

RAPID COMMUNICATION

Low utility of plasma Nociceptin/orphanin FQ in the diagnosis of hepatocellular carcinoma

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Abstract

AIM: The utility of serum alpha-fetoprotein (α -FP) in the detection of hepatocellular carcinoma (HCC) is questionable. Very high circulating levels of nociceptin/orphanin FQ (N/OFQ), a ligand for a novel opioid receptor, have recently been reported in HCC. The aim of this study was to assess the role of plasma N/OFQ in the diagnosis of HCC arising in patients with liver cirrhosis.

METHODS: Plasma N/OFQ levels were measured by ELISA in 58 patients (28 HCC and 30 liver cirrhosis) and in 25 healthy controls. The values were correlated with clinical and laboratory features including α -FP. Spearman index, biserial correlation coefficient, non parametric combination (NPC) test and discriminant stepwise analysis were used for statistical evaluation of data.

RESULTS: The upper normal limit of nociceptin was 122 pg/mL. Plasma levels above this cut-off were found in 21.4% of patients with HCC, in 23.3% of those with cirrhosis and in 8% of healthy subjects. α -FP serum levels > 200 ng/mL were found in 46.4% of the patients with HCC and in none of those with cirrhosis. No correlation was found between N/OFQ levels and any of the clinical and laboratory features, including α -FP. By NPC test, HCC and cirrhotic patients were different with regard to α -FP ($P = 0.000$) but not in terms of nociceptin ($P = 0.595$). By point biserial correlation, HCC presence was positively correlated with α -FP ($rpb = 0.52$, $P = 0.000$) but not with N/OFQ ($rpb = 0.16$, $P = 0.157$). In a discriminant analysis, α -FP was significant in the Wilks test ($Y = -0.709 + 0.03 \alpha$ -FP) and properly classified

81% of all patients and 61% of HCC. N/OFQ had lower sensitivity, specificity and predictive values than α -FP.

CONCLUSION: Nociceptin is increased in patients with chronic liver disease, independently of the presence of HCC, although the underlying mechanism has yet to be clarified. We conclude it is not a useful marker for HCC.

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Key words: Hepatocellular carcinoma; Nociceptin/orphanin FQ; Liver cirrhosis; Alpha-fetoprotein

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INTRODUCTION

Nociceptin/orphanin FQ (N/OFQ) is a 17-aminoacid neuropeptide with selective binding affinity for a novel opioid receptor-like 1 (ORL1/NOP). N/OFQ is synthesized in the neurons of the central and peripheral nervous system and is present in the blood and in the cerebrospinal fluid. The N/OFQ/NOP system has been implicated in a variety of biological functions at both central and peripheral levels: pain modulation, immunity, memory, learning, feeding, locomotion, thermoregulation and activities of the gastrointestinal, cardiovascular, renal and respiratory systems^[1,2]. However, its precise biological role in humans has not been fully defined. The presence of N/OFQ/NOP has also been reported in human neuroblastoma cell-lines^[3]. N/OFQ is detectable in the blood and high circulating levels have been reported in incidences of acute and chronic pain, where they are correlated with the duration of symptoms^[4]. In Wilson disease, probably in relation to altered catabolism caused by liver and/or brain deposits of copper^[5], and in cirrhosis of the liver^[6] slightly increased circulating levels of N/OFQ have recently been found. Moreover, very high

levels have been reported in patients with hepatocellular carcinoma (HCC) suggesting that plasma N/OFQ level might represent a specific marker for HCC^[6].

HCC is the leading cause of death in patients with cirrhosis of the liver^[7]. The most commonly used serological marker for HCC surveillance, alpha-fetoprotein (α -FP), has highly variable sensitivity and specificity^[8] while the diagnostic utility of other markers, such as des- γ -carboxyprothrombin^[9], human hepatocyte growth factor^[10], and serum chromogranin-A^[11] remains unclear.

The aim of this study was to investigate the utility of plasma N/OFQ levels in the diagnosis of HCC in patients with liver cirrhosis.

MATERIALS AND METHODS

Patients and healthy subjects

Fifty-eight consecutively observed patients, 28 with HCC and 30 with liver cirrhosis, and 25 healthy members of the medical staff were included in the study. Informed written consent was obtained from patients and healthy controls. The diagnosis of HCC was based on imaging findings and/or histological confirmation. The diagnosis of liver cirrhosis was clinical and/or histological. The demographic and clinical characteristics of the populations studied are shown in Table 1.

Parameters examined

Clinical features (age, gender, etiology, Child-Pugh score, HCC size, presence of pain) and laboratory parameters as reported in Table 2 (plasma N/OFQ, serum α -FP, blood glucose, blood urea, serum creatinine, K, Na, Ca, bilirubin, aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transpeptidase, alkaline phosphatase, lactic dehydrogenase, creatine phosphokinase, amylases, cholinesterase, albumin, erythrocyte sedimentation rate, C-reactive protein, fibrinogen, international normalized ratio, hemoglobin, red blood cells, white blood cells, platelets) were evaluated through statistical analysis.

N/OFQ assay

Blood drawn from fasting subjects between 7.30 and 9.30 AM was collected in tubes containing K-EDTA. Aprotinin was immediately added to inhibit proteases. Plasma samples, stored at -80°C for less than one month, were loaded onto C-18 containing Sep-columns (Phoenix Europe GmbH, Karlsruhe, Germany), washed with 0.1% trifluoroacetic acid, eluted with 60% acetonitrile in 1% trifluoroacetic acid and freeze-dried. ELISA assay of N/OFQ was performed using a commercially available kit (Phoenix Europe GmbH, Karlsruhe, Germany). Briefly, the lyophilized samples reconstituted with assay buffer were placed in microwells, together with rabbit anti-orphanin FQ serum and biotinylated peptide. After 2 h incubation at room temperature, streptavidin-horseradish peroxidase was added followed by a 1 h incubation. After washing, 100 μ L of substrate solution was added and the reaction was terminated after 1 h with 100 μ L of 2N HCl. Absorbance was read at 450 nm and results were compared to a standard curve ranging from 10 to 100.000 pg/mL.

Table 1 Clinical-Demographic characteristics of the populations studied

	HCC	Cirrhosis	Controls
Number of patients	28	30	25
Mean age \pm SD (yr)	70 \pm 4.8	65.3 \pm 11.1	35.4 \pm 13.9
Male/Female	20/8	21/9	10/15
Etiology <i>n</i> (%)			
HCV	24 (85.8)	15 (50)	
HBV	-	3 (10)	
Alcohol	2 (7.1)	8 (26.7)	
Cryptogenic	2 (7.1)	4 (13.3)	
Child - Pugh score <i>n</i> (%)			
A	22 (78.6)	19 (63.3)	
B	6 (21.4)	7 (23.3)	
C		4 (13.3)	
Patients with pain (%)	4 (14.3)	5 (16.6)	6 (24)
HCC diameter min-max (cm)	1.2-12.5		

HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma.

Statistical analysis

Correlations among the variables studied were calculated using non-parametric Spearman index and biserial correlation coefficient^[12], as appropriate. Differences between groups were evaluated using the Non Parametric Combination Test, NPC^[13]. $P < 0.05$ was considered as statistically significant. Variables discriminating the groups of patients were identified with discriminant stepwise analysis (Wilks test and Classification results)^[14]. The upper normal limit of N/OFQ was defined as the 95th percentile of the values in healthy controls. Software packages used were Methodologica S.R.L. (2001) for non-parametric analysis NPC test, Confidence Interval Analysis (CIA) Windows version 2.0 (2000) for sensitivity and specificity evaluation, and SPSS, Windows 11.0 (2001) for correlation index and discriminant analysis.

RESULTS

The upper normal limit of N/OFQ was 122 pg/mL. Plasma levels above this cut-off were found in 6 patients with HCC (21.4%), in 7 patients with cirrhosis (23.3%) and in 2 healthy subjects (8%). α -FP serum levels > 200 ng/mL, reported in the literature as highly suggestive for HCC^[20], were found in 13 patients with HCC (46.4%) and in none of the patients with cirrhosis (Figure 1).

In all the groups, N/OFQ levels were not correlated with any of the demographic-clinical features nor with the laboratory parameters mentioned in the materials and methods section. In particular, N/OFQ levels were not correlated with α -FP levels.

The NPC test showed that patients with HCC and patients with cirrhosis were significantly different with regard to α -FP ($P = 0.000$), but not according to N/OFQ levels ($P = 0.595$). Point biserial correlation showed that HCC presence was positively correlated with the levels of α -FP ($rpb = 0.52$ $P = 0.000$) but not with those of N/OFQ ($rpb = 0.16$ $P = 0.157$). Within the discriminant

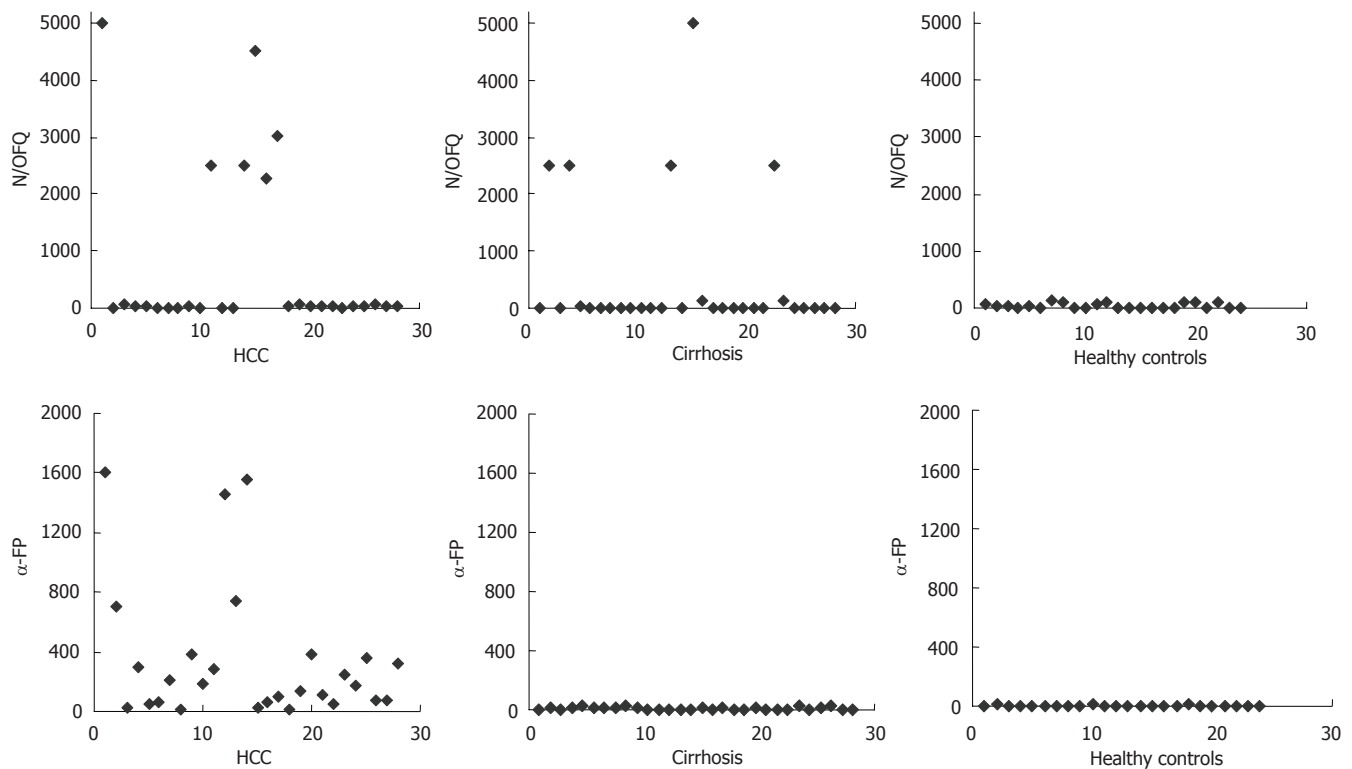


Figure 1 N/OFQ (pg/mL) and α -FP (ng/mL) values in patients with HCC, with cirrhosis and in healthy controls.

Table 2 Laboratory parameters included in the statistical analysis

Mean values \pm SD	HCC	Cirrhosis	Controls
α -FP (ng/mL)	346 \pm 461 ^b	9.2 \pm 8	3.9 \pm 0.4
N/OFQ (pg/mL)	728 \pm 1460	515 \pm 1205	138 \pm 493
Aspartate aminotransferase (U/L)	60 \pm 28.5	54 \pm 35	26 \pm 9
Alanine aminotransferase (U/L)	37 \pm 15	53 \pm 50.5	33 \pm 10.5
γ -glutamyl transpeptidase (U/L)	111 \pm 58	78 \pm 60	28 \pm 11
Alkaline phosphatase (U/L)	325 \pm 113.5	285 \pm 185	122 \pm 75
Cholinesterase (U/L)	3695 \pm 1910	4105 \pm 2891	9680 \pm 2899
Bilirubin (mg/dL)	1.6 \pm 1.3	3.7 \pm 7	0.5 \pm 0.3
Albumin (g/dL)	3.2 \pm 0.4	3.4 \pm 0.6	3.7 \pm 0.4
Lactic dehydrogenase (U/L)	418 \pm 53.5	347 \pm 89	263 \pm 78
Creatine phosphokinase (U/L)	136 \pm 61	72 \pm 18	87 \pm 52
Amylases (U/L)	107 \pm 19	90 \pm 33	76 \pm 45
Blood urea (mg/dL)	44 \pm 16	71 \pm 42.5	32 \pm 11
Creatinine (mg/dL)	1.1 \pm 0.3	1.2 \pm 0.5	0.9 \pm 0.2
Na (mmol/L)	135 \pm 3.8	136 \pm 6.8	139 \pm 5.1
K (mmol/L)	4.4 \pm 0.3	4.5 \pm 0.5	4.3 \pm 0.5
Ca (mg/dL)	9.1 \pm 0.5	8.7 \pm 0.4	4.9 \pm 0.3
Red blood cells (10^6 /mm ³)	3.8 \pm 0.6	1.9 \pm 1.7	4.2 \pm 0.2
With blood cells (10^3 /mm ³)	4.9 \pm 2	5 \pm 1.8	7 \pm 1.3
Hemoglobin (g%)	11.7 \pm 1.5	10.6 \pm 2.2	13.6 \pm 0.9
Platelets (10^3 /mm ³)	116 \pm 48	99 \pm 47	249 \pm 58
Blood glucose (mg/dL)	96 \pm 14	111 \pm 38	78 \pm 11
Erythrocyte sedimentation rate (mm)	66.6 \pm 17.7	49.4 \pm 17.1	7.4 \pm 4.1
C-reactive protein (mg/L)	18.4 \pm 29.7	10.3 \pm 9.7	2.7 \pm 1.4
International Normalized Ratio	1.22 \pm 0.2	1.31 \pm 0.3	0.8 \pm 0.1
Fibrinogen (mg/L)	288 \pm 103	243 \pm 68	291 \pm 93

^b $P < 0.001$ vs cirrhosis and controls.

Table 3 Diagnostic accuracy tests

	N/OFQ		α -FP	
Sensitivity % (CI)	21.4	(0.06 - 0.36)	46.4	(0.29 - 0.64)
Specificity % (CI)	76.7	(0.61 - 0.91)	100	(0.88 - 1.00)
Positive predictive value % (CI)	46.2	(0.19 - 0.73)	100	(0.77 - 1.00)
Negative predictive value % (CI)	51.1	(0.36 - 0.65)	66.7	(0.52 - 0.78)
Positive likelihood ratio (CI)	0.92	(0.35 - 2.40)	∞	(4.01 - ∞)
Negative likelihood ratio (CI)	1.02	(0.77 - 1.35)	0.53	(0.33 - ∞)

analysis, the Wilks test showed that only α -FP was significant in the model ($Y = -0.709 + 0.03 \alpha$ -FP). The classification of results demonstrated that α -FP properly classified 81% of all patients and 61% of patients with HCC. Sensitivity, specificity and predictive values of N/OFQ and α -FP in the diagnosis of HCC are reported in Table 3.

DISCUSSION

HCC is the third leading cancer-related cause of death worldwide^[15]. In western countries, HCC arises in cirrhotic livers with an annual incidence of 3%-5%. Early detection of HCC allows optimal application of curative treatments. The increased survival, after radical treatment in the last decade, recommends surveillance for detection of early HCC in cirrhosis^[16]. Ultrasonography plays a key role in the detection of HCC but its sensitivity for small

nodules is low^[17] and other imaging techniques, such as CT and MRI, are too expensive for screening programs. An elevation in serum α -FP is associated with HCC but it is also elevated in non-hepatic malignancies^[18] and in hepatitis^[19]. Although the diagnostic accuracy of serum α -FP is highly variable in the reported series depending on the cut-off level^[20], it remains the most commonly used serological marker for HCC surveillance. The diagnostic value of other markers proposed so far, such as des- γ -carboxyprothrombin^[9], human hepatocyte growth factor^[10], serum chromogranin-A^[11], is not well defined. It has recently been published that plasma levels of N/OFQ are very high in all patients with HCC, including those with normal α -FP^[6]. With the aim to define if plasma N/OFQ values represent an indicator of HCC even in absence of increased α -FP, we studied circulating N/OFQ and α -FP levels in patients with liver cirrhosis, with and without HCC, comparing the diagnostic value of the two markers.

Mean N/OFQ values were higher both in patients with liver cirrhosis alone and in those with HCC than in controls, but did not differ significantly between the two patient groups, with a similar percentage of values above the normal range. On the other hand, mean α -FP levels in HCC patients were higher than in cirrhotics, with a higher percentage of values > 200 ng/mL. The NPC test showed significant differences between patients with and without HCC with regard to α -FP but not for N/OFQ. Such data are in accordance with the results of Szalay *et al.*^[6] with respect to the increase in plasma N/OFQ values seen in cirrhotic patients, but do not confirm the unique finding of extremely elevated values in the entire population with HCC. Since the demographic and clinical features, including size of the tumor and presence of pain, are similar among the patients enrolled in the two studies, we are not able to suggest any explanation other than possible genetic differences for the discordant results.

Receptors for N/OFQ are normally present in the liver as shown by detection of mRNA for the ORL1/NOP receptor^[21]. Therefore, an increase in plasma N/OFQ values in some patients with chronic liver disease, with and without HCC, might be due to the chronic liver disorder with a reduced ability to bind N/OFQ.

Transcription of the N/OFQ gene is enhanced by estrogen^[22], therefore another explanation for the increase of N/OFQ plasma levels in cirrhotic livers, independently of the presence of HCC, might be the increase of estrogen hormones, common in patients with advanced liver disease.

A higher N/OFQ content has been shown in HCC tissue as compared to tumor-free liver tissue in one patient with PBC^[23] and in rats with experimentally induced tumors^[24], suggesting that the HCC cells might produce N/OFQ or give signals for neuronal production. However, we were not able to find any correlation between plasma N/OFQ levels and tumor size, in agreement with the finding of Szalay *et al.*^[6].

Peripheral blood neutrophils express and secrete N/OFQ following degranulation^[25]; N/OFQ stimulates chemotaxis^[26] and is present at sites of inflammation, such as synovial exudates^[25]. However, in this study, we were not able to correlate the amounts of circulating N/OFQ with

any of the inflammatory indices (erythrocyte sedimentation rate, C-reactive protein, peripheral leukocytes, fibrinogen, platelets).

It has been demonstrated that N/OFQ plays a role in the perception of pain, inhibiting the release of various neurotransmitters, including pain related peptides^[27,28]. However, no correlation between plasma values of N/OFQ and the presence of pain in any of the groups studied was found.

When we compared the diagnostic utility of N/OFQ and α -FP in HCC, α -FP had better sensitivity (46% *vs* 21%), better specificity (100% *vs* 77%), higher positive predictive value (100% *vs* 46%) and negative predictive value (67% *vs* 51%).

In conclusion, from our experience, N/OFQ cannot be recommended as a marker for early detection of HCC in patients with liver cirrhosis. α -FP is very specific when a high cut-off (200 ng/mL) is adopted but its sensitivity is low. N/OFQ is increased in patients with chronic liver disease, independent of the presence of HCC, although the underlying mechanism needs to be clarified. In the diagnosis of HCC, further research is needed in order to find serum markers more sensitive than α -FP.

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