

Annexin A2 Versus Afp as An Efficient Diagnostic Serum Marker for Hepatocellular Carcinoma

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ABSTRACT

AIM: To assess the annexin A2 versus AFP as an efficient Serum marker for Hepatocellular carcinoma diagnosis. Hepatocellular carcinoma is the third leading cause of cancer-related death, HCC prognosis is poor, and early detection is of the utmost importance. Although serum AFP is a useful biomarker for the detection and monitoring of HCC, the false-negative rate using the AFP level alone may be high. Several biomarkers are under research, one of them is annexin A2 (Annexin11, ANXA2) is a 36 kDa calcium-dependent phospholipid-binding protein that is presented on the surface of most eukaryotic cells, involving in several biological processes.

METHODS: 105 patients were included, 70 of them were diagnosed as HCC, these patients were divided into group 1 early stage HCC ($n=37$; male=23, female=14), and group 2 late stage HCC ($n=43$; male=29, female=14) and the other 35 patients (Group 3) were cirrhotics (20 males and 15 females) beside that there were about 20 healthy control (Group 4). All subjects underwent a full history, physical examination and investigations, including: liver function tests, viral markers (HCV Ab and HBs Ag), AFP and evaluation of serum annexin A2 level and abdominal ultrasonography. On the other hand abdominal CT (was done for patients groups) and liver biopsy was done for diagnosis of hepatocellular carcinoma (group 1).

RESULTS: The mean level of serum annexin A2 in HCC patients

were significantly higher than in the cases with liver cirrhosis or normal control ($P<0.001$), while no significant difference between liver cirrhosis and normal control. There was no significant relation with age, gender, S. albumin, S. bilirubin, S. ALT and hepatitis C infection. However there was a significant relation with hepatitis B infection. The specificity and sensitivity of serum annexin A2 and AFP measurements were represented by ROC curves. Area under ROC curve for AFP (AUC) was 0.84 with 0.75 to 0.910, 95% confidence interval with $p<0.0001$, the AUC for Annexin A2 was 0.89 with 0.81 to 0.95, 95% confidence interval with $P<0.0001$, AUC for both markers was 0.818 with 0.723 to 0.890, 95% confidence interval with $P<0.0001$, when comparing the sensitivity and specificity of annexin A2 with AFP or both markers they were; (78% & 91% for annexin A2 and 70.1% & 65.5% for AFP and 91.9% & 63.6% for both).

CONCLUSION: Serum level of annexin A2 can be a good marker for HCC, with higher sensitivity, specificity, positive and negative predictive value than AFP. Complementary diagnostic value was achieved by measuring both annexin A2 & AFP for HCC diagnosis, we found sensitivity and negative predictive value were higher than in either both.

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Key words: Annexin; Marker; Hepatocellular carcinoma

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INTRODUCTION

Liver cancer is the fifth most common fatal cancer worldwide with more than 90% mortality. Hepatocellular carcinoma (HCC) represents the most important, its prevalence approximately 85% of all primary liver cancer^[1], there is estimation that almost 20% chronic-viral infected cases would progress to cirrhosis, and above 80% of tumors developed from cirrhotic livers^[2]. The geographic pattern of HCC incidence is parallel to exposure to viral etiologic

factors. Its incidence is increasing, ranging from 3% to 9% depending on the geographical location^[3]. In Egypt, the growing incidence of HCC, which is nearly doubled over the last decade^[4-7] is parallel with that Egypt are plugged with highest prevalence of HCV in the world, ranging from 6 to 28%^[8-10]. HBV, HCV and other factors are the etiology of HCC. Although HBV is considered worldwide as a major risk factor for liver cirrhosis and HCC, the prevalence of HBV infection in Egypt has been declining over the last two decades^[11].

Due to the asymptomatic nature of early HCC and lack of effective diagnostic and screening strategies, most patients (>80%) present with overt advanced stage of the disease. Currently, the most utilized surveillance laboratory methods for patients with cirrhosis are serum alpha-fetoprotein (AFP) level and ultrasonography with some limits^[12-14]. There is about 30% HCC cases with normal serum AFP levels are hardly diagnosed before any clinical manifestations appear, so, AFP is limited and not efficient for early HCC^[15]. Therefore, it highlights the need for new early detection biomarkers more useful and accurate for HCC. Detection of tumor markers in human serum is the most effective method because it is convenient, noninvasive, inexpensive and accurate. The plasminogen activation system known to be involved in thrombolysis and wound healing plays a major role in cancer progression. The inactive enzyme plasminogen is converted to the active serine protease plasmin by plasminogen activators; tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA)^[16]. A well studied protein which is an important mediator in the plasminogen activator system is annexin A2^[17]. Annexin A2, a calcium-binding cytoskeletal protein is localized at the extracellular surface of endothelial cells and various types of tumor cells^[18-19]. The increased expression of annexin A2 has been reported in cancers of the breast, liver, prostate, pancreas^[20-23] and ovary^[24]. Recent studies showed that annexin A2 (ANXA2) plays an important role in the hepatocyte malignant transformation and HCC development^[25-27]. So it may be used as a serological marker for HCC to enable early diagnosis.

Study the annexin A2 versus AFP as an efficient Serum marker for Hepatocellular carcinoma diagnosis.

METHODS

This diagnostic study was performed from March, 2012 till December, 2012 in Zagazig University hospitals, and included 105 patients, 70 of them were diagnosed as HCC, these patients were divided into group 1 early stage HCC ($n=37$; male=23, female=14), and group 2 late stage HCC ($n=43$; male=29, female=14) and the other 35 patients (Group 3) were cirrhotics (20 males and 15 females) beside that there were about 20 healthy control (Group 4).

Study steps

A consent from all subjects participate in the study was taken. As regards patients groups, 37 patients were diagnosed as early stage HCC (Group 1) patients (23 male and 14 female) and group 2 late stage HCC ($n=43$; male=29, female=14) and 35 patients (Group 3) were cirrhotic (20 males and 15 females). Twenty healthy subjects (Group 4) were included as a control group (10 males and 10 females). They had normal liver function tests negative viral marker and normal Abdominal ultrasonography.

All subject underwent full history (HBV, HCV infection)-asymptomatic or with positive history of fatigue, abdominal pain or dyspepsia, hematemesis, melena, hepatic encephalopathy, jaundice and lower limb edema, clinical examination (general examination as, blood pressure, low grade fever, pallor, jaundice, foeter hepatics,

face examination, flapping tremors, Palmar erythema, echymosis, purpura, spider nevi and local abdominal examination (hepatomegaly, splenomegaly, abdominal mass and ascites).

Investigations were done for patients and control include: liver function tests, viral markers (HCV ab and HBVs antigen), AFP and abdominal ultrasonography. Computed tomography (CT) was done for patients groups only, beside that when the previous investigations suggested a possible diagnosis of HCC, liver biopsy was done for proving the diagnosis of hepatocellular carcinoma (group 3 only). Evaluation of serum annexin A2 level for all subjects (Group 1, Group 2 and Group 3) was done by ELISA.

Although the diagnosis of HCC is confirmed by any of the following criteria histological evidence, demonstration of a focal lesion >2 cm in size and with arterial hypervascularisation by two imaging techniques showing this morphological aspect with an AFP level of ≥ 400 ng/mL^[28], our research depended on histological evidence through liver biopsy as a golden standard test to confirm the diagnosis of all HCC patients (Group 1 and Group 2). Early stage of HCC were defined as BCLC stage 0 and A, and late stage was defined as combination of BCLC stage B and stage C^[29].

Exclusion criteria

History of other cancers as cancer pancreas, ovary, breast, and prostate (in which annexin A2 serum level is elevated).

ELISA method for detection of serum annexin level

Evaluation of serum annexin A2 level for all subjects (Group 1, Group 2, Group 3 and Group 4) by using a human ANXA2 ELISA kit (Usen Life Science Inc., USA) according to the manufacturer's instructions. To each well was added 100 μ L of serum sample or standard separately, and then 100 μ L of detection reagent A was added and incubated for 1 h at 37°C. Subsequently, 100 μ L of detection reagent B was added and incubated for 0.5 h at 37°C. Then, 90 μ L of substrate solution was added and incubated for 25 min at 37°C. Finally, 50 μ L of stop solution was added to each well, and absorbance was read at 450 nm. During the procedure, washing the plate was according to the ELISA routine method.

Sensitivity and specificity of the assay; the minimum detectable dose of human Annexin A2 is typically less than 0.256 ng/mL. This assay has high sensitivity and excellent specificity for detection of human Annexin A2. No significant cross-reactivity or interference between human annexin A2 and analogues was observed.

Additionally, serum viral markers (HBs Ag and HCV Ab) and serum α -fetoprotein. AFP were measured by commercially available chemiluminescence detection methods (Immulite, Roche, Germany).

Statistical analysis

Samples were grouped into HCC, liver cirrhosis and normal group. Statistical analyses were conducted using SPSS version 10.0 (SPSS Inc, Chicago, IL). The levels of annexin A2 in the sera were shown as the mean \pm standard deviation (mean \pm SD), groups were compared using parametric (ANOVA and T student test) and non parametric method (Kruskal-Wallis, Mann-Whitney and Spearman coefficient of correlation tests) according to normal distribution acceptance or rejection. The levels of annexin A2 and AFP were additionally evaluated by receiver-operating characteristic curve (ROC) analysis to determine the diagnostic performance. The optimal cutoffs were determined using the maximum sum of sensitivity and specificity. $P < 0.05$ was considered to be statistically significant.

RESULTS

Circulating annexin A2 and AFP level in patient groups: The levels of circulating Annexin A2 and AFP in 115 patients with liver diseases and 20 normal controls are shown in table (3). The mean level of serum annexin A2 expression in HCC patients were significantly higher than in the cases with liver cirrhosis (Group 3 or normal control (Group 4) ($P < 0.001$), there was significant difference between early stage HCC and liver cirrhosis (t ; 5.8 and $P < 0.001$) and normal control (t ; 5.7 and $P < 0.001$) while no significant difference between liver cirrhosis and normal control (t ; 0.4 and p ; 0.3) The diagnostic cutoff value was more than 18 ng/ml and the incidence of circulating annexin A2 was 80.4% in the HCC patient, 14.3% in the liver cirrhosis, and zero in the normal control group. With comparison of AFP median in studied groups, there is a significant difference between HCC group 2 and each of HCC group1 (Mann-Whitney Z ; 2.7 and p ; 0.007) and liver cirrhosis group3 (Mann-Whitney Z ; 3.1 and p ; 0.005) Normal control group 4 (Mann-Whitney, Z ; 5.7 and $P < 0.001$), also between group 3 and group 4 (Mann-Whitney, Z ; 5.1 and $P < 0.001$) While no significant difference between early stage HCC group and liver cirrhosis (Z ; 0.25 and P ; 0.7). The diagnostic cutoff value for AFP was more than 32 ng/mL and the incidence of serum AFP in the HCC patients (56 of 37; 70.1%) and in liver cirrhosis (18 of 35; 51.4%) and zero in normal control.

Annexin A2 and AFP as complementary tumor markers

The rank correlation coefficient between serum annexin A2 and AFP values in HCC patients was not significant (ρ ; 0.12 and P ; 0.27) indicating that measuring both markers in serum can improve the diagnostic value (Table 2).

Relation of circulating Annexin A2 to clinical and serological finding in HCC group

There was no significant relation with age, gender, S. albumin, S. bilirubin, S.ALT and hepatitis C infection (Table 3). However there was a significant relation with hepatitis B infection, ($t=6.04$, $P < 0.001$) as a significant difference when comparing annexin A2 values among positive and negative HBsAg.

ROC Curve

The specificity and sensitivity of serum annexin A2 and AFP measurements were represented by ROC curves. Area under ROC curve for AFP (AUC) was 0.84 with 0.75 to 0.910, 95% confidence interval with $p < 0.0001$, the AUC for Annexin A2 was 0.89 with 0.81 to 0.95, 95% confidence interval with $P < 0.0001$, AUC for both markers was 0.818 with 0.723 to 0.890, 95% confidence interval with $P < 0.0001$, when comparing the sensitivity and specificity of annexin A2, AFP or both markers they were; (80.4% and 91% for Annexin A2 and 70.1% and 65.5% for AFP and 91.9% and 63.6% for both).

Table 1 Serum annexin A2 and α -fetoprotein in studied groups.

	Group	N	Mean \pm SD	Median	P value
Annexin A2 ng/mL	Group1	37	35 \pm 19	34	F : 26.8 <0.001 ¹
	Group 2	43	66.3 \pm 47	46	
	Group 3	35	14.4 \pm 5.5	13	
	Group 4	20	10.9 \pm 4.7	9.0	
α-fetoprotein U/L	Group1	37	32.2 \pm 23	23.0	Ht: 58.0 <0.001 ²
	Group 2	43	126 \pm 133	80.0	
	Group 3	35	32.5 \pm 24	25.0	
	Group 4	20	12.3 \pm 5.9	12.0	

¹ There is no significant difference in comparison of Group 3 and Group 4 for Annexin A2 levels; ² There is no significant difference in comparison of Group 1 and Group 3 for AFP levels.

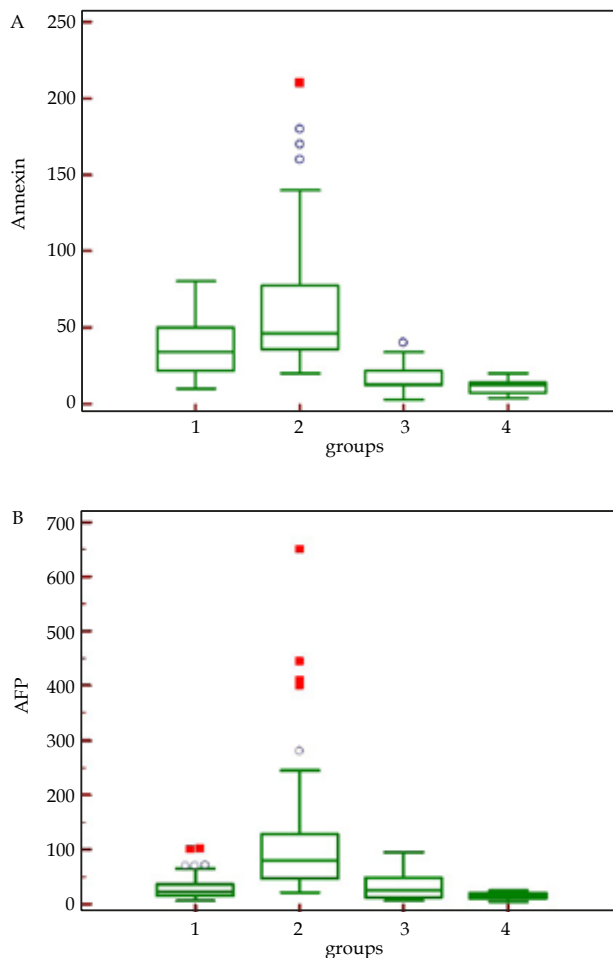


Figure 1 A: comparison of S. annexin A2 levels in studied groups. There is significant difference among studied groups, while there is no significant difference between liver cirrhosis (group3) and normal control (group 4), (t ;1.4 and p ; 0.3); B: comparison of S. α -fetoprotein levels in studied groups, There is significant difference among studied groups while no significant difference between group 1 and group3 (z ; 0.25 and P ; 0.7).

Table 2 Correlation coefficient between serum annexin A2 and AFP values in HCC patients.

Parameter	Rho	P
AFP	0.12	0.27

Table 3 Relationship of Serum Annexin A2 (mean \pm SD) with other parameters in hepatocellular carcinoma group.

Parameter	Character	N	ng/mL	t-test	P value
Sex	Male	14	68 \pm 51	0.77	0.4
	Female	14	54 \pm 52		
Age	<50 y	38	65 \pm 54	0.34	0.7
	\geq 50 y	42	59 \pm 51		
Serum ALT	<80 u/L	35	61 \pm 60	0.12	0.9
	\geq 80 u/L	45	63 \pm 47		
Serum Alb	<3 g/dL	48	62 \pm 55	0.4	0.6
	\geq 3 g/dL	32	55 \pm 50		
S. Bilirubin	<2 mg/dL	28	65 \pm 46	0.53	0.5
	\geq 2 mg/dL	52	55 \pm 54		
HBs Ag	Positive	12	118 \pm 47	6.04	<0.001
	Negative	68	36 \pm 32		
HCV Ab	Positive	56	68 \pm 62	0.7	0.46
	Negative	24	55 \pm 40		

HbsAg; Hepatitis B surface antigen; HCV Ab: Hepatitis C virus antibodies.

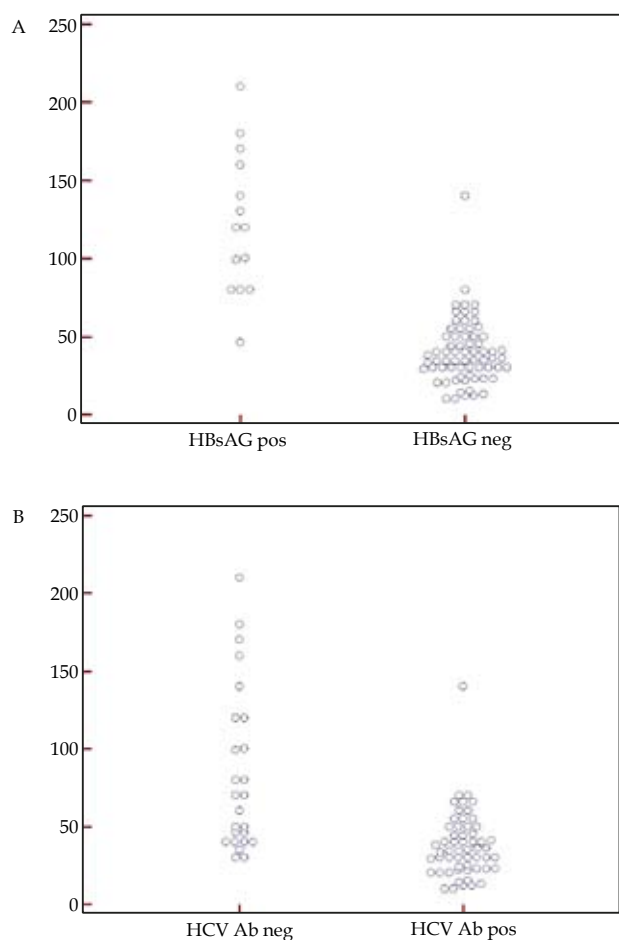


Figure 2 A: Relationship between serum annexin A2 level and hepatitis B virus infection in hepatocellular carcinoma patients. The scatter figure indicated that serum annexin A2 level was obviously correlated with hepatitis B virus (HBV) infection. The serum annexin A2 level in hepatocellular carcinoma (HCC) patients was higher ($t=6.04$, $P<0.001$) in the positive-surface antigen of the HBV (HBs Ag) group than that in the negative-HBs Ag group; B: Relationship between serum annexin A2 level and hepatitis C virus infection in hepatocellular carcinoma patients. The scatter figure indicated that serum annexin A2 level was not correlated with hepatitis C virus (HCV) infection.

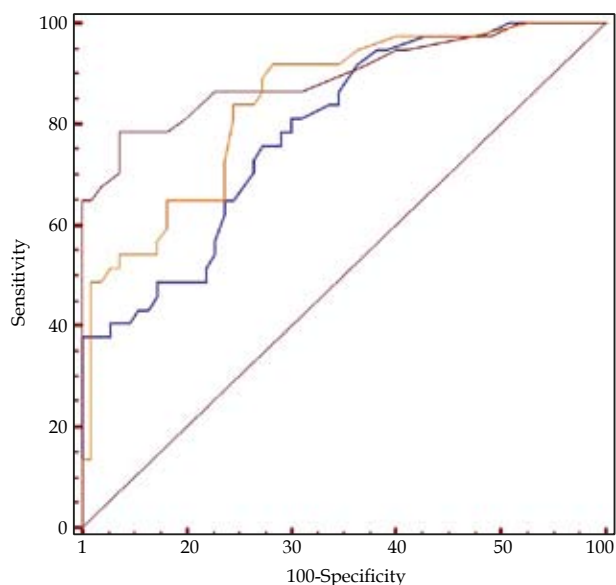


Figure 3 ROC curve for comparison between annexin A2 and AFP and both markers in sensitivity and specificity.

Table 4 The accuracy and efficiency of serum annexin A2 and/or AFP levels for hepatocellular carcinoma diagnosis.

Parameter	AFP %	Annexin A2 %	Both %
Sensitivity	70.1	80.4	91.9
Specificity	65.5	91	63.6
PPV	61.8	84	63
NPV	79.1	86	92

PPV: Positive predictive value; NPV: Negative predictive value; Serum annexin A2 (ANXA2) level >18 ng/mL or α -fetoprotein (AFP) level >32 ng/mL was abnormal.

DISCUSSION

Hepatocellular carcinoma (HCC), as one of the most malignant tumors, is the third leading cause of cancer-related death. HCC prognosis is poor, and early detection is of the utmost importance. Although serum AFP is a useful biomarker for the detection and monitoring of HCC, the false-negative rate using the AFP level alone may be high, so, AFP levels have low sensitivity and specificity, particularly during the early stages of cancer. Several biomarkers, such as des- γ -carboxyprothrombin/ protein induced by vitamin K absence-II (PIVKA II), lens culinaris agglutinin-reactive (AFP-L3) and glypican-3 have been examined for their ability to detect early HCC^[30,31,32] but, these markers also have poor sensitivity and specificity, many studies tended to detect another laboratory markers helping in diagnosis of HCC more sensitive and more specific was up regulated in HCC and it could be a useful molecular marker for HCC^[22,33,34]. Present study detected that serum annexin A2 was significantly increased in HCC patients compared with healthy controls and cirrhotics groups, which agreed with Sun *et al.*^[35].

ANXA2 induces cell migration and neoangiogenesis via tissue plasminogen activator dependent plasmin generation^[36] represents metastatic potential^[37], and promotes invasion and migration of HCC in vitro via its interaction with HAB18G/CD147^[38].

Moreover, Tyr23 phosphorylation-dependent cell-surface localization of ANXA2 is required for invasion and metastases^[39]. The clinicopathologic features of circulating ANXA2 expression in HCC patients demonstrated that there is a very close relationship between ANXA2 level and invasion and metastasis.

Although the mechanisms of hepatocarcinogenesis have not been elucidated, a long-lasting inflammation induced by hepatitis virus infection is a definite risk for neoplastic degeneration and the accumulation of genetic alterations^[40]. Our results detected there was no significant relationship between age and sex and the level of annexin A2 in control and patients group, this result is agreed with SUN *et al.*^[35].

Our Study dealt with underlying infection with HBV and HCV infection with HCC patients, it detected high significant increasing annexin A2 level in positive HBV hepatocellular carcinoma patients but there were no significant increasing in positive HCV hepatocellular carcinoma patients. Annexin A2 was found as up-regulated gene in HCC tissue and HBV-associated hepatocellular carcinoma patients^[41]. Importantly, this study reported that HBV-related HCC cells express approximately 3-fold higher annexin A2 compared with hepatitis C virus-related HCC, another study reported that higher level of Annexin A2 expression in HCC patients was correlated with HBV infection^[35].

One explanation showed that annexin A2 upregulated in viral HBx gene transfected HepG2, suggesting that it might play roles in HBV-induced HCC^[42]. This study found no significant relationship between annexin A2 and liver enzymes (ALT and AST), and this agreed with SUN *et al.*^[35] who detected serum ALT level were not correlated with annexin A2, also he suggested that liver injury was not the major

cause to release annexin A2 in HCC. lastly, our results detected the specificity and sensitivity of annexin A2 in HCC patients is 91% and 80.4% respectively with good negative predictive value(86%),beside that complementary diagnostic value of AFP and annexin A2 was more and more sensitive and higher negative predictive value (91.9% and 92% respectively) however with decreased specificity (63.6%). Many studies dealt with accuracy and efficacy of annexin A2 alone and with AFP as serum marker for HCC as One of them, Zhang *et al*^[40] detected that sensitivity and specificity of annexin A2 of HCC for diagnosis of HCC was 86.96% and 66.67% respectively,and also with AFP was 96.52% and 68.67% (sensitivity and specificity respectively). In conclusion our research detected that serum annexin A2 has higher sensitivity, specificity and negative predictive value than AFP alone and complementary diagnostic value in combination with AFP for HCC diagnosis as regard sensitivity and negative predictive value.

We recommended for other studies to make examinations on a large scale of patients, and for other ethnic groups.

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