Identification of novel autoantigens in patients with liver autoimmune diseases by Protein MicroArray

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ABSTRACT

The characterization of autoimmune disease-specific biomarkers is of primary importance for the development of diagnostic tools and the comprehension of pathogenetic mechanisms leading to autoimmunity. To identify new autoantibodies in the sera of patients with liver autoimmune diseases (AutoImmune Hepatitis (AIH) and Primary Biliary Cirrhosis (PBC)), we developed a protein microarray containing more than 1600 poorly-known recombinant human surface-exposed proteins. We assessed serum samples from 30 autoimmune liver disease patients and 78 healthy subjects and found that 17 of these poorly-known human proteins were preferentially recognized by sera of patients with liver autoimmune diseases. Six of the 17 autoantigens were validated by DELFIA analysis with an indipendent set of sera from 100 patients with liver autoimmune diseases and 50 healthy donors. These 6 autoantigens showed individual sensitivities ranging from 44% to 74% of the autoimmune liver disease patients. Most importantly, combinations of the 6 autoantigens achieves a 81% (±1%) sensitivity and 93% (±6%) specificity, thus displaying much higher sensitivity and specificity than CYP2D6 and ASGPR, the benchmark autoantigens

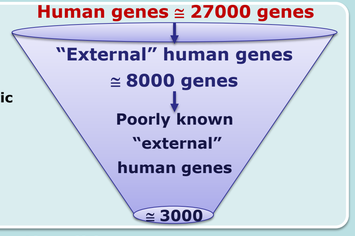
EXPERIMENTAL APPROACH

Proteins selection and array preparation

In *silico* identification of the human genes of interest

1

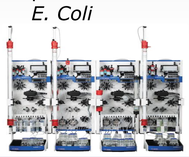
Proteins were selected through a bioinformatic analysis of the whole human genome as translated sequences carrying:
i) a signal peptide,
ii) at least one transmembrane domain,
iii) having unknown biological function



Cloning, Expression* and Purification^

2

*Gene Cloning as His-tagged products and Expression in *E. Coli*

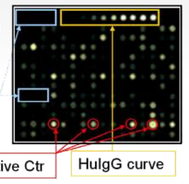


^Purification in denaturing conditions (Urea 6M) on IMAC resins

Printing proteins on the slides

3

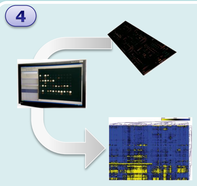
>1500 recombinant human proteins were spotted in quadruplicate onto nitrocellulose coated slides.



Technical and biological control spots were printed in each grid.

Data analysis and results interpretation

4



All signals were background subtracted.

Positive hits were defined as proteins reacting with a serum with a normalized Mean Fluorescence Intensity (MFI) above a threshold of 4000.

Sera stratification

More than 300 sera samples were analyzed using in-house recombinant human proteins microarray. The samples were diveded into four groups as shown in Tab. I:

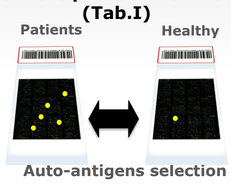
Tab. I

DISCOVERY							
	Training set			Test set			
Group	Healthy Donors	Liver Autoimmune disease patients		Healthy Donors	Liver Autoimmune disease patients		Viral hepatitis
Sub-group	HD	AIH	PBC	HD	AIH	PBC	HCV
# samples	39	8	7	39	7	8	109

VALIDATION							
Group	Healthy Donors	Liver Autoimmune disease patients		System Autoimmune disease		Viral hepatitis	
Sub-group	HD	AIH	PBC	SLE		HBV	HCV
# samples	50	50	50	50		24	50

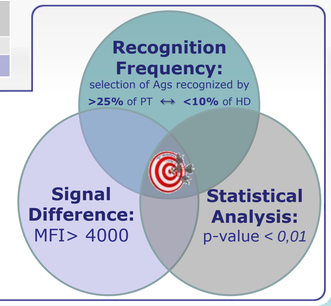
***HD**: Healthy Donor*
***PBC**: Primary Billiary Cirrhosis;*
***AIH**: Autoimmune hepatitis;*
***SLE**: Systemic Lupus Erythermatosus;*
***HCV**: Hepatitis C Virus;*
***HBV**: Hepatitis B Virus*

Microarrays were probed with discovery sera (Tab.I)



Multiple criteria to score potential autoantigens

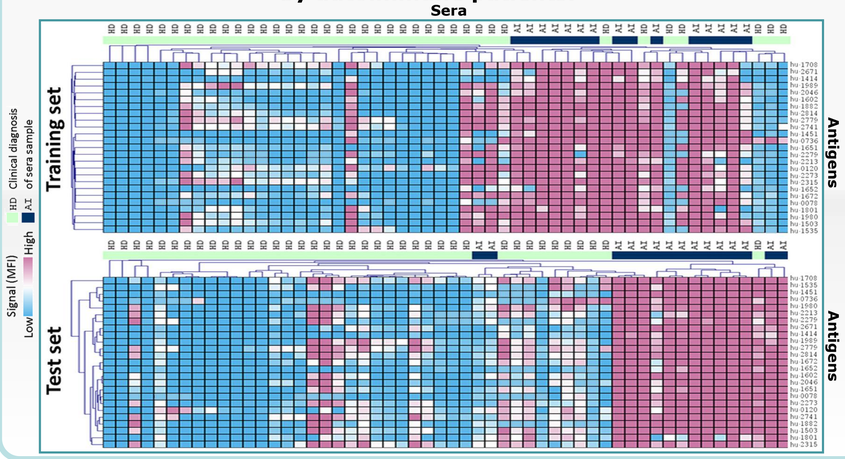
Following normalization after microarray analysis, individual autoantigens from protein microarray were ranked according to:



RESULTS

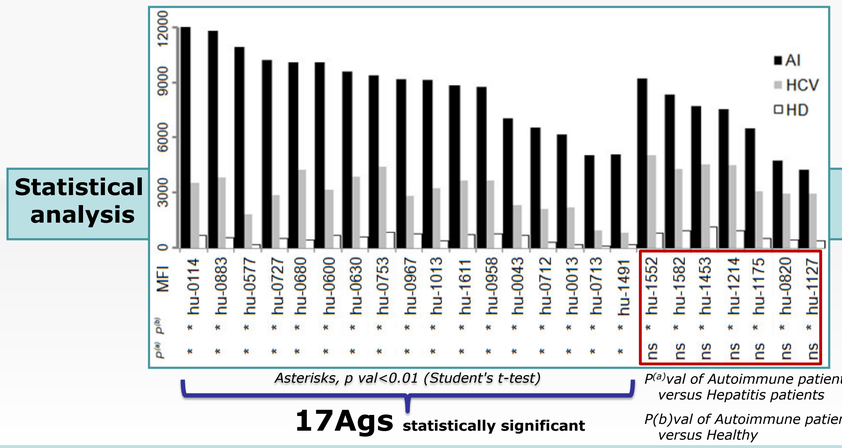
17 autoantigens out of 25 display significant autoreactivity when comparing AI with HCV patients or with HD

Heat maps of the 25 autoantigens selected as specifically recognized by autoimmune patients.



MFIs of the 25 autoantigens selected in a whole Discovery sample set

No significant differences were observed between the group of patients sera for seven antigens (red box).



Validation of selected candidates by DELFIA assay

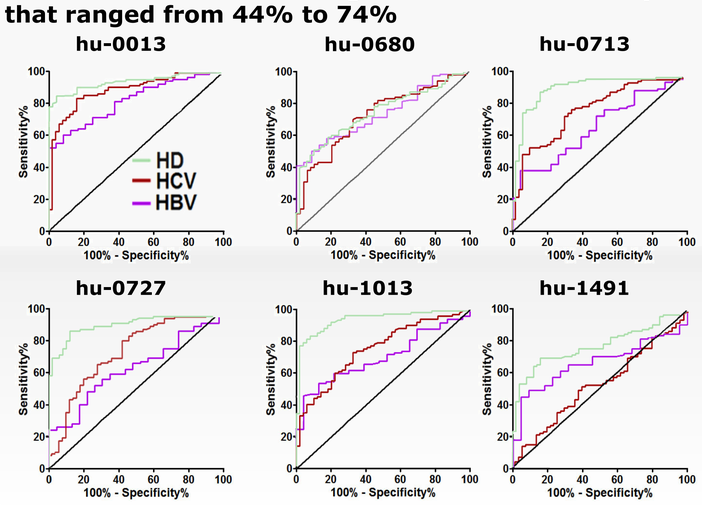
6 candidates were confirmed to be specifically recognized by patients sera with regard to healthy donors

	Prot. Id	[a]SE (AI) %	[b]SP (HD) %	[c]SP (HCV) %	[d]SP (LES) %	[e]SP (HBV) %
Known AutoAgs	AGPR	70	48	48	36	46
	Cyp450	70	50	42	30	54
New our AutoAgs	hu-0013	74	100	90	44	75
	hu-0680	44	94	84	64	92
	hu-0713	48	96	92	62	67
	hu-0727	48	100	82	44	71
	hu-1013	63	98	74	50	63
	hu-1491	54	94	48	46	71

Cytochrome P450(CYP2D6) and Asialoglycoprotein receptor (ASGR-1), liver specific autoantigens were used as references biomarkers (2).

[a]**SE**nsitivity is defined as % of positive autoimmune patients
SPecificity is defined as % of negative patients: [b]healthy, [c]HCV [d]LES [e]HBV

6 new candidates autoantigens showed sensitivity value for the autoimmune samples that ranged from 44% to 74%



Combo Serology

The combinations of the 6 autoantigens achieves a 81% (±1%) sensitivity and 93% (±6%) specificity

	SE (%)		SP (%)		
Prot_ID	[a](AI)	[b](HD)	[c](HCV)	[d](LES)	[e](HBV)
COMBO-Known	76	38	30	26	38
COMBO-6	82	86	30	12	42
COMBO-4	81	96	62	22	50
COMBO-3	79	98	62	22	50

The performance of the six autoantigens.

Receiver-operating-characteristic (ROC) curves are based on multiplex analysis of the patient with autoimmune liver diseases from a total of 100 samples (50 from patients with AIH and 50 from PBC).

CONCLUSIONS

- **A panel of 17 (poorly known) potential novel autoantigens identified in patients with liver autoimmune diseases (AIH & PBC) by protein microarray**
- **6 of the 17 novel autoantigens validated in patients with liver autoimmune diseases with individual sensitivities that ranged from 44% to 74% by DELFIA method. The combined assessment of the six autoantigens displays 81% (±1%) sensitivity and 93% (±6%) specificity**
- **Superior Sensitivity and Specificity (Vs HD, HCV and HBV) compared to benchmarks (CYP2D6 & ASGPR)**
- **Protein Microarray technology has the potential to rapidly identify new biomarkers useful to improve the diagnosis and/or prognosis of autoimmune diseases, and at the same time to identify new pathogenetic proteins**

ACKNOWLEDGMENTS

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REFERENCES

1) Muratori L. *et al.,* Dig Liver Dis., 2010, 2) Song L. *et al.*, J Proteome Res, 2009, 3) Bombaci M. *et al.*, PLoS One, 2009, 4) Wang X. *et al.*, New Engl J Med, 2005,