Unraveling the molecular mechanism governing the tissue specific expression of IFNλR1

Hashaam Akhtar[1], Ole Jensen Hamming[2], Syed Umer Jan[3], Samar Akhtar[4], Ewa Terczyn´ska-Dyla[2], Piotr Siupka[2], Adeena Shafique[1], Rune Hartmann[2] and Hajra Sadia[1]

[1]Atta-Ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad, Pakistan
[2]Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark
[3]Faculty of Pharmacy, University of Balochistan, Sariab Road, Quetta, Pakistan
[4]Riphah Institute of Pharmaceutical Sciences, Riphah International University, 7th Avenue, Islamabad, Pakistan

Abstract: The functional receptor for type III interferons (IFNs) is a heterodimer of IFNLR1 and IL10R2. IFNLR1 is expressed in a highly tissue specific manner, with epithelial and liver tissue as the prime expressing tissues in humans. However, knowledge about the molecular pathways responsible for regulating the expression of IFNLR1 is yet unknown. In this study, various bioinformatics tools were used to predict the scores of signal peptides of IFNλR1 and IFNαR1, which was considered as an important difference in the expression of both receptors or participation in regulating the IFNLR1 gene. *In silico* study revealed that the signal peptide of IFNαR1 had more potential than the signal peptide of IFNλR1 but changing the signal peptide of wild type IFNλR1 with the signal peptide of IFNαR1 in wet lab had barely shown any differences. Selective expression of IFNλR1 was considered to be a plus point towards the targeted anti-viral activity of IFNλs but artificial control on its expression will surely make IFNλs a better drug with enhanced activity. The results of this study may help us in contributing some understanding towards the mechanisms involved in the selective expression of IFNLR1 and exceptionalities involved.

Keywords: IFNLR1, signal peptide, interferon lambda, expression, transcription factor binding sites (TBS).

INTRODUCTION

Interferons (IFN) are a group of cytokines that has shown its antiviral potentials since its discovery in 1957 (Isaacs and Lindenmann, 1957). They were called interferons because of their interference in viral replications (Thibault and Utz, 2003). In year 2003, type III IFNs, commonly known as IFN-λs, were added into the category of antiviral drugs by Kotenko and Sheppard (Kotenko *et al*., 2003; Sheppard *et al*., 2003). All three, in fact all four after the addition of the fourth (IFN-λ4) last year signals through a heterdimeric receptor, consisting of a private chain (IFNλR1) and a shared chain (IL-10R2). (Booth and George, 2013; Commins *et al*., 2008; Dumoutier *et al*., 2004; Hamming *et al*., 2010; Hamming *et al*., 2013; Pestka *et al*., 2004; Sheppard *et al*., 2003; Witte *et al*., 2009).

All four members of type III IFNs show fairly similar anti-viral, anti-neoplastic and anti-proliferative activities as shown by type I interferons but the only advantage of type III IFNs over type I IFNs is the selective expression of the private chain of their own receptor (Gad *et al*., 2009; Hamming *et al*., 2010; Hamming *et al*., 2013; Kotenko *et al*., 2003; Liu *et al*., 2011). The underline pathways activated by both types of interferons are unique to interferons and are called interferon stimulated genes (ISGs), which includes many transcription factors like signal transducers and activators of transcription family of transcription factors (STAT), that activate various overlapping pathways and genes, hence performing different useful functions to boost immunity (Hamming *et al*., 2013; Hashaam *et al.,* 2013; Kotenko *et al*., 2003; Sheppard *et al*., 2003; Hashaam *et al*., 2013a).

IFNλR1 (alpha) is a member of class II cytokine receptor family and it functions after making a heterodimer with IL-10R2 beta chain. Its heterodimeric composition and selective expression has made IFN-λs superior in the selection for drug of choice against hepatitis C, because it has shown fewer side effects as compared to IFN- α in clinical trials (Keller, 2011; Muir *et al*., 2010; Torres *et al*., 2014). Reduction in thrombocytopenia or anemia during IFN-λ therapy is the advantage of the selective expression of IFNλR1 but on the other side, single nucleotide polymorphisms (SNP) in IFNλR1 are also been found to be helpful in predicting the outcomes of IFNα and ribavirin therapy (Aparicio *et al*., 2010; Fukuhara *et al*., 2010; Suppiah *et al*., 2009; Tanaka *et al*., 2009) and has opened a new direction for the research in interferon resistant patients (Hashaam *et al.,* 2015)

IFNλR1 has three splice variants (SV), SV-1 being complete is fully functional and the other two are not studied much (Witte *et al*., 2009b). SV-2 is rarely found and is deficient of a sequence, resulting in deletion of 29-amino acids in the intracellular domain, at the head fragment of exon VII. It lacks downstream signaling domains, whereas SV-3 lacks the sequence that matches

**Corresponding author:* e-mail: hashaamakhtar@gmail.com

with the transmembrane domain of exon VI and consequence is replacement of an unconventional premature stop codon. It is a soluble receptor, which is believed to halt IFN-λ signaling at some point but this still remains controversial (Witte *et al*., 2009b). Expression of SV-1 and its balance with SV-3 can be the story behind the selectivity of responding and non-responding cells of IFN- λ, but signal peptide also play an important role in the expression of any receptor. In the present study we have compared the signal peptides of IFNλR1 and IFNαR1 and have shown that though the signal peptide of IFNλR1 seems weaker than the signal peptide of IFNαR1, but the expression potential of both the signal peptides are same.

MATERIAL AND METHODS

In vitro and in silico analysis

Analyzing signal peptide and exchanging it

We analyzed the signal peptides of interferon lambda receptor and interferon alpha receptor through SignalP 4.1 (Petersen *et al*., 2011) and then used following primers to mutate BamH1 sire and attach HA-Tag (5'-TACCCATACGATGTTCCAGATTACGCT-3', 5'-GCA TTACATGGC-CAGGGGATCCTCTGCAGATATCC-3' 5'-GGATATCTGCAGAGGA-TCCCCTGGCCATGTAA TGC-3'). We further more used IFN_αR1sig_pepseq as sense primer and IFN-λR1 _HA-Tag rev as anti-sense primer to exchange the signal peptides of IFN-λR1 with the signal peptide of IFN_αR1. Sequencing of our construct confirmed our exchanging success.

Sequence of the signal peptide of IFN_αR1 is ATGCTTTTGAGCCAGAATGCCTTCATCTTCAGAT CACTTAATTTGGTTCTCATGGTGTATATCAGCCTC GTGTTTGGT and for the signal peptide of IFN-λR1 is ATGGCGGGGCCCGAGCGCTGGGGCCCCCTGCTCC TGTGCCTGCTGCAGGCCGCTCCAGGG

Expression of mut IFNλR1 in HEK-293 cells

Confocal microscopy

We seeded 1-2 x 10^5HEK 293 cells in 6 well plate in plain Dulbecco's Modified Eagle Medium (DMEM) and 10 % FBS. Place them with coverslip. On day 2, make concentrations of both mutated construct, wild type and an empty vector and lipofectamine 2000 (invitrogen) was used for transfection with the help of user's manual. Immunofluorescence was performed on day three after washing the cells twice with PBS at room temperature (RT); we fixed them with 4% formaldehyde. Again washed them twice with phosphate buffered salineand permeabalised them with 0.1% Triton x100 (in PBS) for 90 sec (RT). Washed again and Block coverslip by adding 1%Bovine serum albumin (BSA) (in Phosphate buffered saline (PBS) and incubate for 10-30 min (RT). 1x HA (Abcam), 1x calreticulin and 1x Giatin were prepared in PBS. incubated 1x HA (Abcam) on coverslip after placing it on a parafilm for 1 hour (RT). Washed with PBS and incubated again for an hour in dark with 20µl of secondary antibodies [in dark] 2x goat (invitrogen) + 2x rabbit (invitrogen). Added DAPI without washing and incubated for 3 min. Wash 2x with PBS and mount coverslip using prolong gold on microscope slides. Store microscope slides with coverslip in the dark or take images on confocal microscope.

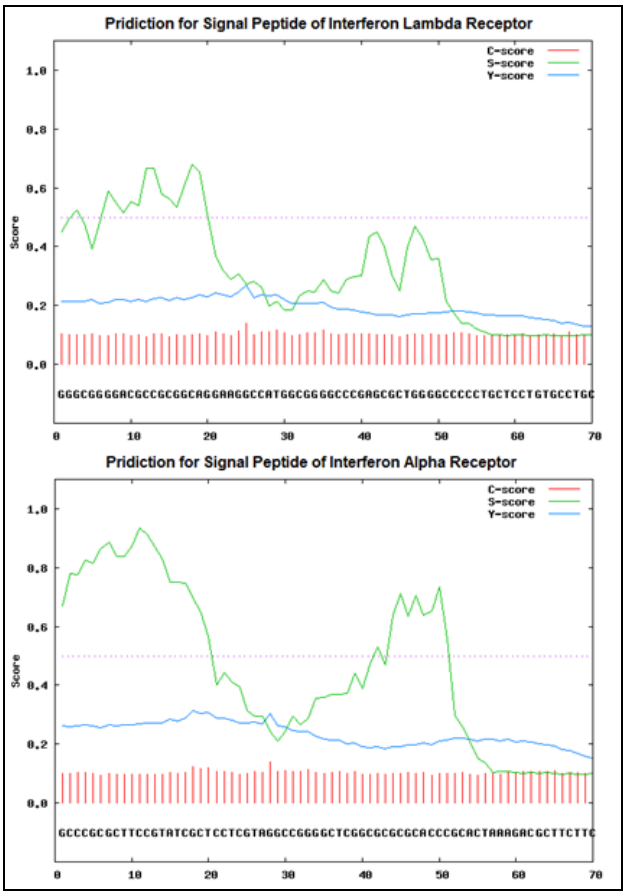

Fig. 1: C-scores, S-scores and Y-scores of the signal peptides of IFN-λR1 and IFN_αR1 attained through Signal P.

Luciferase assay

4×10^5 HEK 293 cells were seeded in a 24 well plate and transfection with lipofectamine of positive control, negative control, tagged wild type and tagged mutant was performed on day two. A dual-luciferase reporter assay was performed according to the author's instruction and previously described protocol on day three (Bruce A. Sherf, 1996; Hamming *et al*., 2013) (Dual-Luciferase Reporter Assay System, Promega).

RESULTS

The results attained through the computational analysis shows that the mean S- score for the signal peptide of IFN-λR1 and IFN_αR1 is 0.513 and 0.821 respectively. Fig. 1 shows that the C-scores for IFN-λR1 and IFN_αR1

were same with a small difference in its position. Table 1 shows the differences in different parameters measured and proves that the signal peptide of IFN_αR1 is much stronger than the signal peptide of IFN-λR1.

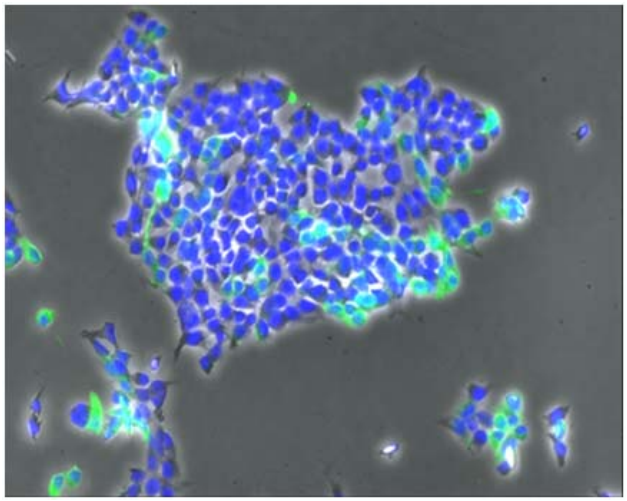

Fig. 2: Image through confocal microscopy showing expression of mutated IFN-λR1 in HEK 293 cells.

DISCUSSION

C-score is a raw cleavage site score of any protein and it distinguishes cleavage site of the signal peptide from everything else. The red line in fig. 1 shows that the cleavage site for IFNλR1 is at position 25, whereas the cleavage site for IFNαR1 is at position 28. This score is not very crucial in evaluating the strength of any signal peptide, though its position plays an important role. The maximum C value was 1.39 for both of the compared proteins.

S-score defines the signal peptide score and it distinguishes the positions within signal peptides from positions in mature part of the proteins and from proteins without signal peptides. It is an imperative measurement of any protein and the results shows a clear difference within the scoring of both proteins; the mean S-score of IFNαR1 is 0.821 with the highest peak of 0.934, whereas the mean S-score of IFNλR1 is 0.513 with the elevation up to 0.681, as shown in table 1. This scoring clearly states a marked difference within the strength of the signal peptides of both proteins.

Y-score is actually a combination of geometric average of C-score and the slope of the S-score. It is a better predicting tool to get cleavage site as compared to C-score alone. Maximum Y-score of IFNλR1 was found to be 0.268 at position 25 and it was 0.313 for IFNαR1 at position 18 (table 1). C-score can have multiple peaks in one sequence but the cleavage site is always one, so Y-score comes up with the best option by distinguishing between the peaks of C-score by selecting the one where the slope of the S-score is sudden or steep.

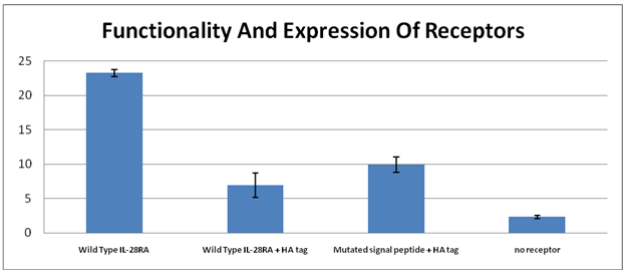

Fig. 3: Luciferase assay showing the functionality of receptors in HEK 293 cells

D-score is the discrimination score and it is the average of mean s-score and maximum Y-score reached. D-score is vital in differentiating signal peptide from mature protein and all scores are either negative or near 0.1 on average if the protein is a non-secretory protein (Petersen *et al*., 2011).

Wild type IFNλR1 and mut-IFNλR1 were expressed in HEK 293 cells and were checked for their expression and functionality. HEK 293 cells do not express IFNλR1 naturally but following an assay developed in our lab (Hamming *et al*., 2013), we expressed these receptors in HEK 293 cells and verified its expression in a confocal microscope (figs. 2& 3). Both wild type IFNλR1 and mut IFNλR1 were able to reach the cell membrane and express themselves. Their functional and quantitative assay was performed through Dual-Luciferase Reporter Assay System (Promega). Both HA-tagged wild type IFNλR1 and mut IFNλR1 were able to show similar results and have proved that the selective expression of IFNλR1 is independent of the quality of its signal peptide, as Ding *et al*., has recently found that expression of IFNλR1 is dependent on histone deacetylase (HDAC) and few transcription factors (TF) like NF-Y and E2F (Ding *et al*., 2014). Infect they were able to express IFNλR1 in U-87 cells, which are naturally a non-responder cell lines with reference to IFNλs (Ding *et al*., 2014). Expression of IFNλR1 was also claimed to be depending on TFs in 2010 by Liming *et al*., in an *in silico* study but none of their claimed TF has yet been confirmed in wet lab (Liming Yang, 2010).

We get another clue from macrophages (Mø1 and Mø2) and dendritic cells (pDC), as they share common progenitor; Monocytes (Liu *et al*., 2010). Monocytes lack IFNλR1, but when they differentiate into Mø1 and Mø2, they express IFNλR1 and respond to IFNλR1, whereas when they differentiate into pDC, they don't express IFNλR1.However, the debates are still there to resolve the common precursor for both types of cells, because there are some pathways that up or/and down regulate their signaling receptors (Newman *et al*., 1980).

Table 1: Various scoring parameters of the signal peptides of IFN-λR1 and IFN_αR1 attained through computational analysis.

IFNλR1 Measure	Position	Value	IFN-AR1 Measure	Position	Value
max. C	25	0.139	max. C	28	0.139
max. Y	25	0.268	max. Y	18	0.313
max. S	18	0.681	max. S	11	0.934
mean S	1-24	0.513	mean S	1-17	0.821
D	1-24	0.400	D	1-17	0.587
SP	NO		SP	YES	Cleavage site between pos. 17 and 18: ATC-GC
D	0.400		D		0.587
D-cutoff	0.450		D-cutoff		0.450

CONCLUSION

This study aimed at revealing the molecular mechanism that governs the expression of IFN-λRα. The expression of IFN-λRα is highly tissue specific with epithelial and liver tissues being the major tissues that express it in humans. This study employed various bioinformatics tools to assess the signal peptide scores of IFN-λRα and IFN-αR1, which revealed that the signal peptide of IFN-αR1 had more potential over the signal peptide of IFN-λRα. Contrary to this, wet-lab studies showed no difference between the two. Owing to its selective expression, IFN-λRα can have targeted anti-viral activity with artificial control on its expression leading to enhanced activity.

REFERENCES

Akhtar H, Islam G , Jan SU, Nawaz A, Akhtar S, Hartmann R and Sadia H (2015). Identification of essential regulatory elements responsible for the explicit expression of IL-28Rα and their effect on critical SNPs using *in-Silico* methods. *Pak. J. Pharm. Sci.,* **28**(4)(Suppl): 1523-1532.

Aparicio E, Parera M, Franco S, Perez-Alvarez N, Tural C, Clotet B and Martinez MA (2010). IL28B SNP rs8099917 is strongly associated with pegylated interferon-alpha and ribavirin therapy treatment failure in HCV/HIV-1 coinfected patients. PLoS One **5**: e13771.

Booth D and George J (2013). Loss of function of the new interferon IFN-lambda4 may confer protection from hepatitis C. *Nat. Genet,* **45**: 119-120.

Bruce A, Sherf SLN, Rita R Hannah and Keith V Wood (1996). Dual-luciferase reporter assay: An advanced Co-reporter technology integrating firefly and renilla luciferase assays, promega notes Magazine. *Promega Corporation,* p.**7**.

Commins S, Steinke JW and Borish L (2008). The extended IL-10 superfamily: IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29. *J. Allerg. Clin. Immunol.,* **121**: 1108-1111.

Ding S, Khoury-Hanold W, Iwasaki A and Robek MD (2014). Epigenetic reprogramming of the type III interferon response potentiates antiviral activity and suppresses tumor growth. *PLoS Biol.,* **12**: e1001758.

Dumoutier L, Tounsi A, Michiels T, Sommereyns C, Kotenko SV and Renauld JC (2004). Role of the interleukin (IL)-28 receptor tyrosine residues for antiviral and antiproliferative activity of IL-29/interferon-lambda 1: Similarities with type I interferon signaling. *J. Biol. Chem.,* **279**: 32269-32274.

Fukuhara T, Taketomi A, Motomura T, Okano S, Ninomiya A, Abe T, Uchiyama H, Soejima Y and Shirabe K *et al*. (2010). Variants in IL28B in liver recipients and donors correlate with response to peg-interferon and ribavirin therapy for recurrent hepatitis C. *Gastroenterology,* **139**: 1577-1585, 85 e1-3.

Gad HH, Dellgren C, Hamming OJ, Vends S, Paludan SR and Hartmann R (2009). Interferon-lambda is functionally an interferon but structurally related to the interleukin-10 family. *J. Biol. Chem.,* **284**: 20869-20875.

Hamming OJ, Terczynska-Dyla E, Vieyres G, Dijkman R, Jorgensen SE, Akhtar H, Siupka P, Pietschmann T, Thiel V *et al*. (2013). Interferon lambda 4 signals via the IFNlambda receptor to regulate antiviral activity against HCV and corona viruses. *EMBO J.,* **32**: 3055-3065.

Hamming OJ, Gad, HH, Paludan S and Hartmann R (2010). Lambda Interferons: New Cytokines with Old Functions. *Pharmaceuticals,* **3**: 795-809.

Hashaam A, Samar A, Ummar R, Muhammad F, Muhammad A, Muhammad Y and Najam us Sahar SZ (2013). Interferons as immune regulators: A rivalry between HCV and interferons. *J. Clin. Cellu.Immunol.,* **04**: 36-42.

Hashaam A, Samar A, Jan SU, Azka K, Najam us Sahar SZ and Ishtiaq Q (2013). Over expression of a synthetic gene encoding interferon lambda using relative synonymous Codon usage bias in *Escherichia coli. Pak. J. Pharm. Sci.,* **26**(6): 1181-1188.

Isaacs A and Lindenmann J (1957). Virus interference. I. The interferon. *Proc. R. Soc. Lond. B. Biol. Sci.,* **147**: 258-267.

Keller DM (2011). Interferon lambda beats interferon alfa-2a for HCV infection, Medscape medical news Medscape, WebMD, LLC.

Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA, Sheikh F and Dickensheets H *et al*. (2003). IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat. Immunol.,* **4**: 69-77.

Liming Yang, Y.L., Jifu Wei and Shaoheng He (2010). Integrative genomic analyses on IL28RA, the common receptor of interferon lambda-1, lambda-2 and lambda-3. *Int. J. Molecu. Med.,* **25**: 807-812.

Liu BS, Janssen HL and Boonstra A (2011). IL-29 and IFN alpha differ in their ability to modulate IL-12 production by TLR-activated human macrophages and exhibit differential regulation of the IFNgamma receptor expression. *Blood,* **117**: 2385-2395.

Muir AJ, Shiffman ML, Zaman A, Yoffe B, de la Torre, A, Flamm S, Gordon SC, Marotta P and Vierling JM *et al*. (2010). Phase 1b study of pegylated interferon lambda 1 with or without ribavirin in patients with chronic genotype 1 hepatitis C virus infection. *Hepatology,* **52**: 822-832.

Newman SL, Musson RA and Henson PM (1980). Development of functional complement receptors during *in vitro* maturation of human monocytes into macrophages. *J. Immunol.,* **125**: 2236-2244.

Pestka S, Krause CD and Walter MR (2004). Interferons, interferon-like cytokines and their receptors. *Immunol Rev.,* **202**: 8-32.

Petersen TN, Brunak S, von Heijne G and Nielsen H (2011). Signal P 4.0: Ddiscriminating signal peptides from transmembrane regions. *Nat. Methods,* **8**: 785-786.

Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, Kuestner R, Garrigues U and Birks C *et al*. (2003). IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat. Immunol.,* **4**: 63-68.

Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U and Dore GJ *et al*. (2009). IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat. Genet,* **41**: 1100-1104.

Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M and Hino K *et al*. (2009). Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat. Genet,* **41**: 1105-1109.

Thibault DL and Utz PJ (2003). Interpreting interest in interferon-alpha. *Arthritis Res. Ther.,* **5**: 246-248.

Torres C, Brahm J and Venegas M (2014). Lambda interferon serum levels in patients with chronic hepatitis C virus infection according to their response to therapy with pegylated interferon and ribavirin. *J. Interferon Cytokine Res.,* **34**: 106-110.

Witte K, Gruetz G, Volk HD, Looman AC, Asadullah K, Sterry W, Sabat R and Wolk K (2009). Despite IFN-lambda receptor expression, blood immune cells, but not keratinocytes or melanocytes, have an impaired response to type III interferons: Implications for therapeutic applications of these cytokines. *Genes Immun.,* **10**: 702-714.