Detection of antibodies anti-ARFp as biomarker of liver fibrosis progression in patients with chronic HCV

Suiane Lima de Souza[1,2], Luãnna Liebscher Vidal [1,3], Renata de Mello Perez [1], João Marcello de Araújo Neto [1,2], Maria Chiara Chindamo[1],Henrique Sérgio Moraes Coelho [1,4], Hilton Jorge dos Santos[3], Penelope Mavromara[5], Marcelo Alves Soares [1,2], André Felipe Santos [1]

[1] Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil; [2] National Institute of Cancer (INCA), Rio de Janeiro, Brazil [3] Farmanguinho, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil; [4] D'Or Institute for Research and Education (IDOR), Rio de Janeiro, Brazil; [3] rHellenic Pasteur Institute, Athens, Greece

INTRODUCTION

- It is estimated that 71 million people are infected with Hepatitis C virus (HCV) and per year 400,000 people die from diseases related to HCV infection (WHO, 2018);
- In chronic Hepatitis C, there is progression of liver fibrosis, which can develop to cirrhosis and hepatocellular carcinoma (HCC) (WHO, 2018);
- Fibrosis can be classified in a five level severity scale, from F0, without fibrosis, to F4, which is cirrhosis (Nguyen-Khac and Capron, 2006);
- Although available treatments have high cure rates, HCC can develop even after clearance of infection;
- The HCV genome has a open reading frame (ORF) that generates a precursor polyprotein, but in the alternative reading frame -2/+1, the protein ARFp (Alternative Reading Frame protein) is synthetized (Xu *et al*., 2001);
- ARFp is not necessary for the viral replication or assembly. Recently some of its carcinogenic mechanisms were elucidated, as cellular cycle acceleration and increase of the tumor foci (Moustafa *et al*., 2018);
- Previous studies have shown a higher prevalence of anti-ARFp antibodies in patients with HCC compared to patients without HCC, and in patients with advanced cirrhosis compared to mild cirrhosis, but did not consider the different stages of fibrosis within the non-HCC group (Dalagiorgou *et al*., 2011, Kassela *et al*., 2017).

OBJECTIVES

- Detection of anti-ARFp antibodies in HCV patients with chronic infection in different degrees of fibrosis (F0 to F4) as a biomarker of progression of hepatic disease in hepatitis C;
- Detection of anti-ARFp antibodies in HCV patients with cirrhosis (F4) with and without HCC symptoms;
- Association of ARFp expression with clinical factors (age, AST and ALT).

METHODS

Plasmids — 2 plasmids were donated by contributors of Hellenic Pasteur Institute (Athens/Greece), containing the core and ARFp nucleotide sequence (genotype 1a), followed by a histidine tail.

Expression — The plasmids were transformed into E.coli BL21 (DE3) cells to express the proteins using IPTG for 4h at 37ºC.

Purification — The proteins were purificated using nickel column by affinity chromatography. Western-blot was performed to confirm that it was no contamination among plasmids.

ELISA — The proteins were used to sensitize ELISA plate and the patients sera were tested for core (positive control) and ARFp in triplicate.

Patients — 279 patients HCV+ from UFRJ University Hospital and/or National Institute of Cancer (INCa) and 28 blood donors samples HCV- were enrolled in this study. Patients were classified according the fibrosis level and HCC presence.

Data Analyzes — The proportions of patients expressing antibodies anti-ARFp among different patient groups were compared using chi-square or T student two-tailed test in R project version 2.14. p value ≤ 0.05 were considered significant.

RESULTS

Disease stage	F0	F1	F2	F3	F4	HCC	Total
N	4	74	54	51	83	13	279
Age (median)	39	52	58	58	55	68	56
Gender							
Female	3 (75%)	39 (53%)	27 (50%)	33 (65%)	47 (57%)	2 (15%)	152 (54%)
Male	1 (25%)	35 (47%)	27 (50%)	18 (35%)	36 (43%)	11 (85%)	127 (46%)
Genotype							
1	4 (100%)	54 (90%)	45 (96%)	46 (96%)	78 (98%)	3 (50%)	230 (94%)
2	---	1 (2%)	---	---	---	---	1 (0.4%)
3	---	5 (8%)	2 (4%)	2 (4%)	1 (1%)	3 (50%)	13 (5%)
4	---	---	---	---	1 (1%)	---	1 (0.4%)
ND		14	7	3	3	7	34

Table 1: Patients HCV+ clinical data. ND: Not determinated.

Patients	n	Core +	ARFp+
F0	4	4 (100%)	2 (50%)
F1	74	73 (99%)	15 (21%)
F2	54	51 (94%)	9 (18%)
F3	51	51 (100%)	20 (39%)
F4	83	81 (98%)	34 (42%)
HCC	13	11 (85%)	10 (91%)
Total	279	271 (97%)	90 (33%)
Blood donors	28	0	0

Table 2. Proportion of anti-core and anti-ARFp by patient group. ARFp chi-square test between F1 and F2 and between F3 and F4 had p = 0.8184 and p = 0.8561, respectively.

Patients	n	ARFp +	p
F0/F1/F2	128	26 (20%)	0.0004
F3/F4	132	54 (41%)	

Table 3. Proportion of anti-ARFp in patients with mild (F0-F2) and advanced (F3-F4) fibrosis.

Patients	n	ARFp +	p
F4 without HCC	81	34(42%)	0.0028
F4 with HCC	11	10 (91%)	

Table 4. Proportion of anti-ARFp in patients with cirrrhosis with and without HCC.

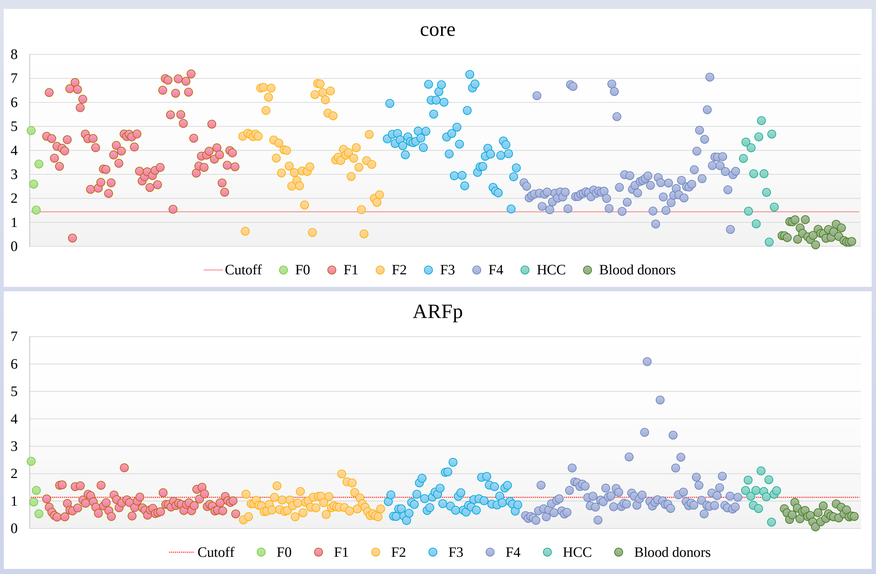

Figure 1:Optical density value of ELISA. Patients were grouped by liver damage level. a) Detection of anti-core antibodies (cutoff = 1.44). b) Detection of anti-ARFp antibodies (cutoff = 1.13).

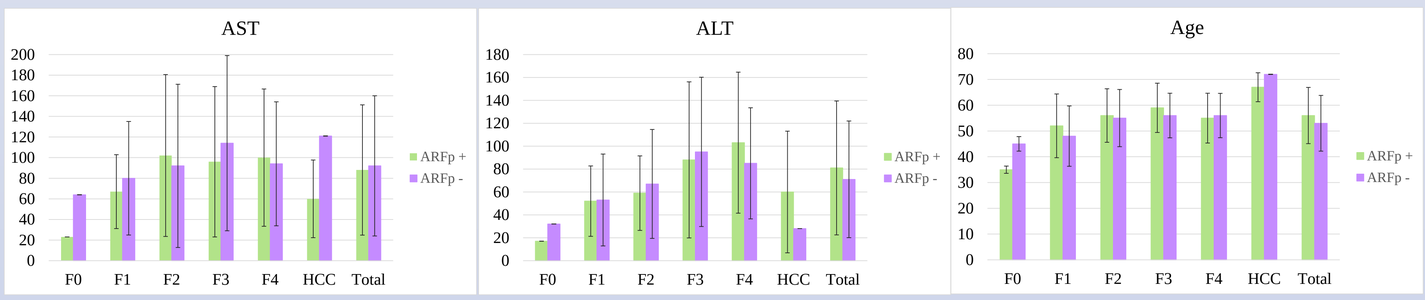

Figure 2: ALT and AST levels and age avarage in patients with different liver damage level according with the presence of anti-ARFp antibodies. p > 0,05 from T student test.

CONCLUSIONS

- There was no difference of antibodies proportions of individuals anti-ARFp 1a+ between levels F1 and F2, as well as between levels F3 and F4, but when comparing the groups with mild fibrosis (F0 to F2) and advanced fibrosis (F3 and F4) , the proportion of anti-ARFp antibodies was significantly higher in patients with increased liver damage.
- A significantly higher antibodies proportion of individuals with anti-ARFp 1a+ in the group with F4 level and HCC were observed than in the F4 group and without HCC. In the F4 without HCC group, the OD values were significantly higher than other groups (p = xxx).
- There was no association between anti-ARFp antibodies presence and a higher expression of ALT and AST, as between anti-ARFp antibodies and age.

ACKNOWLEDGEMENTS