RESEARCHS / INVESTIGACIÓN

Gene expression profile in cervical carcinoma cells treated with HeberFERON.

Perfil de expresión génica en células de carcinoma cervical tratadas con HeberFERON.

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DOI. 10.21931/RB/2018.03.04.3

Abstract: Interferon-alpha (IFN-α) and gamma (IFN-γ) are important cytokines with multiple functions. HeberFERON is a co-formulation of recombinant IFN-α2b and IFN-γ that shows improved pharmacodynamics properties and stronger antitumor response than individual IFNs. The aim of this study was to investigate the differentially expressed genes by HeberFERON in relation to their IFN components by a Suppressive Subtractive Hybridization (SSH) study. Two subtractive cDNA libraries were constructed from HEp-2 cells independently treated with recombinant IFN-α2b and IFN-γ for 72 hours as tester, and cells treated with HeberFERON as driver and vice versa. Near 300 cloned PCR products were sequenced and compared to the database in GenBank and BLAST. We obtained homology to 36 known proteins coding genes. Genes for ribosomal proteins and translation factors besides rRNAs 18S and 28S, cytoskeleton related proteins and proteins participating in antigen presentation and immune responses were mainly identified, using DAVID and GeneCodis tools. Validation of differential gene expression ($p< 0.05$) of genes from the main biological processes components by quantitative PCR (qPCR) showed diverse gene signature by individual IFNs or HeberFERON.

Key words: gene expression, HEp-2, interferon alpha, interferon gamma, quantitative PCR, suppressive subtractive hybridization.

Resumen: El interferón-alfa (IFN-α) y el gamma (IFN-γ) son importantes citoquinas con múltiples funciones. HeberFERON es una co-formulación de IFN-α2b recombinante e IFN-γ que muestra propiedades farmacodinámicas mejoradas y una respuesta antitumoral más fuerte que los IFN individuales. El objetivo de este estudio fue investigar los genes expresados diferencialmente por HeberFERON en relación con sus componentes de IFN mediante un estudio de Hibridación Subtractiva Supresiva (SSH). Se construyeron dos bibliotecas de ADNc sustractivas a partir de células HEp-2 tratadas independientemente con IFN-α2b recombinante e IFN-γ durante 72 horas, y las células tratadas con HeberFERON como conductor y viceversa. Cerca de 300 productos de PCR clonados fueron secuenciados y comparados con la base de datos en GenBank y BLAST. Obtuvimos homología con 36 proteínas conocidas que codifican genes. Se identificaron principalmente genes para proteínas ribosómicas y factores de traducción además de los ARNr 18S y 28S, proteínas relacionadas con citoesqueleto y proteínas que participan en la presentación de antígenos y respuestas inmunitarias, utilizando las herramientas DAVID y GeneCodis. La validación de la expresión génica diferencial ($p <0.05$) de los genes de los principales componentes de los procesos biológicos mediante PCR cuantitativo (qPCR) mostró una firma de genes diversa por IFN individuales o HeberFERON.

Palabras clave: expresión génica, HEp-2, interferón alfa, interferón gamma, PCR cuantitativa, hibridación sustractiva supresiva.

Introduction

Interferons (IFN-α, -β, -λ and -γ) are a multigene family of cytokines that possess a wide range of biological functions including antiviral, anti-proliferative, pro-apoptotic, anti-angiogenesis, anti-fibrotic, neuromodulators and other effects[1,2], through activation of related pathways[3]. These pathways involve specific IFN type I and type II receptors, which initiate activation through JAK-STAT cascades. Type I IFNs interact with the IFNα/β receptor (IFNAR) subunits composed by IFNAR1 and IFNAR2 associated with tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1); while IFN-γ binds to the IFN-γ receptor (IFNGR) receptor subunits composed by IFNGR1 and IFNGR2 associated with JAK1 and JAK2[4].

Thus, IFNs induce the expression of hundreds of IFN-regulated genes (IRGs) via the JAK-STAT pathway[5]. Some of IRGs are regulated by both types of IFNs, whereas others are selectively induced by distinct IFNs through drastic changes in genomic binding locations in a manner dependent on the combinational involvement of STAT1 and STAT2[6]. Depending of doses, treatment time and other factors, IFN-α and IFN-γ signaling may interfere or potentiate each other[7]. Contemporary studies as ChIP-chip analysis of STAT1 and STAT2 targets coupled to quantitative gene-specific PCR (ChIP-qPCR), screening of DNA microarrays or tiling arrays (ChIP-chip), or high-throughput DNA sequencing (ChIP-seq) methods have permitted a more comprehensible picture of how the complex machinery composed by transcription factors, transcriptional co-regulators, histone modifiers, and other players is working for the regulation of IFN target genes[4].

HeberFERON is a co-formulation of IFN-α2b and IFN-γ , with improved pharmacodynamics properties[8] that has demonstrated better results than the individual IFNs in the treatment of basocellular and spinocellular carcinomas[9, 10]. In an attempt to evaluate the gene expression pattern promoted by HeberFERON and potential distinctive regulation of the combination with respect to separated IFNs, we performed a SSH experiment[11] linked to qPCR in HEp-2 cell line, representing

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cervical carcinoma tissue. The understanding of the biological effects that distinguish HeberFERON from their individual components will aid to the optimal clinical application of this formulation in the future.

Materials and methods

Biological Reagents

Recombinant (r) interferons, rIFN-a2b and rIFN-g, and the pharmaceutical co-formulation of both rIFN-a2b and rIFN-g, HeberFERON, were produced at CIGB, Havana, Cuba.

Cell treatment for suppression subtractive hybridization (SSH) experiment

HEp-2 (ATCC-CCL23) cell line, (human cervix carcinoma), was grown in MEM-CANE (Gibco, USA) containing gentamycin (50 mg/mL) (Gibco, USA) and 10% fetal bovine serum (FBS) (Gibco, USA). Cells were seeded into 75cm^2 dishes at 3-4 x10^4 cells/ mL in culture medium containing 10% FBS, incubated at 37 oC and 5% of CO_2. Twenty four hours later, the medium of the treatment groups were refreshed with rIFN-α2b (75 IU/ml), rIFN-g (5 IU/ml) or HeberFERON while the control cells received only cell culture medium with antibiotic and serum. Cells were incubated for another 72h.

Construction of SSH library

Total RNA was extracted from cells by TriReagent procedure (Sigma, USA) and DNase I treatment (Promega, USA). First-strand cDNA synthesis was carried out following SuperScript II reverse transcriptase Invitrogen kit instructions (Invitrogen, USA) from 5µg of total RNA. The second strand cDNA synthesis (dscDNA) was carried out from the first strand and a mixture with DNA polymerase I, RNase H and dNTPs (Promega, USA) at 14 ºC overnight. The dscDNA from HeberFERON -treated (population 1) and [IFN-α2b + IFN-γ]-treated (population 2) HEp-2 cells were used as tester and driver, respectively, in a first hybridization (SSH1) and they were exchanged in a second hybridization (SSH2) to obtain both genes upregulated and downregulated. Subtracted cDNA libraries were constructed using Clontech PCR-Select cDNA subtraction kit (Clontech Laboratories, USA), following the manufacturers protocol. The subtracted tester dscDNA was amplified in suppression and nested PCR to enrich only the "differential population". The nested PCR products from libraries SSH1 and SSH2 were ligated into pGEM T-Easy Vector (Promega, USA), transformed into DH10B *E. coli* cells and screened on LB plates containing ampicillin/X-gal/IPTG at standard concentrations. More than 3000 white colonies were obtained. Plasmids from selected clones were purified using a Qiagen plasmid mini kit (Qiagen, USA).

EST sequencing and bioinformatics analysis

About 300 clones were sequenced using an automated sequencer (Macrogen, Korea) and submitted to GenBank for homology analysis. Nucleic acid homology searches were performed using the BLAST program (National Institutes of Health, Bethesda, Md.). DAVID and GeneCodis were used for Gene Annotation and Functional clustering analysis[12, 13]. Web sites for IFNs, INTERFEROME V1.0 and V2.01 were consulted to find genes described as IRGs in our list (http://interferome.its.monash.edu.au/interferome/home.jspx and https://interferome-v1.erc.monash.edu.au) (October 22, 2018)[14, 15].

Quantitative PCR validation

We validated gene expression differences among treatment conditions for a subset of transcripts derived from SSH by qPCR as described[16]. The design included two biological replicates of untreated, IFN-α2b- treated, IFN-γ- treated and HeberFERON - treated cell samples; two replicates of cDNA reactions (from 1µg of total RNA) from each and three technical replicates. As a result, we had 12 data per sample per gene. Primers are listed in Table 1S (*Supplemental Materials*). Statistically significant results were considered for $p<0.05$ after reference gene normalization.

Results

Identification of differentially expressed genes after HeberFERON treatment in HEp-2 cells

From 288 clones sequenced, 215 clones were found to be highly homologous (92%-100%, E value near 0) with 36 known genes. High numbers of hits were obtained for 18S and 28S ribosomal RNAs (rRNA); four genes had more than 10 hits (RHOA, RPS21, C19orf42, and RAB7L1). Other 30 genes were also identified (Table 1).

Using DAVID and GeneCodis we annotated genes in relation to biological process, molecular function and cell compartment and obtained functional clusters (Table 2). The identified genes code for: structural proteins constituent of ribosome (RPL4, 7, 10A, 24, RPS3A, 16, 19, 21 and 27A); proteins participating in protein synthesis (EIF4A3, EEF1A1), in regulation of actin cytoskeleton (ACTG1, ACTB, RHOA) and in antigen processing and presentation and immune response (B2M, HLA-C, HLA-B, HSPD1, HSPA5, HSP90AB1).

Cross-referencing the gene list (Table 1) with the INTERFEROME V1.0 and V2.01 databases[14, 15] highlighted C2orf50 gene as the only one that had not been reported as IFN response gene in humans.

We selected transcripts from coding genes participating in the main biological processes for validation by qPCR in the HEp-2 cells untreated or treated with individual IFNs or HeberFERON. As described before[16], GAPDH and HMBS genes were the least variable and were used for qPCR normalization. Table 3 shows the factor of change for each gene in each experimental condition (treatment with IFN-α2b, IFN-γ or HeberFERON) respect to the untreated control after normalization with the two reference genes.

Using RT-qPCR fourteen genes were validated as HeberFERON response genes (Table 3). B2M and ACTB genes were up-regulated by IFN-α2b, IFN-γ and HeberFERON, confirming they are IRGs as it has been reported before[5, 17-19].

In the cases of a group of 18SrRNA, 28SrRNA, EIF4A3, EEF1A1, RPL10A, RPS3A, and RPS19 genes, non-static significant differences were detect for HeberFERON gene expression regulation (see Table 3). Conversely, both IFNs up-regulated 18SrRNA and EIF4A3 gene expressions; while RPL10A, 28SrRNA, RPS3A and RPS19 genes were upregulated solely by IFN-α2b or IFN-γ. EEF1A1 gene was the only gene down-regulated by the treatment with IFN-γ. This gene regulation behavior is an evidence of a differential gene expression pattern of HeberFERON with respect to separated IFNs. Another differential gene signature was detected for RPL4 and RPS21 or RHOA genes that were solely down-regulated or up-regulated, respectively, by HeberFERON. A more intriguing gene regulation pattern was observed for RPL7 and RPS27A genes. In both cases, the separated IFNs (IFN-α2b, no effect; IFN-γ up-regula-

Gene	GenBank No		Oligonucleotide sequence (5'....3)	Intron Spanning
GAPDH	NM_002046	R	CAAAGTTGTCATGGATGACC	Yes
		F	CCATGGAGAAGGCTGGGG	
HMBS	NM_000190	R	CCTGACTGGAGGAGTCTGGAGT	Yes
		F	GGAATGTTACGAGCAGTGATGC	
ACTB	NM_001101	R	AATGTGGCCGAGGACTTTGATT	No
		F	GGACTGGGCCATTCTCCTTAGA	
B2M	NM_004048	R	CCTGGAGGCTATCCAGCGTACT	Yes
		F	TCAATGTCGGATGGATGAAACC	
18SrRNA	NR_003286	R	GAACGCCACTTGTCCCTCTA	No
		F	CTCAACACGGGAAACCTCAC	
28srRNA	NR_003287	F	GCAAAAGCTCGCTTGATCTTGA	No
		R	CACAAGCCAGTTATCCCTGTGG	
EEF1A1	NM_002954.3	F	GTCCACCACTACTGGCCATCTG	Yes
		R	TCCAAGACCCAGGCATACTTGA	
EIF4A3	NM_004048.2	F	TCAAGCAATTTTTCGTGGCAGT	Yes
		R	TCATTTTCTCCGTCAGCCAGTC	
RPL10A	NM_080725.1	F	GCCATGAGCAGCAAAGTCTCTC	Yes
		R	CTGATCTGCAACTCCACCGTCT	
RPL4	NM_182500.1	F	CCAAGGAAGCTGTTTTGCTCCT	Yes
		R	CGGTTTCTCATTTTGCCTTTGC	
RPL7	NR_003286.1	F	CGAGGATGGCAAGAAAAGCTG	Yes
		R	TGAAGATTTGACGAAGGCGAAG	
RPS19	NR_003287.1	F	GAACCAGCAGGAGTTCGTCAGA	Yes
		R	CCAGTTCTCATCGTAGGGAGCA	
RPS21	NM_001022.3	F	CGAGTTCGTGGACCTGTACGTG	Yes
		R	GCCATTAAACCTGCCTGTGACC	
RPS27A	NM_001024.3	F	TCGTGGTGGTGCTAAGAAAAGG	Yes
		R	CGACGAAGGCGACTAATTTTGC	
RPS3A	NM_001664.2	F	GCATGGATCTTACCCGTGACAA	Yes
		R	CCAACACAGAACAGACGAAGCA	
RHOA	NM_014740.2	F	AGGCCCCTCTCCTACCCAGATA	Yes
		R	CGTTGGGACAGAAATGCTTGAC	

Table 1S. Genes evaluated and Primers information. A summary of the Gene symbols and GenBank Number (No), Sequences for both oligonucleotides (5´...3´; Forward:F and Reverse: R) and intron spanning characteristic are provided.

tion) and HeberFERON (down-regulation) promoted different regulation pattern.

The carefully observation of these transcriptional signatures identified potential antagonistic or synergistic effect of HeberFERON.

Antagonism between IFN-α2b and IFN-γ is expected for those genes where each IFN regulates the gene differently. These are the cases of: 28SrRNA, EEF1A1, RPL7, RPL10A, RPS3A, RPS19 and RPS27A genes. Another kind of antagonist was observed where both IFNs up-regulated the gene expression but HeberFERON, unexpectedly, had no effect on the mRNA expression of these genes. We have the cases of 18SrRNA and EIF4A3F as examples. Additionally, a clear antagonist effect between IFNα-2b and IFN-γ is observed for the regulation of RPL4 and RPS21 genes, where separately IFNs had no effect on regulation, while HeberFERON down-regulated the expression of both genes.

Additive or synergistic effect of the combination of both IFNs could be the cause of the significant increase in gene expression of RHOA gene by HeberFERON.

Discussion

A subtractive hybridization assays experiment was carried out in HEp-2 cell line with sensitivity to growth arrest by IFN-γ[20], IFN-α[21] and their co-formulation as HeberFERON[10]. In this study, we used this cell line as a model to firstly understand what distinguish HeberFERON from individual IFNs actions, at the transcript level.

As it has been reported in previous microarrays studies[5, 17-19] and compiled in IFN Databases INTERFEROME V1.0[14, 22] and V2.01[15], we identified HeberFERON differentially expressed genes encoding proteins that participate in Antigen processing and presentation and Immune Response (B2M, HLA-C, HLA-B, HSPD1, HSPA5, HSP90AB1), Cytoskeleton regulation (ACTB, ACTG1, RHOA) and a high proportion in protein translation (RPL10A, RPL24, RPL4, RPL7, RPS16, RPS19, RPS21, RPS27A, RPS3A, EEF1A1 and EIF4A3). High percentage of hits was for rRNAs 18S and 28S.

IFN Database INTERFEROME V1.0[14, 22] shows 69% of the IFN-g–regulated genes are also induced by type I IFNs. In com-

	Accession	Description	GENE ID	Name	No Hits
1	NR_003286.1	Homo sapiens 18S ribosomal RNA (LOC100008588)	100008588	LOC100008588	38
2	NM_001664.2	Homo sapiens ras homolog gene family, member A (RHOA), mRNA	387	RHOA	35
3	NM_001024.3	Homo sapiens ribosomal protein S21 (RPS21), mRNA	6227	RPS21	34
4	NR_003287.1	Homo sapiens 28S ribosomal RNA (LOC100008589)	100008589	LOC100008589	23
	NC_001807.4	Homo sapiens mitochondrion, complete genome			23
5	NM_024104.3	Homo sapiens chromosome 19 open reading frame 42 (C19orf42), mRNA	79086	C19orf42	18
6	NM_003929.1	Homo sapiens RAB7, member RAS oncogene family-like 1 (RAB7L1), mRNA	8934	RAB7L1	11
7	NM_182500.1	Homo sapiens chromosome 2 open reading frame 50 (C2orf50), mRNA	130813	C2orf50	2
8	NM_001020.4	Homo sapiens ribosomal protein S16 (RPS16), mRNA	6217	RPS16	2
9	NM_080725.1	Homo sapiens sulfiredoxin 1 homolog (S. cerevisiae) (SRXN1), mRNA	140809	SRXN1	2
10	NM_001101.2	Homo sapiens actin, beta (ACTB), mRNA	60	ACTB	1
11	NM_001614.2	Homo sapiens actin, gamma 1 (ACTG1), mRNA	71	ACTG1	1
12	NM_005139.2	Homo sapiens annexin A3 (ANXA3), mRNA	306	ANXA3	1
13	NM_004048.2	Homo sapiens beta-2-microglobulin (B2M), mRNA	567	B2M	1
14	NM_001344.1	Homo sapiens defender against cell death 1 (DAD1), mRNA	1603	DAD1	1
15	NM_001402.5	Homo sapiens eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), mRNA	1915	EEF1A1	1
16	NM_014740.2	Homo sapiens eukaryotic translation initiation factor 4A, isoform 3 (EIF4A3), mRNA	9775	EIF4A3	1
17	NM_000146.3	Homo sapiens ferritin, light polypeptide (FTL), mRNA.	2512	FTL	1
18	NM_001136557.1/ NM_001136558.1	Homo sapiens G protein-coupled receptor 107 (GPR107), mRNA	57720	GPR107	1
19	NM_003922.3	Homo sapiens hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC1 (CHC1)-like domain (RLD) 1 (HERC1), mRNA	8925	HERC1	1
20	NM_005514.6	Homo sapiens major histocompatibility complex, class I, B (HLA-B), mRNA	3106	HLA-B	1
21	NM_002117.4	Homo sapiens major histocompatibility complex, class I, C (HLA-C), mRNA	3107	HLA-C	1
22	NM_001077442.1 /NM_001077443.1	Homo sapiens heterogeneous nuclear ribonucleoprotein C (C1/C2) (HNRNPC), transcript variant 3 and 4, mRNA	3183	HNRNPC	1
23	NM_007355.2	Homo sapiens heat shock protein 90kDa alpha (cytosolic), class B member 1 (HSP90AB1), mRNA	3326	HSP90AB1	1
24	NM_005347.3	Homo sapiens heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa) (HSPA5), mRNA	3309	HSPA5	1
25	NM_002156.4/ NM_199440.1	Homo sapiens heat shock 60kDa protein 1 (chaperonin)	3329	HSPD1	1
26	NM_001144831.1/ NM_007273.3	Homo sapiens prohibitin 2 (PHB2), mRNA	11331	PHB2	1
27	NM_006775.1	Homo sapiens quaking homolog, KH domain RNA binding (mouse) (QKI), transcript variant 1, mRNA	9444	QKI	1
28	NM_007104.4	Homo sapiens ribosomal protein L10a (RPL10A), mRNA	4736	RPL10A	1
29	NM_000986.3	Homo sapiens ribosomal protein L24 (RPL24), mRNA	6152	RPL24	1
30	NM_000968.2	Homo sapiens ribosomal protein L4 (RPL4), mRNA	6124	RPL4	1
31	NM_000971.3	Homo sapiens ribosomal protein L7 (RPL7), mRNA	6129	RPL7	1
32	NM_002954.3	Homo sapiens ribosomal protein S27a (RPS27A), mRNA	6233	RPS27A	1
33	NM_001006.3	Homo sapiens ribosomal protein S3A (RPS3A), mRNA	6189	RPS3A	1
34	NM_014220.2	Homo sapiens transmembrane 4 L six family member 1 (TM4SF1), mRNA	4071	TM4SF1	1
35	NM_001130145.1/ NM_006106.3	Homo sapiens Yes-associated protein 1, 65kDa (YAP1), mRNA	10413	YAP1	1
36	NM_001022.3	Homo sapiens ribosomal protein S19 (RPS19), mRNA	6223	RPS19	1

Table 1. Homology analysis results of positive clones with Gen-Bank database. GenBank Accession Number, Gene Description, Gene ID and Name are provided. Number (No) of hits for each gene is also included.

parison to type I IFNs used alone, the addition of type II IFN caused enhanced expression not only of many of the genes correlated with the direct antiviral state but also of genes involved in Antigen Presentation to cytotoxic T lymphocytes (CTLs) and Apoptosis[22].

SSH confirmed 36 differentially expressed genes from 215 sequenced clones, representing a 17%, in consistence with previous publications where the number of genes obtained by SSH represents less than 25% of the number of sequenced clones[23, 24].

The differences in gene regulation after 72 hour of incubation time with IFNs could explain, at least in part, the differential gene expression patterns detected. A recent study examining gene expression in human cervical cancer cell line found that although IFN-α and IFN-β induced comparable levels of transcription at early time points, IFN-α induced transcription declined after 8 hours[25]. This decrease was associated with the expression of the IFN stimulated gene USP18 (UBP43), which interacts with the IFNAR2 and inhibits signaling through JAK1 26. Moreover, treatment with IFN-γ for 72 hours markedly inhibits IFN-α-activated STAT1, STAT2 and STAT3; whereas a 24 hours' treatment with IFN-γ slightly enhanced IFN-α-activated STAT1[4].

HeberFERON antagonizes the effect of IFN-α2b and IFN-γ on the expression of 18SrRNA, EIF4A3 (helicase that promotes tumorigenesis[27]), RPL4 (inhibitor of normal physiological

Category	Term	Count	p Value	Genes
Annotation Cluster 1	**Enrichment Score: 8.309666159633796**			
GOTERM_BP_FAT	GO:0006414~translational elongation	10	2.10E-13	EEF1A1, RPS19, RPS16, RPL7, RPS3A, RPL24, RPL4, RPL10A, RPS21, RPS27A
KEGG_PATHWAY	hsa03010:Ribosome	9	5.65E-10	RPS19, RPS16, RPL7, RPS3A, RPL24, RPL4, RPL10A, RPS21, RPS27A
SP_PIR_KEYWORDS	protein biosynthesis	9	6.17E-10	EIF4A3, EEF1A1, RPS19, RPS16, RPL7, RPS3A, RPL24, RPS21, RPS27A
SP_PIR_KEYWORDS	ribosomal protein	9	6.17E-10	RPS19, RPS16, RPL7, RPS3A, RPL24, RPL4, RPL10A, RPS21, RPS27A
GOTERM_BP_FAT	GO:0006412~translation	10	8.97E-09	EEF1A1, RPS19, RPS16, RPL7, RPS3A, RPL24, RPL4, RPL10A, RPS21, RPS27A
Annotation Cluster 2	**Enrichment Score: 3.741836482838242**			
SP_PIR_KEYWORDS	protein biosynthesis	9	6.17E-10	EIF4A3, EEF1A1, RPS19, RPS16, RPL7, RPS3A, RPL24, RPS21, RPS27A
GOTERM_BP_FAT	GO:0042254~ribosome biogenesis	5	9.41E-05	EIF4A3, RPS19, RPS16, RPL7, RPL24
Annotation Cluster 3	**Enrichment Score: 1.258818852316191**			
GOTERM_BP_FAT	GO:0032989~cellular component morphogenesis	5	0.007511	ACTG1, ACTB, RHOA, RPL24, RPS27A
KEGG_PATHWAY	hsa04810:Regulation of actin cytoskeleton	3	0.206259	ACTG1, ACTB, RHOA
SP_PIR_KEYWORDS	cytoskeleton	3	0.285929	ACTG1, ACTB, RHOA
GOTERM_CC_FAT	GO:0005856~cytoskeleton	3	0.850258	ACTG1, ACTB, RHOA
Annotation Cluster 4	**Enrichment Score: 0.3007635785334165**			
GOTERM_BP_FAT	GO:0006955~immune response	3	0.403777	HLA-C, HLA-B, HSPD1, B2M
Annotation Cluster 5	**Enrichment Score: 0.22659942488551563**			
KEGG_PATHWAY	hsa04612:Antigen processing and presentation	4	0.003912	HSP90AB1, HLA-C, HLA-B, HSPA5, B2M

Table 2A. Gene Annotation Clustering by DAVID. Each annotation cluster has an Enrichment Score associated, a Category and Term from the Databases consulted, a list of Genes included in categories and the p value associated.

Biological Process	Number	Hyp_c	Genes
Gene expression	11	8.93E-13	RPS16,HNRNPC,RPL7,RPS19,EEF1A1,RPS21,RPL24,RPS27A,RPS3A,RPL4, RPL10A
Antigen processing and presentation	5	1.01E-08	B2M,HLA-C,HLA-B,HSP90AB1,HSPA5
Immune response	3	1.18E-08	B2M,HLA-C,HLA-B
Regulation of actin cytoskeleton	3	1.18E-08	RHOA,ACTG1,ACTB

Table 2B. Gene Annotation Clustering by GeneCodis. Each Biological Process included a Number and list of Genes. P values were obtained through Hypergeometric analysis corrected by FDR method, showing the significance of each process (Hyp_c).

levels of p53[28]), RPL7 (associated with an increased risk factor at early stages of colon recto carcinoma development[29]), RPS27A (promotor of proliferation, cell cycle progression and inhibitor of apoptosis in solid tumors, advanced-phase chronic myeloid leukemia (CML) and acute leukemia (AL) patients[30, 31]), and RPS21 (its reduction is coupled to antitumor effect of ruthenium compound[32]). Anti-cancer effect of HeberFERON at blocking translation would be more effective when multiple intervening factors can be inhibited in combination.

Antagonism between IFN-α and IFN-γ has been reported by several authors. The antagonism could involve regulation of IFN receptor expression, as observed by Rayamajhi et al, when IFN type I reduced the expression of IFNGR1 in macrophages infected with *L. Monocytogenes*, with the corresponding suppression of host responsiveness to IFN-γ[33]. In macrophages, interferon consensus sequence binding protein (ICSBP) mRNA and protein are strongly induced by IFN-γ, but only marginally by IFN type I. When both IFNs are present, IFN type I antagonizes IFN-γ-induced ICSBP mRNA and protein synthesis[34].

Regulation of phosphorylation of transcriptional factors involved in IFN type I and type II signaling could also explain the antagonism observed for HeberFERON with respect to separated IFNs. For example, overexpression of protein tyrosine phosphatase Shp1 in endothelial cells abrogated IFN type I signaling through a GAS site, suggesting a role of level of Shp1 on the interference between IFN types I and II signaling pathways[35].

The increased gene expression of RHOA stimulated by HeberFERON could indicate an additive or synergic effect. IFN-α[36] and IFN-γ[37] have been involved in the reorganization of the cell cytoskeleton through RHOA, with impact in the cell growth. The upregulation of RHOA by HeberFERON could be beneficiated from the described crosstalk between both type of IFNs[38] and the further regulated expression of STAT1 via c-Jun-mediated production of basal levels of IFN-β[39]. In this context, we could remark the facts that STAT1 and the stimulation of c-Jun expression could be involved in the regulation of RHOA gene expression[39, 40].

The diverse mode of gene regulation revealed in this work by the combination of IFN-α2b and IFN-γ (HeberFERON), is congruent with the recent study of the ENCODE project performed on genomic binding sites suggesting that transcription factors often show different co-association patterns in binding sites, and the binding of one transcriptional factor affects the preferred binding partners of others[41]. Furthermore, efficient transcriptional activation of STAT1 target genes requires posttranslational modification of STAT1 and the recruitment of coactivators and histone and chromatin modifying complexes[4].

As part of this work we obtained the gene C2orf50 regulated by individual IFNs or their combination that was not previously described as IRGs in humans[14, 15]. This gene is poorly characterized [https://www.uniprot.org/uniprot/Q96LR7] and the understanding of their participation in the mechanism of action of HeberFERON could be an interesting point in the future.

In spite of our study examined only a small number of

		IFN Alpha		IFN gamma		HeberFERON		
Function/Process	**Gene**	**FC**	**p**	**FC**	**p**	**FC**	**p**	
Ribosome/ translation								
	18SrRNA	**1,89**	0.000	**1,67**	0.000	**-1,03**	0.760	**
	28SrRNA	1,33	0.075	**1,39**	0.020	1,03	0.810	
	EIF4A3	**1,26**	0.002	**1,35**	0.005	**-1,02**	0.764	**
	EEF1A1	-1,27	0.092	**-1,47**	0.011	-1,15	0.303	
	RPL4	-1,04	0.523	1,04	0.574	**-1,25**	0.006	**
	RPL7	-1,08	0.223	**1,25**	0.000	**-1,22**	0.000	**
	RPL10A	**1,15**	0.021	1,18	0.114	-1,02	0.689	
	RPS3A	-1,11	0.447	**1,47**	0.007	-1,09	0.488	
	RPS19	1,09	0.482	**1,38**	0.022	-1,11	0.358	
	RPS21	1,04	0.591	1,19	0.062	**-1,35**	0.000	**
	RPS27A	-1,02	0.834	**1,31**	0.005	**-1,19**	0.004	**
Immune Response								
	B2M	**2,60**	0.000	**4,60**	0.000	**5,62**	0.000	
Cytoskeleton								
	ACTB	**1,42**	0.001	**1,94**	0.000	**1,28**	0.002	
	RHOA	-1,06	0.436	-1,05	0.520	**1,56**	0.000	**

Table 3. Gene expression (mRNA) following treatment with IFNα (Alpha), IFNγ (gamma) and the combination HeberFERON. Factor of Change (FC) respect to untreated cells, calculated by REST 2009, and the p values associated to each comparison are shown. In Bold and Shadows formats we show genes which have statistically significant differences ($p < 0.05$). Genes are grouped by Function and Process. Double asterisks pointed to the genes showing new regulation pattern of HeberFERON respect to IFNα (Alpha) and IFNγ (gamma).

IRGs, it suggests that compared with transcriptional patterns of separated IFNs, HeberFERON induces a unique transcriptional signature after 72 hour of cell treatment. The meaning of this new signature should be taken into account for clinical translation.

Competing interests and Funding

The authors declare that they have no competing interests. All Authors are (were) employees of the Center for Genetic Engineering and Biotechnology (CIGB), Havana where IFNa2b, IFNg and HeberFERON are produced. The study was financed by CIGB.

Acknowledgements

We would like to thank Amanda Colarte, Adelaida Villarreal and Tamara Díaz for their technical support.

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Recibido: 15 october 2018
Aprobado: 17 november 2018