

Expression analysis of protein inhibitor of activated *STAT* (*PIAS*) genes in IFN β -treated multiple sclerosis patients

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Objectives: Protein inhibitors of activated *STAT* (*PIAS*) are transcription co-regulator of the Janus kinase/signal transducer and activator of transcription signaling pathway as well as nuclear factor- κ B family of transcription factors. Both of them are involved in cytokine release during inflammatory response.

Patients and methods: Considering the role of cytokine imbalance in the pathogenesis of multiple sclerosis (MS), we compared blood expressions of *PIAS1-4* genes in 48 interferon β (IFN β)-treated MS patients with those of healthy subjects by means of real time PCR.

Results: Although the expression levels of these genes were not significantly different between MS patients and healthy subjects, significant inverse correlations have been found between *PIAS1* expression and age at disease onset. *PIAS2* and *PIAS3* expressions were inversely correlated with Expanded Disability Status Scale (EDSS) in patients. Moreover, *PIAS3* expression was correlated with disease duration in patients and with age in controls. In addition, *PIAS4* expression was inversely correlated with EDSS and age at disease onset while it was positively correlated with disease duration.

Conclusion: The present study provides evidences for altered expression of *PIAS* genes in IFN β -treated MS patients compared with healthy subjects. However, future studies are needed for elaboration of their exact function in this disorder.

Keywords: multiple sclerosis, JAK/STAT pathway, protein inhibitors of activated *STAT*, *PIAS*

Introduction

Multiple sclerosis (MS) is a multifactorial autoimmune disorder of central nervous system.¹ Among several factors implicated in this demyelinating disorder, changes in cytokines levels have crucial roles in MS pathogenesis and response to therapies.² The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway is a fundamental pathway for induction of cytokine functions and regulation of immune responses.³ Impairment of the JAK/STAT pathway regulation leads to imbalance between production of T helper 1 (Th1)- and Th2-type cytokines and is considered as an underlying cause of MS.² Among transcriptional co-regulators of this pathway are *protein inhibitors of activated STAT (PIAS)*.⁴ The mammalian *PIAS* family includes proteins encoded by four genes: *PIAS1*, *PIAS2 (PIASx)*, *PIAS3*, and *PIAS4 (PIASy)*.⁵ These proteins interact with several diverse proteins implicated in transcription and exert inhibitory effects through suppression of the DNA-binding activity of a transcription factor, enrollment of other co-regulators, or enhancing the sumoylation of a transcription factor.⁶ The individual interactions of *PIAS1*, *PIAS3*, *PIASx*, and *PIASy* with *STAT1*, *STAT3*, *STAT4*, and *STAT1* have been demonstrated,

respectively.⁷ In addition to regulation of STAT pathway through which PIAS proteins were first identified, nuclear factor- κ B (NF- κ B) family of transcription factors are targets of regulation by PIAS family.⁶ NF- κ B transcription factors are extensively applied by cytokines to alter gene expression⁶ and their dysregulation has been regarded as a principal culprit in the pathogenesis of MS.⁸ Considering the role of PIAS proteins in regulation of two important pathways in modification of immune response that are altered during MS development, we aimed at expression analysis of *PIAS* genes in MS patients compared with healthy subjects. To the best of our knowledge, this is the first study to examine expression of *PIAS* genes in MS patients.

Materials and methods

Study participants

A total of 100 persons, including 48 MS patients and 52 healthy subjects participated in the current case-control study. All patients were diagnosed as relapsing-remitting MS patients based on the revised McDonald criteria.⁹ Patients were selected from the earlier human leukocyte antigen (HLA)-typed MS patients who were at remission due to daily injections of interferon β (IFN β) (CinnoVex, Cinagen Company, Tehran, Iran). Based on the role of HLA-DRB1*15 as a principal risk factor for MS,¹⁰ we just included HLA-DRB1*15 negative patients in the current study. Fifty-two age- and sex-matched volunteers were enrolled in the study as control subjects. The existence of any neurological disorders was ruled out in these subjects through physical examination and history taking. The study was approved by the Ethical Committee of Shahid Beheshti University of Medical Sci-

ences. Written informed consents were obtained from all participants. The study was conducted in accordance with the Declaration of Helsinki.

Sampling and RNA extraction

A total of 5 mL of peripheral blood was gathered from all study participants in EDTA tubes. Total RNA extraction was carried out using Hybrid-resin transfer molding blood RNA extraction Kit (Geneall Biotechnology Co., Ltd, Seoul, South Korea) from these blood samples. Subsequently, Nanodrop equipment (Thermo Scientific, Waltham, MA, USA) was applied for evaluation of RNA quantity and quality.

cDNA synthesis and quantitative reverse transcription-PCR

First strand cDNA was produced from extracted RNA samples using High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) as instructed by the manufacturer. Specific primers and probes for expression analysis of *PIAS1-4* genes were designed by using Allele ID 7 for x64 windows software (Premier Biosoft, Palo Alto, CA, USA). Table 1 shows the nucleotide sequence of primers and probes used in the study. *HPRT1* gene was used as normalizer.

Statistical analysis

Bayesian estimation was used for evaluation of significance of difference in mean values between MS patients and healthy subjects. A normal prior distribution was supposed for parameters with 200,000 iterations. Spearman rank order correlation test was used for the evaluation of the correlation between genes expressions as well as gene expression and

Table 1 Nucleotide sequence of primers and probes

Gene name	Primer and probe sequence	Primer and probe length	Product length
<i>HPRT1</i>	F: AGCCTAAGATGAGAGTTC	18	88
	R: CACAGAACTAGAACATTGATA	21	
	FAM -CATCTGGAGTCTATTGACATCGC- TAMRA	24	
<i>PIAS1</i>	F: AGCCTAAGGGAAAGCCATAGC	21	83
	R: ATGTCTGGTATGATGCCAAAGATG	24	
	FAM-TGCCGTGTCCGTGCTGCTCCTGT- TAMRA	24	
<i>PIAS2</i>	F: CACGAACTCTTGAAGGACTTTCTG	24	126
	R: AAGTGAAGGCAACGAGTGG	20	
	FAM - CCAGCCACGGCCAAGTCAGGTTCT -TAMRA	24	
<i>PIAS3</i>	F:GCCCTACCTGGAAGCAAAGG	20	120
	R: GTACTCATGTAGTGGGAGACTGG	23	
	FAM- CCCACCCAACGTGCCCATAGCAGG - TAMRA	24	
<i>PIAS4</i>	F: GAGAAGAAGCCCACCTGGATG	21	77
	R: AGGAGCCCGTCGATGATGAG	20	
	FAM- CCCGTGTGCGACAAGCCAGCCC - TAMRA	22	

clinical data. The analysis of covariance (ANCOVA) was used to control the effects of possible confounding variables. P -values <0.05 were considered as significant.

Results

General information of study participants

Table 2 shows the characteristics of study participants.

Relative transcript levels of PIAS genes in MS patients and controls

Relative expression levels of PIAS genes were not significantly different between MS patients and healthy subjects or between sex/age-based subgroups (Figure 1). Tables 3–6 show the detailed statistical parameters.

When we used ANCOVA test to control confounding effects of sex and age, we did not detect significant difference in PIAS genes expressions between MS patients and healthy subjects. Moreover, the interactions of both age and sex with disease status were insignificant.

Spearman correlation analysis showed significant pairwise correlations between expression levels of PIAS genes

in all study subgroups (Spearman correlation coefficients range: 0.4–0.88) except between PIAS3 and PIAS4 in control group. PIAS1 expression was inversely correlated with age at disease onset. PIAS2 and PIAS3 expressions were inversely correlated with Expanded Disability Status Scale (EDSS) in patients. Moreover, PIAS3 expression was correlated with disease duration in patients and with age in controls. In addition, PIAS4 expression was inversely correlated with EDSS and age at disease onset while it was positively correlated with disease duration. Table 7 shows the results of correlation analysis.

We also compared expression of PIAS genes between male and female and found no significant difference between males and females either in patient or in control groups (Table 8).

Finally, we assessed sex-specific associations between PIAS genes and clinical variables, such as EDSS and age at disease onset (Table 9). Median regression analysis showed association between PIAS3 expression and age of patients ($P=0.045$, 95% CI: -0.25 to -0.01). No other significant association was found after controlling the effect of sex.

Table 2 Characteristics of study participants

Variables	MS patients (n=48)	Healthy subjects (n=52)
Female/male (no., %)	39 (81.2%)/9 (18.7%)	37 (71.1%)/15 (28.8%)
Age (mean \pm SD, Y)	36.2 \pm 2.9	35.3 \pm 2.1
Age range (Y)	17–55	22–60
Age of onset (mean \pm SD, Y)	31.41 \pm 2.8	–
Relapsing–remitting course (no., %)	100 (100%)	–
Disease duration (mean \pm SD, Y)	4.58 \pm 3.2	–
EDSS (mean \pm SD)	3.07 \pm 2.7	–

Abbreviations: EDSS, Expanded Disability Status Scale; MS, multiple sclerosis; Y, years.

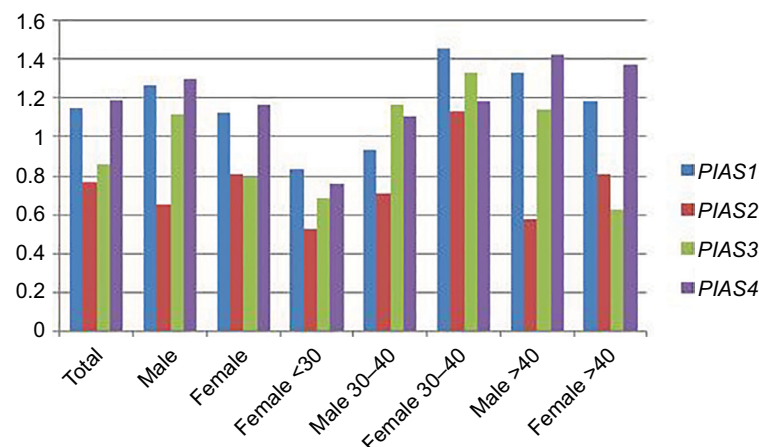


Figure 1 Relative expression levels of PIAS genes in multiple sclerosis patients compared with healthy subjects.

Note: Each sex-/age-based subgroup of patients are compared with the corresponding subgroup of healthy subjects.

Table 3 *PIAS1* expression in MS patients compared with healthy subjects

	Control numbers	MS patient numbers	Expression ratio	$\Delta\Delta CT$	SD	P-value	95% highest density interval	
Total	52	48	1.1566	0.205	-0.23	0.163	-0.23 to 0.63	
Male	15	9	1.2754	0.495	-0.58	0.29	-0.6 to 1.5	
Female	37	39	1.1270	0.123	-0.12	0.304	-0.364 to 0.62	
<30	Male	0	-	-	-	-	-	
	Female	8	9	0.8340	-0.352	0.99	0.744	-2.24 to 1.61
30-40	Male	2	3	0.9361	-	-	-	
	Female	7	15	1.4601	0.549	-0.06	0.253	-0.66 to 1.7
>40	Male	11	6	1.3332	0.53	-0.83	0.325	-1.25 to 2.28
	Female	22	15	1.1821	0.327	-0.34	0.25	-0.339 to 1

Abbreviation: MS, multiple sclerosis.

Table 4 *PIAS2* expression in MS patients compared with healthy subjects

	Control numbers	MS patient numbers	Expression ratio	$\Delta\Delta CT$	SD	P-value	95% highest density interval	
Total	52	48	0.7710	-0.552	-0.28	0.06	-1.06 to -0.02	
Male	15	9	0.6585	-0.822	-0.32	0.093	-2 to 0.3	
Female	37	39	0.8108	-0.459	-0.3	0.234	-1.07 to 0.15	
<30	Male	0	-	-	-	-	-	
	Female	8	9	0.5381	-1.17	-0.66	0.19	-3.23 to 0.894
30-40	Male	2	3	0.7113	-	-	-	
	Female	7	15	1.1310	0.055	0.46	0.733	-1.5 to 1.6
>40	Male	11	6	0.5846	-1.7	-1.21	0.116	-3.4 to 1.23
	Female	22	15	0.8144	-0.493	-0.449	0.336	-1.4 to 0.43

Abbreviation: MS, multiple sclerosis.

Table 5 *PIAS3* expression in MS patients compared with healthy subjects

	Control numbers	MS patient numbers	Expression ratio	$\Delta\Delta CT$	SD	P-value	95% highest density interval	
Total	52	48	0.8615	-0.397	-0.43	0.485	-1.05 to 0.26	
Male	15	9	1.1217	0.217	-0.86	0.646	-0.95 to 1.47	
Female	37	39	0.7977	-0.53	-0.23	0.403	-1.34 to 0.28	
<30	Male	0	-	-	-	-	-	
	Female	8	9	0.6876	-0.83	-1.16	0.583	-3.54 to 1.93
30-40	Male	2	3	1.1799	-	-	-	
	Female	7	15	1.3370	0.393	0.44	0.422	-1.3 to 2.1
>40	Male	11	6	1.1479	0.258	1.6	0.683	-2.07 to 2.56
	Female	22	15	0.6357	-0.825	-0.25	0.253	-2.08 to 0.42

Abbreviation: MS, multiple sclerosis.

Table 6 *PIAS4* expression in MS patients compared with healthy subjects

<i>PIAS 4</i>	Control numbers	MS patient numbers	Expression ratio	$\Delta\Delta CT$	SD	P-value	95% highest density interval	
Total	52	48	1.1912	0.371	-0.22	0.226	-0.302 to 1.08	
Male	15	9	1.3012	0.644	-0.65	0.45	-0.99 to 2.31	
Female	37	39	1.1740	0.324	-0.08	0.301	-0.46 to 1.08	
<30	Male	0	-	-	-	-	-	
	Female	8	9	0.7655	-0.614	-0.34	0.716	-3.15 to 2.04
30-40	Male	2	3	1.1178	-	-	-	
	Female	7	15	1.1824	0.206	1.33	0.707	-2.2 to 2.6
>40	Male	11	6	1.4277	0.797	-0.96	0.394	-1.7 to 3.28
	Female	22	15	1.3703	0.6	-0.41	0.174	-0.46 to 1.7

Abbreviation: MS, multiple sclerosis.

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Table 7 The results of Spearman correlation analysis between *PIAS* genes expressions and clinical data

	<i>PIAS1</i>				<i>PIAS2</i>				<i>PIAS3</i>				<i>PIAS4</i>			
	Age	EDSS	Age at onset	Disease duration	Age	EDSS	Age at onset	Disease duration	Age	EDSS	Age at onset	Disease duration	Age	EDSS	Age at onset	Disease duration
Male	-0.8	-0.66*	-0.14	0.09	-0.29	-0.55	0.07	-0.1	-0.14	-0.77**	-0.08	0.1	0.01	-0.77**	-0.35	0.29
Female	-0.16	0.24	-0.33*	0.1	-0.04	-0.29	-0.21	0.26	-0.07	-0.14	-0.22	0.3	0.02	-0.19	-0.29	0.28
Case	-0.25	-0.35*	-0.28*	0.16	-0.02	-0.3*	-0.14	0.23	-0.08	-0.3*	-0.24	0.3*	-0.18	-0.34*	-0.31*	0.35*
Control	-0.12	-	-	-	-0.05	-	-	-	0.76**	-	-	-	-0.00	-	-	-

Notes: **Correlation is significant at the 0.01 level; *correlation is significant at the 0.05 level.

Abbreviation: EDSS, Expanded Disability Status Scale.

Discussion

Considering the role of the JAK/STAT pathway as a target of drug design in MS,³ identification of factors that modify its function is of practical value. Among regulatory mechanisms of this pathway are PIAS proteins. Notably, PIAS1 has been suggested to discriminatively decrease expression of some cytokine-responsive genes which potentiates it as a target of cytokine-based treatment modalities.⁶ Based on the participation of STAT and NF- κ B in MS pathogenesis, we aimed at evaluation of the effects of PIAS dysregulation in MS as a prototype of autoimmune disorders. Consequently, we assessed *PIAS* genes expressions in IFN β -treated MS patients and healthy subjects. We found significant correlations between the expressions of all of these genes in all blood study subgroups except for lack of correlation between *PIAS3* and *PIAS4* expressions in control group. This concordant expression implies the presence of a single regulatory mechanism for expression of these four genes in blood tissue or a kind of cooperative function for them. Such cooperation has been previously found between PIAS4 and PIAS1 in the regulation of the specificity and amount of NF- κ B/STAT1-mediated gene stimulation.¹¹ However, we could not find any significant difference in expression of *PIAS* genes between MS patients and healthy subjects. This might be due to the fact that all MS patients who participated in the current study were under treatment with IFN β and were in remission. PIAS4 has been shown to diminish both IFN transcription and IFN-induced gene expression via various mechanisms.¹² However, the effects of IFN on expression of PIAS genes have not been evaluated yet.

We have found significant inverse correlation between the expression level of *PIAS1* and age at disease onset. This is in line with the previously reported role of PIAS1 in differentiation of regulatory T cells. PIAS1 has been shown to induce and retain a repressive chromatin state in the Foxp3 promoter leading to hampering the differentiation of natural regulatory T cells. *Pias1* knocked-out mice have been shown to be resilient to experimental autoimmune encephalomyelitis due to the existence of high proportion of these cells.¹³ However, another study has shown that PIAS1 selectively decreases the expression of some STAT1- or NF- κ B-dependent genes, resulting in downregulation of expression of proinflammatory cytokines and chemokines.¹⁴ Besides, we have found significant inverse correlations between EDSS and expression levels of *PIAS2*, *PIAS3*, and *PIAS4*. However, in spite of the observed inverse correlation between *PIAS4* expression and EDSS, the expression level of this gene was inversely correlated with age at disease onset while it was positively

Table 8 Comparison of *PIAS* genes expression levels between male and female subjects

Study groups	Male numbers	Female numbers	<i>PIAS1</i>				<i>PIAS2</i>		
			Expression ratio	SD	P-value	95% highest density interval	Expression ratio	SD	P-value
Total	50	50	0.09	0.29	0.559	-0.49 to 0.64	-0.05	0.34	0.147
Case	11	39	0.18	0.46	0.348	-0.72 to 1.06	-0.31	0.56	0.735
Control	13	37	-0.04	0.37	0.495	-0.76 to 0.69	0.21	0.4	0.366
<50	12	59	0.37	0.41	0.472	-0.45 to 1.15	-0.12	0.48	0.079
>50	12	17	-0.11	0.34	0.137	-0.78 to 0.53	-0.1	0.47	0.084

Notes: Reference group: male, P-values computed from nonparametric frequentist methods.

Table 9 Results of median regression for analysis of association between *PIAS* genes expression and independent variables in patients

Variables	<i>PIAS1</i>					<i>PIAS2</i>			
	Beta	SE	t	P-value	95% CI	Beta	SE	t	P-value
Gender (M/F)	-0.64	0.69	-0.93	0.356	-2.02 to 0.75	-0.05	0.69	-0.07	0.942
EDSS	-0.12	0.19	-0.63	0.529	-0.5 to 0.26	-0.2	0.19	-1.07	0.289
Age	-0.04	0.05	-0.78	0.44	-0.13 to 0.06	-0.01	0.05	-0.15	0.879
Disease duration	-0.04	0.05	-0.76	0.451	-0.14 to 0.07	0.03	0.05	0.55	0.588
Age at onset	-0.04	0.03	-1.51	0.138	-0.09 to 0.02	0.01	0.03	0.23	0.82

Abbreviation: EDSS, Expanded Disability Status Scale.

correlated with disease duration. Such correlations might indicate certain roles of *PIAS* proteins in distinct phases of MS development. Moreover, it is possible that each *PIAS* protein exert distinctive role in the pathogenesis of MS. Future studies are needed to elaborate the effects of this gene on MS course and clinical symptoms.

In brief, in the present study, we detected significant correlations between *PIAS* genes expression and some clinical data of MS patients. Assessment of *PIAS* genes expression in a group of drug-naïve patients is necessary to elaborate the involvement of these genes in the pathogenesis of MS. Moreover, longitudinal sampling from MS patients would help to explore the effects of IFNβ on the expression of *PIAS* genes.

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Disclosure

The authors report no conflicts of interest in this work.

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	PIAS3				PIAS4			
95% highest density interval	Expression ratio	SD	P-value	95% highest density interval	Expression ratio	SD	P-value	95% highest density interval
-0.7 to 0.62	0.17	0.47	0.482	-0.74 to 1.09	0.57	0.43	0.136	-0.29 to 1.42
-1.39 to 0.85	0.63	0.7	0.491	-0.71 to 2.03	0.56	0.66	0.162	-0.75 to 1.87
-0.55 to 0.99	-0.31	0.67	0.169	-1.57 to 1.02	0.54	0.56	0.294	-0.58 to 1.62
-1.06 to 0.81	0.5	0.56	0.146	-0.6 to 1.61	0.55	0.72	0.51	-1.03 to 1.79
-1 to 0.85	-0.4	0.89	0.779	-2.23 to 1.33	0.32	0.64	0.476	-0.95 to 1.55

	PIAS3					PIAS4				
95% CI	Beta	SE	t	P-value	95% CI	Beta	SE	t	P-value	95% CI
-1.44 to 1.34	-0.64	0.92	-0.7	0.491	-2.48 to 1.21	-0.41	1.07	-0.38	0.706	-2.57 to 1.76
-0.58 to 0.18	-0.33	0.25	-1.33	0.192	-0.83 to 0.18	-0.56	0.3	-1.89	0.065	-1.14 to 0.04
-0.1 to 0.09	-0.13	0.07	-2.07	0.045	-0.25 to -0.01	-0.06	0.08	-0.83	0.412	-0.21 to 0.09
-0.08 to 0.13	0.03	0.07	0.39	0.696	-0.11 to 0.16	0.05	0.08	0.62	0.541	-0.11 to 0.21
-0.05 to 0.06	-0.02	0.04	-0.57	0.572	-0.09 to 0.05	-0.05	0.04	-1.23	0.224	-0.13 to 0.03

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