Immunobiology 224 (2019) 560–564

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

# *GAS8* and its naturally occurring antisense RNA as biomarkers in multiple sclerosis

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ARTICLE INFO

*Keywords:*
*GAS8*
*GAS8-AS1*
Multiple sclerosis

ABSTRACT

Expressions of the *Growth arrest specific 8* (*GAS8*) and its naturally occurring anti-sense RNA (*GAS8-AS1*) have been assessed in tumoral tissues of different origins. However, their association with immune-related disorders has been poorly understood. In the current study, we evaluated expression levels of these genes in 50 relapsing-remitting multiple sclerosis (RRMS) patients compared with age- and sex-matched controls. Expressions of both genes were significantly higher in total MS patients compared with controls ($P = 0.001$ and $P < 0.0001$ respectively). The difference in *GAS8* expression was also significant in total female patients and females aged less than 50 when compared with the corresponding control subjects ($P = 0.002$ and 0.006 respectively). *GAS8-AS1* was higher in male patients in both age-based subgroups compared with the corresponding healthy subjects ($P < 0.0001$). Expressions of both genes were inversely correlated with age of male study participants but no other subgroups. *GAS8-AS1* transcript levels had 99.6% accuracy in diagnosis of disease status in male subjects. The current study shows significance of *GAS8* and *GAS8-AS1* in the pathogenesis of MS and the putative role of *GAS8-AS1* as a diagnostic biomarker in a subset of patients.

## 1. Introduction

Multiple sclerosis (MS) is a devastating disorder of the central nervous system (CNS) whose underlying mechanism has not clarified despite extensive efforts. DNA and RNA-based studies have suggested functional roles for several genes and transcripts in the pathogenesis of this autoimmune disorder (Taheri et al., 2017; Rezazadeh et al., 2018). The *growth arrest specific 8* (*GAS8*) gene encodes an element of the nexin-dynein regulatory complex that contributes in ciliary movement (Colantonio et al., 2009). Although hematopoietic cells do not have primary cilia, some proteins with crucial roles in ciliary assembly are expressed in both lymphoid and myeloid cells of human and are suggested to participate in the construction of immune synapse (IS) (Finetti et al., 2009). IS might provide a platform for mutual interactions between T cells and antigen-presenting cells (APCs). Dys-regulation of these interactions might participate in immune cell autoreactivity in MS (Shapiro et al., 2003). The *GAS8* locus in human genome contains an open reading frame in opposite direction of the *GSA8*. The long non-coding RNA (lncRNA) transcribed from this region is named *GAS8-antisense RNA 1* (*GAS8-AS1*). Few studies have evaluated the role of this lncRNA in the pathogenesis of cancer. For instance, Pan et al. have identified *GAS8-AS1* as one of the most commonly mutated genes in papillary thyroid carcinoma (PTC) (Pan et al., 2016) and Zhang et al. have proposed plasma levels of this lncRNA as a potential biomarker in PTC patients (Zhang et al., 2017). However, the role of this lncRNA and its sense transcript in the pathogenesis of MS is largely unknown. Recent studies have reported dys-regulation of several lncRNAs in MS patients among them is another growth arrest specific gene namely *GAS5* (Gharesouran et al., 2018). Based on the results of previous studies regarding the role of lncRNAs in the pathogenesis of MS and the availability of peripheral blood as a source of biomarker discovery in these patients, we conducted the current study to evaluate expression of *GAS8* and *GAS8-AS1* in the peripheral blood of relapsing-remitting MS (RRMS) patients and healthy subjects to find their potential roles as diagnostic biomarkers for MS.

## 2. Material and methods

### 2.1. Study participants

A total of 50 MS patients with relapsing-remitting course and 50 age- and sex-matched healthy subjects enrolled in the current study. All

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https://doi.org/10.1016/j.imbio.2019.04.005
Received 12 February 2019; Received in revised form 7 April 2019; Accepted 9 April 2019
Available online 13 April 2019

of patients were under treatment with daily injections of IFN-β (CinnoVex, Cinagene Company, Iran) and were in remission phase when samples were obtained. Persons enlisted in the control group were healthy volunteers without any neurological or autoimmune conditions. The study protocol was approved by the Ethical Committee of Shahid Beheshti University of Medical Sciences. Written informed consents were obtained from all participants.

### 2.2. Expression study

Expression assays were performed on peripheral blood samples of study participants following extraction of total RNA and cDNA synthesis using Hybrid-RTM blood RNA extraction Kit (GeneAll, Seoul, Korea) and High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Gent, Belgium) respectively. TaqMan® Universal PCR Master Mix (ThermoFisher Scientific, Gent, Belgium) was used for expression analysis. *HPRT1* was used as normalizer based on the previous reports regarding stability of its expression in peripheral blood cells (Valceckiene et al., 2010; Vandesompele et al., 2002) and similar transcript levels between MS patients and healthy subjects (Gharesouran et al., 2018; Taheri et al., 2018). The primers and probes sequences and PCR product length are shown in Table 1. We included positive control in each qPCR run to certify that the method is capable of sufficiently amplifying the target. PCR negative controls were also used to confirm that no contaminating nucleic acid is present into the master mix.

### 2.3. Statistical analyses

The efficiency of the PCR assays has been determined and considered in the calculation of the relative expression levels. The differences in mean values of genes expressions were compared between two groups using Multilevel Bayesian model. This method was used as the distribution of expression data was not normal and the sample size was low. Different possible distributions were fitted to the data and the final model was selected based on the WAIC and LOO indices. The observation effects were considered as random in this model. A t student/Gaussian prior distribution was assumed for parameters with 8000 iteration and 1000 warm-up. The adequacy of the fit of the model to the data and model convergence were checked by R-hat and Gelman-Rubin diagnostics available in Shynistan. Gelman-Rubin diagnostics method draws simulated values from the joint posterior predictive distribution of replicated data and compares these samples to the observed data. According the results, there was no deviation of posterior distribution assumptions. The effects of possible confounding variables were measured through application of Quantile regression. The Box-Cox transformation was used to normalize data. The ROC (Receiver Operating Characteristic) regression model was used to estimate optimal cut-off points of transcript levels of genes for prediction of disease status. The area under ROC curve (AUC), sensitivity (Se), specificity (Sp), and optimal cut-off point based on the Youden index J (JI) were estimated from ROC regression model. The Bayesian multilevel model was estimated using Hybrid Monte Carlo with 6000 iterations and 1000 warm-up in RStan C++ library. The pROC, Stan, loo, and shynistan packages were used in R 3.5.1 environment. The statistical significance was assessed by 95% credible interval (95%CrI) and P-values < 0.05.

**Table 1**
Nucleotide sequences of primers and probes used for expression study.

| Gene name | Primer and probe sequence | Primer and probe length | Product length |
|---|---|---|---|
| *HPRT1* | F: AGCCTAAGATGAGAGTTC | 88 | 18 |
| | R: CACAGAACTAGAACATTGATA | 21 | |
| | FAM -CATCTGGAGTCCTATTGACATCGC-TAMRA | 24 | |
| *GAS8* | F: CTACAACGACATCACCCTCAAC | 22 | 121 |
| | R: GTTCTGCCCAGACACCTCTG | 20 | |
| | FAM- TCTCCCTCTCCAGGTGGTCCTCCT -TAMRA | 24 | |
| *GAS8-AS1* | F: CCCATAGCCTGCCCCGTAAG | 20 | 144 |
| | R: CGTTGTCCCAGCATGTGAGC | 20 | |
| | FAM -CCCGTCTCCCTGTCCGCTTCCCAT-TAMRA | 24 | |

**Table 2**
General demographic and clinical information of study participants.

| Variables | MS patients | Controls |
|---|---|---|
| Female/Male [no. (%)] | 35 (70%) / 15 (30%) | 35 (70%) / 15 (30%) |
| Age (mean ± SD, Y) | 36.2 ± 2.7 | 35.3 ± 2.4 |
| Age (Median [Interquantile range], Y) | 39 [30–45] | 45 [31–55] |
| Age of onset (mean ± SD, Y) | 31.41 ± 2.8 | – |
| Disease duration (Median [Interquantile range], Y) | 5 [3-10.25] | – |
| EDSS score (mean ± SD) | 3.07 ± 2.5 | – |

## 3. Results

### 3.1. General data of study participants

General demographic and clinical information of study participants are summarized in Table 2.

### 3.2. Relative expression of genes in patients compared with healthy individuals

*GAS8* expression was significantly higher in total MS patients compared with controls (P = 0.001). The difference in its expression was also significant in total female patients and females aged less than 50 when compared with the corresponding control subjects (P = 0.002 and 0.006 respectively). *GAS8-AS1* expression was significantly upregulated in total MS patients compared with controls (P < 0.0001). Its expression was also higher in male patients in both age-based subgroups compared with the corresponding healthy subjects (P < 0.0001).

Fig. 1A and B show the relative expression of *GAS8* and *GAS8-AS1* in MS patients and healthy subjects.

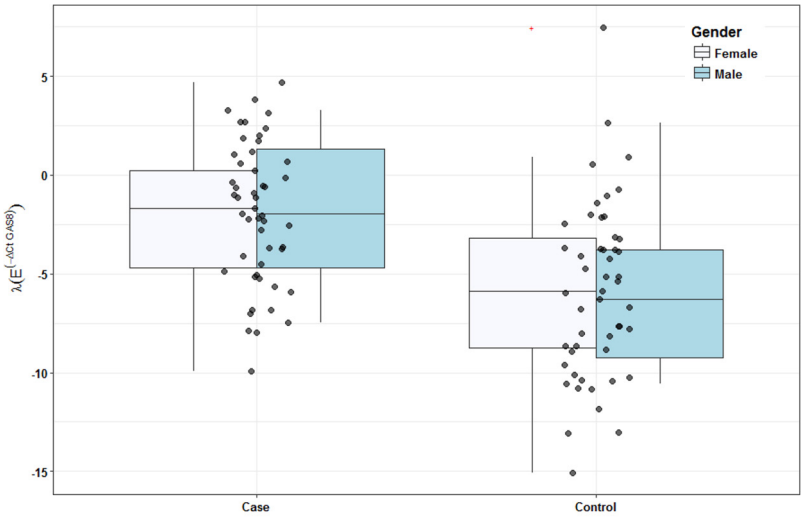
Table 3 shows the results of Multilevel Bayesian model for association between genes expression and MS disease.

Based on the Quantile regression model and after controlling the effects of age and sex, there were significant differences in expressions of *GAS8* and *GAS8-AS1* between cases and controls (P = 0.011 and P < 0.0001 respectively). The interaction between *GAS8-AS1* expression and sex was significant (P = 0.001). Table 4 shows the results of Quantile regression.

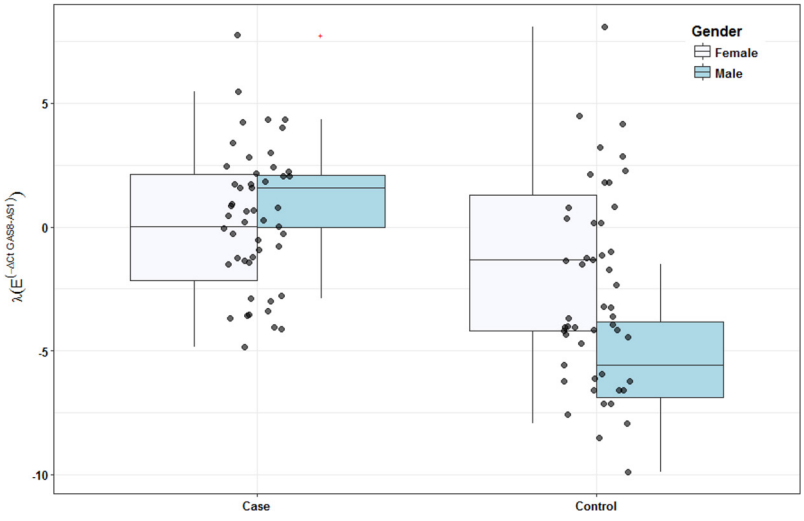
Expressions of both genes were inversely correlated with age of male study participants but no other subgroups (Table 5).

### 3.3. ROC curve analysis

Based on the AUC values, *GAS8-AS1* transcript levels had 99.6% accuracy in diagnosis of disease status in male subjects (Fig. 2). However, the diagnostic power of this gene in total patients were lower (AUC = 0.746, sensitivity = 0.92, specificity = 0.52). *GAS8* transcript levels were more effective in differentiation of disease status in female subjects (AUC = 0.757, sensitivity = 68.5, specificity = 71.4) than in male subjects (AUC = 0.533, sensitivity = 73.3, specificity = 80).




A




B

**Fig. 1.** A and B. The relative expression of *GAS8* and *GAS8-AS1* in MS patients and healthy subjects.

**Table 3**
Multilevel Bayesian results of association between genes expression and disease (CrI: Credible Intervals, P-values were estimated from Frequentist method).

| | | | | *GAS8* | | | | | *GAS8-AS1* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Controls Number | Patients Number | Posterior expression difference | SE | P-value | 95% CrI for expression difference | Power | Posterior expression difference | SE | P-value | 95% CrI for expression difference | Power |
| Total | | 50 | 50 | 4.105 | 0.87 | 0.001 | [2.46, 5.83] | – | 3.084 | 0.71 | < 0.0001 | [1.71, 4.46] | – |
| Male | | 15 | 15 | 4.739 | 2.09 | 0.181 | [0.58, 8.75] | – | 7.883 | 1.24 | < 0.0001 | [5.54, 10.33] | – |
| Female | | 35 | 35 | 3.761 | 0.97 | 0.002 | [1.96, 5.76] | – | 1.495 | 0.84 | 0.304 | [-0.17, 3.12] | – |
| ≤50 | Male | 5 | 13 | 3.795 | 2.3 | 0.075 | [-0.84, 8.34] | 0.49 | 7.207 | 1.68 | < 0.0001 | [3.87, 10.4] | 0.991 |
| | Female | 27 | 30 | 4.506 | 1.16 | 0.006 | [2.19, 6.8] | 0.968 | 1.595 | 0.86 | 0.339 | [0.03, 3.26] | 0.71 |
| > 50 | Male | 10 | 2 | 4.223 | 3.91 | 0.273 | [-3.9, 12.03] | 0.35 | 7.21 | 1.73 | < 0.0001 | [3.79, 10.6] | 0.999 |
| | Female | 8 | 5 | 0.791 | 2.49 | 0.932 | [-4.33, 5.74] | 0.065 | 1.224 | 3.35 | 0.68 | [-5.48, 7.81] | 0.066 |

**Table 4**
Results of Quantile regression model for controlling the effects of age and sex.

| | *GAS8* | | | | | *GAS8-AS1* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Variable | Beta | SE | t | P-Value | 95% CI | Beta | SE | t | P-Value | 95% CI |
| Group (Case/Control) | 5.15 | 1.99 | 2.59 | 0.011 | [1.2, 9.11] | 7.96 | 1.64 | 4.84 | < 0.0001 | [4.7, 11.22] |
| Sex | 0.85 | 1.78 | 0.48 | 0.632 | [-2.68, 4.38] | 5.49 | 1.47 | 3.75 | < 0.0001 | [2.58, 8.4] |
| Age | 0.04 | 0.05 | 0.95 | 0.342 | [-0.05, 0.14] | 0.06 | 0.04 | 1.69 | 0.094 | [-0.01, 0.14] |
| Group*Sex | −1.05 | 2.30 | −0.46 | 0.649 | [-5.62, 3.52] | −6.65 | 1.90 | −3.50 | 0.001 | [-10.42, -2.87] |

## 4. Discussion

MS as a demyelinating disorder of the CNS has been associated with alteration of several molecules and signaling pathways in the peripheral blood and CNS (Gironi et al., 2000). MS has a diverse pathological course with an initial, principally inflammatory relapsing–remitting phase, which over a heterogeneous interval progresses into a gradually degenerative phase leading to neurological deficits (Harris and Sadiq, 2014). Any detected peripheral biomarker in the remission phase of RRMS is potentially associated with the pathogenesis of disease. In the present study, we evaluated expression of *GAS8* and *GAS8-AS1* in peripheral blood of RRMS patients and found significant up-regulation of both genes in total MS patients compared with healthy subjects. The association between GAS8 protein and Golgi apparatus (Colantonio et al., 2006) and the physical interactions between Toll like receptors and this cellular apparatus (Hornef et al., 2002) might provide the functional link between GAS8 and MS pathogenesis. Moreover, NS1 protein as the sole non-structural protein of the influenza A virus has been shown to interact with GAS8 protein (Zhao et al., 2009). The remarkable role of NS1 protein in the development of viral infection (Zhao et al., 2009) and the significant contribution of influenza virus in the relapse of MS (Oikonen et al., 2011) might be regarded as other missing pieces of this puzzle.

Notably, we found a gender specific pattern of expression in both genes in a way that difference in *GAS8* expression was also significant in female patients, while *GAS8-AS1* expression difference was significant in male patients compared with the corresponding healthy subjects. A former a whole-genome gene expression analysis of RRMS patients has shown diverse molecular mechanisms in females and males. Importantly, they reported a critical sex-specific participation of non-coding RNA in this disorder (Irizar et al., 2014). Our findings along with the results of this high throughput expression assessment further emphasizes the previously reported role of gender in determination of the activity, amplitude and skewing of immune responses (Klein, 2012). The fact that females induce greater innate and adaptive immune responses than males (Correale et al., 2010), might be reflected in our observed significant interaction between *GAS8-AS1* expression and sex in the current study. The established gender bias in the prevalence of MS (Eikelenboom et al., 2009) in addition to the sex-specific behavior of genetic risk factors of this disorder (Camiña-Tato et al., 2010) further support the interaction between gender and genes expression in the context of MS.

Of note, we found significant inverse correlation between expressions of mentioned genes and age of study participants only in male subjects. Lack of correlation between expression of *GAS8* and *GAS8-AS1* and age in female subjects, supports sex-specific regulations of these genes on one hand and potentiates these genes as age-independent markers for MS in female subjects on the other hand. Previous studies have detected age-independent MRI-based markers of disease progression in MS patients (Juha et al., 2007). Our results might open a new research era in this regard.

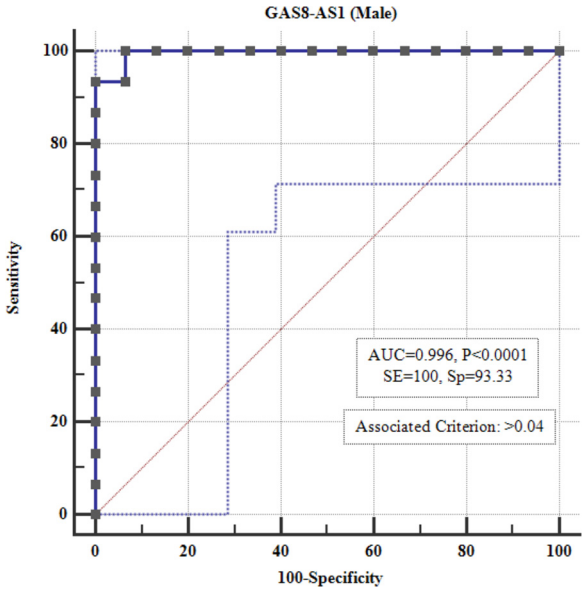



**Fig. 2.** ROC curve analysis for assessment of diagnostic power of GAS8-AS1 transcripts in male subjects.

Pan et al. have shown that in hepatocytes *GAS8-AS1* keeps the *GAS8* promoter in an open chromatin configuration through employing the MLL1/WDR5 complex (Pan et al., 2018). In spite of the expected role of *GAS8-AS1* in the regulation of *GAS8* expression, we found no significant correlation between expressions of these genes in peripheral blood of patients or healthy subjects which might imply a distinct regulatory mechanism for their expression regulation in the peripheral blood cells versus hepatocytes. The independence in regulation of expression has also reported for sense-antisense pairs whose expression is intensely correlated. This emphasizes the necessity of conduction of distinct experimental researches for each antisense pair to unravel their regulatory processes (Goyal et al., 2017). Alternatively, the effects of *GAS8-AS1* on expression of *GAS8* in peripheral blood cells might be similar to its effects in hepatocytes, but the degree of such positive

**Table 5**
Spearman correlation coefficient between genes expressions and age (*Correlation is significant at the 0.05 level).

| | Group | | | | Gender | | | |
|---|---|---|---|---|---|---|---|---|
| | Case | P value | Control | P value | Male | P value | Female | P value |
| *GAS8* | −0.038 | 0.791 | 0.109 | 0.452 | −0.369* | 0.04 | .085 | 0.486 |
| *GAS8-AS1* | −0.163 | 0.259 | 0.222 | 0.122 | −0.369* | 0.039 | .085 | 0.658 |

No significant correlation was found between expressions of these genes in the peripheral blood of patients or healthy subjects.

effects might be less than to be detected by our experimental and statistical analyses.

Finally, we assessed diagnostic power of *GAS8* and *GAS8-AS1* in all subgroups of patients and healthy subjects and found the highest performance for *GAS8-AS1* transcript levels in diagnosis of disease status in male subjects. As expected from expression analysis, *GAS8* transcript levels were more effective in differentiation of disease status in female subjects compared with male patients. Although our results suggest preliminary clues for designing sex-specific biomarker panels for MS, based on the low number of samples evaluated in this study, this data should be interpreted with caution.

Our study had the strength of simultaneous assessment of an mRNA coding gene and its antisense in peripheral blood of study participants and evaluation of their diagnostic power in definite subsets of patients. However, we state small sample size as a limitation of our study which might affect the power of study especially when referring to certain subgroups of the study participants.

In brief, in the current study we found significant alterations in expression of *GAS8* and *GAS8-AS1* in peripheral blood of RRMS patients and suggested them as potential diagnostic biomarkers.

All included patients in the present study were responsive to IFN-β. However, the level of expression of mentioned genes were different between patients and controls. This difference could be either due to the course of MS or the effects of the therapy. Previous studies have shown the distinctive effects of IFN-β on gene expression signature in monocytes and T cells (Henig et al., 2013). Based on the readiness of total blood, we used this kind of sample as a source for expression analysis to decrease preparation steps and suggest a cost-effective and simple method for further biomarker discovery steps. IFN-β-inducible genes are naturally expressed in the peripheral blood cells of drug-naive MS patients (Yamaguchi et al., 2008). Moreover, elevated expressions of these genes have been associated with better disease control (Hesse et al., 2011). However, no study has reported *GAS8* and *GAS8-AS1* as IFN-β-inducible genes yet. To unravel the role of these genes in the MS course and therapeutic response, it is necessary to assess their expression in drug-naïve as well as IFN-β non-responder MS patients. Such studies would help in suggestion of these genes as markers of therapeutic response.

## Conflict of interest

The authors declare they have no conflict of interest.

## Acknowledgment

The study was financially supported by Shahid Beheshti University of Medical Sciences.

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