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YRNAs overexpression and potential implications in allergy

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ABSTRACT

Background: Small non-coding RNAs (snRNAs) develop important functions related to epigenetic regulation. YRNAs are snRNAs involved in the initiation of DNA replication and RNA stability that regulate gene expression. They have been related to autoimmune, cancer and inflammatory diseases but never before to allergy. In this work we described for the first time in allergic patients the differential expression profile of YRNAs, their regulatory mechanisms and their potential as new diagnostic and therapeutic targets.
Methods: From a previous whole RNAseq study in B cells of allergic patients, differential expression profiles of coding and non-coding transcripts were obtained. To select the most differentially expressed non coding transcripts, fold change and p-values were analyzed. A validation of the expression differences detected was developed in an independent cohort of 304 individuals, 208 allergic patients and 96 controls by using qPCR. Potential binding and retrotransponibility capacity were characterized by *in silico* structural analysis. Using a novel bioinformatics approach, RNA targets identification, functional enrichment and network analyses were performed.
Results: We found that almost 70% of overexpressed non-coding transcripts in allergic patients corresponded to YRNAs. From the three more differentially overexpressed candidates, increased expression was independently confirmed in the peripheral blood of allergic patients. Structural analysis suggested a protein binding capacity decrease and an increase in retrotransponibility. Studies of RNA targets allowed the identification of sequences related to the immune mechanisms underlying allergy.
Conclusions: Overexpression of YRNAs is observed for the first time in allergic patients. Structural and functional information points to their implication on regulatory mechanisms of the disease.

Background

Epigenetic modifications are inheritable and reversible changes in gene expression that do not involve changes in the DNA sequence.[1] An increasing amount of evidence suggests that epigenetic mechanisms act as a link between genetic and environmental factors in allergy.[1] In the first epigenomic study performed in B cells from allergic patients, we identified a differential methylation pattern in allergy.[2] In addition to DNA methylation, histone modifications and RNAs are also involved in epigenetic processes.[1] Most of the transcribed RNA does so in the form of non-coding RNAs (ncRNA).[3,4] ncRNAs, are grouped into housekeeping

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List of abreviations

ADAM33	Disintegrin and metalloproteinase domain-containing protein 33
AIM1	Absent in melanoma-1
AKAP13	A-Kinase Anchoring Protein 13
CD19	Cluster of Differentiation 19
cDNA	Complementary DNA
Ct	Cycle threshold
DENND5B	DENN Domain Containing 5B
DNA	Deoxyribonucleic acid
EAACI	European Academy of Allergy and Clinical Immunology
FPRP	False positive report probability
GAPDH	Gliceraldehído-3-fosfato deshidrogenasa
gDNA	Genomic DNA
GEF	Guanine nucleotide Exchange Factor
HLA	Human Leukocyte Antigen
hYRNA	Human cytoplasmic RNAs
IL4R	Interleukin-4 receptor
IL10	Interleukin-10
KCNMA1	Potassium Calcium-Activated Channel Subfamily M Alpha 1
K-W test	Kruskal–Wallis test
LINE-1	Long interspersed nuclear element-1
mRNA	Messenger RNA
miRNA	Micro ribonucleic acid
ncRNA	Non-coding RNAs
NGS	Next-Generation Sequencing
NPV	Negative Predictive Value
OAS1	2'-5'-Oligoadenylate Synthetase 1p
iRNA	Piwi ribonucleic acid
PCR	Polymerase chain reaction
PPV	Positive Predictive Value
qPCR	Quantitative real time PCR
RhoA	Ras homolog gene family, member A
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing or whole transcriptome shotgun sequencing
RNP	Ribonucleoprotein
rRNA	Ribosomal Ribonucleic acid
RT	Reverse transcription
scRNA	Small cytoplasmic RNA
sgRNA	Small guide fragments
siRNA	Small interference ribonucleic acid
SINE	Short interspersed nucleotide elements
SLE	Systemic Lupus Erythematosus
snRNA	Small non-coding ribonucleic acid
SP	Statistical power
tRNA	Transfer ribonucleic acid
TSLP	Thymic Stromal Lymphopoietin
VARS	Valyl-TRNA Synthetase
YRNA	Cytoplasmic RNAs

RNAs, long-ncRNAs and small-ncRNAs (sncRNA).[5,6] sncRNAs are smaller than 200 nucleotides (nt) and include miRNAs (micro RNAs), siRNAs (small interfering RNAs), and piRNAs (Piwi-interacting RNAs), many of which have been involved in pathological processes.[1]

miRNAs have been involved in asthma and other respiratory diseases.[7–9] Previous studies have shown different miRNA expression profiles in the bronchial epithelium of asthmatic patients, involved in epithelial cell proliferation, differentiation, and apoptosis.[10] The role of other ncRNAs in allergy remains largely unknown yet. Among sncRNAs, YRNAs have been described as a new group of cytoplasmic sncRNAs (as opposed to those with nuclear localization or URNAs), originally described in autoimmune diseases.[11,12] The human YRNA family (hYRNA) has four members, namely Y1, Y3, Y4, and Y5 (Y2 is a truncated form of Y1)[13] and appears highly conserved in animal phylogeny.[14] Orthologous genes exist in prokaryotes,[15] suggesting important biological functions. Although hYRNAs have been associated to immunological and inflammatory diseases,[16] they have not been directly linked to allergy so far.

hYRNAs are transcribed by the RNA polymerase III from a cluster of genes located in chromosome 7q36,[17] ranging from 83 to 112 nt.[13,18,19] Ro/SSA and La/SSB proteins interact with YRNA to form active ribonucleoproteins (RNP), although some RNPs can also be formed independently of Ro/SSA and/or La/SSB proteins.[20] RNP function ultimately depends on the YRNA species and the proteins that form each specific RNP.[21] YRNAs have been associated to the transcription start complex,[22] Ro/SSA-mediated chaperone activity regulation (RNA quality control),[2,23] 5S RNA quality,[24] or stress resistance.[19] In addition, it has even been speculated with the possibility that YRNAs might be a source of miRNAs.[25] Furthermore, YRNAs are degraded during apoptosis in a fast and specific caspase dependent process,[26] generating YRNA-derived small RNAs that have been associated with atherosclerosis, cardiovascular diseases[27,28] and cancer.[29,30] Recently, it has been described that RNA fragments derived from YRNAs can function as small guide fragments (sgRNA) that can direct endoribonucleases to their target RNAs, working as signaling molecules between cells.[31]

From a functional point of view, hYRNAs are flanked by L1 retrotransposition machinery sequences, constituting a new class of non-autonomous L1-dependent elements (retrotransposons).[32] The movable genome elements L1 (long interspersed nuclear element-1, LINE-1) retrotransposon family represents approximately 17% of the genome.[33] The L1 retrotransposing machinery participates in the integration into the genome of processed pseudogenes, chimeric retrogenes, *cis* retrotransposable elements, and *trans* non-autonomous sequences.[32,34,35] Potential functions have been described for retrotransposons in posttranscriptional regulation[32] as well as in human pathology such as cancer.[29] Their overexpression deregulates innate immunity, escaping to DNA-RNA sensor regulation and leading to the development of autoimmune diseases.[36,37]

In our previous RNAseq studies we identified differential expression patterns of several B-cell coding genes, such as *IL4R*,[38] which might have a pathological impact on allergy. Here, we aimed to identify the differential expression of non-coding sequences in allergic patients not previously been described in allergy. We also present novel evidences that suggest a role for hYRNAs in regulatory mechanisms of allergic responses, providing a new insight into the potential implications of hYRNAs as biomarkers or therapeutic targets in allergy.

Methods

Transcriptomic RNAseq study

We have previously investigated six representative samples, corresponding to unrelated Caucasian individuals[38] including 3 patients with house dust mite allergic asthma diagnosed by an allergist and 3 controls. The controls fulfilled the following criteria: (i) absence of symptoms or history of asthma or other pulmonary diseases; (ii) no symptoms or history of allergy; (iii) negative skin prick tests to a locally adapted battery of common aeroallergens; and (iv) absence of familial history of asthma or allergic diseases. $CD19^{+}$ B lymphocytes were isolated from peripheral blood samples and RNA extraction was performed. RNAseq was developed using the IlluminaHiSeq 2000 platform (San Diego, California, USA), and differential expression was analyzed by DESeq package as previously described.[38]

Sequences selection and characterization

From the most differentially expressed transcripts,[38] those that appeared with YRNA notation after alignment with genomic databases[39] were selected. For a confirmatory study, the best candidate sequences were selected according to p-value and fold change. Sequences were mapped using the ENSEMBL-GRCh38/hg38 database[39] and Genome Browser database[40] as a confirmation tool. For the sequence homology, YRNAs were studied and aligned with the YRNA3 sequence by BLAT (BLAST-like alignment tool). For the retrotransposition analysis, the RT Analyzer software[41] was employed.

Validation analyses

For the validation analysis, 304 Caucasian individuals were recruited from the Department of Allergy. Among them there were 208 allergic patients diagnosed by allergists and 96 control individuals that fulfilled the previously mentioned criteria.[42] The characteristics of patients and controls are described in Table 1 and the clinical entities and severity of the diseases are reflected in Table 2. The sample size was calculated with the statistical power (SP)[43] to obtain a minimum of 80% of statistical power for a 0.05 alpha error. Skin prick tests were performed following The European Academy of Allergy and Clinical Immunology (EAACI) allergen standardization and skin test recommendations.[44]

RNA isolation and reverse transcription

Total RNA was isolated using the RiboPure-Blood kit (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). DNAse treatment was performed using Turbo DNAse (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). Concentrations and RNA quality ratios were measured in a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription (RT) was performed on 500 ng of total RNA using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) using a thermal cycler (MultiGene OptiMax, Labnet International Inc., Edison, NJ, USA) in a total volume of 20 µl, with a single cycle and incubation periods of 65 °C for 5 min, 25 °C for 10 min, 50 °C for 50 min, 85 °C for 5 min and 37 °C for 20 min.

qPCR analysis

Relative qPCR was performed with a LightCycler480® Instrument and SYBR Green I Master (Roche, Basel, Switzerland). Fold induction was calculated using the Livak method.[47] YRNA primers were designed using the primer analysis software Primer 3.0[48] and the Beacon Designer Software[49] for Y_RNA.269-201 (ENSG00000201555) (forward primer 5′-cgagtgcag tggtgttgac-3′ and reverse primer 5′-gctagtgggagtggagaagg-3′), Y_RNA.231-201 (ENSG00000201228) (forward primer 5′-agcaggtccgagtgcagt-3′ and reverse primer 5′-gccagtcaagtgaagcagtg-3′), and Y_RNA.575-201 (ENSG00000207499) (forward primer 5′-ctggtctgagtgcagtggtg-3′ and reverse primer 5′-ttaaggcaagtcaagtgaagca-3′). *GAPDH* reference gene primers (forward 5′-ctctgctcctcctgttcgac-3′ and reverse 5′-acgaccaaatccgttgactc-3′) were chosen from *The Real Time ready Human Reference GenePanel* (Roche Applied Science, Indianapolis, IN, USA). Primers were used at 300 nM and cDNA at 20 ng in 15 µl reactions. Conditions for PCR included 10 min at 95 °C followed by 45 cycles of real-time PCR with 3 segments amplification, (10 s at 95 °C denaturation, 10 s at 60 °C annealing, and 10 s at 72 °C polymerization). The dissociation protocol to determine the melting curve from 60 °C to 95 °C was added after thermocycling to verify that each primer pair produced only a single product. PCR generated only one amplicon and no primer/dimer was formed. qPCR efficiencies were analyzed by the amplification of standardized dilution series of the template cDNA and determined based on the slope of the standard curve according to the equation: $E = (10^{-1/slope}-1) \times 100$. All efficiencies ranged from 90% to 110%. Reactions were performed in triplicate, with non-template controls and calibrator and assessed for gDNA contamination with ValidPrime® (TATAA Biocenter, Goteborg, Sweden). The study was performed blindly regarding control or patient status. All procedures followed MIQE guidelines.[50]

Table 2
Distribution of patients with concomitant asthma and rhinitis regarding the severity of the symptoms according to the GINA (Global Initiative for Asthma)[45] and the ARIA (Allergic Rhinitis and its Impact on Asthma)[46] classifications.

	Allergic Asthma and Rhinitis	Age	Female
Asthma classification	n = 142	32.0±13.0	72
Intermittent	68	27.9±11.3	33
Mild persistent	22	35.4±9.8	11
Moderate persistent	45	35.95±15.3	24
Severe persistent	7	39.4±12.9	4
	Allergic Asthma and Rhinitis		
Rhinitis classification	n = 142	32.0±13.0	72
Mild Intermittent	23	32.8±14.7	9
Moderate/severe intermittent	26	30.0±12.2	14
Mild Persistent	14	30.36±13.4	7
Moderate/severe persistent	79	32.7±12.9	42

Statistics

Descriptive analysis was performed by central and dispersion tendency measurements, followed by a bivariate and multivariate analysis. The distribution and homogeneity of variances were assessed before applying statistical tests. Statistical controls were applied for statistically significant associations: the analysis of binary logistic regression adjusted for potential confounding variables such as sex and age; the statistical power (SP)[43] to evaluate the sample size and the false positive report probability (FPRP) using the method described by Wacholder and colleagues[51] to identify potential type I error. In addition, the Bonferroni correction was applied when appropriate.

In silico mRNA-ncRNA Binding Prediction

For the *in silico* binding prediction between mRNA-ncRNA, the IntaRNA algorithm ***version 2.3.0*** was applied.[52,53] This method is an RNA-RNA interaction predictor and interrogates different structural and thermodynamics parameters that affect the possible stabilization of RNA complex. We consider the Gibbs energy how final score for the mRNA-ncRNA complex stabilization estimation.

Each interesting YRNA was evaluated against all human mRNAs described in the NCBI nucleotide database [https://www.ncbi.nlm.nih.gov/nucleotide/] using "in house" programs based on Perl and Python. With this strategy, we obtain for each target, a vector with the Gibbs

Table 1
Characteristics of the studied population.

	N	Age	Female	Allergic Asthma	Allergic Rhinitis
Control	96	55.9±18.1	55	0	0
Allergic patients	208	33.1±13.9	107	149	198
Monosensitized to pollens	52	34.0±15.2	31	37	51
Monosensitized to mites	38	33.9±15.9	17	24	34
Polisensitized	118	32.1±12.6	59	88	113

energy of all human mRNAs described in the database. We selected the statistical low confidence interval of 95% as threshold for the selection of the most probable mRNA-ncRNA complexes. This process was carried out with R platform, version 3.4.3.

For the reduction of the possible technical bias, an YRNA control was used applying the same process described earlier. All possible mRNAs-ncRNAs selected complex using this control were eliminating of analysis.

Functional enrichment and target genes network analysis

The functional ncRNA role was analyzed by the functional enrichment of selected mRNA-ncRNA complexes using topFun.[54] The p-values were adjusted using the FDR method.[55] Network analysis of selected targets for each ncRNAs was performed using the GeneMANIA algorithm.[56] For the specific tissue expression analysis of targets genes, the DAVID method was used.[57] This method provides functional clusters based on different functional sources; in this case, clusters associated to blood and $CD19^+$ cell tissues expression were selected.

Results

Based on our previous RNAseq transcriptomic data from B cells in allergic patients,[38] we focused now on the group of non-coding transcripts with differential expression profile. Interestingly, from the 50 transcripts showing the highest differential expressions, 32 were non-coding. From these, 22 (69%) were YRNAs (Table 3). Differences in YRNAs expression levels were statistically significant with a p-value below 0.05 and a fold ranging change from 2.2 to 9 between controls and patients.

Using the Repeat Mask platform, we verified the homology of our three selected YRNAs with the human YRNA3 (hYRN3) consensus sequence. Several genetic variants in the main YRNA3 recognition sites[32] were detected: CG dinucleotide at position 9; the central pyrimidine enriched region ACTGC; and the polyU tail (Fig. 1). The YRNA.269-201 (ENSG00000201555) exhibited the greatest differences in the sequence, compared to the canonical YRNA3. Using the RT Analyzer, the Y_RNA.269-201 (ENSG00000201555) presented the highest total retrotransposition score: 70 out of 100. These values were 64 for Y_RNA.575-201 (ENSG00000207499) and 54 for Y_RNA.231-201 (ENSG00000201228), strongly suggesting a retrotransposable character (cut-off 40). The analysis of the differences between allergy-related YRNAs and canonical YRNAs suggested a potential decrease in their ability to bind proteins in favor of an increased retrotransposition capacity. The disperse location of the allergy-related YRNAs throughout the genome together with specific modifications in relation to hYRN3 also support this notion.

To confirm the differences observed in the RNAseq study, a validation qPCR study was performed in peripheral blood using an independent cohort of 304 individuals (Table 1). Indeed, all three hYRNAs showed a statistically significant increased expression in allergic patients monosensitized to pollens ($p < 0.005$) (Fig. 2). These results were confirmed by logistic regression adjusted by age and sex ($p = 0.044$) for Y_RNA.269-201 (ENSG00000201555). In addition, for this YRNA we detected a PPV of 40.8% and a NPV of 77.8% for a cutoff of 0.138. As a comparative example, total IgE levels presented a PPV of 85% and a NPV of 52.8%. No differences were observed among the three YRNAs according to severity of asthma (Table 4).

According to these parameters and the Ct levels obtained in the qPCR analysis, the best three candidates [Y_RNA.269-201 (ENSG00000201555), Y_RNA.231-201 (ENSG00000201228) and Y_RNA.575-201 (ENSG00000207499)] (Fig. 1) were selected. Y_RNA.269-201 (ENSG00000201555) was located in the reverse strand of chromosome 6, having a length of 89bp. Aligned in the same chromosome is Y_RNA.575-201 (ENSG00000207499), which generated a

Table 3
Top 50 transcripts differentially expressed ($p < 0.025$) between control and allergic groups.

CODING				NON CODING				
Ensemble ID	External ID Gene	Log_2Fold Change	p value	Ensemble ID	External ID Gene	Log_2Fold Change	p value	YRNA/no YRNA
ENSG00000077238	*IL4R*	0.81	0.024	ENSG00000207281	*FR040863*	2.40	3.18E-05	YRNA
ENSG00000100721	*TCL1A*	1.27	0.001	ENSG00000206967	*FR065554*	1.83	0.007	YRNA
ENSG00000187231	*SESTD1*	1.28	0.002	ENSG00000200521	*FR066841*	1.95	2.14E-04	YRNA
ENSG00000134909	*ARHGAP32*	1.36	0.011	ENSG00000201498	*FR098651*	1.73	0.001	YRNA
ENSG00000185015	*CA13*	1.58	0.014	ENSG00000201867	*FR112219*	2.20	0.019	YRNA
ENSG00000139193	*CD27*	−1.06	0.025	ENSG00000201309	*FR121426*	2.09	0.010	YRNA
ENSG00000173210	*ABLIM3*	4.74	0.01	ENSG00000200888	*FR125594*	2.15	1.91E-04	YRNA
ENSG00000196374	*HIST1H2BM*	−1.67	0.002	ENSG00000207123	*FR164405*	2.11	1.75E-06	YRNA
ENSG00000154380	*ENAH*	2.50	0.003	ENSG00000199751	*FR175800*	1.40	0.004	YRNA
ENSG00000105507	*CABP5*	4.73	0.012	ENSG00000199911	*FR179474*	1.88	0.006	YRNA
ENSG00000150625	*GPM6A*	−1.59	0.003	ENSG00000200849	*FR211833*	1.81	1.39E-05	YRNA
ENSG00000122970	*IFT81*	2.45	0.024	ENSG00000201555	*FR224257*	2.26	0.024	YRNA
ENSG00000151838	*CCDC175*	5.11	0.021	ENSG00000207499	*FR225656*	2.09	3.22E-05	YRNA
ENSG00000107104	*KANK1*	−1.64	0.023	ENSG00000206808	*FR231980*	2.07	0.009	YRNA
ENSG00000064886	*CHI3L2*	−1.75	0.012	ENSG00000200118	*FR269876*	1.81	0.009	YRNA
ENSG00000185666	*SYN3*	3.92	0.006	ENSG00000206659	*FR287842*	1.79	3.20E-04	YRNA
ENSG00000174599	*TRAM1L1*	[a]	0.023	ENSG00000201228	*FR290186*	2.22	9.20E-06	YRNA
ENSG00000042317	*SPATA7*	−3.30	0.012	ENSG00000200314	*FR345774*	1.67	0.017	YRNA
				ENSG00000202536	*FR356123*	2.05	1.89E-04	YRNA
				ENSG00000207387	*FR386105*	1.50	0.017	YRNA
				ENSG00000200164	*FR396408*	1.73	0.001	YRNA
				ENSG00000201955	*RNY3P1*	1.69	0.007	YRNA
				ENSG00000204872	*AC092653.5*	4.05	0.024	no YRNA
				ENSG00000254703	*FLI1-AS1*	[a]	0.023	no YRNA
				ENSG00000215483	*LINC00598*	[a]	0.014	no YRNA
				ENSG00000234456	*MAGI2-AS3*	4.22	0.016	no YRNA
				ENSG00000210195	*MT-TT*	1.96	0.014	no YRNA
				ENSG00000260816	*RP11-319G9.3*	2.73	0.018	no YRNA
				ENSG00000262902	*RP11-750B16.1*	1.29	0.014	no YRNA
				ENSG00000255885	*RP11-815D16.1*	[a]	0.017	no YRNA
				ENSG00000238594	*snoU13*	−1.16	0.024	no YRNA
				ENSG00000223551	*TMSB4XP4*	1.46	0.002	no YRNA

[a] Transcripts not expressed in controls.

Ensemble ID	External ID Gene	Base Mean Expression		Fold Change	p-Value	Chromosome	Strand	Length (bp)
		Controls	Patients					
ENSG00000201555	***FR224257***	6.8	32.7	4.8	0.024	6	Reverse	89
ENSG00000201228	***FR290186***	21.8	101.4	4.6	9.20E-06	12	Reverse	102
ENSG00000207499	***FR225656***	84.4	360.5	4.3	3.22E-05	6	Forward	102

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ENSG00000201555      GGCUGGUCCGAGUGCAGUGGUGUUGACAACUAAUUGAUCGUAACCAGUUACAGAUUUCUU
ENSG00000201228      GGCAGGUCCGAGUGCAGUGGUGUUGAUAACUAAUUCAUUACAAUCAGUUACAGAUUUCUU
ENSG00000207499      GGCUGGUCUGAGUGCAGUGGUGUUUACAACUAAUUGAUCACAACCAGUUACAGAUUUCAU
ENSG00000202354      GGCUGGUCCGAGUGCAGUGGUGUUUACAACUAAUUGAUCACAACCAGUUACAGAUUUCUU
                     *** **** *************** * ******** **   ** ************** *

ENSG00000201555      UGUUCCUUCUCCACUCCCACU-------------AGCCUUAA
ENSG00000201228      UGUUCCUUCUCCACUCCCACUGCUUCACUUGACUGGCCUUUA
ENSG00000207499      UGUUCCUUCCCCACUCCUACUGCUUCACUUGACUUGCCUUAA
ENSG00000202354      UGUUCCUUCUCCACUCCCACUGCUUCACUUGACUAGCCUUUU
                     ********* ******* ***              *****
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Fig. 1. Characteristics of Y_RNAs selected as best candidates. In the Clustal W2 Alignment the hYRNA3 more frequently mutated positions are marked in blue and the bases that vary with respect to hYRNA3 are marked in bold. 1: ENSG00000201555 (Y_RNA.269-201), 2: ENSG00000201228 (Y_RNA.231-201), 3: ENSG00000207499 (Y_RNA.575-201), and 4: ENSG00000202354 (hYRNA3 consensus sequence).

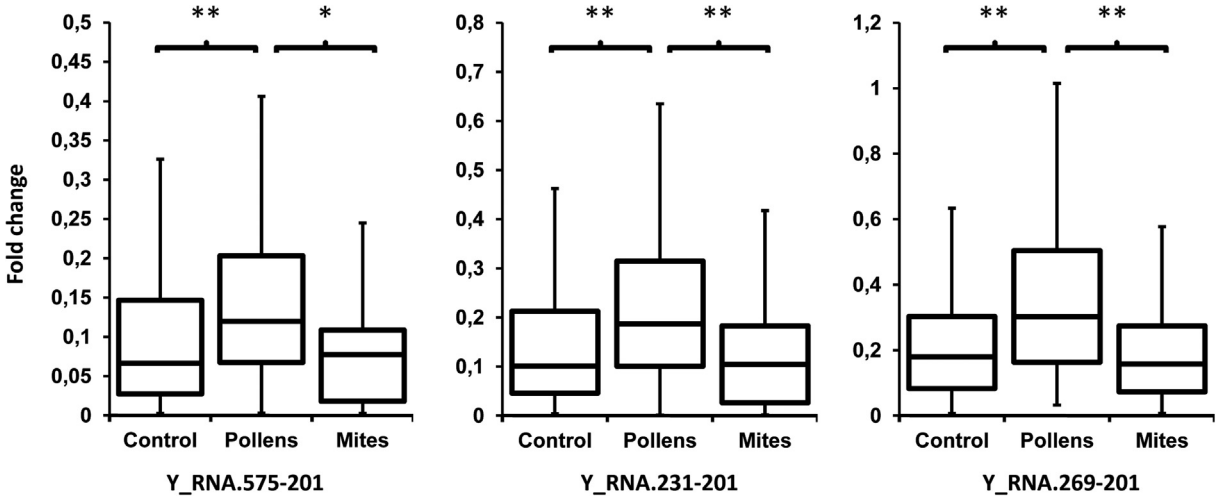

Fig. 2. qPCR validation study of YRNAs overexpression in allergic patients. Distribution of the fold change expression levels of Y_RNA.575-201 (ENSG00000207499), Y_RNA.231-201 (ENSG00000201228) and Y_RNA.269-201 (ENSG00000201555) in the population of controls (n = 96), patients monosensitized to pollens (n = 52) and patients monosensitized to mites (n = 38). K–W test was performed, and significant differences were found between control individuals and those monosensitized to pollens and between individuals monosensitized to pollens and monosensitized to mites. (*$p < 0.05$, **$p < 0.01$).

Table 4
Expression of YRNAs in patients with allergy and asthma, according to severity of symptoms.

	Allergic Asthma and Rhinitis	Y RNA.269-201 (Mean ± SD)	Y RNA.231-201 (Mean ± SD)	Y RNA.575-201 (Mean ± SD)
Asthma classification	n = 142	$p = 0.410$	$p = 0.515$	$p = 0.192$
Intermittent	68	0.276±0.247	0.236±0.372	0.184±0.323
Mild persistent	22	0.236±0.163	0.196±0.133	0.128±0.102
Moderate persistent	45	0.301±0.291	0.236±0.326	0.162±0.196
Severe persistent	7	0.162±0.074	0.439±0.870	0.343±0.687
	Allergic Asthma and Rhinitis	Y RNA.269-201 (Mean ± SD)	Y RNA.231-201 (Mean ± SD)	Y RNA.575-201 (Mean ± SD)
Rhinitis classification	n = 142	$p = 0.590$	$p = 0.408$	$p = 0.422$
Mild Intermittent	23	0.211±0.211	0.165±0.243	0.125±0.196
Moderate/severe intermittent	26	0.344±0.297	0.301±0.381	0.183±0.211
Mild Persistent	14	0.266±0.212	0.142±0.134	0.114±0.128
Moderate/severe persistent	79	0.274±0.248	0.258±0.441	0.201±0.350

SD: standard deviation. p = Anova test.

transcript of 102 bp. Finally, Y_RNA.231-201 (ENSG00000201228) aligned on chromosome 12, with a transcript of 102 bp. These sequences were annotated as "new" in databases. The sequences were located in intronic regions of known genes, Y_RNA.269-201 (ENSG00000201555) in intron 28 of *VARS* gene, Y_RNA.231-201 (ENSG00000201228) in intron 1 of *DENND5B*, and Y_RNA.575-201 (ENSG00000207499) in intron 1 of *AIM1*.

Interestingly, no differences in expression of these three YRNAs were found between control individuals and those monosensitized to mites. In addition, similar statistic significant results were observed when compared the population of allergic patients monosensitized to pollens with the population of patients monosensitized to mites ($p < 0.05$) (Fig. 2). Finally, no differences were found among patients untreated (62.5%), treated with topical corticoid (17.3%) or under immunotherapy. No patient included in this study was under treatment with systemic corticoids.

Considering the potential decrease in protein binding ability of Y_RNA.269-201 (ENSG00000201555) (inferred by the sequence modifications) and under the hypothesis that YRNAs could act as single guide RNA sgRNA directing endoribonucleases to RNA targets,[58] the functional prediction analysis was focused on its capacity of binding RNA targets. We developed a new model to study different parameters that affect stabilization, union force, accessibility and structure to confront YRNAs to more than 100,000 transcripts. The potential target genes for Y_RNA.269-201 (ENSG00000201555) resulting from this complex *in silico* mRNA-ncRNA binding prediction analysis are shown in Fig. 3.

As Y_RNA.269-201 (ENSG00000201555) differential expression was detected both in peripheral blood (PB) and in $CD19^+$ cells, a specific *in silico* tissue filtering analysis using the DAVID algorithm was performed among all potential targets in both $CD19^+$ cell and PB cells (Fig. 3). In addition, to interrogate the functional relationship among all Y_RNA.269-201 (ENSG00000201555) potential targets in both PB and $CD19^+$cells, the Gene MANIA algorithm was applied (Fig. 4). In the $CD19^+$ target analysis, several genes associated to immune response pathways were identified: *KCNMA1*, associated to the nitric oxide pathway, which has been involved in remodeling processes; *TSLP*, which regulates eosinophil migration and has been implicated in treatment of rhinitis; and *OAS1*, which has also been involved in innate immunity and interferon pathways. Remarkably, we identified *AKAP13* a *RHOA* GEF (Guanine nucleotide Exchange Factor) that has been involved in anaphylaxis and asthma.[59,60] The analysis of Y_RNA.269-201 (ENSG00000201555) potential targets developed in peripheral blood, not only confirmed the previous pathways related RHO and *AKAP13*, but also the retinoic acid and *ADAM33* pathways, with important functions described in asthma.[61]

Discussion

Our previous transcriptomic study, performed by RNAseq on B cells of allergic patients was initially focused on differential expression of coding sequences.[38] In this study, we undertook a detailed analysis of differential expression patterns of non-coding sequences that allowed the identification of YRNA overexpression patterns, potentially associated with allergic responses. In fact, out of the top 50 differentially expressed genes, almost 70% of the non-coding sequences corresponded to YRNAs. This impressive percentage arises the question of whether we are facing a regulatory mechanism not previously identified in allergy.

From the 22 YRNAs overexpressed in B cells, the best three candidates, Y_RNA.269-201 (ENSG00000201555), Y_RNA.231-201 (ENSG00000201228) and Y_RNA.575-201 (ENSG00000207499), were selected according to p-value; fold change and Ct expression level. This overexpression was confirmed in peripheral blood by qPCR in an independent cohort of allergic patients. Peripheral blood was selected due to its non-invasive nature, ease and accessibility.

A regulatory role of YRNAs has been previously described in other pathological processes, such as autoimmune diseases,[25] cancer[29,30] and cardiac diseases.[27,28] They have been related to the modulation of the expression of some cytokines, such as IL10.[62] Small fragments derived from YRNAs have been identified in different studies.[29] Here, we identified complete YRNAs, as confirmed by RT-PCR by using primers that

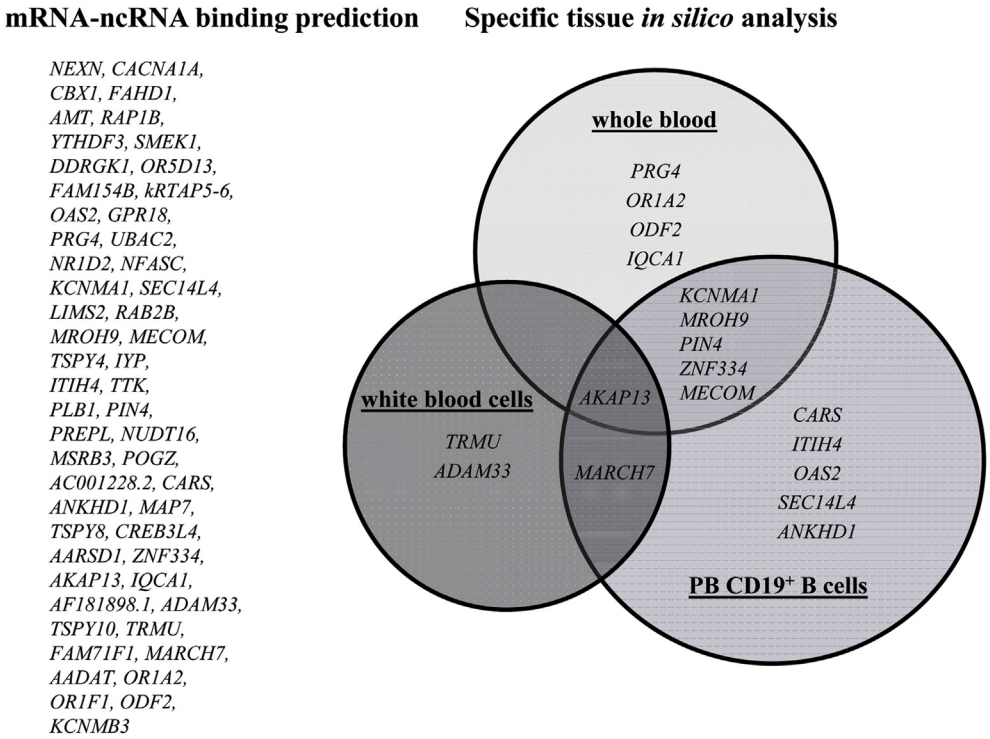

Fig. 3. Y_RNA.269-201 *In silico* analyses.

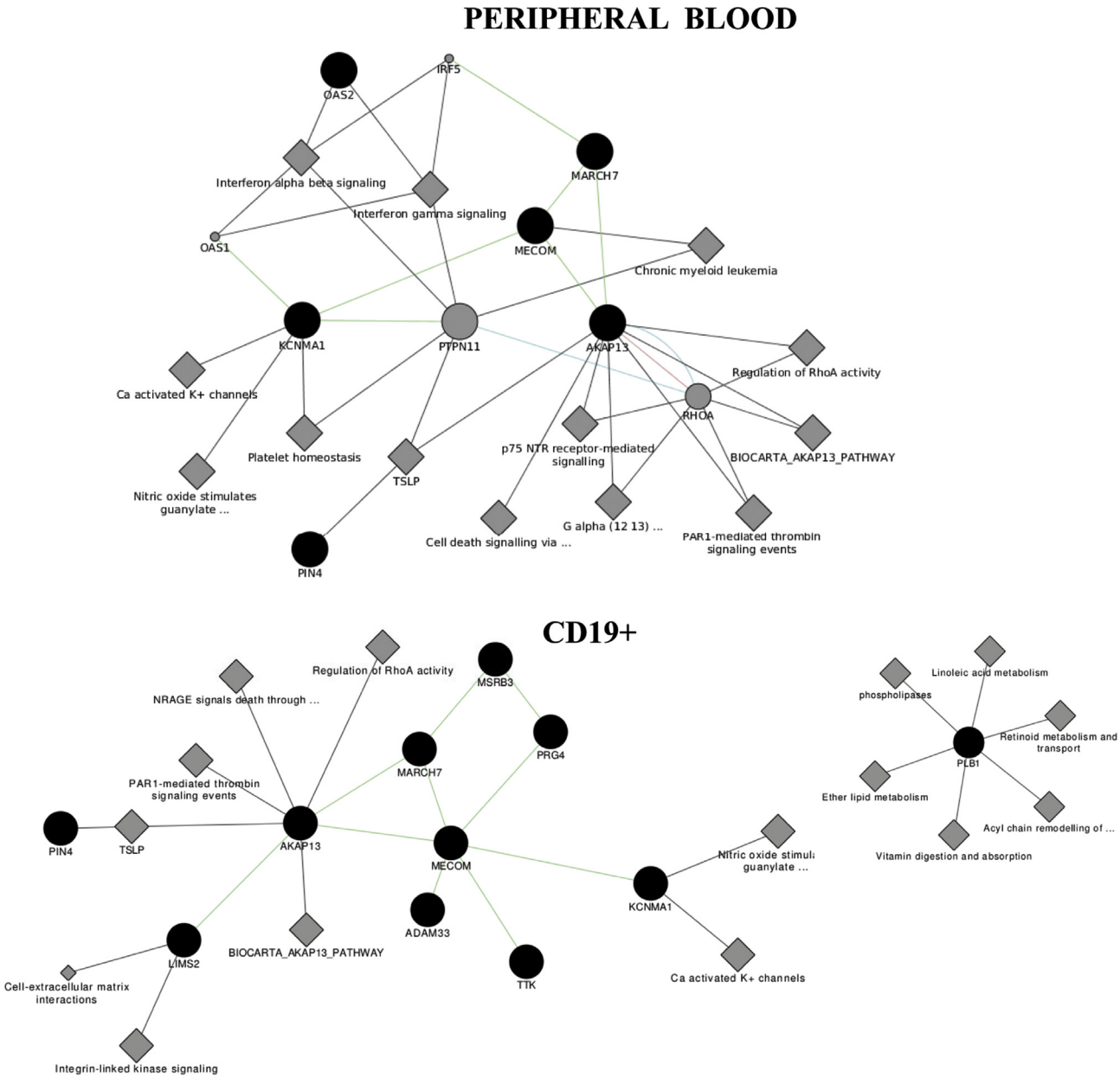

Fig. 4. Functional relations of Y_RNA.269-201 (ENSG00000201555) potential targets. GeneMANIA algorithm prediction. Black circles represent gene targets. Grey circles represent associated genes to network inference. Grey rhombuses represent pathways. The green edges represent genetics relationship. Pink and blue edges represent to protein-protein interaction.

amplify the entire YRNA sequence. This permits the identification of complete YRNA sequences but also validates the differential expressions previously obtained by NGS in allergic patients.

We have detected an increased expression of Y_RNA.269-201 (ENSG00000201555) in allergic patients, particularly in those patients sensitized to pollen, obtaining better NPV specifications than total IgE levels. Although we are aware that IgE is not the biomarker regarding NPV, our results arise the question of whether YRNAs could be considered potential clinical biomarkers in allergic disease. To assess their potential as biomarkers, they should allow for the differentiation of two biological states, showing significant differences between patients and controls, being robust, analyzable by a practicable technique, detected in a biological sample (preferentially non-invasively), and having greater clinical utility than other markers currently used. Although our study seems to point in this sense, larger studies are needed to confirm whether these molecules could be considered biomarkers in allergy.

There is not a clear explanation for the finding regarding to patients monosensitized to pollens. In this sense, we analyzed several factors that could have interfered, such as the time of evolution of the disease, finding no statistically significant differences (allergic asthma, 12.94±11.77 years for patients monosensitized to pollen and 10.0±9.35; for patients monosensitized to mites, $p = 0.336$; allergic rhinitis, 12.86±11.89, versus 9.4±10.07, respectively, $p = 0.406$). Also, no differences were observed according to the severity of patients monosensitized to pollen or to mites (data not shown). Concerning pollen allergic patients, we did not find statistically significant differences between patients that were analyzed during the pollen seasons and out of it (data no shown). Finally, it could be related to differences in the interaction of mites and pollens with the immune system.[63,64]

To interrogate the potential role of YRNAs as regulatory mechanisms in allergy, exhaustive structural and functional analyses were performed. The structural analysis of these sequences allowed us to characterize

specific differences that predicted modifications on the capacity of binding to specific proteins for RNP formation. Thus, the increased expression of these YRNAs has the potential to impair the balance of classical RNP formation or determine a different protein binding pattern that could affect their function. In the future, it could be highly interesting to identify specific proteins able to bind to these allergy-related YRNAs.

The analysis of the differences between allergy-related YRNAs and canonical YRNAs suggested a potential decrease in their ability to bind to proteins in favor of an increased capacity for retrotransposition. This "retrotransposable character" was reinforced by the scores obtained in the *in silico* analysis. The disperse location of the allergy-related YRNAs throughout the genome, together with specific modifications in relation to hYRN3, likewise supports this notion. A role for endogenous genome retroelements in the development of diseases has been strongly related to autoimmune processes, where retroelements have been described as constitutively synthesized elements that can alter the immune system and trigger pathological states when deregulated.[35,36] Our study opens new insights regarding whether this process affects immune mechanisms underlying allergic diseases and could reinforce the linking of allergy with autoimmune processes. Consistent with the hypothesis that the retrotransposable capacities of YRNAs might play a role in immunomodulation, Y_RNA.269-201 (ENSG00000201555) is located at intron 28 of the *VARS* gene, inside region III of the human histocompatibility principal complex (HLA). In this sense, molecules such as Y_RNA.269-201 (ENSG00000201555), may be developing a "Trojan horse" strategy[35,36] maintaining its expression at basal level until the conditions necessary to escape the control of immune system and being overexpressed in pathological process.

Considering the potential decrease of the ability of the Y_RNA.269-201 (ENSG00000201555) to bind proteins, deduced by its sequence modifications and under the hypothesis that YRNAs could act as sgRNAs for directing endoribonuclases to RNA targets,[31] the functional prediction analysis was focused on its capacity of binding RNA targets. We developed a new model to study different parameters that affect stabilization, union force, accessibility and structure to confront YRNAs to more than 100,000 transcripts that could confirm potential union targets. The analysis of these targets allowed the identification in $CD19^+$ cells of transcripts associated with the nitric oxide pathway, such as *KCNMA1*, which has been involved in remodeling processes; *TSLP,* released by epithelia and able to induce allergic responses; and *OAS1,* which has also been involved in innate immunity and interferon pathways.[65] YRNAs, in combination with miRNAs, have been previously reported to be able to regulate the innate immune response in human parasite secretory vesicles.[37] Remarkably, we identified *RHO* (associated with *AKAP13*), which has been previously described involved in anaphylaxis and asthma.[59,60] Silencing studies of SOC3 have demonstrated downregulation of RhoA/Rho-kinase protein expression, pointing to potential therapeutic applications in chronic asthma.[66] In the last years, several studies have indicated the involvement of the RhoA-ROCK pathway in a wide spectrum of immune-mediated diseases.[60] The bioinformatics analysis developed in peripheral blood not only confirmed the previous pathways of *RHO* and *AKAP13,* but also the retinoic acid and *ADAM33* pathways, which have important functions in asthma.[61]

In summary, ncRNAs are important elements of epigenetic regulation. In this study, we described for the first time a validated differential expression pattern of hYRNAs in peripheral blood of allergic patients. We present an *in silico* structural analysis that allowed us to infer a decrease in the protein binding capacity in favor of acquiring additional retrotransposable potential. Furthermore, we performed a functional *in silico* screening for potential RNA sequences that might be targeted by these hYRNAs, affecting immunological pathways that might have an ultimate impact on allergy. Although additional analyses and functional studies to confirm the precise biological role of YRNAs are needed, this study opens exciting new insights of ncRNAs (other than miRNAs) as potential biomarkers, therapeutic targets and molecules with latent pathological impact on allergy.

Conclusions

In this study, we described for the first time the different YRNA expressions and their potential role in allergy. Structural and functional *in silico* analyses point to their implication in regulatory mechanisms of the disease.

Declarations

Ethics approval and consent to participate

The approval of the Ethical Committee of the University Hospital of Salamanca was obtained.

Consent for publication

Not applicable.

Availability of data and materials

Please contact author for data requests.

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Competing interests

The authors declare that they have no competing interest.

Authors' contributions

I-G M, G-S A, S C, E M, M-V E, P M, R S, M-G F, T JC, and D I have contributed in designing research studies, conducting experiments, acquiring data, analyzing data and writing the manuscript. All authors read and approved the final manuscript.

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