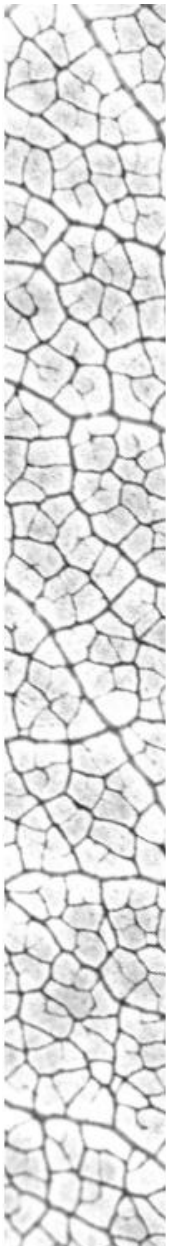

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Gender specific mRNA expression of *HSPA8* in Parkinson's disease

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Abstract
HSPA8, is a molecular chaperone that plays a crucial role in assuring the protein quality. *HSPA8*, has therefore been hypothesized to be associated with Parkinson's risk because the pathogenesis of this disease is characterized by intracellular protein misfolding and inclusion body formation. Also, epidemiological data suggest that the male gender is one of the risk factors for the development of PD. However, the molecular mechanisms underlying gender specificity in PD is less explored. Hence, we investigated the *HSPA8* expression profile of male and female in a south Indian population and whether *HSPA8* expression levels correlate with *HSPA* genetic variants and disease. Using quantitative real-time reverse transcription PCR (qRT-PCR) we quantified *HSPA8* mRNA expression in peripheral blood lymphocytes (PBL) of thirty cases of Parkinson's patients (PD) with anti-PD medications (20 males aged 65.85±1.19 and 10 females aged 65.7±1.202) and 30 age matched healthy people (20 males aged 68.45±1.282 and 10 females aged 65.8±1.133). Further, polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) analysis was performed to detect gene variants in *HSPA8*. We observed that *HSPA8* expression was markedly reduced in PD patients compared with control ($p<0.05$), but *HSPA8* mRNA in males was reduced to greater degree (-48.56%) than in females (-38.28%). SSCP analysis detected polymorphisms at exon 4 of *HSPA8*, in both male and female patients, No significant difference in SSCP patterns were observed between genders of control and PD. On the molecular level, our results provide evidence that the expression profiles of *HSPA8* of age matched normal and PD are gender specific. Altogether, we believe that our data provide a platform for investigating peripheral markers and understanding the role of gender in PD pathogenesis.

Keywords: Parkinson's disease, *HSPA8*, Peripheral blood lymphocytes, Gender, qRT-PCR

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INTRODUCTION

Parkinson's disease is a progressive neurodegenerative disorder of unknown etiology characterized by clinical motor symptoms like bradykinesia, tremor and akinesia; but also impairment of cognitive capabilities (Mhyre et al. 2012). The manifestation of these symptoms are subsequent to 70-80% loss of mesencephalic dopaminergic neurons (mDA) in the substantia nigra pars compacta (SNc) affecting up to 1-2% of the population over 60 years of age (DeMaagd and Philip 2015; Adda, et a, 2016).

Multiple epidemiological studies have suggested that aside of age, the male gender is more vulnerable for the development of PD. However, the reasons for variations between genders in PD are not clear and most likely involve combinations of different patterns of deregulation of genes between gender in mDA of SNc and hormonal and reproductive factors (Smith et al. 2014).Various multi leveled studies using PD-relevant pathway analysis, computational and gene term association networks as well as comparative data mining from published literature on PD pathogenesis established that key cellular pathways associated to oxidative phosphorylation, apoptosis, synaptic transmission and transmission of nerve impulse showed different patterns of deregulation between males and females (Simunovic et al. 2010).

Heat shock 70-kDa protein 8 (*HSPA8*), a cytosolic molecular chaperone specifically maintains normal protein homeostasis within the cell by assisting protein folding, inhibiting protein aggregation, and modulating protein degradation pathways. Substantial evidence supports the involvement of *HSPA8* in PD pathogenesis (Molochnikov et al.2012). *HSPA8* was reported to be co-localized with αsynuclein (αSyn), the major component of Lewy bodies (LBs), within the intraneuronal inclusions

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in PD brains (Klucken et al. 2004, Muchowski et al. 2000). Perturbed expression of *HSPA8* was demonstrated in the SNc of the PD brains. Considerable data derived from cell culture and animal model studies support the protective role of *HSPA8* against αSyn aggregation and toxicity, considered to be vital in the etiology of the PD (Sala et al. 2016, Bandopadhyay et al. 2010). An exploratory study suggests that during healthy aging, *HSPA8* in cerebrospinal fluid decrease, perhaps due in part to an increase in oxidative stress (Loeffler et al. 2010). However, the gender-related changes in *HSPA8* in the human PD are unknown.

We recently measured transcription factors viz., *NURR1* and *FOXA1 that* mediate mDA neuron development and maturation in both male and female PD patients in a small Chennai population of India (Tippabathani et al. 2017). We concluded in that study that *NURR1* and *FOXA1* expression in peripheral blood lymphocytes differ greatly between male and female PD patients with a near difference in gene variants between genders. In this study, we present our findings with regard to gender related changes in peripheral *HSPA8* and the association of genetic variants with PD.

METHODS

Studied Groups

The subjects for the current study were those described previously (Tippabathani et al. 2017). In the previous study, we included 20 male PD patients aged 65.85±1.19, 10 female PD patients aged 65.7±1.20, and 30 healthy controls (HC) matched by age, gender and origin that were collected from Malar hospital, Chennai. The study was approved by the Institutional Human Ethics Committee, Sathyabama University and was conducted with the informed consent of all patients (Sathyabama University/IHEC/Study No 7).

RNA Extraction and cDNA synthesis

As stated, the previous study collected Human peripheral blood (PBL) (1ml) from cubital vein in citrated or EDTA-containing tubes and isolated Human peripheral blood mononuclear cells (PBMCs) using Hisep LSM medium (Himedia). Total RNA was isolated from PBMCs using the TRIzol Reagent method (Invitrogen) and then cDNA synthesis was performed according to the manufacturer's protocol (Applied Biosystem). The cDNA was then assigned to perform the quantitative measurement of *HSPA8* gene expression.

Analysis of *HSPA8* gene expression by qRT-PCR assay

The fluorescent real-time PCR reaction was carried out in the Step One System Applied Biosystem) with a final volume of 25 µl for each reaction containing with the specific primers targeting human *HSPA8* (forward: 5'-TGATGTGTCAATCCTCACTA -3'; reverse: 5'-ACTGATGTCCTTCTTATGCT -3' (NC_000011.10) and human Beta-actin (β-actin), forward: 5'-TCGTGCGTGACATTAAGG-3', reverse: 5'-AAGGAAGGCTGGAAGAGT-3' (X00351.1). Human β-actin gene was used as internal control. After 95°C for 3 min, the experimental reaction consists of 40 cycles of denaturation for 30 sec at 95^0C, annealing for 45 sec at 61.5^0C and extension for 45 sec at 72^0C. The relative quantification of gene expression among the different groups was determined by the formation of $2^{-\Delta\Delta Ct}$ (Schmittgen et al. 2008).

Single strand conformation Polymorphism (SSCP) analysis

Genomic DNA was isolated from each blood sample according to the standard protocol (Russell et al.2001). The target regions were amplified using different primers using Takara Master Mix and Single Strand Polymorphism (SSCP) was performed as defined by (Orita et al. 1989). Briefly, samples of purified double-stranded PCR have been added to formamide dye (95 percent formamide, 0.025 percent xylene cyanol, 0.025 percent bromophenol blue, 0.5 M EDTA), denatured for 10 minutes at 95°C, and immediately snapped cooled on ice for 15 minutes. Aliquots were mounted on 12% polyacrylamide gel and subjected to electrophoresis at 200 V, 4°C for 10 hours. The gel was colored with 0.1 percent silver nitrate to represent the products.

Statistical analysis

The X^2 test was used to test for differences between the PD patients and the control subjects in the distributions of gender. One- or two-way ANOVA was performed to evaluate the differences in the mean value of the relative *HSPA8* gene expression. Unconditional multi variable logistic regression was performed to control for possible confounding by age and gender.

RESULTS

We analyzed the mRNA levels of *HSPA8* gene in the peripheral blood of PD patients and age-matched controls across genders. Prior to the analysis of gene expression profiles related to PD, we investigated whether gender-specific gene expression occurred in healthy control individuals. The most prominent observation was that the abundance of *HSPA8* mRNA in female controls was significantly greater than those in male controls (1.442±0.082 vs. 1.159±0.039 $p<0.05$) (**Table 1**). Focusing on *HSPA8* mRNA levels in PD samples, in contrast to healthy female controls, *HSPA8* gene expression was down regulated by 38.28% in female PD ($p<0.05$) and 48.56% in male PD compared to healthy male controls ($p<0.05$) (**Fig. 1** and **Table 1**). Interestingly, a statistically significant difference of *HSPA8* mRNA between male PD and female PD was reported (0.596±0.088 vs. 0.890±0.038 $p<0.05$) (**Table 1**).

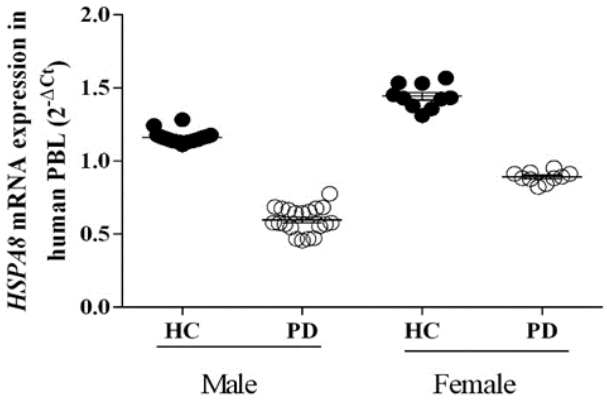

Fig. 1. Scatter plot showing *HSPA8* mRNA expression on PBL in different study groups. Q-PCR data was quantitatively analyzed by determining the difference of Ct between Ct of *HSPA8* and β-actin. mRNA using the formation of $2^{-\Delta Ct}$. Data represent mean ± SME. $p < 0.05$. HC-Healthy Control, PD-Parkinson's Disease

Table 1. The mRNA levels of *HSPA8* gene normalized to β-Actin. Each value represents the mean +SEM of at least three independent experiments. Statistical Significance was assessed by using ANOVA. n=number of individuals used in the current study. HC-Healthy Control, PD-Parkinson's Disease

GROUP	GENDER	*HSPA* expression	*p* value
HC (n=20)	MALE	1.159±0.039	
PD (n=20)	MALE	0.596±0.088	*<0.05*
HC (n=10)	FEMALE	1.442±0.082	
PD (n=10)	FEMALE	0.890±0.038	*<0.05*

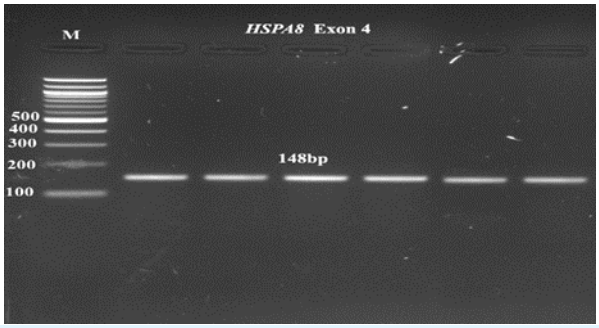

Fig. 2. Electrophoresis pattern of PCR product (148bp) for exon 4 of the *HSPA8* in 1.5% Agarose gel. M-Marker 100bp

SSCP Polymorphisms

The data of current study involved gene polymorphism of *HSPA8* in PD comparison with control group. The products of PCR for exon 4 of the *HSPA8* in both males and females (**Fig. 2**) were subjected to SSCP analysis. The data of haplotype polymorphism in *HSPA8* gene reveal three patterns of haplotype written as (H1, H2 and H3) (**Table 2**, **Fig. 3**).

The data of *HSPA8* polymorphism which display in **Table 2** and **Fig. 3** explained the difference of haplotypes in studied groups, there were three patterns (H1,H2 and H3), In current study two (H1 and H2)

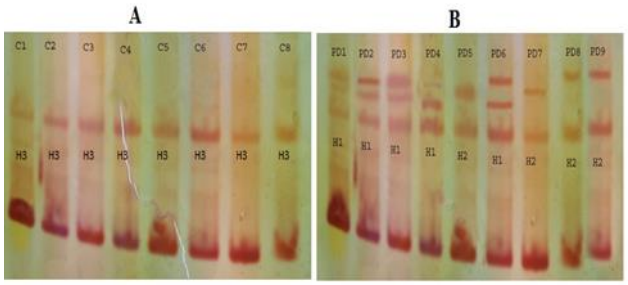

Fig. 3. Electrophoresis Pattern of PCR-SSCP of *HSPA8* gene polymorphism for patients and control on 12% acrylamide. Conformational Patterns of the bands found in A: Healthy controls (C1-C5 in male and C6-C8 in female) and B: PD patients (PD1-PD5 male, PD6-PD9 female) respectively

Table 2. Haplotype frequency of *HSPA8* (exon 4) gene in patients and Controls

Haplotype	*Healthy Control (%)*	*PD patients (%)*	*P value*
Haplotype- H1	100	-	0.05
Haplotype- H2	-	33	0.05
Haplotype- H3	-	66	0.05

haplotype association with PD in small South Indian population. While other pattern (H3) did not show association with PD.

Table 2 showed that most abundant haplotype was H3 and H2 among patient groups (66 %, 33%) and H3 in control groups (100%) respectively. On other hand there was H2, H3 pattern polymorphism shows highly significant differences between studied Healthy control and PD groups (p= 0.05). While migration of band pattern did not show any significant difference across genders in PD and control group.

DISCUSSION

The clinical and experimental evidence have shown that males have greater susceptibility to PD, compared with females. These sex differences may be largely determined, by biological sex differences in the nigrostriatal DA system which in turn arise from hormonal, genetic and environmental factors (Gillies et al.2014). Gene expression studies revealed a sex biased genome signature in human SNc dopamine neurons. In normal brain, the genes mainly involved in signal transduction and neuronal maturation were up regulated in females, while genes involved in PD pathogenesis such as alpha-synuclein and *PINK1* were up regulated in males (Cantuti-Castelvetri et al.2007)

In post-mortem substantia nigra (SN) obtained from PD patients, transcripts of *HSPA8* were down-regulated by a factor of ≥1.5, compared to non-diseases controls (Molochnikov et al. 2012). Notably, evidences indicate that the peripheral blood tissue shares significant gene expression similarities to inaccessible brain tissues, thus offer valuable surrogate markers for PD. In a microarray gene profiling study with blood PD tissue, it was demonstrated that *HSPA8* is associated with PD risk. So

far, to the best of our knowledge, participation of the *HSPA8* gene polymorphism at exon 4 and mRNA expression as the risk factor for the gender specific PD development has not been investigated. This is the first report to identify a gender difference in the expression levels of *HSPA8* in the PBL of PD patients and healthy controls. We demonstrated that the levels of *HSPA8* mRNA expression was significantly higher in the healthy female controls than in male controls. Whereas, *HSPA8* was more down regulated in the male PD patients than in the female PD patients. It is possible that the decreased levels of *HSPA8* observed in our study may be related to a decrease in plasma alpha-synuclein concentration in men as PD progressed, with consequent greater intracellular accumulation of this protein in the brains of men than in women (Caranci et al. 2013). Furthermore, the absence of these chaperones plays a role in the neuronal death due to deposition of ubiquitinated protein aggregates such as α-synuclein and phosphorylated tau in DA neurons (Mahul-Mellier et al. 2020). It seems that the male predominance in the development of PD would be caused due to differential basal transcription of *HSPA8* gene and subsequent changes with the disease duration. That is, estrogen would play a protective role against the development of PD by regulating molecular chaperones expression in neuronal cells (Hou et al.2010). However, we would like to acknowledge that the females in our current study might have lower sex hormones as they are in their postmenopausal phase which might have down regulated the *HSPA8* expression, a risk factor for PD (Lee YH et al.2019).

Variants of *HSPA8* gene could affect *HSPA8* levels and/or function. In Taiwanese PD patients, 1 –110 A/C functional polymorphism in the 5' promoter region of *HSP70* gene may effect susceptibility to PD (Wu YR et al. 2004). So far, to the best of our knowledge, participation of the *HSPA8* gene polymorphism at exon 2 and mRNA expression in the PD development has not been investigated. Through the comparison of genotyping results between PD patients and healthy individuals, the impact of the investigated polymorphism on PD development was estimated. Conversely, we could show that the investigated genetic variants for exon 4 of the *HSPA8* gene were differentially distributed between PD patients and healthy controls, although the differences were statistically insignificant across genders in PD and control group. It seems that *HSPA8* polymorphisms might contribute to an increased susceptibility for the disease, nevertheless, these polymorphisms alone not necessarily could represent the risk factor for the gender specific PD development and progression. Well-designed large-scale studies will be necessary to reveal the possible gender discrepancy in PBL levels of *HSPA8* is related to gene variants, may contribute for the disease progression.

CONCLUSION

In conclusion, our findings suggest that gender differences in the expression of *HSPA8* assessed in the PBL, is a marker of PD progression across genders. However, no important variants of the *HSPA8* gene at exon 2 were observed across the genders, suggesting that other mechanism may be involved in controlling *HSPA8* expression. Altogether, we believe that our data provide a platform for future investigations in a larger population on understanding the role of gender in PD pathogenesis, and the development of appropriately targeted-treatment strategies.

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REFERENCES

Adda, A., Mohammed, C., Djamel, S., Abdelkader, B., & Khalladi, M. (2016). Remote Sensing of Evapotranspiration in a Southern Mediterranean Forest. Application to Bissa Forest, Algeria. The International Journal of Biotechnology, 5(3), 35-44.

Bandopadhyay R, de Belleroche J (2010) Pathogenesis of Parkinson's disease: emerging role of molecular chaperones. Trends in molecular medicine 16(1):27-36. http://dx.doi.org/10.1016/j.molmed.2009.11.004

Cantuti-Castelvetri I, Keller-McGandy C, Bouzou B, Asteris G, Clark TW, Frosch MP, Standaert DG (2007) Effects of gender on nigral gene expression and parkinson disease. Neurobiology of disease 26(3):606-614. http://dx.doi.org/doi: 10.1016/j.nbd.2007.02.009

Caranci G, Piscopo P, Rivabene R, Traficante A, Riozzi B, Castellano AE, Ruggieri S, and Vanacore N, Confaloni A (2013) Gender differences in Parkinson's disease: focus on plasma alpha-synuclein. Journal of Neural Transmission 120(8):1209-1215. http://dx.doi.org/10.1007/s00702-013-0972-6

DeMaagd G, Philip A (2015) Parkinson's disease and its management: part 1: disease entity, risk factors, pathophysiology, clinical presentation, and diagnosis. Pharmacy and Therapeutics 40(8):504-532.

Gillies GE, Pienaar IS, Vohra S, Qamhawi Z (2014) Sex differences in Parkinson's disease. Frontiers in neuroendocrinology. 35(3):370-384. http://dx.doi.org/10.1016/j.yfrne.2014.02.002

Hou Y, Wei H, Luo Y, Liu G (2010) Modulating expression of brain heat shock proteins by estrogen in ovariectomized mice model of aging. Experimental gerontology 45(5):323-330. http://dx.doi.org/10.1016/j.exger.2009.10.006

Klucken J, Shin Y, Masliah E, Hyman BT, McLean PJ (2004) Hsp70 reduces α-synuclein aggregation and toxicity. Journal of Biological Chemistry 279(24):25497-25502. http://dx.doi.org/10.1074/jbc.M400255200

Lee YH, Cha J, Chung SJ, Yoo HS, Sohn YH, Ye BS, Lee PH (2019) Beneficial effect of estrogen on nigrostriatal dopaminergic neurons in drug-naïve postmenopausal Parkinson's disease. Scientific reports 9(1):10531. http://dx.doi.org/10.1038/s41598-019-47026-6

Loeffler DA, Klaver AC, Coffey MP, Aasly JO, LeWitt PA (2016) Age-related decrease in heat shock 70-kDa protein 8 in cerebrospinal fluid is associated with increased oxidative stress. Frontiers in aging neuroscience 8:178. https://doi.org/10.3389/fnagi.2016.00178

Mahul-Mellier AL, Burtscher J, Maharjan N, Weerens L, Croisier M, Kuttler F, Leleu M, Knott GW, Lashuel HA (2020) The process of Lewy body formation, rather than simply α-synuclein fibrillization, is one of the major drivers of neurodegeneration. Proceedings of the National Academy of Sciences 117(9):4971-4982. http://dx.doi.org/10.1073/pnas.1913904117

Mhyre TR, Boyd JT, Hamill RW, Maguire-Zeiss KA (2012) Parkinson's disease. Subcell Biochem 65:389-455. http://dx.doi.org/10.1007/978-94-007-5416-4_16

Molochnikov L, Rabey JM, Dobronevsky E, Bonuccelli U, Ceravolo R, Frosini D, Grünblatt E, Riederer P, Jacob C, Aharon-Peretz J, Bashenko Y (2012) A molecular signature in blood identifies early Parkinson's disease. Molecular neurodegeneration 7(1):26. http://dx.doi.org/10.1186/1750-1326-7-26

Muchowski PJ, Schaffar G, Sittler A, Wanker EE, Hayer-Hartl MK, Hartl FU (2000) Hsp70 and hsp40 chaperones can inhibit self-assembly of polyglutamine proteins into amyloid-like fibrils. Proceedings of the National Academy of Sciences. 97(14):7841-78416. http://dx.doi.org/10.1073/pnas.140202897

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proceedings of the National Academy of Sciences 86(8):2766-2770. http://dx.doi.org/10.1073/pnas.86.8.2766

Russell DW, Sambrook J (2001). Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory. https://doi.org/10.1016/0167-7799(91)90068-S

Sala G, Marinig D, Riva C, Arosio A, Stefanoni G, Brighina L, Formenti M, Alberghina L, Colangelo AM, Ferrarese C (2016) Rotenone down-regulates HSPA8/hsc70 chaperone protein in vitro: a new possible toxic mechanism contributing to Parkinson's disease. Neurotoxicology. 54:161-169. http://dx.doi.org/10.1016/j.neuro.2016.04.018

Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C T method (2008). Nature protocols 3(6):1101-1108. http://dx.doi.org/10.1038/nprot.2008.73

Simunovic F, Yi M, Wang Y, Stephens R, Sonntag KC (2010) Evidence for gender-specific transcriptional profiles of nigral dopamine neurons in Parkinson disease. PloS one 5(1). https://doi.org/10.1371/journal.pone.0008856

Smith KM, Dahodwala N (2014) Sex differences in Parkinson's disease and other movement disorders. Experimental neurology 259:44-56. http://dx.doi.org/10.1016/j.expneurol.2014.03.010

Tippabathani J, Nellore J, Radhakrishnan V, Banik S, Kapoor S (2017) Identification of NURR1 (Exon 4) and FOXA1 (Exon 3) haplotypes associated with mRNA expression levels in peripheral blood lymphocytes of parkinson's patients in small Indian population. Parkinson's disease: 2017. https://doi.org/10.1155/2017/6025358

Wu YR, Wang CK, Chen CM, Hsu Y, Lin SJ, Lin YY, Fung HC, Chang KH, Lee-Chen GJ (2004) Analysis of heat-shock protein 70 gene polymorphisms and the risk of Parkinson's disease. Human genetics 114(3):236-241. http://dx.doi.org/10.1007/s00439-003-1050-1