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 \textit{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.\textsuperscript{1} An increasing amount of evidence suggests that epigenetic mechanisms act as a link between genetic and environmental factors in allergy.\textsuperscript{1} In the first epigenomic study performed in B cells of allergic patients, we identified a differential methylation pattern in allergy.\textsuperscript{2} In addition to DNA methylation, histone modifications and RNAs are also involved in epigenetic processes.\textsuperscript{3,4} Most of the transcribed RNA does so in the form of non-coding RNAs (ncRNA).\textsuperscript{3,4} ncRNAs, are grouped into housekeeping
RNAs, long-ncRNAs and small-ncRNAs (snRNAs). SnRNAs 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.2–4 Previous studies have shown different miRNA expression profiles in the bronchial epithelium of asthmatic patients, involved in epithelial cell proliferation, differentiation, and apoptosis.5 The role of other ncRNAs in allergy remains largely unknown yet. Among snRNAs, YRNAs appears highly conserved in animal phylogeny.14 Orthologous genes exist in animals,25 Furthermore, YRNAs are degraded during apoptosis in a fast and specific caspase dependent process,26 generating YRNA-derived small RNA fragments that have been associated with atherogenesis, cardiovascular diseases27,28 and cancer.29,30 Recently, it has been described that these 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, YRNAs are transcribed by the RNA polymerase III from a cluster of genes located in chromosome 7q36,13 ranging from 83 to 112 nt.13,18,19 hYRNAs are transcribed by the RNA polymerase III from a cluster of transposable elements, and potential functions have been described for retrotransposons in 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 regulation32 as well as in human pathology such as cancer.26 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 individuals38 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, those that appeared with YRNA notation after alignment with genomic databases 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-GRC38/hg38 database and Genome Browser database 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 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. 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) 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.

**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 Optimizer, 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. YRNA primers were designed using the primer analysis software Primer 3.0 and the Beacon Designer Software for Y_RNA.269-201 (ENSG00000201555) (forward primer 5’-cgagtgcag tgggtgtcgc-3’ and reverse primer 5’-gcagcttgtagaggaag-3’), Y_RNA.23 1-201 (ENSG00000201228) (forward primer 5’-agagctgctgagctgc-3’ and reverse primer 5’-gacgtaactgtagaag-3’) and Y_RNA.142 3-201 (ENSG00000207499) (forward primer 5’-tcggtctgagctggggt-3’ and reverse primer 5’-aaggaagtcagctggta-3’). GAPDH reference gene primers (forward 5’-ctgtcctgctgctgtc-3’ and reverse 5’-aaggaagtcagctggta-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) × 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 (TATTA Biocenter, Göteborg, Sweden). The study was performed blinded regarding control or patient status. All procedures followed MIQE guidelines.

**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) to evaluate the sample size and the false positive report probability (FPRP) using the method described by Wacholder and colleagues 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 was applied. 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 using “in house” programs based on Perl and Python. With this strategy, we obtain for each target, a vector with the Gibbs

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**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 pollen | 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              |

**Table 2**

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

|               | Allergic Asthma and Rhinitis | n = 142 | Age | Female |
|---------------|-----------------------------|---------|-----|--------|
| Asthma class  |                             |         |     |        |
| Intermittent  |                             | 68      | 27.9±11.3 | 33 |
| Mild persistent |                           | 22      | 35.4±9.8  | 11  |
| Moderate persistent |                      | 45      | 35.9±15.3 | 24  |
| Severe persistent |                         | 7       | 39.4±12.9 | 4   |
| Rhinitis class |                             |         |     |        |
| 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  |
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. The p-values were adjusted using the FDR method. Network analysis of selected targets for each ncRNAs was performed using the GeneMANIA algorithm. For the specific tissue expression analysis of targets genes, the DAVID method was used. 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, we focused now on the group of non-coding transcripts with differential expression profile. Several genetic variants in the main YRNA3 recognition sites32 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 increase in allergic patients mono-sensitized 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.

| Ensemble ID      | External ID Gene | Log2Fold Change | p value | NON CODING | Ensemble ID      | Log2Fold Change | p value | YRNA/no YRNA |
|------------------|------------------|-----------------|---------|------------|------------------|-----------------|---------|--------------|
| ENSG00000077238  | I4AR             | 0.81            | 0.024   | NON CODING | ENSG0000007281  | 2.40            | 3.18E-05| YRNA         |
| ENSG00000100721  | TClIA            | 1.27            | 0.001   | NON CODING | ENSG0000006967  | 1.83            | 0.007  | YRNA         |
| ENSG00000187231  | SEDST1           | 1.28            | 0.002   | NON CODING | ENSG000000521   | 1.95            | 2.14E-04| YRNA         |
| ENSG00000134909  | ARHGAp2          | 1.36            | 0.011   | NON CODING | ENSG000001498   | 1.73            | 0.001  | YRNA         |
| ENSG00000185015  | CAL3             | 1.58            | 0.014   | NON CODING | ENSG0000021667  | 2.20            | 0.019  | YRNA         |
| ENSG00000139193  | CD27             | -1.06           | 0.025   | NON CODING | ENSG000001309   | 2.09            | 0.010  | YRNA         |
| ENSG00000173210  | ABLIM3           | 4.74            | 0.01    | NON CODING | ENSG0000008888  | 2.15            | 1.91E-04| YRNA         |
| ENSG00000196374  | HST1H2BM         | -1.67           | 0.002   | NON CODING | ENSG0000027123  | 2.11            | 1.75E-06| YRNA         |
| ENSG00000154380  | ENAH             | 2.50            | 0.003   | NON CODING | ENSG0000019110  | 1.40            | 0.004  | YRNA         |
| ENSG00000105507  | CABP5            | 4.73            | 0.012   | NON CODING | ENSG0000019911  | 1.88            | 0.006  | YRNA         |
| ENSG00000150625  | GPM6A            | -1.59           | 0.003   | NON CODING | ENSG0000020399  | 2.18            | 1.36E-05| YRNA         |
| ENSG00000122970  | IFF1             | 2.45            | 0.024   | NON CODING | ENSG00000201555 | 2.26            | 0.024  | YRNA         |
| ENSG00000151838  | CCDC175          | 5.11            | 0.021   | NON CODING | ENSG0000027499  | 2.09            | 3.22E-05| YRNA         |
| ENSG00000107104  | KANK1            | -1.64           | 0.023   | NON CODING | ENSG0000026808  | 2.07            | 0.009  | YRNA         |
| ENSG0000064866   | CHEL2            | -1.75           | 0.012   | NON CODING | ENSG00000200118 | 1.81            | 0.009  | YRNA         |
| ENSG00000185666  | SYN3             | 3.92            | 0.006   | NON CODING | ENSG0000020659  | 1.79            | 3.20E-04| YRNA         |
| ENSG00000174599  | TRAM1L1          | 2.03            | 0.023   | NON CODING | ENSG0000021228  | 2.22            | 9.20E-06| YRNA         |
| ENSG00000042317  | SPATA7           | -3.30           | 0.012   | NON CODING | ENSG00000200314  | 1.67            | 0.017  | YRNA         |
| ENSG00000025236  | RAB5D            | 2.05            | 0.007   | NON CODING | ENSG00000215483  | 1.50            | 0.017  | YRNA         |
| ENSG0000002000164 | MAGI2-AS3       | 4.22            | 0.016   | NON CODING | ENSG0000024456  | 1.73            | 0.001  | YRNA         |
| ENSG00000021955  | RNY3P1           | 1.69            | 0.007   | NON CODING | ENSG0000020987  | 4.05            | 0.024  | no YRNA      |
| ENSG000000204872 | ACO29653.5       | 4.05            | 0.024   | NON CODING | ENSG0000026816  | 2.73            | 0.018  | no YRNA      |
| ENSG000000254703 | IL11-AS1         | 4.05            | 0.024   | NON CODING | ENSG0000026202  | 1.29            | 0.014  | no YRNA      |
| ENSG000000255885 | RP11-81D16.1     | 4.05            | 0.024   | NON CODING | ENSG00000023551 | 1.46            | 0.002  | no YRNA      |

* Transcripts not expressed in controls.
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 hYRNA 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).

![Alignment figure](#)

**Table 4**

|     | Y_RNA.575-201 (Mean ± SD) | Y_RNA.231-201 (Mean ± SD) | Y_RNA.269-201 (Mean ± SD) |
|-----|-----------------------------|-----------------------------|-----------------------------|
| **Asthma classification** |                             |                             |                             |
| Intermittent | n = 142 | 0.276±0.247 | 0.236±0.372 | 0.184±0.323 |
| Mild persistent | n = 22 | 0.236±0.163 | 0.196±0.133 | 0.128±0.102 |
| Moderate persistent | n = 45 | 0.301±0.291 | 0.236±0.326 | 0.162±0.196 |
| Severe persistent | n = 7 | 0.162±0.074 | 0.439±0.870 | 0.343±0.687 |

**Rhinitis classification**

|     | Y_RNA.269-201 (Mean ± SD) | Y_RNA.231-201 (Mean ± SD) | Y_RNA.575-201 (Mean ± SD) |
|-----|-----------------------------|-----------------------------|-----------------------------|
| Intermittent | n = 142 | 0.211±0.211 | 0.165±0.243 | 0.125±0.196 |
| Mild Intermittent | n = 26 | 0.344±0.297 | 0.301±0.381 | 0.183±0.211 |
| Mild Persistent | n = 14 | 0.266±0.212 | 0.142±0.134 | 0.114±0.128 |
| Moderate/severe persistent | n = 79 | 0.274±0.248 | 0.258±0.441 | 0.201±0.350 |

SD: standard deviation. *p < 0.05, **p < 0.01.
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 rhinitic; 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 cancer29,30 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

**mRNA-ncRNA binding prediction**

- **NEXN**, CACNA1A, CBX1, FAHD1, AMT, RAP1B, THD2F3, SMEK1, DDKRG1, OR5D13, FAM154B, KRTAP5-6, OAS2, GPR18, PRG4, UBAC2, NR1D2, NFASC, KCNA1, SEC14L4, LIMS2, RAB28, MROH9, MECOM, TSPY4, IYP, ITIH4, TTK, PLB1, PIN4, PREPL, NUDT16, MSR3, POGZ, AC001212.2, CARS, ANKHD1, MAP7, TSP56, CREB3L4, AARS1, ZNF334, AKAP13, IQCA1, AF181898.1, ADAM33, TSPY10, TRMU, FAM71F1, MARCH7, AADAT, OR1A2, ORIF1, ODF2, KCNMB3

**Specific tissue in silico analysis**

- **PB CD19+ B cells**: TRMU, ADAM33
- **white blood cells**: TRMU, ADAM33
- **whole blood**: KMOH9, PIN4, ZNF334, MECOM

**Fig. 3. Y_RNA.269-201 In silico analyses.**
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 YRNA.269-201 (ENSG00000201555) in allergic patients, particularly in those patients sensitized to pollen, obtaining better NPV specificities 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 not 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 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 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” strategy35,36 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 endonucleases 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 contain 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.32 Remarkably, we identified RHO (associated with AKAP13), which has been previously described involved in anaphylaxis and asthma.39,60 Silencing studies of SOC3 have demonstrated downregulation of RhoA/Rho-kinase protein expression, pointing to potential therapeutic applications in chronic asthma.56 In the last years, several studies have indicated the involvement of the RhoA-ROCK pathway in a wide spectrum of immune-mediated diseases.59 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.

In summary, ncRNAs are important elements of epigenetic regulation. 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.

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

J-G M, G-S A, S C, E 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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References

1. Isidoro-García M, Dávila-González I, Pascual de Pedro M, Sanz-Lozano C, Lorente-Toledano F. Interactions between genes and the environment. Epigenetics in allergy. Allergol Immunopathol. 2007;35(6):254-258.
2. Pascual M, Suzuki M, Isidoro-García M, et al. Epigenetic changes in B lymphocytes associated with house dust mite allergic asthma. Epigenetics. 2011;6(9):1–7.
3. Santosh B, Varshney A, Yadava PK. Non-coding RNA: biological functions and applications. Cell Biochem Funct. 2015;33(1):1-22.
4. Hall AE, Turnbull C, Dalmay T. YRNAs: recent developments. Biomol Concepts. 2013;4(2):103–110.
5. Ernst C, Morton CC. Identification and function of long non-coding RNA. Front Cell Neurosci. 2013. https://doi.org/10.3389/fncel.2013.00168.
6. NONCODE. An integrated knowledge database dedicated to ncRNA, especially IncRNAs. http://www.noncode.org/index.php. Accessed 6 Sep 2017.
7. Brook PO, Perry MM, Adcock IM, Durham AL. Epigenome-modifying tools in asthma. Epigenomics. 15(7):1017–1032.
8. Reumann G, Chavez L, Gerastimova A, et al. Epigenomic analysis of primary human T cells reveals enhancers associated with TH2 memory cell differentiation and asthma susceptibility. Nat Immunol. 2014;15(8):777–788.
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28. Repetto E, Lichtenstein L, Hizir Z, et al. RNY-derived small RNAs as a signature of dysregulated in asthma controls IL-6 production in bronchial epithelial cells. PLoS ONE 2014;9(10):e111659.

29. Solberg OD, Ostrin EJ, Love MI, et al. Airway epithelial miRNA expression is altered in asthma. Am J Respir Crit Care Med. 2012;186(10):965–974.

30. Lerner MR, Boyle JA, Hardin JA, Steitz JA. Two novel classes of small ribonucleoproteins detected by antibodies associated with lupus erythematosus. Proc Natl Acad Sci USA. 1979;74:5495–5499.

31. Wolin SL, Steitz JA. Genes for two small cytoplasmic ro RNAs are adjacent and appear to be single-copy in the human genome. Cell. 1983;32:735–744.

32. Lerner MR, Boyle JA, Hardin JA, Chung J, Wolin SL. The subcellular distribution of an RNA quality control protein, the Ro autoantigen, is regulated by noncoding Y RNAs. Nucleic Acids Res. 2006;34(21):1328–1339.

33. Sim S, Weinberg DE, Fuchs G, Choi K, Wolin SL. The functional requirement of noncoding Y RNAs for human chromosomal DNA replication. Mol Biol Cell. 2009;20:1555–1564.

34. Mosig A, Guofeng M, Stadler BMR, Stadler PF. Evolution of the vertebrate Y RNA gene families. Bioessays. 2016;38(5):415–419.

35. Christov CP, Gardiner TJ, Szüts D, Krude T. Functional requirement of noncoding Y RNAs derived from a human Y RNA gene in patients with asthma. Allergy. 2006;61(5):543–548.

36. Volkman HE, Stetson DB. The enemy within: endogenous retroelements and RNA degradation of the small cytoplasmic Y RNAs during apoptosis. J Mol Med (Berl). 2004;82:232–247.

37. Stadler PF, Mosig A. Y RNA genes: evolution, function, and conservation. Annu Rev Genet. 2007;41(1):423–446.

38. Pascual M, Roa S, García-Sánchez A, et al. Genome-wide expression profiling of B lymphocytes reveals IL-4R increase in allergic asthma. J Allergy Clin Immunol. 2014;134:972–975.

39. Ensembl. http://www.ensembl.org/index.html. Accessed 1 Oct 2018.

40. Genome Browser. https://genome.ucsc.edu/. Accessed 16 Nov 2018.

41. RT-Analyzer. http://biotools.riboclub.org/cgi-bin/RTAnalyzer/index.pl?page=r1_final. Accessed 1 Apr 2016.

42. Sanu C, Isidoro-García M, Davila I, et al. Promoter genetic variants of prostanoid DP receptor (PTGDR) gene in patients with asthma. Allergy. 2006;61(5):543–548.

43. Proportion Difference Power/Sample Size Calculation. http://statpages.info/proppower.html. Accessed 30 Oct 2009.

44. Dreborg S. The skin prick test in the diagnosis of atopic allergy. J Am Acad Dermatol. 1989;21(4):820–821.

45. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention. NHLBI/WHO Workshop Report. 2006. NIH Publication number 02-3659.

46. Bouquet J, Khaltaev N, Cruz AA, et al. Allergic rhinitis and its impact on asthma (ARIA) 2008 update (in collaboration with the world health organization, GAC/LEN and AllerGen). Allergy. 2008;63:8–160.

47. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402–408.

48. Primer3.O. http://bioinfo.ut.ee/primer3-0.4.0/. Accessed 20 Nov 2012.

49. Beacon Designer. http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1. Accessed 14 Jul 2017.

50. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55(4):611–622.

51. Wacholder S, Rothman N, Caporaso N. Population stratification in epidemiologic studies of common genetic variants and cancer: quantification of bias. J Natl Cancer Inst. 2004;96(2):1151–1158.

52. Wright PR, Georg J, Mann M, et al. CopraRNA and IntaRNA: predicting small RNA targets, networks and interaction domains. Nucleic Acids Res. 2014;42(W1):W119–W123.

53. Busch A, Richter AS, Backofen R. IntaRNA: efficient prediction of bacterial sRNA targets incorporating target site accessibility and seed regions. Bioinformatics. 2008;24(24):2849–2856.

54. Chen J, Bardes EE, Aronow BJ, Jegga AG. ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. Nucleic Acids Res. 2009;37:W363–W368.

55. Proportion Difference Power/Sample Size Calculation.http://statpages.info/proppower.html. Accessed 30 Oct 2009.

56. Mostafavi S, Ray D, Warde-Farley D, Grouios C, Morris Q. GeneMANIA: a real-time interaction network inference tool. Bioinformatics. 2008;24(24):2849–2856.

57. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nat Genet. 2009;41(1):44–57.

58. Ninomiya S, Kawano M, Abe T, et al. Potential small guide RNAs for tRNase ZL from D. radiodurans. J Biol Chem. 2006;281(26):15648–15654.

59. Sadler AJ, Williams BRG. Interferon-inducible antiviral effectors. J Biol Chem. 1999;274(24):17499–17507.

60. Ricker E, Chowdhury L, Yi W, Perins AB. The RhoA-ROCK pathway in the regulation of epithelial barrier function. J Exp Med. 2016;16(4):207–219.

61. Vishweswaraiah S, Veerappa AM, Mahesh PA, Jayaraju BS, Krishnarao CS, Ramachandra NB. Molecular interaction network and pathway studies of ADAM33 potentially relevant to asthma. Ann Allergy Asthma Immunol. 2014;113(4):368–374.

62. Seumos G, Chavez L, Geraminova A, et al. Epigenomic analysis of primary human T cells reveals enhancers associated with TH2 memory cell differentiation and asthma susceptibility. Nat Immunol. 2014;15(8):777–788.

63. Ricker E, Chowdhury L, Yi W, Perins AB. The RhoA-ROCK pathway in the regulation of T and B cell responses. J Immunol Research. 2016;2016:1–9.

64. Vishweswaraiah S, Veerappa AM, Mahesh PA, Jayaraju BS, Krishnarao CS, Ramachandra NB. Molecular interaction network and pathway studies of ADAM33 potentially relevant to asthma. Ann Allergy Asthma Immunol. 2014;113(4):368–374.

65. Gon Y, Hashimoto S. Role of airway epithelial barrier dysfunction in pathogenesis of asthma. Allergy. 2016;71:12–17.

66. Traidl-Hoffmann C, Mariani V, Hochrein H, et al. Pollen-associated propeptidases inhibit dendritic cell interleukin-12 production and augment T helper type 2 cell polarization. J Exp Med. 2005;201(4):457–459.

67. Sadler AJ, Williams BRG. Interferon-inducible antiviral effectors. Nat Rev Immunol. 2008;8(7):559–568.

68. Zafra MP, Mazzeo C, Gámez C, et al. Gene silencing of SOCS3 by siRNA intranasal delivery inhibits asthma phenotype in mice. PLoS One. 2014;9(4):e91996.