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Research Article

Deregulated Homocysteine Metabolism in Friedreich's Ataxia Patients: A Risk Factor?

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Abstract

Friedreich's ataxia (FRDA) is a progressive neurodegenerative disorder primarily caused by sub-optimal levels of mitochondrial protein, frataxin, and is characterized by hypersensitivity to oxidative stress. Homocysteine (Hcy) is well acknowledged to mediate oxidative stress-induced toxicity and mitochondrial dysfunction in pathogenesis of several cardiovascular and neurological diseases. Here, we tried to explore the correlation between plasma Hcy levels with various biochemical, genetic and clinical parameters in FRDA. Assessment of Hcy levels in blood plasma of FRDA patients (N = 25) and healthy controls (N = 25) showed that mean levels of Hcy were significantly elevated (9.78 ± 2.7 µmol/l) in FRDA as compared to those of controls (7.0 ± 2.05 µmol/l). The clinical severity of FRDA, as reflected by the (GAA) expansion number in DNA (extent of genetic error), the decreased levels of frataxin protein (primary pathogenic marker), longer disease duration and the high score of Friedreich's Ataxia Rating Scale (measure of neuromuscular activity) revealed positive correlation with plasma Hcy levels in FRDA patients. As deficiency of Vitamin B12 is linked to hyperhomocysteinemia, we analyzed the plasma Vitamin B12 levels and found an inverse correlation with those of Hcy, which supports their deregulated metabolic homeostasis in FRDA. Present results highlight the association of Hcy with the pathogenesis of FRDA thus suggesting Hcy as a potential biomarker for disease progression and/therapeutic intervention.

Keywords: Friedreich's Ataxia (FRDA); Homocysteine; Vitamin B12; Oxidative Stress; Neurodegeneration

Abbreviation

FRDA: Friedreich's Ataxia; GAA: Guanine-Adenine-Adenine; Hcy: Homocysteine; FXN: Frataxin gene

Introduction

Friedreich's ataxia (FRDA) is a progressive neuro and cardio degenerative disorder characterized by atrophy of large sensory neurons and hypertrophic cardiomyopathy [1,2]. The other clinical features of this disease are steady gait and limb ataxia, absence of lower limb reflexes and loss of vibration sense [2,3]. The homozygous DNA triplet (GAA) expansion in the *FXN* gene in FRDA patients results in decreased frataxin protein expression (www.curefa.org). In healthy individuals, the number of (GAA) repeats are generally between 6-36, whereas in FRDA patients, repeats observed are from 70 to 1700 [4]. However, most commonly the repeat number 600-900 [4]. Human *FXN* gene is mapped on chromosome 9. its major transcript (1.3 kb) encodes a 210 amino acid precursor frataxin protein [5]**.** Precursor protein undergoes maturation by the mitochondrial processing peptidase (MMP) to form the functional 14kDa protein [6-8]. Even though frataxin is constitutively expressed, its mRNA and protein levels display tissue specificity with highest expression being observed in heart, spinal cord and cerebellum [9].

Frataxin is known to localize in the mitochondrial matrix and its role in iron homeostasis and mitochondrial function is well established [7,8]. It is vital for iron-sulphur-cluster biogenesis; however its precise mechanism is still not clear [7].

Integral functioning mitochondria is vital because of its central role in ATP production, apoptosis and iron metabolism [8]**.** The deficiency of ATP production is reported in FRDA [9]. Frataxin deficiency causes mitochondrial dysfunction which leads to increased reactive oxygen species (ROS) production associated with FRDA [6,7]. Based on their study in drosophila, Shidara., *et al.* reported that elevated ROS generation combined with a feeble antioxidant defence system are two widely held factors associated with majority of neurodegenerative diseases [10]. The brain is very susceptible to free radicals because of its high oxygen consumption rate, higher levels of unsaturated lipids in the metabolic pool (which are very labile to peroxidation and oxidative damage) and relatively lower antioxidants contents [11,12]. Therefore, it is unequivocally accepted that mitochondrial dysfunction and oxidative stress play a critical role in neurodegeneration of FRDA. It appears that the accumulation of ROS over a long time in neurons could account for the late on set FRDA. Homocysteine (Hcy) is a naturally occurring non-proteinogenic sulphur amino acid. It is cysteine homologue, structurally different from the same by an additional methylene group. Hcy is formed as an intermediate metabolite in the tissues as a result of methyl group transfer from S-adenosyl methionine (SAM) to various molecules, namely proteins, lipids, and nucleic acids. In plasma Hcy is mainly present as oxidised state in the bound form via disulfide bridges to proteins, primarily to albumin (80%) or to itself (20%), and a very less amount (<1%) as low molecular weight free thiols.

Hcy's potential role in pathogenesis of many clinical complications such as cardiovascular disease, osteoporosis and pregnancy issues [13] has gained remarkable interest. Studies have suggested that increased Hcy contributes to the pathogenesis of neurodegenerative disorders by inducing oxidative injury and apoptosis in neurons and is also believed to increase the neuronal vulnerability to excitotoxicity [14]. Recent studies in multiple sclerosis [15], dementia [16], ischemic brain [17] and other neurological disorders like Parkinson's disease (PD) and Alzheimer's disease (AD) patients [18] also supports its proposed role in neurodegeneration. Elevated levels of Hcy in endothelial, glial or neuronal cells, interferes with the redox equilibrium which results in increased production of free radicals and thus oxidative damage. Neurons are specifically very vulnerable to the presence of high levels of Hcy as they cannot metabolize Hcy either by transsulfuration or by remethylation pathway [14,16]. Chronic high levels of Hcy contribute to increased neuronal lipoperoxidation and cellular protein oxidation, with all products obviously identified in neurotoxicity and brain damage procedures [16,17]. Homocysteine is metabolised either by the trans-sulfuration pathways which requires pyridoxine as a cofactor, or the remethylation pathways which requires folate and vitamin B12 together as a cofactor [19,20]. The correlation between the levels of Hcy and vitamins B12 and B6 is of clinical relevance as reported in ALS patients [21].

Purpose of the Study

The purpose of this research is to prospect Hcy's role in FRDA pathogenesis. To this end, we assessed Hcy and vit B12 plasma concentrations in FRDA patients and examined their correlation with the clinical severity of the disease.

Material and Methods

Patient recruitment

FRDA suspected patients showing specific clinical symptoms of FRDA with early age of onset were selected for this case control study. Suspected patients were recruited from the Department of Neurology and Department of Paediatrics, All India Institute of Medical Sciences, New Delhi, India. However, after the genetic analysis only those patients who showed expanded (GAA) repeats in pathogenic range were recruited in the study (n = 25). Age and gender matched individuals who have no past history of any neuromuscular symptoms were enrolled in the study as healthy controls (n = 25). Written informed consent was obtained from both patients and healthy controls. The study was ethically approved by the Institute Ethic Committee, AIIMS (Ethical Clearance No. IESC/T-45/21.01.2015). All experiments were performed in accordance with relevant guidelines and regulations of the ethical committee.

FARS scoring for clinical severity

Clinical severity of FRDA was assessed according to the FARS (Friedreich's Ataxia Rating Scale). FARS scale was developed by Subramony., *et al*. in 2006 to specifically assess the severity of Friedreich's ataxia [22]. The FARS consists of three subscales, constituting of a Functional staging for ataxia (score 0 to 6), an assessment of the activities of daily living (score 0 to 36) and neurological assessment (maximum score 117), composed of bulbar

(maximum score 11), upper limb (maximum score 36) and lower limb (maximum score 16), peripheral nerve (maximum score 26) and upright stability/gait (maximum score 28) sub scores making a total score ranging from 0 to 159.

Blood collection

Peripheral whole blood (8 ml) was collected into K2 EDTA vacutainer tubes (BD Biosciences, San Jose, CA) from each patient and healthy control in the morning. Within 1 hour of blood collection, samples were centrifuged at 700g at 4ºC for 15 minutes in order to separate plasma from the cellular fraction. Collected plasma was further centrifuged at 18000g at 4ºC for 15 minutes to ensure complete removal of any residual cellular component and resulting plasma was stored at - 80ºC. For the purpose of genetic analysis, the peripheral blood mononuclear cells (PBMC) were isolated from the residual cellular part using Ficoll density gradient centrifugation method. Further Genomic DNA was extracted from PBMCs using the Qiamp blood DNA mini kit (Qiagen GmbH, Germany).

Genetic analysis for (GAA) repeats

Analysis of (GAA) repeats was carried out using long-range PCR [23-26]. Long range PCR Kit together with a set of FRDA primers were procured from Thermo fisher scientific, USA. PCR amplification was performed twice on an MJ Mini Thermocycler (BioRad Laboratories, USA) for all samples. Forward primer, 5'GGAGGGATCCGTCTGGGCAAAGG-3' and reverse primer 5'CAATCCAGGACAGTCAGGGCTTT-3' were used for the PCR. Thermocycling steps followed for the long range PCR were: 94°C for 20s, 68°C for 2.5min, followed by 17 cycles in which the length of the 68°C step was increased by 15s/cycle [27]. (GAA) repeats numbers were calculated by evaluating the band size of amplified fragments on 1.2% agarose gel.

Total protein extraction from PBMCs

PBMC pellets were resuspended in 500 ml lysis buffer (7M urea, 2 M thiourea, 4% chaps, 40 mM tris-base, 1% dithiothreitol (DTT)) with 1% protease inhibitor and incubated on ice for 20 minutes followed by sonication. Samples were then centrifuged (25 minutes, 12000g) and supernatant was discarded. The samples were then desalted by acetone precipitation followed by protein quantification using Bradford method.

Estimation of frataxin levels using ELISA

The comparative levels of frataxin proteins in PBMC cells of FRDA patients and healthy individuals was measured using human frataxin *in vitro* Simple Step ELISA (Enzyme-Linked Immunosorbent Assay) kit (Abcam, UK). Briefly 25 µg of protein was added to immune affinity anti-tag antibody coated 96-well plate with affinity tag labelled capture antibody and a reporter conjugated detector antibody which immune captured the sample analyte in solution. After a 1-h immunocapture incubation period, a signal was generated using a TMB-HRP reaction (3, 30, 5, 50-tetramethylbenzidine: horseradish peroxidase) solution that produced a colorimetric signal proportional to the amount of bound analyte (frataxin); the signal was measured at 600 nm.

Biochemical analysis

Assessment of plasma homocysteine

Hcy levels were measured by an Enzymatic cycling method using immunoassay kit (Elabsciences, USA).Homocysteine, present in various biological states in the samples, are reduced to free Hcy by the reducing reagent, tri-ethyl phosphine (TCEP); free homocysteine is then converted to S-adenosyl homocysteine and measured by bioanalyzer (Immulite 2000 system, Siemens Healthcare Diagnostics, UK).

Assessment of plasma vitamin B12

Serum B12 was measured by chemiluminescence enzyme immunoassay (VitB-12 assay kit Bio Vision) involving an automated alkaline denaturation procedure (Immulite 2000, Siemens Healthcare Diagnostics, UK).

Statistical analysis

The comparisons of the mean inter-quartile range between FRDA and control group was done using the Mann-Whitney U test. Logistic regression predicted the odds ratio (OR) and the confidence interval (CI) of 95 percent. Statistical analysis was carried out using the SPSS 14.0 software. All tests were two-tailed and $p < 0.05$ was considered as statistically significant. Whisker plot was made using Graph pad prism 8.0 software.

Results

Genomic DNA of healthy controls carries a range 20 ± 4 (GAA) repeats (60 bp) yielding a ~1.5 kb band on agarose gel. Size of (GAA) repeats in FRDA patients were more than 2 kb (above 200 repeats). Representative gel of molecular analysis of (GAA) repeats is shown in figure 1 (obtained from G:BOX Chemi gel imager). Lane 1contains 1 kb DNA marker, lane 2 is showing a FRDA positive patient with a band size of ~3kb; lane 3 presents a ~1.5 kb band for

a suspected patient who turn out negative for FRDA while lane 4 showing a healthy control with band size below 1.5 kb.

Severity of FRDA in genetically confirmed patients was assessed on Friedreich Ataxia Rating Scale (FARS). In this study the average FARS score was found to be 64.4 ± 24 (Table 1). The mean value of subscales was found to be 3.4(out of 6) for functional staging, 29.5 (out of 36) for ADL, and 94.7 (out of 117) for neurological examination. ELISA assay showed a significantly decreased level of frataxin in the PBMC of FRDA patients (mean ± SD) 48 ± 26 as compared to control group (mean ± SD) 82 ± 36.

The plasma Hcy level in patient group was significantly higher than that in controls (9.78 ± 2.7µmol/l vs. 7.0 ± 2.05µmol/l, p = 0.002).

The plasma B12 concentrations in the FRDA group were also significantly lower than in the controls (542.8 ± 208.2pg/ml vs. 697.3 ± 157 pg/ml, p < 0.05) (Table 1). For patient group, Odds ratio (OR) of Hcy was measured to be 1.7(95% CI = 1.2-2.41, p = 0.020), and that of vitamin B12 was 0.994 (95% CI = 0.990-0.997, p < 0.05). A whiskers box plot presentation of differences found in the plasma levels of Hcy and vitamin B12 between FRDA patients and controls is shown in figure 2.

Correlation analysis revealed positive interrelationship between the levels of Hcy with FARS score (r = 0.23, p = 0.008), GAA repeat numbers (r = 0.28, p = 0.004) and duration of disease (r = 0.311, p = 0.06). However, the correlation between the levels of Hcy and the frataxin concentration were found to be negative (r= -0.27, p = 0.014). Similar inverse correlation trend was observed between levels of Hcy and vitamin B12 levels (r = -0.41, p = 0.02) (Figure 3).

Clinical and Molecular parameters	FRDA	Control
Onset age (years)	11 ± 5 (4-22)	--
Frataxin (pg/ml)	48 ± 26	82 ± 36
Disease duration (years)	6 ± 4 (1-13)	--
(GAA)n	884 ± 75 (800-1000)	--
FARS score	64.4 ± 24 (32-103)	--
Hcy (µmol/l) Mean ± SD (range) IQR	 9.78 ± 2.7 (4.8-16.8) 8.4-10.9	 7.0 ± 2.05 (3.2-10.8) 5.3-8.6
Vitamin B12 (pg/ml) Mean ± SD (range) IQR	 542.8 ± 208.2(132-889.4) 302-790	 697.3 ± 157(446-957) 758.6-957.3

Table 1: Clinical and molecular parameters in FRDA Patients and healthy controls.

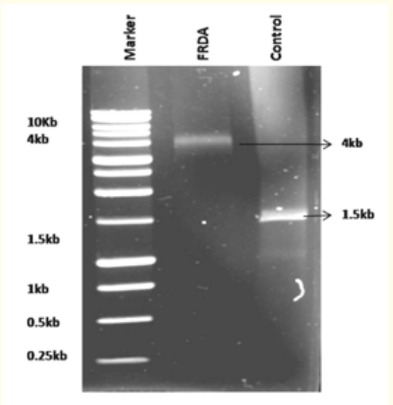

Figure 1: Representative image of the agarose gel of the PCR products for FRDA patients and healthy controls. Lane 1- 1Kb DNA Ladder; Lane 2- Homozygous FRDA patient with 4kb bands; Lane 3-Healthy control (PCR products were analysed using G:BOX chemi gel imager).

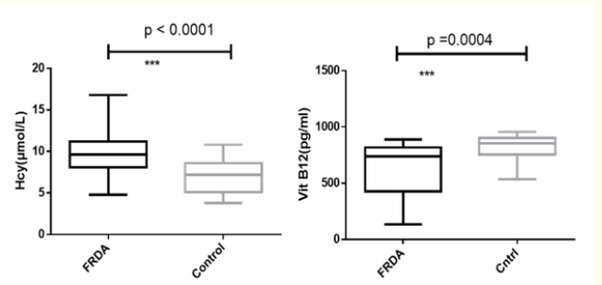

Figure 2: Whisker box plot representing the total plasma levels of Hcy and vitamin B12 in FRDA patients (n = 25) in comparison with Healthy controls (n = 25). (a) Total Hcy level and (b) Total B12 levels in FRDA patients and controls. Line inside the box represent the median value (p < 0.001) obtained using Mann-Whitney U test. (Graph pad prism 8.0 was used to plot whisker box plots).

Discussion

Oxidative stress is believed to be one of the major contributors to the pathology of FRDA disease [9]. Increased levels of ROS (Hydroxyl, peroxide and superoxide ions) have been reported in the FRDA models of yeast [28] and drosophila [10] as well as in the FRDA patient cells [28,29]. Previous studies have shown that frataxin deficiency results in compromised antioxidant defence mechanism and increased cellular sensitivity to ROS species which leads to oxidative stress, mitochondrial DNA damage and finally cellular death in FRDA patients [30,31]. Factors like elevated oxygen consumption, high iron and lipid concentration, and the relatively less efficient antioxidant defence mechanism makes the central nervous system (CNS) extreme vulnerable to oxidative stress. Given their ability to cause oxidative stress, elevated Hcy level are believed to have an adverse effect on neuronal cells and triggering neurodegeneration process.

Two separate case-control studies in 1998 by McCaddon [32] and Clarke R [33], and more recently by Morris., *et al.* [34] reported an elevated levels of homocysteine in patients with Alzheimer's disease (AD). Since then many studies have reported the role of Hcy in neurotoxicity and neuronal death. It has been shown in animal models of hyperhomocysteinemia that high plasma Hcy concentrations compromise the integrity of the blood brain barrier resulting in leakage and, in serious instances, disruption [35]. Although the mechanism of homocysteine in the pathology of neurodegeneration is not fully known, it is hypothesized that a high homocysteine levels alters the redox signalling pathways thus causing the disruption of cellular redox homeostasis [36,37]. Hcy with its thiol group can undergo auto-oxidation to produce hydrogen peroxide and other reactive oxygen species, causing oxidative stress [38]. It can further up hold the oxidative damage though reduced activity of antioxidants such as glutathione, probably by increasing SAH level. Hcy induced neuronal toxicity is also supposed to involve DNA strand breakage, and activation of p53 and poly-ADP-ribose polymerase (PARP) [39].

Under physiological conditions, Hcy is formed during methionine metabolism. It is eliminated by two pathways, either remethylated to regenerate methionine or it can be changed to cysteine via the transsulfuration pathway. With methionine synthase, a vitamin B12 dependant enzyme, Hcy gains a methyl group of 5-methyltetrahydrofolate (5-methyl THF). Methionine is transformed into S-adenosyl methionine (SAM), a universal methyl group donor which is then demethylated to form S-adenosyl homocysteine (SAH), which then get converted to Hcy, reversibly. The transsulfuration pathway is vitamin B6-dependent and is catalyzed by cystathionine-b-synthase. A combination of folic acid, vitamin B12 and B6 can lower Hcy concentrations by activating its methylation and transsulfuration [19]. Based on the reports on the role of Hcy in the oxidative stress in neurodegeneration a hypothetical view of biochemical pathways that takes place in pathological condition is depicted schematically in figure 4.

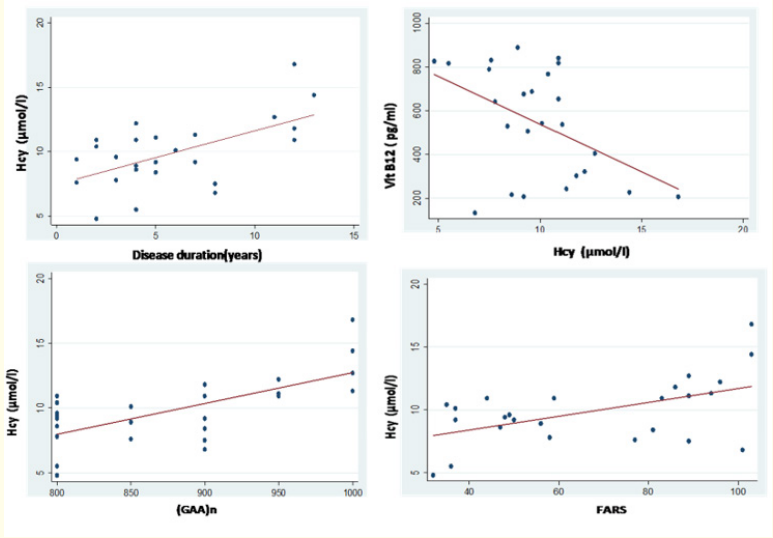

Figure 3: Correlation plots between Hcy and and various clinical and biochemical parameters of FRDA patients: (a) duration of disease, (b) plasma vitamin B12; (c) (GAA) repeats and (d) FARS score Plasma Hcy levels were in significant positive correlation with disease duration, (GAA)n repeat number and FARS score. However the plasma Hcy levels were negatively correlated with plasma vitamin B12 levels. (SPSS 14.0 software was used for the correlation graph plotting).

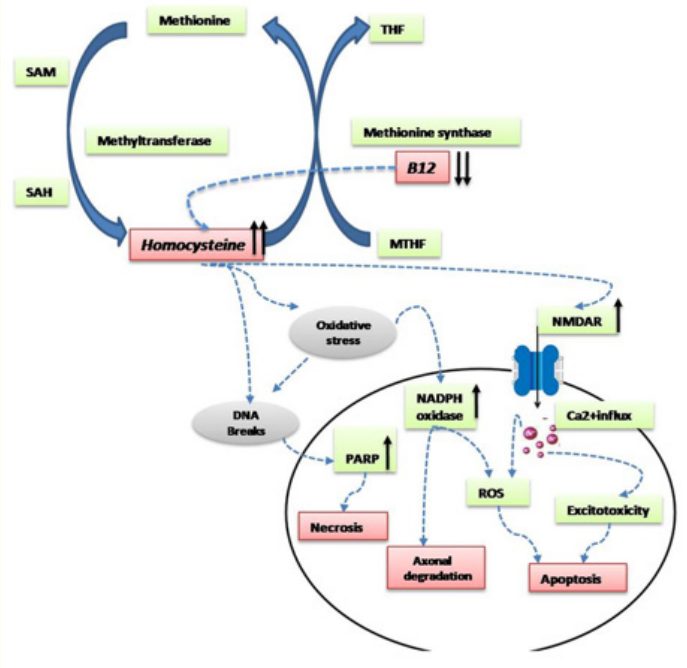

Figure 4: Schematic representation of vitamin B12 dependent Homocyteine - Methionine cycle. Bold arrows represent normal physiological condition while the dotted arrows represent the hypothetical pathways involved in the oxidative damage induced neurodegeneration in FRDA patients caused by the elevated plasma Hcy. Upward and downward arrows represent the elevated and decreased levels respectively.

The most studied pathways by which Hcy is believed to causes oxidative damage is by stimulation of N-methyl-D-aspartate (NMDA) receptors pathway (See figure 4). Hcy acts as NMDA receptor agonists and can trigger calcium overload and glutamate excitotoxicity by overstimulating NMDA receptors in the cortical neurons, resulting in persistent activation of ERK MAP kinase downstream. This might activates the pro-apoptotic signalling leading to neuronal death (STUART A., *et al*. 1997). Recent *in vitro* studies have supported this hypothesis by showing the decreased effect of Hcy when neuronal cells were treated with glutamate receptor antagonists (including MK801and MSOP) [41]. Moreover, Hcy can over-stimulate poly ADP ribosome polymerase (PARP) and thus lead to oxidative neuronal stress and apoptosis by damaging DNA. Hcy may also induce Oxidative damage through NADPH oxidase or through the activation of c-Jun N-terminal kinases (JNKs).

To the best of our knowledge, this is the first study investigating the role of Hcy and vitamin B12 in FRDA pathogenesis. We report here that patients suffering from FRDA have higher levels of Hcy as compared to those of healthy people. Moreover, the patients group also showed decrease concentrations of B12 as compared to the control group. Therefore, the observed elevated levels of plasma Hcy in FRDA can be a sign of its metabolic dysfunction in the brain or it simply suggests an overall deregulated Hcy homeostasis. This can due to either deficient vitB12 or by some other molecular changes in FRDA.

The relation between the individual experimental parameter with the levels of Hcy in FRDA patients has been depicted in figure 3. As expected, the levels of plasma Hcy was found to be inversely correlated with the plasma levels of vitamin B12, supporting its well documented role as biomarker of Hcy concentration (Panel b, Figure 3). Also, one can clearly see a positive correlation between levels of Hcy and disease duration which suggests that Hcy levels in FRDA reflect the progression of the disease (Panel a, Figure 3). The above statement is supported by the changes in the neurological/physiological findings (as FARS) (Panel d, Figure 3), the extent of genetic fault (as GAA repeat numbers) (Panel c, Figure 3) and

molecular aberration (as concentrations of frataxin) (Panel e, Figure 3) with reference to the concentration of Hcy in FRDA patients.

Interestingly, a small group of patients (n = 5) with more pronounced physical disability, history of longer disease duration (1 - 9 yrs) and higher clinical severity (FARS ≥ 80); carrying larger repeat numbers (GAA) ≥ 900) as compared to the rest of the patient group revealed a greater elevation in Hcy levels and relatively lower vitamin B12 levels.

Conclusion

In conclusion, our study revealed increased levels of Hcy in plasma of FRDA patients and presented correlation of Hcy levels with GAA repeat numbers, FARS score, disease duration and vitamin B12 levels. It remains unclear whether elevated Hcy levels directly contributes to the pathogenesis of the diseases or it represents some kind of metabolic aberrations in FRDA patients which deteriorates with disease progression. Further investigation about Hcy in FRDA will help to verify its efficacy and validity as a diagnostic or prognostic biomarker of FRDA.

Author Contributions

MRR: Conceived the idea and wrote the manuscript. AKS and SG: Provided the FRDA patient samples. DP: Designed and executed the experiments, acquired the raw data and wrote the first draft of the manuscript. All authors were involved in interpreting the data. All authors approved the final manuscript.

Declaration of Conflicting Interest

The author (s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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Ethical Approval

This study was ethically approved by the Institute Ethic Committee, AIIMS (Ethical Clearance No. IESC/T-45/21.01.2015). All experiments were performed in accordance with relevant guidelines and regulations of the ethical committee.

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