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TRUNCATED IFI16 mRNA TRANSCRIPTS CAN CONTROL ITS VIRAL DNA DEFENSE ACTIVITY

Jorge Martinez-Laso[1,*], Isabel Cervera[1], Marina S. Martinez-Carrasco[1,2], Clara Sánchez-Menéndez[3,4], Manuel Remesal[5],Guiomar Casado-Fernández[3,6], Elena Mateos[3,7], Luis Lemus-Aguilar[3,4], Montserrat Torres[3,7], Mayte Coiras[3,7]

[1]Immunogenetics Unit. National Center of Microbiology, Instituto de Salud Carlos III, 28220 Madrid, Spain.

[2]Pediatrics Department. Hospital Universitario 12 de Octubre. Avda de Córdoba s/n 28041 Madrid

[3]Immunopathology and Viral Reservoir Unit, National Center of Microbiology, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain.

[4]PhD Program in Biomedical Sciences and Public Health, Universidad Nacional de Educación a Distancia (UNED), Madrid, Spain.

[5]Department of Pharmacy and Nutrition. Faculty of Biomedical and Health Sciences. Universidad Europea de Madrid. Villaviciosa de Odón, 28670 Madrid, Spain.

[6]PhD Program in Health Sciences, Faculty of Sciences, Universidad de Alcalá, Alcalá de Henares, Madrid, Spain.

[7]Biomedical Research Center Network in Infectious Diseases (CIBERINFEC), Instituto de Salud Carlos III, Majadahonda, Madrid, Spain.

Correspondence: Jorge Martinez-Laso. Unidad de Inmunogenetica. Centro Nacional de Microbiología. Instituto de Salud Carlos III. Ctra Majadahonda-Pozuelo K2,2, 28220 Majadahonda, Madrid. e-mail: jmlaso@isciii.es; Tel.: 34918223751

ABSTRACT

One of the most well-known viral receptors of the group called named ALRs is IFI16 (interferon-inducible protein 16) that are responsible for responses against viral dsDNA. A pyrin domain (PYD), two HIN domains, a NLS (nuclear localization sequence), and S/T/P repeats region form the structure of IFI16. Five alternatively transcripts have been described (V1, V2, V9, V4 and Vβ) that encode five isoforms (IFI16-iso1, 2, 3, 4 and β) with different structure, localization, and function. Another four transcripts (V3, V5, V6, and V8) and 12 predicted transcripts (VX1-VX7, VX1.1-VX5.1) have also been registered in the Genebank without any structural study. In the present study, we have performed a complete study of the presence of the IFI16 transcripts in a healthy population. All the alternative transcripts described except six of the so-called predicted transcripts were found, furthermore, two new transcripts (V10, V11) were described. The main mechanisms for the regulation of mRNA from IFI16 expression are due to the insertion of non-coding regions and the loss of almost all exons. A total of nine different isoforms were found and the corresponding protein models were constructed to establish the modification of its functionality to form inflammasomes or the binding to viral DNA.

Keywords: IFI16; ALRs; alternative RNA splicing; isoforms.

1. INTRODUCTION

IFI16 (Interferon inducible protein 16), AIM2 (absent in melanoma 2), IFIX (interferon-inducible protein X), and MNDA (myeloid cell nuclear differentiation antigen) are DNA viral receptors and constitute a gene family named HIN-200 or PYHIN. These groups of receptors are also named as ALRs (AIM2-like receptors) and the corresponding genes are located in the chromosome band 1q23.1. The structure of these receptors is made up by an N-terminal domain named PYD (pyrin domain) and another one consisting of 200 amino acids motif named HIN-200. IFI16 has two HIN domains (HIN-A, HIN B) while AIM2, MNDA, and PYHIN1 has only one (HIN C) (Borucka et al., 2022; Brunette et al, 2012; Cridland et al., 2012; Veeranki & Choubey, 2012). HIN-200 domain is in charge of the viral dsDNA binding, and in IFI16, HIN-A bind ssDNA and dsDNA and HIN-B GC-rich DNA and the pyrin domain regulate protein interactions (Bertin and DiStefano, 2000; Li et al., 2012; Ni et al., 2016; Schattgen and Fitzgerald, 2011; Unterholzner et al., 2010).

In the case of AIM2 and IFI16, once the receptor recognizes the viral DNA, two activation pathways occur. On the one hand, the formation of an inflammasome that gives rise to the production of IL1b and IL18 and cell lysis by pyroptosis as in the case of HIV proviral DNA case killing infected CD4 T lymphocytes (Mishra et al., 2022; Monroe et al.., 2014: Munoz-Arias et al., 2015; Schattgen and Fitzgerald, 2011). On the other hand, the pathway corresponding to STING is activated, which through TBK1 and IRF3 generates increased expression of interferon α and β (Jønsson et al., 2017; Almine et al., 2017).

The main viruses recognized by IFI16 are vaccinia virus (VACV), Kaposi sarcoma-associated herpesvirus (KSHV), Epstein-Barr virus (EBV) and herpes virus type 1 (HSV-1) that mainly activate inflammasome formation (Ansari et al., 2015; Iqbal et al., 2016; Kerur et al., 2011; Roy et al., 2016; Unterholzner et al., 2010), and HIV type 1 and

hepatitis B virus that activate the STING pathway (Ansari et al., 2015; Kerur et al., 2011; Monroe, et al., 2014; Roy et al., 2016; Unterholzner et al., 2010; Yang et al., 2020). IFI16 also has other functions besides that of viral receptor since it is involved in the processes of suppression of cellular expression genes (Johnstone et al.., 1998a; Johnson et al., 2014), proliferation (Ong et al., 2022), differentiation (Piccaluga et al., 2015) and inflammation (Ansari et al., 2013).

In addition, the presence of anti-IFI16 autoantibodies or the increased levels of IFI16 have been found in several autoinmune diseases as systematic lupus erythematosus (Antiochos et al., 2022), rheumatoid arthritis (Alunno et al., 2016), inflammatory bowel disease (Vanhove et al., 2015), systemic scleroderma (McMahan et al., 2016), psoriasis (Cao et al., 2016), and Sjogren syndrome (Baer et al., 2016) without any clear explanation of the participation of this receptor in the molecular pathogenesis of diseases.

Alternatively spliced transcript variants encoding five isoforms (IFI16-iso1, 2, 3, 4, and beta) have been described for the IFI16 gene, the corresponding transcripts have also been named as V1, V2, V9, V4, and Vβ. Transcript V1 consists of 11 exons of which there is one duplicated (named as 7A, later exon 9; Johnstone et al., 1998b). In addition, it lacks one exon later named as 4 so the total number of exons would be 12 (for this reason the exon 7 and 7A would be later as 8 and 9 respectively; Figure 1). This transcript encoding a protein of 729 amino acids (previously named IFI16A; Li et al., 2021). This variant lacks an in-frame exon near the 59-coding region that encodes the corresponding NLS motifs 2, 3, and 4 in V2, but it has an additional equal-length in-frame exon in the 39-coding region (Figures 1 and 3) (Li et al., 2012, Johnstone et al., 1998b, Trapani et al., 1992).

Transcript V2 has the exon 4 but lacks the duplicated exon 7A (which would later be exon 9; Figure 1) encoding a protein of 729 amino acids (previously named IFI16B, Li et al., 2021). This variant is the most abundantly expressed and extensively studied as a tumor suppressor and as a DNA sensor (Johnstone et al., 2000; Kerur et al., 2011; Liao et al., 2011; Unterholzner et al., 2010). Transcript V4 contains the 12 exons including exons 4 and 7A (now 9, see figure 1) and encodes a protein of 785 aa giving rise to the complete protein. Transcript V9 is similar to V4 but without duplicated exons 7 and 7A (now named 8 and 9) that encode a 672 aa protein (previously named IFI16C; Li et al., 2021). Transcript Vβ is similar to V2 but lacks exons 1, 2, and 7A (later exon 9) and 714 nucleotides of intron 2 are transcribed forming a protein of 571 amino acids (Johnstone et al., 1998b; Wang et al., 2018). The resulting protein has two HIN domains lacking the PYD domain and has been found in several human tissues and cells. Besides, this isoform inhibits AIM2 inflammasome activation (Griffante et al., 2022). Four other transcripts (V3, V5, V6, and V8) and 12 predicted transcripts (VX1-VX7, VX1.1-VX5.1) have been deposited in the Genbank.

In this work, we have described two novel shorter transcript isoforms of human IFI16, with a domain architecture different from those previously published. Each variant has been tested in an unrelated healthy population and compared to the other ones described to establish the different mechanism of diversity and the corresponding isoforms generated for IFI16.

2. MATERIALS AND METHODS

2.1.Samples

IFI16 mRNA transcripts were studied in a total of 15 healthy, anonymous, unrelated blood donors obtained from Transfusion Center of the Comunidad de Madrid (Madrid, Spain). Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coat bags with EDTA by centrifugation in a Ficoll-Hypaque density gradient (Corning, NY, USA). PBMCs were cryopreserved and stored in liquid nitrogen until the moment of analysis.

2.2.Total RNA isolation

Total RNA was extracted from PBMCs using the RNeasy Mini kit (Qiagen Iberia, Madrid, Spain), as stated by the manufacturer's protocols. After lysing the cells and adding ethanol, the lysate was added to a silica membrane removing all possible contaminants. A specific process to eliminate contaminating genomic DNA was carried out with the RNAse-Free DNAse Set Kit and the RNA concentration was measured with the NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.3.RT-PCR amplification

One μg of total RNA was reverse transcribed to cDNA using the NZY first-strand cDNA synthesis RT kit with dual primer, oligo (dT), and random hexamers (Nzytech, Portugal), according to the manufacturer's protocol. Then, samples were treated with NZY RNase H to eliminate the binding of RNA to cDNA. This procedure is mainly recommended when using cDNA in PCR amplification, especially for some targets (>1 kb) that may require RNA-free DNA as a template. Removing the RNA will increase the sensitivity of the PCR step. The obtained cDNA was used directly in PCR or stored at −20 °C. cDNA PCR was performed using NZYTaq II 2x Colorless Master Mix (Nzytech, Portugal), according to the manufacturer's protocol. The primers shown in Table 1 were used for general and specific amplification of IFI16 to determine expression of the alternative

transcripts previously described. Amplification was performed at 5 min at 95ºC for initial denaturation, followed by 35 cycles of 94ºC (45 s, denaturation), 60ºC (45 s, annealing) and 72ºC (3 min, elongation), and 30 min at 72ºC for the final elongation. PCR products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide (0.5 mg/ml). NZY DNA Ladder VIII (Nzytech, Portugal) was used as a molecular weight reference. Gel images were captured using the G:BOX F3 gel imaging system (Syngene, Cambridge, UK).

2.4.Sequencing of PCR products

The obtained IFI16 PCR products were sequenced using the same specific amplification primers (Table 1). PCR products yielding one band were purified using the EXOSAP-IT® methodology (Life technologies, Thermofisher, Waltham, MA, USA). Extraction and gel purification were performed for each gel band using IlustraTM GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, United Kingdom). The obtained products were sequenced in both directions in ABI PRISM 3730 genetic analyzer (Applied Biosystems) using BigDye terminator cycle sequencing kit (Applied Biosystems, Thermofisher, Waltham, MA, USA). To avoid PCR errors, each sample was sequenced from two different PCR amplifications. The obtained sequences were compared with reference IFI16 cDNA sequences obtained from Genbank (see below).

Sequence analysis and accession numbers of the sequences used

The comparisons between the different transcripts sequences and the corresponding translation amino acid sequences were studied using the Blast© program (National Center for Biotechnology Information, Bethesta, MD, USA).

The alignment of the obtained sequences was carried out using the MEGA 11 software (Tamura et al., 2021) with the corresponding published sequences.

2.6. Protein structure predictions

The tertiary structures of the IFI16 protein isoforms were predicted using the AlphaFold Protein Structure Prediction Server (https://alphafoldserver.com). Amino acid sequences of each isoform were submitted in FASTA format, and predictions were performed using the default template and multiple sequence alignment (MSA) settings described in the AlphaFold 3 (Abramson et al., 2024). Specifically, template structures were automatically retrieved from the Protein Data Bank (PDB) with a cutoff date set to September 30, 2021. The default databases and parameters for MSA searches were also employed, as detailed in the AlphaFold 3 methodology. Model confidence was assessed using predicted local distance difference test (pLDDT) scores, categorized as follows: very high confidence (pLDDT > 90, dark blue), high confidence (90 ≥ pLDDT > 70, cyan), low confidence (70 ≥ pLDDT > 50, yellow), and very low confidence (pLDDT ≤ 50, orange). On this basis, a pLDDT above 90 would be taken as the highest accuracy category, in which both the backbone and side chains are typically predicted. In contrast, a pLDDT above 70 usually corresponds to a correct backbone prediction with misplacement of some side chains. Some regions below 50 pLDDT may be unstructured in isolation.

On the other hand, TED (The encyclopedia of Domains) was used to obtain the Domains and Predicted Aligned Error (PAE). TED is a large-scale classification of structural domains derived from AlphaFold predictions. It uses multiple domain boundary prediction methods to identify independent folding units within proteins. This integration of TED into the (AlphaFold Protein Structure Database) AFDB provides domain-level annotations, simplifying the interpretation of complex protein structures and enhances the definition of functional units, improving structural analysis (TED domains are integrated with the Predicted Aligned Error (PAE) plot), and facilitating comparative studies.

Twenty-one alternative transcripts for IFI16 have been described in humans, and twelve of them have been predicted according to Genbank: V1: NM001206567.2; V2:

NM005531.3; V3: NM001364867.2; V4: NM001376587.1; V5: NM001376588.1; V6: NM001376589.1; V8: NM001376591.1; V9: NM001376592.1; Vbeta: MH445452; VX1: XM047419399.1; VX2: XM047419406.1; VX3: XM047419411.1; VX4: XM047419413.1; VX5: XR007059344.1; VX6: XM054336306.1; VX7: XR008486020.1; VX1.1: XM054336307.1; VX2.1: XM054336308.1 ; VX3.1: XM054336309.1; VX4: XM054336310.1; VX5.1: XR008486021.1 (the letter "V" has been added to the names to represent the corresponding variant; in addition, ".1" has been added to differentiate two sequences with the same name but different Genbank accession numbers).

3. RESULTS AND DISCUSSION

3.1.Description of new alternative transcript variants

Two new alternative transcripts of IFI16 have been described in which neither part of exon 5, nor exon 6, 7, 8, 9, 10 nor part of exon 11 were transcribed (Figure 1). Even so, a protein of 209 amino acids can be produced corresponding to the 615 nucleotides that can be translated. This would lead to the loss of the two HIN domains of the protein and therefore the lack of its functionality with respect to these domains. The transcripts found have been named V10 (Genbank accession number OQ970153) and V11 (Genbank accession number OR060637) and the only difference between them is the different transcription with respect to the 5UT/exon 1 region. V10 had the complete exon 1 like the previously described transcripts V1, V2, V4 and V9, whereas V11 had a transcript with a 420-nucleotide 5UT fragment (called 5UT.5, see Supplementary Figure 1) located 9522 nucleotides from exon 1. Furthermore, a partial transcript of 158 nucleotides of the 254 nucleotides of exon 1 (see below) (Figure 1) previously described in transcripts V3, V5 and V8 was found.

3.2. *mRNA IFI16 transcripts characterization and their comparisons*

Other four transcripts named V3, V5, V6, and V8 and twelve "predicted transcripts" VX1-VX7, VX1.1-VX5.1 ("dot 1" has been added to sequences that have the same name but a different nucleotide sequence) had been deposited in Genbank (see Materials and Methods). V3 is the same as V4 but a partial fragment of exon 1 is transcribed (it is named as E1s and has 158 nucleotides of the 254) and a fragment of the 5UT region, which we have named 5UT.5 of 420 nucleotides located 9522 nucleotides from exon 1, giving rise to the complete protein of 785 amino acids; V5 is the same as V2 but with the E1S and the same 5UT.5 fragment, giving rise to a 729 amino acid protein; V6 is the same as V2 but with the E1S fragment and another part of the 5UT region that we have named 5UT.1

of 235 nucleotides and located 682 nucleotides from exon 1, giving rise to a 729 amino acid protein; V8 is the same as V9 but with E1S and 5UT.5 like V3 and V5 giving rise to a shorter protein of 672 amino acids (Figures 1 and 2).

The predicted transcripts are: VX1 equal to V4 but with E1S and a different part of the 5UT region called 5UT.3 of 79 nucleotides and 3865 nucleotides from exon 1; VX2 equal to V4 but with E1S and a part of the shorter 5UT.1 region (named 5UT.1S) and the 5UT.3 region; VX3 equal to V9 but with E1S the sequences 5UT.3, 5UT.1 and the region called 5UT.2 of 2948 nucleotides and 917 nucleotides from exon 1: VX4 equal to V9 but with E1S and 5UT.3 like VX1; VX5 loses the last two exons and the regions E1S, 5UT.1, 5UT.2, 5UT.3 and partially intron 9 are transcribed into two fragments, one named I9.1 of 971 nucleotides and a second fragment called I9.2 of 432 nucleotides; VX6 same as V4 but with E1S, 5UT.1 and a partial fragment of 5UT.2 called 5UT.2S1 of 38 nucleotides; VX7 equal to V4 but loses the last 2 exons and fragments I9.1 and I9.2 of intron 9 are transcribed; VX1.1 same as VX1 but a new fragment of the 5UT region called 5UT.4 of 109 nucleotides is inserted at 3946 nucleotides of exon 1; VX2.1 equal to VX2 BUT a shorter piece of 5ut.3 is transcribed, called 5ut.3S of 74 nucleotides; VX3.1 same as VX3 but transcribed from 5UT.2S1; VX4.1 same as VX4 but with the partial transcript of 5UT.4 named 5UT.4S of 38 nucleotides; VX5.1 same as VX5 but with a shorter fragment of 5UT.2 called 5UT.2S2 of 35 nucleotides (Figure 1 and 2). On the other hand, all transcripts described have been found in the studied population except Vx1.1, Vx2, Vx2.1, Vx3, Vx5, and VX5.1.

From the mechanisms described for the generation of alternative transcripts (Baralle and Giudice, 2017; Ghigna et al., 2008), in the gene corresponding to the IFI16 receptor, the transcription of several fragments of the 5UT region, the insertion of the introns 2 or 9 and the alternative deletion of almost all exons: part or all of exon 1, exon 2, exon 4, part

of exon 5, exons 6, 7, 8, 9, 10, partial or total exon 11, exon 12, and exon 13 are the most important to generate Alternative Splicing (AS). Other AS mechanisms such as alternative 3' splice sites, alternative 3' terminal exons and alternative 5' exons were not found in the sample studied.

It has been described that within the AS mechanisms, exon skipping appears in 40% of higher eukaryotes and that the insertions of introns or untranslated regions in the mRNA transcripts are not important for the generation of alternative transcripts in humans (El-seedy et al., 2023; Nilsen and Graveley, 2010; Sakabe and De Souza, 2007; Wang et al., 2008). The data described in the present work and in others on human innate immune receptors such as LGP2 (Martínez-Laso et al., 2024) confirm that this mechanism could be important in the regulation of mRNA and its protein expression corresponding to the presence of several isoforms with different functionality.

3.3. IFI16 Isoforms and Protein Structure

From the transcripts described previously and those obtained in this work the presence of nine different isoforms can be established (Figures 1, 3 and Supplementary Figure 1).

Besides, no non-synonymous single nucleotide polymorphisms (nsSNPs) were found in the population studied in the present work as previously described in IFI16, so molecular dynamics (MD) simulation studies cannot be performed to obtain energy-minimized structures (Sahoo et al., 2024 a, b).

Isoform 1(IS1) (also named IFI16-iso1 or IFI16A) includes the V1 transcript with a total of 729 amino acids and loses the M2, M3 and M4 motifs of the Nuclear Localization Signal (NLS) (Figure 3).

Isoform 2 (IS2) (IFI16-iso2 or IFI16B) with a total of 729 amino acids lacks part of the S/T/P repeats and includes transcripts V2, V5 and V6. 56 amino acids are lost.

Isoform 3 (IS3) of 785 amino acids constitutes the complete protein and includes transcripts v3 and v4.

Isoform 4 (IS4) (previously named IFI16C) of 672 amino acids includes the V8 and V9 transcripts and loses 112 amino acids corresponding to part of the S/T/P repeats region.

Isoform 5 (IS5) (named β) loses 214 amino acids corresponding to the Pyrin domain, the four NLS motifs and part of the S/T/P repeats (Figure 2) and corresponds to the transcript previously described as Vβ.

Isoform 6 (IS6) includes the transcripts described in the present work V10 and V11. A region of 580 amino acids corresponding to the HINA, S/T/P repeats, and HINB domains is lost and cannot bind to the viral DNA. In addition, 4 more amino acids are translated in the N-terminal region as in the isoforms discussed below.

Isoform ISX1 is the same as IS3 but with 4 more amino acids in the N-terminal region like the IS6 isoform with a total protein of 789 amino acids and includes the transcripts VX1, VX2, VX6, VX1,1, VX2.1.

Isoform ISX2 is the same as IS4 but with the 4 amino acids of the previous isoforms in the N-terminal region, resulting in a protein of 676 amino acids, includes the transcripts VX3, VX4 VX3.1, VX4.1.

Isoform ISX3 is made up of 573 amino acids that include the 4 amino acids mentioned above, 14 amino acids are inserted corresponding to a partial transcription of intron 9, and loses the HINB domain. This isoform corresponds to the VX5, VX5.1 and VX7 transcripts.

A total of six isoforms (IS1-IS6) are described corresponding to the transcripts previously described and those of the present article and three (ISX1-ISX3) from the transcripts

described as predicted because the transcripts VX1, VX3.1, VX4, VX4.1, VX6, and VX7 were found in the present work. IS3 and ISX1 have the complete protein with its normal functionality. The only difference between them is the presence of 4 amino acids in the N-terminal region. In fact, this same difference of the four amino acids appears between the isoforms ISX2 and IS4 that have the same sequence and are also present in the isoforms IS6 and ISX3. It is not known whether this 4 amino acid difference results in any change in functionality. Isoform IS1 pierde los motivos 2, 3, 4 y la IS5 los cuatro motivos que constitute the region named NLS (nuclear localization signal) (Figure 3 and supplementary Figure 1). These motifs can be acetylated in lymphocytes and macrophages giving rise to cytoplasmic localization by inhibiting nuclear import. In this way, acetylation would be a tool for the cellular localization of IFI16, being able to detect pathogenic DNA according to its location. As in this case it would not have this region, there would be a disparity in the cellular localization of this isoform (Li et al., 2012) losing its trafficking between cytoplasm and nucleus.

Besides the isoform IS5 (ISβ) loses the PYR preventing the formation of the corresponding inflammasome through the binding of PYR to the adapter protein ASC.

Isoforms 2, 4 and ISx2 lose part of the S/T/P repeats region, which is responsible for the formation of alternative transcripts and therefore the formation of different proteins. The IS6 isoform loses part of the HINA domain, the HINDB domain and the S/T/P repeats region, so there would no longer be the possibility of binding to the viral DNA, limiting the response of the receptor.

Isoform ISX3 loses the HINB domain that in IFI16 is in charge of binding to GC-rich viral DNA regions and binds to both dsDNA strands at the same time while HINA binds to a single dsDNA strand of the virus, so it can also bind to the viral ssDNA, this binding

being weaker than that of HINB. Furthermore, the two HIN domains have different functions in their binding to the virus DNA, such that HINA decreases the expression of IFN-β while HINB increases it. (Bertin and DiStefano, 2000; Cagliani et al., 2014; Jin et al., 2012; Li et al., 2012; Ni et al., 2016; Schattgen and Fitzgerald, 2011; Unterholzner et al., 2010) .

If we only compare the HINA, HINB and PYD domains, the isoforms IS1, IS2, IS3, IS4, ISX1 and ISX2 have the same PYD, HINA and HINB domains. IS5 loses PYD but the HINA and HINB domains are the same as before. IS6 loses the HINA and HINB domains but retains the PYD domain, which is the same as before. The isoform ISX3 loses the HINB domain but retains the same PYD and HINA domains as before. This is in agreement with what was previously stated regarding the functionality of these domains.

Functional experiments to the protein level (isoforms) were not posible due to the lack of specific monoclonal antibodies to the different proteins. However, mRNA quantification "in vivo", of the different transcripts in infectious diseases are being studied yielding a differential contribution of the different mRNA transcripts to the infectious response (Martinez-Laso et al., unpublished).

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Figure legends.

Figure 1. Genetic structure of the different IFI16 mRNA transcripts. Grey boxes are exon sequences transcribed. White boxes are sequences deleted from exons. Boxes with inclined lines represents the introns sequences transcribed. NEW and OLD represents the different nomenclature of the exons at the beginning (OLD) and nowadays (NEW). E1: exon 1, etc..; I1: intron 1,….; 5UT.1: fragment 1 of the 5´UT region.

Figure 2. Structure of the partial 5'UT sequence involved in the constitution of the different IFI16 mRNA transcripts.

Figure 3. Schematic structure of the IFI16 molecule and the protein models of the structure of the differrent isoforms obtained. The different isoforms obtained by the protein modellling used are also shown regarding to the different aminoacid sequence. White boxes indicate the lack of the protein regarding to the complete structure. See Material and Methods for software used.

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CONFLICTS OF INTEREST

The authors declare no financial or commercial conflict of interest of this manuscript. Moreover, the authors also declare that this is our first submission of the manuscript for publication in this journal and all the materials are original.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Jorge Martinez-Laso: Conceptualization, Methodology, Formal analysis, Writing-Original Draft, Supervision, Project administration; Isabel Cervera: Validation, Formal analysis, Investigation; Marina S. Martinez-Carrasco: Formal analysis, Investigation, Software; Clara Sánchez-Menéndez: Formal analysis, Investigation, Software; Manuel Remesal: Formal analysis, Software; Guiomar Casado-Fernández: Validation, Investigation, Software; Elena Mateos: Formal analysis, Investigation; Luis Lemus-Aguilar: Validation, Investigation; Montserrat Torres: Formal analysis, Investigation, Writing-Review & Editing; Mayte Coiras: Conceptualization, Formal analysis, Resources, Writing-Review & Editing, Supervision, Funding acquisition, Project administration.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of the Instituto de Salud Carlos III and Centro de Transfusion de la Comunidad de Madrid. The study also complied with the International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS), the Belmont Report. All participants provided written informed consent.

Table 1. Amplification and sequencing primers used

PRIMER NAME	SEQUENCE (5´→3´)
5UT.5-F1	AGGAAAGACCAAGTCTTGACTGAG
5UT.5/E1S -F2	GAAGCAAGATACTTCATTTTCTTAGCG
5UT.1-F3	TTCGATGCTGTCATGTTGAGAG
E1FU-F2	GCCAGCACTAGTCAGCTAACTAAGTG
E1-F2	GCATTTCTGAAGATCTCAAGATCTG
E2-F1	TGGTTAAGTCCTTACTGAGCAACG
I2-F2	AGGGGGACTGAGACTTCCAG
E3-F2	TGGATGCTACTTCACCTGCAC
E3/5-F1	GAGCTCAGAACCCGAAAACAG
E8-F1	GAATGACTTCATGAGGATGCAG
E10-F1	CCTGAAGAAGTTTCCATAGAAGACAG
E7/10-F1	CACCAAGTTGAAACCAAGACTG
E8/10-F1	TAACCACGTTGAAACCAAGACTG
E1/2N-F1	TTTCCAGTGAGGCTCACTTATGTC
E4/5N-F1	CAACTGAGAACCCGAAAACAG
5UT.1/E1S-F1	AATCTAATCATTGAGATACTTCATTTTCTTAGC
5UT.3-F1	CACTGCGAGGTGGAGATCTG
5UT.3/E1S-F1	GACTGCCTCCTGTATACTTCATTTTCT
5UT.3/5UT.1S-F2	GCCTCCTGTAGAAAAATACACTGC
5UT.3/5UT.2-F1	TGCCTCCTGTGTAAGTTTCTGC
5UT.2-F1	TGCTAGTTGATGCCATTTATTTGAC
5UT.2(660)-F1	CGACCTTCCCAGCTAGAGAGAG
5UT.2(1629)-F1	AAGCCATAACCTCCCAGTGTG
5UT.2S1-2/5UT.1-F1	TGCTAGTTGATGCCATTTATTTGAC
5UT.2S1-2-F1	CTGGGTTAACACCACATCATCTG
5UT.4-F1	TATAGCTATCCCCACCCCTCAG
5UT.4-4S-F1	AGCTTTTCCTTCCTTGTTTCTTCTC
5UT.4-4S/5UT.3-F2	TGTTTCTTCTCACTGTCTGAAAGACTACTC
E8/9N-F1	TCTTAACCACGAAAAGTGAAGACAC
E9/10N-F1	CTTAACCACGTTGAAACCAAGAC
E9/I9.1-F1	TTAACCACGGTACAAGTTCCCTC

PRIMER NAME	SEQUENCE (5´→3´)
E5/11D-R1	TTAGAAGAAAAAGTCTGGGAGTTACCTG
E5-R2(A39)	GAGTTACCTGACATTTGGCCAC
E3/5-R2	CCACTGTTTTCGGGTTCTGAG
E7-R3	GCTGGATGGAGTTGTTGGTG
E8-R1	GAAGTCATTCATTTTGGAGATTGTG
E12-R1	ATACCCCATTCCATAGGATTAACAG
E7/8N-R1	GATTGTGTCTTCACTTTTCTTGGTG
E8/9N-R3	GTGTCTTCACTTTTCGTGGTTAAGA
E9/10N-R2	AGTCTTGGTTTCAACGTGGTTAAG
E2-R1	AATCGTTGCTCAGTAAGGACTTAACC
5UT.2/5UT.1-R1	TCAAATAAATGGCATCAACTAGCAG
5UT.2(728)-R1	CTGCTTGTCTCTTTAATCACCCAG
5UT.2(1784)-R1	TATGCAGGGTCCTCACCTAGTG
E9/I9.1-R1	GAACTTGTACCGTGGTTAAGAAACTG
I9.2/E10-R1	GTCTTGGTTTCAACTTAGAAGGTAGAGAC
I9.1/I9.2-R1	GCAGCTGTCCTGGAAGATGA
E8/10-R2	GGTTTCAACGTGGTTAAGAAACTG
E7/10-R1	TCAGTCTTGGTTTCAACTTGGTG