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The Utility of Serum Neuropilin 1 as a Diagnostic Biomarker for Hepatocellular Carcinoma in Egyptian Cirrhotic Patients

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ABSTRACT

Background: In recent decades, hepatocellular carcinoma (HCC) has become the prevailing type of primary hepatic cancer, with its incidence rising dramatically. Many HCC patients are identified at an advanced, inoperable stage, limiting curative treatment options and resulting in poor survival rates.

Aim: The study aimed to evaluate the role of Neuropilin 1 (NRP1) as a new serum diagnostic marker for hepatocellular carcinoma in cirrhotic Egyptian patients.

Subjects and methods: This case control study encompassed 60 individuals, aged 18 and above, recruited from The Hepatology and Internal Medicine Clinics and Hospital Units between January 2024 and June 2024 at Ain Shams University Hospital. The patients were classified as follows: Group 1 consisted of 30 HCC patients through alpha-fetoprotein testing and imaging. Group 2 comprised 30 cirrhotic patients, devoid of HCC, matched and categorized following the Child-Pugh scoring system.

Results: This investigation of Egyptian patients found that HCC typically emerges in the sixth decade of life and is often linked to liver cirrhosis. Patients with HCC exhibited significantly elevated serum alpha-fetoprotein (AFP) levels. Moreover, NRP1, a recently discovered biomarker, showed a substantial increase in HCC patients compared to cirrhosis patients. This elevation correlated strongly with liver function indicators (AST, ALT, AFP & albumin) and Barcelona and Child-Pugh scores. NRP1 demonstrated superior sensitivity (96.67%) and specificity (80%) in diagnosing HCC, unlike AFP, highlighting its capability as a more effective indicator for diagnosing HCC.

Conclusion: Serum NRP1 levels were notably elevated in HCC patients. In contrast, cirrhotic patients showed only a slight increase in NRP1 levels compared to the HCC group.

Keywords: Hepatocellular carcinoma, Egyptian cirrhotic, Serum neuropilin 1, Diagnostic biomarker.

INTRODUCTION

Hepatocellular carcinoma represents close to 80% of the global incidence of primary hepatic cancer. It is a considerable health challenge and is a primary contributor to cancer-related mortality in numerous areas. Globally, HCC is the fourth prevailing reason for cancer-linked deaths [1]. Alpha-fetoprotein (AFP) is the prevailing blood-based marker utilized for HCC diagnosis and prognosis. Nevertheless, the reliability of AFP has been questioned, as approximately 30% of patients with early-stage HCC do not exhibit detectable AFP levels [2].

While NRP1 has been extensively studied and documented, NRP2 has received less attention. Nevertheless, recent studies have underscored the importance of both neuropilins (NRPs) in cancer, including HCC, due to their robust connection with essential tumor development-derived cytological and molecular functions [3].

The function of NRP1 in the HCC progression remains inadequately elucidated. NRP1 expression has been found in the cellular environment of liver sinusoids including endothelial and stellate cells and the Hepa 129 hepatoma cell line. Its levels have been manifested to rise under shear stress conditions, after partial hepatectomy, and liver cirrhosis, indicating its contribution in NRP1 in hepatic function and its potential association with liver carcinogenesis [4].

SUBJECTS AND METHODS

This case-control study was previously posted as a preprint version on research square (DOI: 10.21203/rs.3.rs-5706222/v1), on 28th of December 2024.

Participants: This study encompassed 60 individuals aged 18 and above who were recruited from the hepatology and internal medicine clinics and hospital units between January 2024 and June 2024 at Ain Shams University Hospital. The 60 Egyptian patients were classified as follows: **Group 1** consisted of 30 HCC patients through alpha-fetoprotein testing and imaging. **Group 2** comprised 30 cirrhotic patients, devoid of HCC matched and categorized following the Child-Pugh scoring system.

Setting of the study: This study was performed in The Hepatology and Internal Medicine Department Outpatient Clinic, and clinical pathology lab at Ain Shams University Hospitals, under the supervision of Dr. Mariem Elsaid, Lecturer of Clinical Pathology at Ain Shams University.

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Inclusion criteria: The study encompassed liver cirrhosis patients over 18 years old, with or without HCC.

Exclusion criteria: Individuals under 18 years old, pregnant women, malignant patients other than HCC, and those who declined participation.

Sample distribution: The 60 patients were allocated into two groups: Group A (Case) comprised of 30 patients experienced both liver cirrhosis and HCC, whereas group B (Control) consisted of 30 liver cirrhosis patients without HCC.

Study tools and procedures: Participants underwent comprehensive medical history taking and thorough clinical examinations. Laboratory tests encompassed coagulation studies (PT, PTT & INR), complete blood count, kidney function tests (BUN & creatinine), electrolytes (Na & K), liver function tests (ALT, direct bilirubin, AST, serum albumin and total bilirubin), and tumor markers (Alpha-fetoprotein and NRP1). Imaging studies involved pelviabdominal ultrasound and any identified liver abnormalities warranting further investigation were assessed using contrast-enhanced triphasic computed tomography or dynamic magnetic resonance imaging. Data analysis assessed the sensitivity and specificity of serum NRP1 in diagnosing HCC. Stringent safety measured and personal protective equipment was employed to ensure participant and staff safety.

Assay principle: Enzyme-Linked Immunosorbent Assay (ELISA) was utilized to quantify Human NRP1 (Bioassay Technology Laboratory (BT Lab), Shanghai, China). The process involved binding sample NRP1 to pre-coated antibodies, followed by addition of biotinylated Human NRP1 antibody, which bind to streptavidin-HRP upon adding it. A substrate solution was added after washing, producing color proportional to NRP1 concentration. Afterward, an acidic stop solution was introduced, halting the reaction, and the absorbance was quantified at 450 nm.

Ethics approval and consent to participate: Ethical protocol: All study participants provided written informed consent. Confidentiality was maintained, with no patient names disclosed in any publications. The study commenced only after approval from The Scientific Research Ethics Committee of Ain Shams University's Faculty of Medicine (Ethical number FWA000017585- Ethical approval date 17-1-2024). The study adhered to Helsinki Declaration through its execution.

Statistical analysis

The collected data were examined, encoded, and put into IBM SPSS version 27. For parametric distributions, quantitative data were summarized using means, standard deviations, and ranges, while medians and interquartile ranges (IQR) were utilized for non-parametric distributions. Qualitative variables were reported using percentages and frequencies. The Chi-square test was employed to compare qualitative data between groups, whereas Fisher's exact test was utilized when expected cell counts fell below 5. To compare two independent groups, the independent t-test and Mann-Whitney test were employed for parametric quantitative and non-parametric data, respectively. The Kruskal-Wallis test was employed for comparisons among many groups with non-parametric quantitative data. Spearman's correlation coefficients were employed to evaluate the correlations between two quantitative variables within a single group. The receiver operating characteristic curve was deployed to ascertain the optimal threshold, in addition to evaluating negative and positive predictive values, specificity, sensitivity, and the area under the curve for marker under examination. A 95% confidence interval and 5% error margin were applied. Significance levels were defined as: $P > 0.05$ (non-significant, NS), $P \leq 0.05$ (significant, S), and $P \leq 0.01$ (highly significant, HS).

RESULTS

Here, we enrolled 60 subjects, evenly allocated into: 30 cirrhotic patients experiencing HCC and 30 cirrhotic patients without HCC. Participants were enrolled from Ain Shams University Hospitals following the acquisition of informed consent. All patients were matched according to sex and age (a mean age: 61 years). Furthermore, HCC patients exhibited a significant rise in AFP and NRP1 level, unlike cirrhotic patients, devoid of HCC. Moreover, NRP1 demonstrated significant positive connections with AST, ALT & MELD among all cases as well as NRP1 & ALT in the cirrhotic group and NRP1 and ALT, AST & Barcelona score in the HCC group. Conversely, among all cases, NRP1 revealed a statistically significant adverse connection with albumin (**Table 1**).

Table (1): Comparison between group A (HCC) and group B (Cirrhotic) regarding (Liver profile, complete blood picture, Kidney function, electrolyte, INR, AFP & neuropilin 1)

	Median (IQR)	Median (IQR)	value	P value	sig.
	Cirrhotic	HCC	Mann Whitney test		
TLC (10^3/mm^3)	4.5 (3.7 - 8.2)	5 (3.9 - 6.4)	z = -0.17	0.865	NS
Hb (g/dl)	10.85 (9 – 12)	11.15 (9.6 - 13)	z = -1.243	0.214	NS
Platelet (10^3/mm^3)	119.5 (64 - 177)	134.5 (90 - 181)	z = -0.92	0.355	NS
BUN (mg/dl_)	21.5 (14 - 34)	25 (19 - 30)	z = -0.8	0.424	NS
CR (mg/dl)	0.9 (0.7 - 1.3)	0.9 (0.7 – 1.1)	z = -0.601	0.548	NS
Na (mg/dl)	135.5 (132 - 137)	132.5 (130 - 135)	z = -1.51	0.13	NS
K (mg/dl)	4.1 (3.5 – 4.6)	4 (3.3 – 4.3)	z = -1.044	0.296	NS
Total bilirubin (mg/dl)	2.2 (0.8 - 5.7)	1 (0.7 - 2.1)	z = -1.47	0.141	NS
Direct bilirubin (mg/dl)	0.85 (0.3 - 2.4)	0.5 (0.3 - 0.7)	z = -1.34	0.18	NS
AST (mg/dl)	39 (29 - 85)	47 (27 - 70)	z = -0.08	0.935	NS
ALT (mg/dl)	26.5 (12 - 53)	35.5 (17 - 51)	z = -1.21	0.228	NS
Albumin (g/dl)	2.85 (2.3 – 3.7)	3.15 (2.46 – 3.6)	z = -0.844	0.398	NS
INR	1.5 (1.20 – 1.6)	1.2 (1.2 – 1.53)	z = -1.492	0.136	NS
AFP (ng/ml)	2.65 (1.65 – 5.6)	39.5 (5.8 - 350)	z = -4.266	<0.001	S
Neuropilin1 (ng/ml)	5.65 (4.23 - 10.65)	42.19 (23.97 - 49)	z = -6.14	<0.001	S

TLC: Total leucocytic count, **Hb:** Hemoglobin, **BUN:** Blood urea nitrogen **CR:** Creatine, **Na:** Sodium, **K:** potassium, **AST:** Aspartate aminotransferase, **ALT:** Alanine transaminase, **INR:** International normalized ratio, and **AFP:** Alpha-feto protein.

No statistically significant correlations were found between NRP1 and Hb, TLC, platelet, BUN, CR, TB, DB, NA, K, INR & child score among all cases. Similarly, no significant correlations were observed between NRP1 and TLC, Hb, Platelet, BUN, CR, DB, AST, INR, CHILD, MELD, NA, K & TB) in the cirrhotic group, or between NRP1 and TLC, Hb, platelets, BUN, CR, DB, TB, INR, CHILD, MELD, NA & K) in the HCC group (Table 2).

Table (2): Correlation of Neuropilin 1 level with other studied parameters among all cases, group A (HCC) and group B (cirrhotic).

	Neuropilin1 (ng/ml)								
	Whole sample (N=60)			Cirrhotic (N=30)			HCC (N=30)		
	r	P-value	Sig.	r	P-value	Sig.	r	P-value	Sig.
TLC	0.049	0.712	NS	0.073	0.7	NS	0.016	0.934	NS
Hb	0.243	0.062	NS	0.332	0.073	NS	0.079	0.68	NS
Platelets	0.104	0.431	NS	0.097	0.611	NS	-0.017	0.931	NS
BUN	0.21	0.107	NS	0.089	0.64	NS	0.324	0.081	NS
CR	-0.079	0.546	NS	-0.048	0.801	NS	-0.028	0.883	NS
NA	-0.194	0.138	NS	0.088	0.642	NS	-0.192	0.308	NS
K	-0.122	0.351	NS	-0.233	0.215	NS	0.149	0.433	NS
Total bilirubin	-0.137	0.296	NS	-0.197	0.297	NS	0.27	0.149	NS
Direct bilirubin	-0.178	0.174	NS	-0.196	0.299	NS	0.091	0.631	NS
AST	0.310*	0.016	S	0.315	0.09	NS	0.671**	0	HS
ALT	0.504**	0	HS	0.601**	0	HS	0.704**	0	HS
Albumin	-0.274*	0.034	S	-0.689**	0	HS	-0.561**	0.001	HS
INR	-0.131	0.318	NS	0.102	0.593	NS	0.05	0.794	NS
CHILD score	0.123	0.35	NS	-0.245	0.192	NS	0.057	0.764	NS
MELD score	0.482*	0	HS	0.149	0.433	NS	0.186	0.324	NS

TLC: Total leucocytic count, Hb: Hemoglobin, BUN: Blood urea nitrogen CR: Creatine, Na: Sodium, K: potassium, AST: Aspartate aminotransferase, ALT: Alanine transaminase, INR: International normalized ratio, and AFP: Alpha-feto protein.

Significant correlation between neuropilin 1 level with BARCELONA score in group A (HCC) ($p<0.001$) (Table 3).

Table (3): Correlation of Neuropilin 1 level with BARCELONA score in group A (HCC)

		Neuropilin1 (ng/ml)	Kruskal-Wallis test		
		Median (IQR)	k	P-value	Sig.
BARCELONA	A	16.94 (13.48 – 19.63)	20.855	<0.001	HS
	B	31.38 (26.63 – 36.12)			
	C	48 (42.09 – 49)			
	D	50 (50 – 51)			

The threshold for NRP1 concentration to distinguish between HCC and cirrhotic cases was determined to be >10.7 ng/ml with 96.67% sensitivity, 80% specificity, and AUC of 96.1%. For AFP, the threshold was >5.6 ng/ml, with 80% sensitivity, 76.67% specificity, and an AUC of 82.1% (**Figure 1 and table 4**).

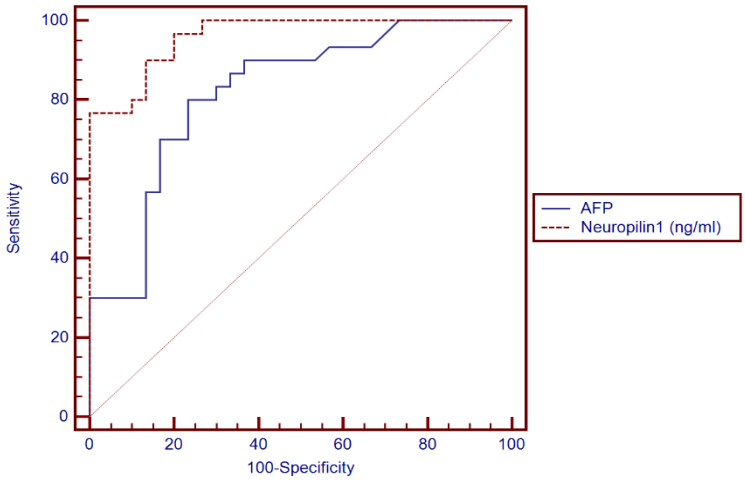

Figure (1): ROC curve to assess neuropilin 1 and AFP levels to differentiate between Group A (HCC) and Group B (Cirrhotic).

Table (4): Comparative Diagnostic Accuracy of AFP and Neuropilin-1 for Detecting Hepatocellular Carcinoma

	Cut off point	AUC	95% CI	Sensitivity	Specificity	+PV	-PV	P-value
AFP	>5.6	0.821	0.700 to 0.908	80	76.67	77.4	79.3	<0.001
Neuropilin1 (ng/ml)	>10.7	0.961	0.877 to 0.994	96.67	80	82.9	96	<0.001

DISCUSSION

This study included 60 subjects aged 28 to 91 years, recruited from Ain Shams University Hospitals following the acquisition of written informed consent. The participants were categorized into (30 subjects/group): Group A (HCC): Cirrhotic patients with HCC and Group B (cirrhotic): cirrhotic patients, devoid of HCC.

Our research revealed a notable elevation in serum AFP levels among HCC patients, unlike cirrhotic patients. This observation aligns with several studies, including one that recognizes elevated serum AFP as a diagnostic indicator for HCC when used in conjunction with triphasic CT abdomen or dynamic MRI [5]. Furthermore, various guidelines suggest combining serum AFP with abdominal ultrasound for screening cirrhotic patients to enable early HCC detection. These outcomes reinforce the ongoing AFP reliability as an HCC indicator.

Regarding serum NRP1, a novel marker examined in this study, our results showed that the HCC group demonstrated significantly elevated NRP1 levels contrasted with the cirrhotic group (*P*-value < 0.001). This is corroborating a previous study by **Mostafa *et al.*** [6], which demonstrated through cross-sectional analyses that HCC patients experienced significantly greater NRP1 levels than cirrhosis patients.

Our research revealed no statistically significant connection between NRP1, and platelet count or INR in both HCC and cirrhotic groups. This finding contradicts a previous study by **Hoda and Dalia** [7], which reported a significant inverse correlation with platelet count and a positive correlation with INR. We found no statistically significant relationship between NRP1 and factors such as total leucocytic count (TLC), hemoglobin (Hb), and creatinine, which aligns with earlier study [(6)]. Furthermore, our study manifested no statistical correlation between NRP1 and bilirubin, which aligns with the findings of **Lin *et al.*** [8].

Our research identified that NRP1 experienced a significant correlation with AST, ALT, AFP, and albumin, which agrees with the findings of **Lin *et al.*** [8]**.** We observed that MELD score had a positive and significant association with serum NRP1 levels, supporting the results of **Hoda and Dalia** [7]. However, no significant connection between NRP1 levels and CHILD score was observed, which is consistent with the findings reported by **Mostafa *et al.*** [6].

Our outcomes revealed that the Barcelona score had a significant correlation with NRP1 levels, which is aligning with the results of **Abdel Ghafar *et al.*** [9] who noted elevated serum NRP1 levels in tumor stages B/C per the Barcelona staging system.

In our research, serum NRP1 manifested high sensitivity (96.67%) and specificity (80%) in detecting HCC, with an AUC of 0.961 at a threshold value > 10.7 ng/ml. This compares to the findings of **Mostafa *et al.*** [6], which reported a sensitivity of 93.3% and specificity of 80% (AUC = 0.842, threshold value = 4030 pg/ml).

Herein, we manifested that AFP exhibited a sensitivity of 80% and specificity of 76.67% in detecting HCC, indicating lower performance compared to NRP1. This supports the findings of **Lin *et al.*** [8]**,** which suggested that serum NRP1 serves as a superior diagnostic indicator compared to AFP, with an AUC of 0.971 for NRP1 versus 0.862 for AFP.

LIMITATIONS

Our study encompassed a small patient sample size and lack of investigations to exclude other malignancies, such as mammograms or colonoscopies.

CONCLUSION

Serum NRP1 levels were markedly greater in HCC patients and slightly elevated in liver cirrhotic patients but remained lower than those in the HCC group. NRP1 exhibited superior sensitivity and specificity compared to AFP. Integrating NRP1 into diagnostic protocols could improve HCC detection and facilitate early identification in surveillance programs, especially when used in conjunction with AFP.

ABBREVIATIONS

AFP: Alpha-feto protein **AST:** Aspartate aminotransferase **ALT:** Alanine transaminase. **AUC:** Area under curve **Cr:** creatinine. **Db:** Direct bilirubin, **HB:** Hemoglobin. **HCC:** Hepatocellular carcinoma. **INR:** International normalized ratio. **K:** potassium. **NRP:** Neuropilin. **Na:** sodium **Tb:** Total bilirubin. **TLC:** Total leucocytic count.

DECLARATIONS

- **Publication Consent:** Not applicable.
- **Data Availability:** Data are accessible from the authors upon request.
- **Conflict of interests:** No Conflict of Interests.
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