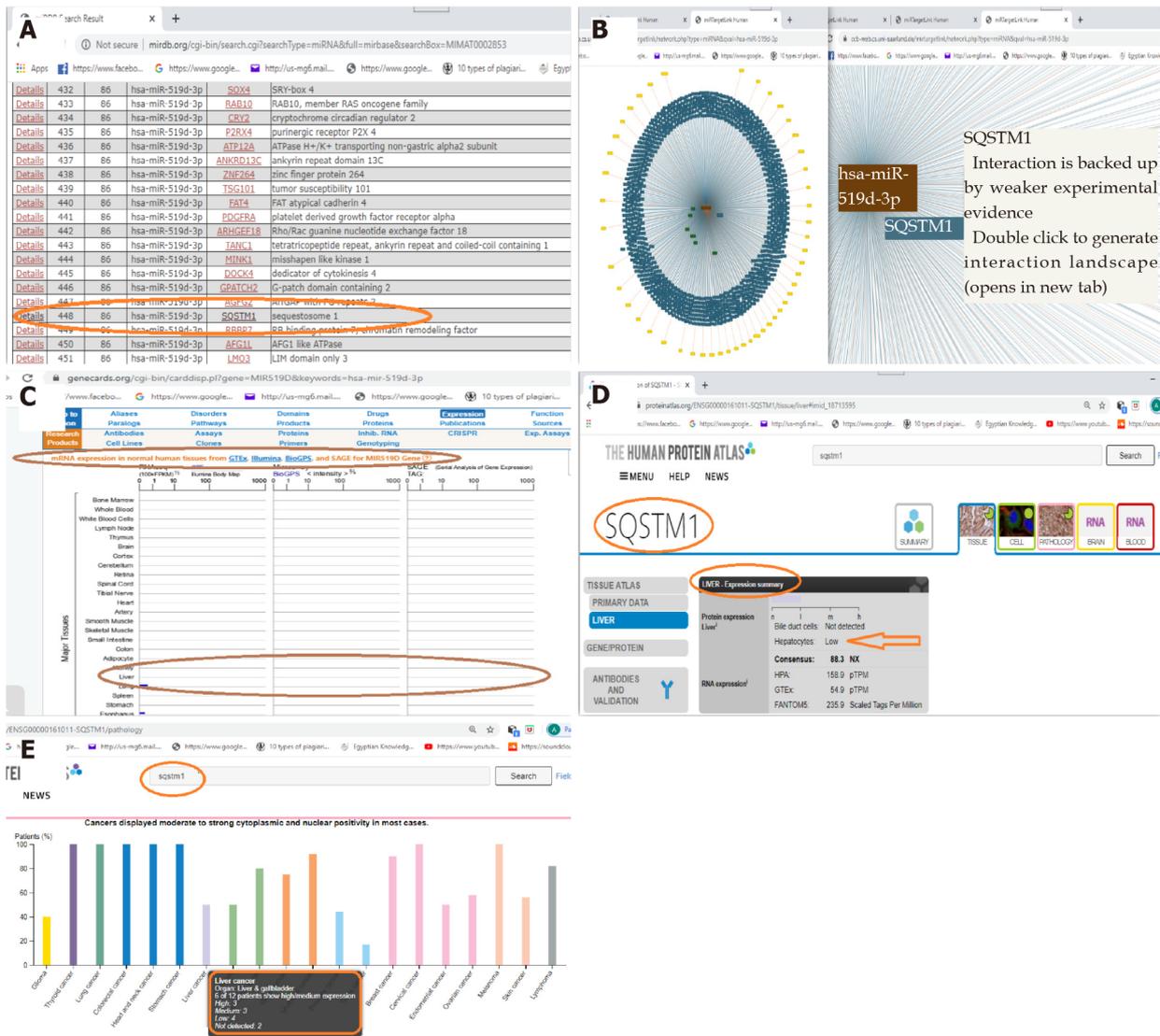
Figure 1 Bioinformatic search and validation of the newly diagnostic biomarkers. A: miR-519d-3p and *SQSTM1* as a targeted mRNA according to miRDB (http://mirdb.org/cgi-bin/search.cgi?searchType=miRNA&full=mirbase&searchBox=MIMAT0002853); B: A network of 923 genes targeted by hsa-miR-519d-3p, along with focusing on *SQSTM1* in the network (miRTargetLink Human) (https://ccb-web.cs.uni-saarland.de/mirtargetlink/network.php?type=miRNA&qval=hsa-miR-519d-3p); C: The expression of miR-519d in liver tissue and other tissues (https://www.genecards.org/); D: The tissue expression of *SQSTM1* is low in hepatocytes of healthy liver tissue (www.proteinatlas.org); E: The expression of *SQSTM1* in cancers and liver cancer specifically (www.proteinatlas.org).

PCR result analysis: The PCR program for the SYBR Green-based qPCR was as follows: Denaturation at 95 °C for 15 min; 40 cycles of denaturation for 10 s at 94 °C; annealing for 30 s at 55 °C; and extension for 34 s at 70 °C. Each reaction was performed in duplicate. A Rotor-Gene manual was used to calculate the threshold cycle (Ct) value of each sample. Any Ct value greater than 36 was considered negative. We used the melting curve analysis software of Applied Biosystems to analyze our results. The melting curves were analyzed to affirm the specificities of the amplicons for the SYBR Green-based PCR amplification. The ($2^{-\Delta\Delta Ct}$) relative quantification RQ technique was used to measure the expression of the RNA-based biomarker panel.

The housekeeping genes, ACTB and RNU-6, were used as an invariant internal control to normalize the raw data of the samples and compare these results with a reference sample.

Statistical analysis

Statistical analyses of the obtained data were performed using SPSS, version 23 (IBM Corp., Armonk, NY, United States).

To describe the studied sample, quantitative data are presented as minimum, maximum, mean, and standard deviation for parametric data and median and interquartile range (IQR) for nonparametric data. Qualitative data are presented as

count and percentage.

Student's t-test was used to compare quantitative data between two independent groups, and the Mann–Whitney U-test was used for nonparametric data.

One-way analysis of variance was performed to compare quantitative data when more than two groups were to be compared; then, a post-hoc test was used to detect the difference between individual groups for parametric data, and the Kruskal–Wallis test was used for nonparametric data.

The chi-square test and Fisher's exact test were used to compare qualitative data between different groups.

The receiver operating characteristic (ROC) curve was used to measure diagnostic validity and determine the best cutoff value for some variables.

P values less than 0.05 denote statistical significance. In addition, concerning the level of significance: *P* values represent the level of significance, *P* values more than 0.05 are non-significant, *P* values less than 0.05 are significant, and *P* values less than 0.01 are highly significant.

RESULTS

We conducted this study on 50 Egyptian participants divided into three groups: 34 patients in the HCC group, 11 patients in the chronic liver infection group, and 5 healthy participants as the control group.

The age of all participants was more than 18 years with a mean of 58.88 ± 8.08 years, 56.18 ± 16.26 years, and 55.40 ± 22.09 years in the HCC, chronic liver infection, and control groups, respectively, with a non-statistically significant *P* value (0.72). In addition, a non-significant difference was observed between the malignant and non-malignant groups (*i.e.* patients in the chronic liver infection group added to the control group) with a *P* value of 0.53.

Gender differences were observed among the study groups—HCC group: Male = 67.6% and female = 32.4%; chronic liver infection group: Male = 81.8% and female = 18.2%); and healthy group: Male = 60% and female = 40%. The difference between the three study groups was statistically non-significant with a *P* value of 0.63 (Table 1). Liver function and laboratory data are shown in Table 2.

Hepatitis C antibody was prevalent in 88.2% of the patients with HCC, whereas all patients with chronic liver disease were positive, and no subjects in the control group were positive for hepatitis C virus antibody. HBsAg was prevalent in 5.9% of the patients with HCC, whereas none of the subjects in the chronic liver disease and control groups were positive for HBsAg. These data are shown in Table 3.

Our results concerning hsa-miR-519d-3p showed data from the qRT-PCR. These data were reported in delta–delta Ct [DDCT or -(ΔΔCT)] and RQ calculated as follows: RQ = 2^{-ddCT} = $2^{-\Delta\Delta CT}$ (Table 4 and Figure 2A).

The results of serum miRNA (miR-519d) in the three study groups, reported in RQ, showed that in the HCC group, serum miRNA was 41.94 (IQR: 18.25–139.10); in the chronic liver infection group, serum miRNA was 5.98 (IQR: 3.14–8.28), and in the control group, serum miRNA was 1.17 (IQR: 1.16–1.21), with a highly significant *P* value (< 0.001) (Table 4). These data suggest that hsa-miR-519d-3p is significantly upregulated in the HCC group compared with the chronic liver infection and control groups. The ROC curve to assess the validity of the results of qRT-PCR of hsa-miR-519d in the serum in differentiating the HCC and chronic liver infection groups with the best cutoff value of ≥ 8.34, sensitivity of 91.2%, and specificity of 81.8% is shown in Figure 3A.

The second part of this study focused on the serum level of *SQSTM1* in HCC and whether it can be used as a significant biomarker. The data we obtained from qRT-PCR using the RQ of the serum *SQSTM1* gene in comparing the three study groups from Table 4 and Figure 2B showed that *SQSTM1* was 33.91 (IQR: 14.83–132.51) in the HCC group, 3.68 (IQR: 2.28–5.50) in the chronic liver infection group, and 0.84 (IQR: 0.76–0.99) in the control group with a highly significant *P* value (< 0.001). The ROC curve to assess the validity of the results of qRT-PCR of *SQSTM1* in the serum to differentiate the HCC and chronic liver infection groups with the best cutoff value of ≥ 7.89, sensitivity of 97.1%, and specificity of 100% is shown in Figure 3B.

When we divided the groups into the malignant and non-malignant groups, we found similar results (Figure 4).

The ROC curve to assess the validity of the RQ results of qRT-PCR of hsa-miR-519d in the serum among the malignant and non-malignant groups with the best cutoff value of ≥ 8.34, sensitivity of 91.2% and specificity of 87.5% is shown in Figure 4A**.** The

Table 1 The ages in the different groups of the study (mean ± SD)

Age	HCC (*n* = 34)	Chronic liver infection (*n* = 11)	Control (*n* = 5)	F[1]	*P* value
	58.88 ± 8.08	56.18 ± 16.26	55.40 ± 22.09	0.34	0.72 NS

[1]One-way analysis of variance. HCC: Hepatocellular carcinoma; NS: Non-significant; SD: Standard deviation.

Table 2 Significance of the differences in laboratory data between the three study groups (mean ± SD)

Variable	HCC (*n* = 34)	Chronic liver infection (*n* = 11)	Control (*n* = 5)	F[1]	*P* value
INR	1.37[a] ± 0.20	1.35[a] ± 0.30	1.07[b] ± 0.08	3.93	0.03 S
Serum albumin (g/dL)	2.94 ± 0.42	3.03 ± 0.73	3.40 ± 0.25	1.95	0.15 NS
AST[2] (IU/L)	50.00[a] ± 38.00–102.00	23.00[b] ± 15.00–39.00	15.00[b] ± 14.00–18.00	16.21	< 0.001 HS
ALT[2] (IU/L)	40.50[a] ± 28.00–73.30	22.00 ± 15.00–38.00	10.00[b] ± 8.00–15.00	12.69	0.002 HS
Total bilirubin[2] (mg/dL)	1.60[a] ± 1.10–2.20	1.10 ± 0.50–1.60	0.40[b] ± 0.30–0.50	14.91	0.001 HS
Direct bilirubin[2] (mg/dL)	0.70[a] ± 0.50–1.10	0.30[b] ± 0.10–0.60	0.10[b] ± 0.10–0.20	15.84	< 0.001 HS

[1]One-way analysis of variance (a, b Post-hoc test).
[2]Kruskal–Wallis test (median and interquartile range).
[a]$P < 0.05$.
[b]$P < 0.01$.
ALT: Alanine transaminase; AST: Aspartate transaminase; HCC: Hepatocellular carcinoma; HS: Highly significant; INR: International normalized ratio; NS: Non-significant; SD: Standard deviation.

Table 3 Hepatitis virus B and C infections in the three study groups

Variable		HCC (*n* = 34), (%)	Chronic liver infection (*n* = 11), (%)	Control (*n* = 5), (%)	(X²)[1]	*P* value
HCVAb	Positive	30 (88.2)	11 (100.0)	0 (0.0)	18.32 FE	< 0.001 HS
	Negative	4 (11.8)	0 (0.0)	5 (100.0)		
HBsAg	Positive	2 (5.9)	0 (0.0)	0 (0.0)	0.78 FE	1.00 NS
	Negative	32 (94.1)	11 (100.0)	5 (100.0)		

[1]The chi-square test (FE: Fisher's exact test). HBsAg: Hepatitis B virus surface antigen; HCC: Hepatocellular carcinoma; HCVAb: Hepatitis C virus antibody; HS: Highly significant; NS: Non-significant.

ROC curve to assess the validity of the RQ results of qRT-PCR of *SQSTM1* in the serum among the malignant and non-malignant groups with the best cutoff value of ≥ 7.89, sensitivity of 97.1%, and specificity of 100% is shown in Figure 4B.

Furthermore, in this study, AFP was 62.60 (IQR: 8.20–600.80) in the HCC group, 3.50 (IQR: 2.50–20.00) in the chronic liver infection group, and 0.70 (IQR: 0.50–1.00) in the control group (Table 5). These results show that AFP is elevated with high statistical significance in patients with HCC as compared to other groups, with a *P* value of < 0.001. The constructed ROC curve to compare AFP results between the HCC and chronic liver infection groups showed an area under the curve (AUC) of 0.794, with the best cutoff value of > 7.30 ng/mL, sensitivity of 76.5%, and specificity of 72.7% (Figure 5A). Meanwhile, the ROC curve assessing the validity of AFP for differentiating between the malignant and non-malignant groups showed an AUC of 0.854, with the best cutoff value of > 7.30, sensitivity of 76.5%, and specificity of 81.2% (Figure 5B).

Most patients had early-stage HCC, except for three patients. The full details of the radiological findings are presented in Table 6.

Table 4 Expression level of hsa-miR-519d-3p, *ACTB*, *RNU6*, and *SQSTM1* between the three study groups (mean ± SD)

Variable	HCC (*n* = 34)	Chronic liver infection (*n* = 11)	Control (*n* = 5)	F[1]	*P* value
Ct (ACTB)	30.65[a] ± 4.21	25.82[b] ± 2.31	27.34[b] ± 2.00	7.69	0.001 HS
Ct (RNU6)	36.03 ± 2.92	36.36 ± 2.82	38.55 ± 0.96	1.78	0.18 NS
Ct (miR-519d)	30.08[a] ± 3.00	33.61[b] ± 2.78	38.05[c] ± 1.08	20.48	< 0.001 HS
mRNA-*SQSTM1*	36.14 ± 3.17	34.89 ± 2.30	38.38 ± 1.86	2.48	0.10 NS
DCT (miR-519d)	−5.95[a] ± 1.98	−2.75[b] ± 0.89	−0.50[c] ± 0.40	31.17	< 0.001 HS
DDCT (miR-519d)	−5.59[a] ± 1.98	−2.39[b] ± 0.89	−0.14[c] ± 0.40	31.17	< 0.001 HS
RQ (miR-519d)[2]	41.94[a] ± 18.25–139.10	5.98[b] ± 3.14–8.28	1.17[c] ± 1.16–1.21	28.46	< 0.001 HS
DCT (*SQSTM1*)	5.49[a] ± 1.83	9.07[b] ± 0.70	11.04[c] ± 0.58	41.08	< 0.001 HS
DDCT (*SQSTM1*)	−5.51[a] ± 1.83	−1.93[b] ± 0.70	0.04[c] ± 0.58	41.08	< 0.001 HS
RQ (*SQSTM1*)[2]	33.91[a] ± 14.83–132.51	3.68[b] ± 2.28–5.50	0.84[c] ± 0.76–0.99	32.54	< 0.001 HS

[1]One-way analysis of variance (a, b post-hoc test).
[2]Kruskal–Wallis test (median and interquartile range).
[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.
Ct: Threshold cycle; HS: Highly significant; NS: Non-significant.

Table 5 Alpha-fetoprotein laboratory results in the three subgroups

Variable	HCC (*n* = 34), median (IQR)	Chronic liver disease (*n* = 11), median (IQR)	Control (*n* = 5), median (IQR)		*P* value
AFP[1] (ng/mL) by ELISA	62.60[a] (8.20–600.80)	3.50[b] (2.50–20.00)	0.70[c] (0.50–1.00)	19.17	< 0.001 HS

[1]Kruskal–Wallis test (median and interquartile range).
[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.
AFP: Alpha-fetoprotein; ELISA: Enzyme-linked immunosorbent assay; HCC: Hepatocellular carcinoma; HS: Highly significant; IQR: Interquartile range.

DISCUSSION

Our results suggest that hsa-miR-519d-3p is upregulated in the serum of the HCC group compared with the chronic liver disease and healthy control groups, with high sensitivity and specificity as a diagnostic marker. Similar results were observed by Fornari *et al*[18], where the miRNA was upregulated and considered an HCC oncogene. The study linked our target miRNA to DNA hypomethylation and p53, both of which are responsible for cell death and apoptosis. However, a recent study by Zhang *et al*[19] has linked miR-519d to the adenosine monophosphate-activated protein kinase pathway in HCC cells, regulating cellular energy metabolism by controlling the Ras-related protein (Rab10)[19]. A recent study has raised the hope of inducing autophagy in hepatoma cells by the administration of metformin through the activation of the mechanistic target of rapamycin pathway[20]. Alternatively, patients with colorectal cancer had improved survival and lower metastasis with upregulated miR-519d-3p by regulating trophinin-associated protein[11].

This study on serum mRNA of *SQSTM1* revealed significant upregulation of its serum level in the HCC group as compared to the levels in the chronic liver disease and healthy control groups. Thus, our results mean that mRNA of *SQSTM1* is upregulated in the serum of patients with HCC. This is compared to the findings of Xiang *et al*[21] who have found higher expression levels of the encoded protein p62 in hepatoma cells of patients with hepatitis B infection or those exposed to aflatoxin B1 [21], whereas our study population was mostly infected with hepatitis C virus.

Table 6 Clinical and radiological characteristics of hepatocellular carcinoma lesions.		
Variable		**mean ± SD**
Child–Pugh score		6.76 ± 1.44
		***n* (%)**
Cirrhosis	Cirrhosis	27 (79.4)
	No cirrhosis	7 (20.6)
BCLC stage	Stage A (early)	31 (91.2)
	Stage D (late)	3 (8.8)
Child–Pugh classification	A	17 (50.0)
	B	14 (41.2)
	C	3 (8.8)
Average tumor size	> 3 cm	3 (8.8)
	< 3 cm	31 (91.2)

BCLC: Barcelona Clinic Liver Cancer; SD: Standard deviation.
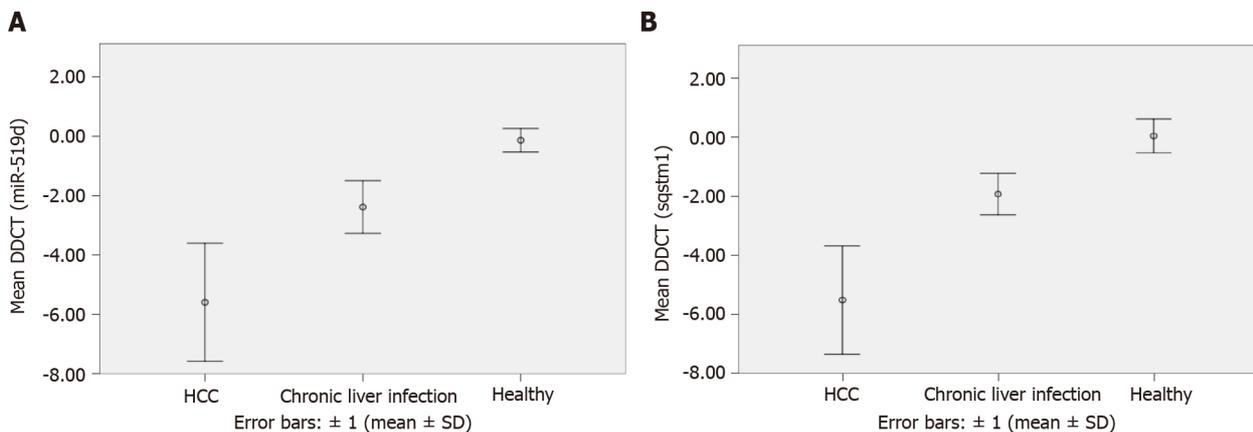
Figure 2 Box-plot figures showing the mean delta–delta threshold cycle in the new diagnostic biomarkers in different groups. A: Illustration of the mean delta–delta threshold cycle (DDCT) of the quantitative real-time polymerase chain reaction (qRT-PCR) results for hsa-miR-519d in the serum of the hepatocellular carcinoma (HCC), chronic liver infection, and control groups using error bars: ± 1 [mean ± standard deviation (SD)]; B: Illustration of the mean DDCT of the qRT-PCR results for mRNA of *SQSTM1* in the serum of the HCC, chronic liver infection, and control groups using error bars: ± 1 (mean ± SD). DDCT: Delta–delta threshold cycle; HCC: Hepatocellular carcinoma.

The *SQSTM1* gene is responsible for coding p62. This protein plays an important role as a receptor in selective autophagy, where specific cell organelles or proteins are degraded selectively by autophagosomes[22,23]. This ubiquitin-binding receptor protein is upregulated in early-stage HCC, as it is responsible for the maintenance of cancerous cells and their survival during stress[24].

In addition, our results show that hsa-miR-519d-3p is upregulated, synchronously with the upregulation of the mRNA of *SQSTM1*; this made us deduce that miRNA 519d stimulates the *SQSTM1* gene and increases the expression of its transcribed mRNA, not just increasing its translated protein level (p62) as previous studies have detected. In this study, we could not define the mechanism by which miR-519d-3p upregulates *SQSTM1*, but we have identified that the gene is upregulated at the transcriptional level, ***not*** at the post-transcriptional level. Besides, we can conclude that miR-519d-3p can affect autophagy and induce the progression of HCC through the targeted upregulation of *SQSTM1*.

In addition to these results, the sensitivity and specificity of hsa-miR-519d-3p, the mRNA of *SQSTM1*, and AFP were 91.2%–81.8%, 97.1%–100%, and 76.5%–72.7%, respectively. Also, the best cutoff values of the three parameters were ≥ 8.34 for miR-519d, ≥ 7.89 for the mRNA of *SQSTM1*, and ≥ 7.30 for AFP. Our results showed that miR-519d and the mRNA of *SQSTM1* showed higher sensitivity and specificity than
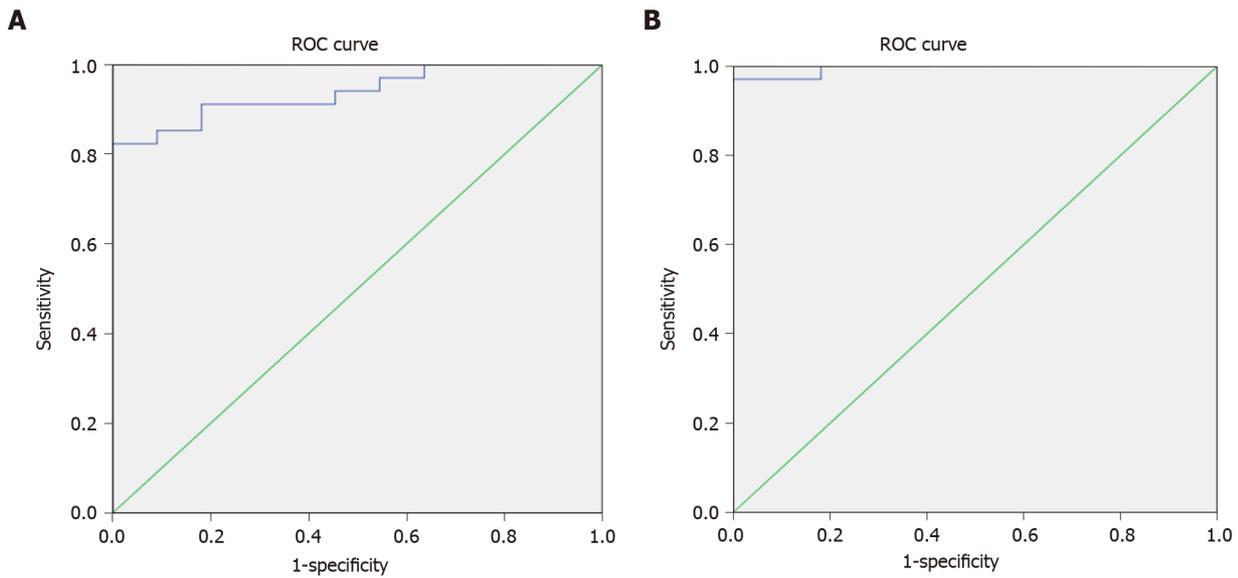
Figure 3 Receiver operating characteristic curves of the new diagnostic biomarkers studied to differentiate between hepatocellular carcinoma and chronic liver infection groups. A: Receiver operating characteristic (ROC) curve for assessing the validity of the RQ results of quantitative real-time polymerase chain reaction (qRT-PCR) for hsa-miR-519d in the serum to differentiate the hepatocellular carcinoma and chronic liver infection groups; B: ROC curve assessing the validity of the RQ results of qRT-PCR for mRNA of *SQSTM1* in the serum between hepatocellular carcinoma and chronic liver infection groups. ROC: Receiver operating characteristic.
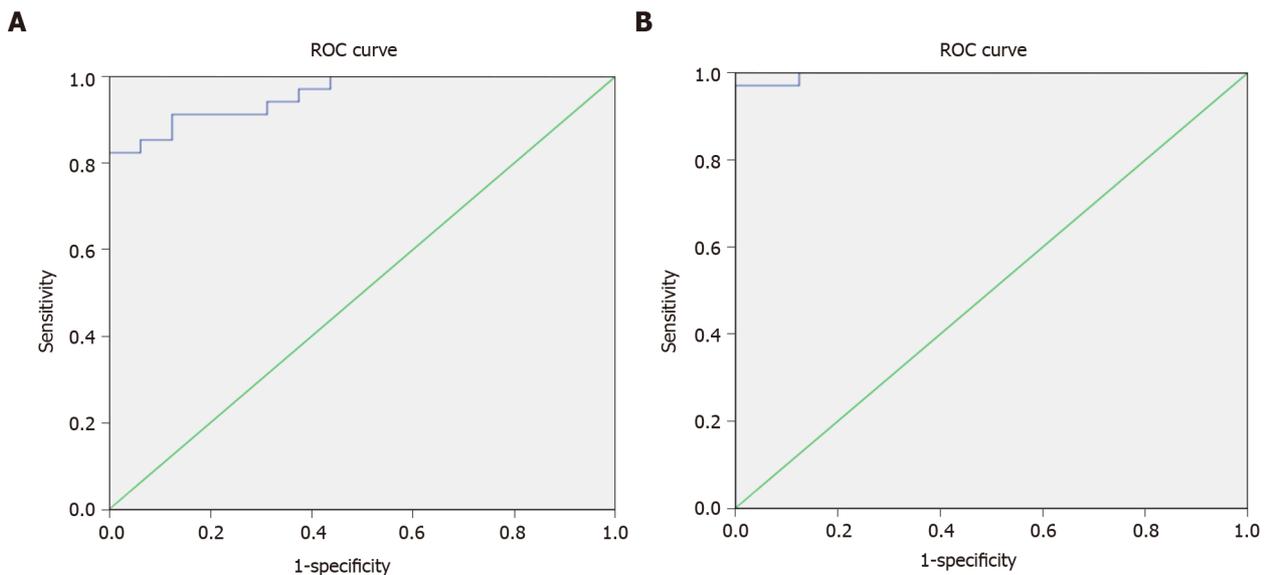
Figure 4 Receiver operating characteristic curves of the new diagnostic biomarkers studied to differentiate between the malignant and non-malignant groups. A: Receiver operating characteristic (ROC) curve assessing the validity of the RQ results of quantitative real-time polymerase chain reaction (qRT-PCR) for hsa-miR-519d in the serum among the malignant and non-malignant groups; B: ROC curve assessing the validity of the RQ results of qRT-PCR for mRNA of *SQSTM1* in the serum among the malignant and non-malignant groups. ROC: Receiver operating characteristic.

AFP, with better detection of early-stage HCC cases; thus can be used as diagnostic biomarkers for early HCC, improving the HCC outcome and prognosis. Moreover, when we compared the HCC group with the chronic liver disease group only or with the combined group of both patients with chronic liver disease and healthy subjects (malignant and non-malignant groups), we found similar results in both categories regarding hsa-miR-519d-3p, the mRNA of *SQSTM1*, and AFP.
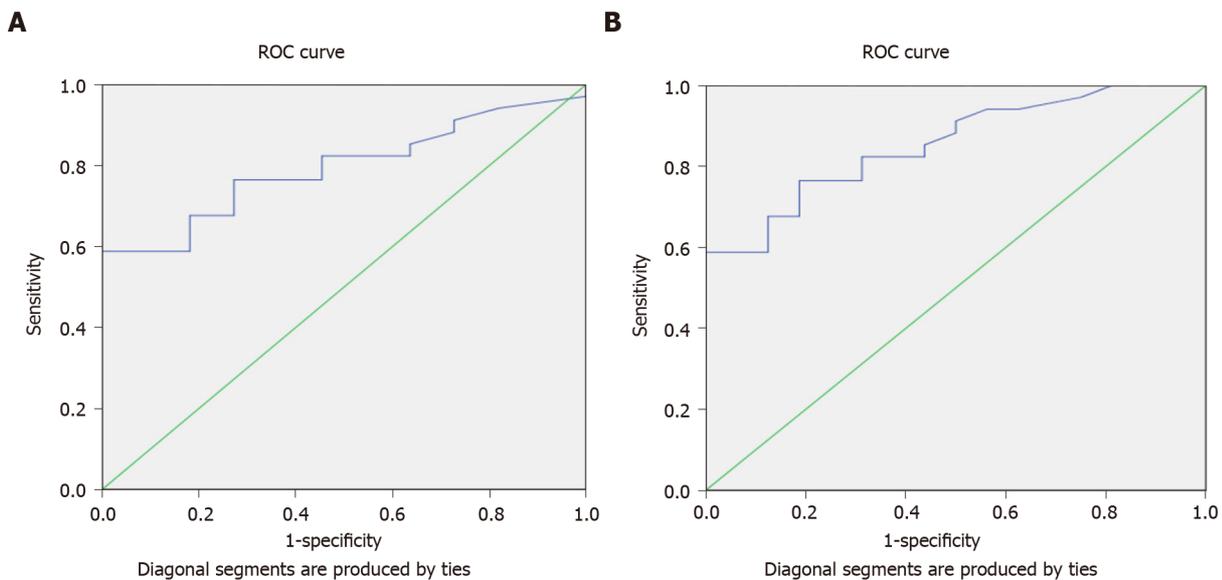
Figure 5 Receiver operating characteristic curves of the alpha-fetoprotein studied to differentiate between the hepatocellular carcinoma and chronic liver disease groups/malignant and non-malignant groups. A: Receiver operating characteristic (ROC) curve to assess the validity of alpha-fetoprotein (AFP) for the differentiation between the hepatocellular carcinoma and chronic liver disease groups; B: ROC curve assessing the validity of AFP for differentiating between the malignant and non-malignant groups. ROC: Receiver operating characteristic.

CONCLUSION

We are the first to establish a link between hsa-miR-519d-3p and the mRNA of *SQSTM1* in HCC. Hsa-miR-519d-3p and the mRNA of *SQSTM1* could be used in the diagnosis of HCC in its early stages. Further studies are needed to detect levels of miR-519d-3p and the mRNA of *SQSTM1* before and after various modalities of treatment to assess their ability to monitor treatment responses and detect recurrences. Multicentric studies with more variability to validate the use of miR-519d-3P and the mRNA of *SQSTM1* as diagnostic biomarkers of HCC on a wide scale are needed.

ARTICLE HIGHLIGHTS

Research background

Autophagy is one of the pathways affected in hepatocellular carcinoma (HCC). Genetic regulation of this pathway through the *SQSTM1* gene was established. Autophagy is responsible for the destruction of cellular components through lysosomal degradation. This process is responsible for cellular recycling and preservation. It protects from cancerous transformation, thus any imbalance in this mechanism will increase the risk of cancer.

Research motivation

We aimed to establish the genetic-epigenetic-phenotypic pathway related to the autophagic process in the pathogenesis of HCC and whether these studied biomarkers could be used as surrogate diagnostic markers for autophagy pathway in HCC.

Research objectives

We examined hsa-miR-519d microRNA effect on HCC and its association with the *SQSTM1* genetic marker. We also examined the sensitivity and specificity of those biomarkers in the diagnosis of early-stage HCC cases.

Research methods

This is an observational study. We evaluated the candidate biomarkers through bioinformatics, and after establishing a computational statistical relation, we proceeded with their clinical association through laboratory validation. We measured the genetic and epigenetic biomarkers in the serum samples taken from HCC patients, chronic liver disease patients, and healthy participants. We used reverse transcription-

polymerase chain reaction and quantitative reverse transcription-polymerase chain reaction.

Research results

We determined the sensitivity and specificity of each biomarker separately and combined as compared to the established alpha-fetoprotein (AFP) biomarker. We found that all the studied biomarkers in our study have better sensitivity and specificity than AFP, when used separately or combined, at the diagnosis of early-stage HCC.

Research conclusions

We could use the autophagy pathway biomarkers in the early-stage HCC diagnosis.

Research perspectives

More autophagy biomarkers could be examined using first *in silico* analysis then clinical laboratory confirmation. Combining computational and clinical validations in clinical studies could benefit the research process immensely.

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