Protein array-based screening of autoantibody signatures in HCV patients with autoimmune manifestations

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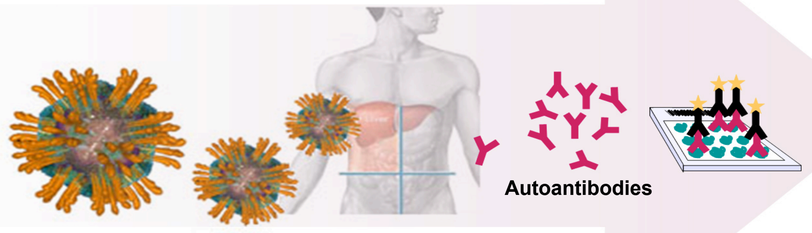
Background

The evidence for an association between autoimmune diseases and chronic Hepatitis C Virus (HCV) infection has been clearly established, although little is known on the mechanism by which HCV infection leads to autoimmunity.

Despite the frequent detection of organ- and non-organ-specific-autoantibodies in patients with chronic HCV infection their clinical significance is not known.

The goal of this study was to identify potential biomarkers that can detect the presence of autoimmune diseases associated with HCV infection by screening a large panel of human antigens. To this aim an in house-developed protein array comprising 1500 poorly characterized proteins was employed to immunoprofile sera of 151 patients and 78 healthy donors. By this approach a panel of antigens with good performance in discriminating among groups of patients was identified. We are currently assessing the possibility to develop novel biomarkers assays that could help both to better categorize autoimmune patients and to predict therapeutic responses.



EXPERIMENTAL APPROACH

Proteins selection and array preparation

1. *In silico* identification of all predicted human surface/secreted proteins

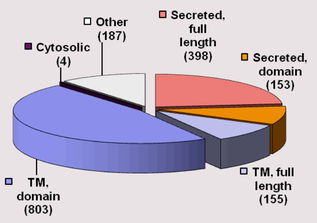
Human genes (≅30000)

Genes encoding for surface/secreted proteins (≅9000)

Genes encoding for unknown surface proteins^(≅3000)

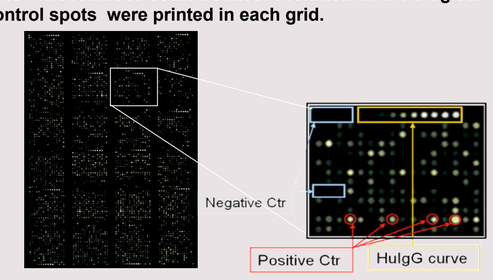
^ "uncharacterized" proteins, i.e., proteins that are still awaiting an assigned function

2. High-throughput Cloning, Expression and Purification* (tot = 1658 proteins)



*cloning as His-tagged products and expression in *E. coli*; purification in denaturing conditions on IMAC resins

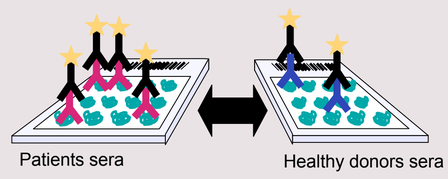
3. Array printing. Proteins were spotted in quadruplicate onto nitrocellulose coated slides. Technical and biological control spots were printed in each grid.



4. Data analysis and results interpretation.

All signals were background subtracted and normalized as described in (1). Positive hits were defined as proteins reacting with a serum with a Normalized Mean Fluorescence Intensity above a threshold of 4000.

Experimental design



Slides were probed with 229 sera as described in table below. Immunoreactivity of patients and healthy donors were compared by frequency and statistical analysis.

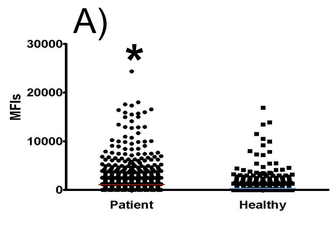
Sera stratification		HCV +	HCV -
Auto Immunity	+	HCV-AI* (43)	AI** (30)
	-	HCV-nAI (78)	HD (78)

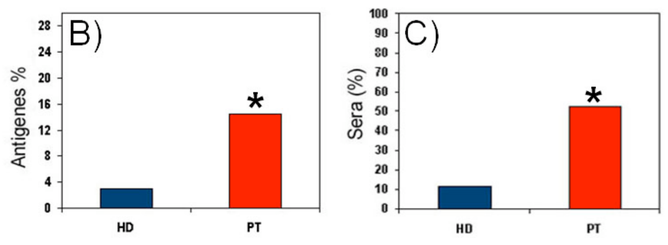
AI=Autoimmune; nAI=non Autoimmune

**Patients with cryoglobulinemia, Thyroid dysfunctions or positive for Non Organ Specific Autoantibodies (NOSA) (ANA, LKM, SMA, anti-DNA)*
*** Patients with liver autoimmunity (Autoimmune Hepatitis or Primary Billiary Cirrhosis*

RESULTS

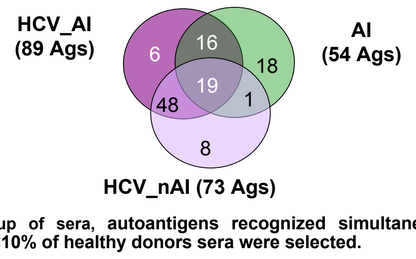
Patients show higher immunoreactivity when compared to Healthy Donors





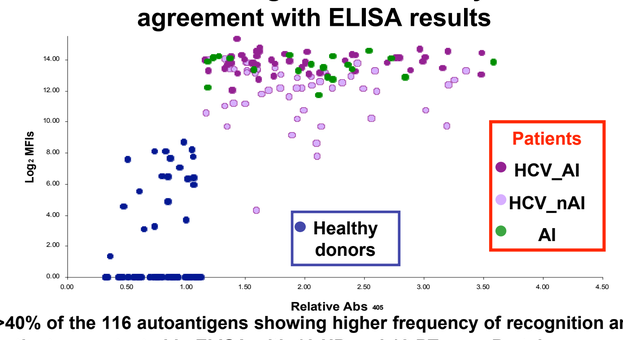
Quantitative analysis of global autoimmunoreactivity:
A) Comparison of Mean Fluorescence Intensity (MFI). Each dot represents the MFI of a protein across the population of sera reported on the X-axis. B) Percent of antigens recognized by more than 10% of Patients or HD sera. C) percent of sera reacting with more than 3% of the proteins spotted. Asterisks: statistical significance, t test and χsquare test (p val <0.0001).

116 autoantigens are recognized with higher frequency by Patients compared to Healthy donors



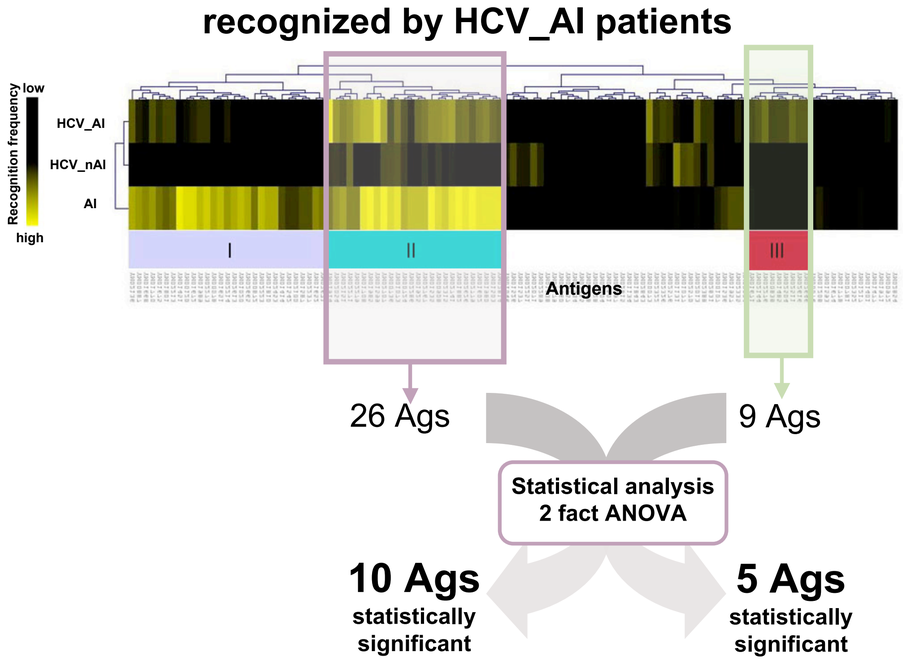
For each group of sera, autoantigens recognized simultaneously by >20% of patients and <10% of healthy donors sera were selected.

For most of the autoantigens Protein array results are in agreement with ELISA results



>40% of the 116 autoantigens showing higher frequency of recognition among patients were tested in ELISA with 10 HD and 10 PT sera. Protein array results were plotted against ELISA results, reported as relative Absorbance (Abs prot/Abs HSA*) in each group of sera. *Human Serum Albumin

35 autoantigens out of the selected 116 are highly recognized by HCV_AI patients



2D Hierarchical CLustering of 116 Ags highlights the presence of 2 clusters of antigens highly recognized by patients with HCV and autoimmunity: cluster II (purple box) comprising Ags with high recognition freq. in both AI and HCV_AI patients and cluster III (green box) comprising Ags specifically recognized by HCV_AI patients. Statistical analysis was performed on antigens belonging to these 2 clustersand 15 antigens were selected for further analysis.

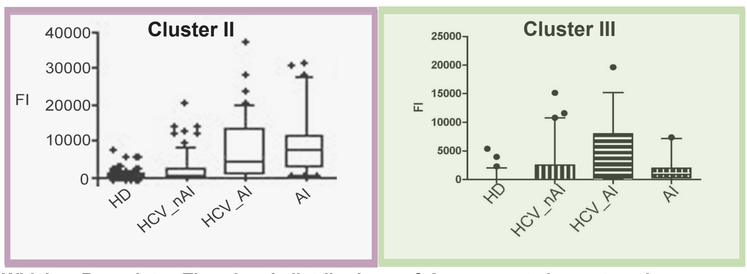
15 autoantigens were identified as potential biomarker candidates

autoantigens recognition frequencies

ProteiID	HCV_AI	AI	HD	HCV_nAI
YM01602	17/43 (39%)	20/30 (67%)	1/78 (1%)	13/78 (17%)
YM01708	23/43 (23%)	20/30 (67%)	5/78 (6%)	10/78 (13%)
YM01882	15/39 (38%)	21/30 (70%)	1/70 (1%)	15/65 (23%)
YM01980	18/43 (42%)	20/30 (67%)	3/78 (4%)	14/78 (18%)
YM01985	14/26 (54%)	23/30 (77%)	4/44 (9%)	4/25 (16%)
YM02315	20/43 (46%)	18/30 (60%)	5/78 (6%)	20/78 (26%)
YM02707	22/43 (51%)	22/30 (73%)	7/76 (9%)	25/78 (32%)
YM01503	16/43 (37%)	20/30 (67%)	2/78 (3%)	15/78 (19%)
YM02741	21/43 (49%)	20/30 (67%)	1/78 (1%)	21/78 (27%)
YM02814	15/43 (35%)	20/30 (67%)	4/78 (5%)	14/78 (18%)
YM01485	14/43 (33%)	3/24 (13%)	0/78 (0%)	10/78 (13%)
YM02504	14/43 (33%)	4/28 (14%)	0/71 (0%)	10/76 (13%)
YM02511	14/37 (38%)	3/30 (10%)	1/63 (1%)	9/59 (15%)
YM02144	15/43 (35%)	7/30 (23%)	1/78 (1%)	9/78 (12%)
YM02646	15/43 (35%)	4/39 (10%)	0/71 (0%)	16/78 (21%)

The recognition frequency of each autoantigen in each group of sera is reported in the table. autoantigens of cluster II are in purple, those of cluster III are in green.

autoantigens MFI is higher respectively in all autoimmune patients (cluster II Ags) or HCV_AI patients (cluster III Ags)



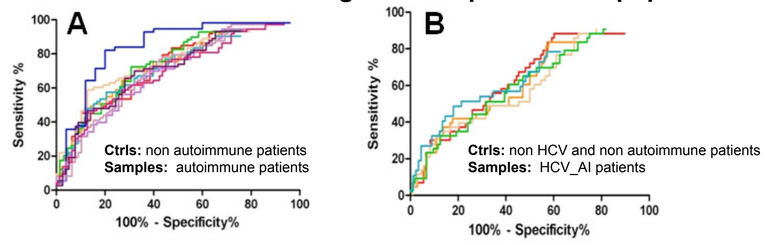
Whisker Box plots: The signal distributions of 2 representative autoantigens, one belonging to cluster II and one to cluster III, reacting with the serum samples of each family (HD, HCV_nAI and HCV_AI. AI) are shown.

The two panels of autoantigens show high sensitivity and high specificity when used all together as predictors for sera classification

Predictor	Sera Classes	Sensitivity	Specificity
10 Ags Cluster II	Positive: AI, HCV_AI Negative: HCV_nAI	72%	73%
5 Ags Cluster III	Positive: HCV_AI Negative: HCV_nAI	49%	75%

The two panels of antigens were used to perform class prediction analysis with Support Vector Machine (One Out Iterative Validation).

The 15-autoantigens show very good performance in discriminating Healthy donors from Patients and good performance in discriminating different patients subpopulations



Receiver-operating-characteristic (ROC) curves to evaluate the performance of:
A. cluster II antigens as markers to discriminate between autoimmune and non-autoimmune patients; all Ags have an Area Under Curve (AUC) significantly above 0.5 (p val <0.0001); **B.** cluster III antigens in discriminating autoimmune HCV patients from non-autoimmune and non-HCV patients; only 3 antigens (YMO2646, YMO2511, YMO2504), have an area under curve significantly above 0.5 (p val <0.01).

When used to discriminate patients from healthy donors all 15 Ags show very good performance (AUC>0.5, p val 0.0001)

CONCLUSIONS

- **10 previously unidentified autoantigens are highly recognized by sera of patients with autoimmune disorders, regardless the concomitant presence of chronic HCV infection.**
- **A subset of 5 previously unidentified autoantigens appear to be better recognized by sera of patients with HCV plus autoimmune complications compared to patients with HCV only or AI only.**
- **Further validation is required to assess the possible employment of the antigens identified as Biomarkers for autoimmunity**

ACKNOWLEDGMENTS

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2. **Wang *et al.*, New England Journal of Medicine, 2005**
3. **Song *et al.*, Journal of proteome Research, 2009**

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