REPORT

Report on participation of the ICMR International Fellow (ICMR-IF) in Training/Research abroad.

1.	Name and designation of ICMR- IF	: Dr. B. Ashokkumar
2.	Address	: Assistant Professor Department of Genetic Engineering School of Biotechnology Madurai Kamaraj University Madurai – 625 021 Tamil Nadu, INDIA.
3.	Frontline area of research in which Training/research was carried out	: Whole exome sequencing and data analysis of Indian patients with Motor Neuron Diseases
4.	Name & address of Professor and host institute	: Department of Molecular Neuroscience UCL Institute of Neurology The National Hospital for Neurology and Neurosurgery Queen Square London WC1N 3BG
5.	Duration of fellowship	: September 09, 2016 – February 05, 2017
6.	Highlights of work conducted	:

i) Technique/expertise acquired:

Clinical whole exome sequencing – Library preparation with the genomic DNA, exome enrichment with SureSelect Human All Exon kit, cluster generation, DNA sequencing, exome data analysis for variant calling; Live cell imaging by Confocal Microscopy

ii) Research results, including any papers, prepared/submitted for publication:

Exome sequencing is an effective array technology used to discover the molecular basis of rare monogenic disorders, since conventional single gene diagnosis has failed to uncover the genetics of many such diseases. Brown-Vialetto-Van Laere syndrome (BVVLS) and Fazio-Londe diseases (FLD) are a class of Motor Neuron Diseases (MNDs) that destroy neuronal cells that control muscle activity such as speaking, walking, breathing, and swallowing. Mutations in the genes *SLC52A2* and *SLC52A3* corresponding to riboflavin transporters (hRFVT-2 and hRFVT-3) are recently known to cause BVVLS (Green et al., 2010 and Johnson et al., 2010). MNDs are common among consanguineous pairings, may pose a major public health challenge in India as consanguineous

marriages are traditional in India. So far there have been several cases of BVVLS and FLD reported among Indian populations (Puri et al., 1996; Nair et al., 2004; Chandran et al., 2015; Varadarajan et al., 2015), while the occurrence of mutations in the riboflavin transporters in the patients and their families has not been explored and ignored in several cases due to poor diagnosis. It is important to highlight here that earlier studies with BVVL/FL patients confirmed that genetics of BVVL is mostly undefined and the genetic basis of the FL phenotype remains unknown. Therefore, we have aimed to diagnose clinical mutations that are prevalent among MND patients of Indian ethnicity by using whole exome sequencing as a diagnostic tool through the collaboration with UCL Institute of Neurology.

Whole exome sequencing

For this, blood genomic DNA of BVVLS and FLD patients and their families among Indian populations (11 no) was isolated, exome capture was performed using SureSelect Human All Exon kit (Agilent Technologies) and the libraries were sequenced to mean >80-100X coverage on Illumina sequencing platform. The sequences obtained were aligned and further analyzed using Picard and Genome analysis toolkit (GATK) to identify clinical variants. Exome sequencing and data analysis of a BVVLS patient has confirmed the existence of previously identified clinical mutations c.C421A (p.P141T) in the hRFVT-2 and c.A62G (p.N21S) in the hRFVT-3 by our recent studies (Udhayabanu et al., 2016). In a FLD patient, we have identified a mutation c. C800T (p.P267L) in the exon-3 of hRFVT-3, which is a known polymorphism that has been shown to reduce the susceptibility to esophageal squamous cell carcinoma. Meantime, in two unrelated BVVLS patients 3 and 4, compound heterozygous mutations c. C833T (p.T278M) and c.A907G (p. I303V) in the exon-3 of hRFVT-3 is identified. Further, Sanger sequencing with the respective exons has validated the presence of identified mutations. We are yet to receive the exome sequence data for five more BVVLS patients. Chromatograms are shown in Fig. 1.

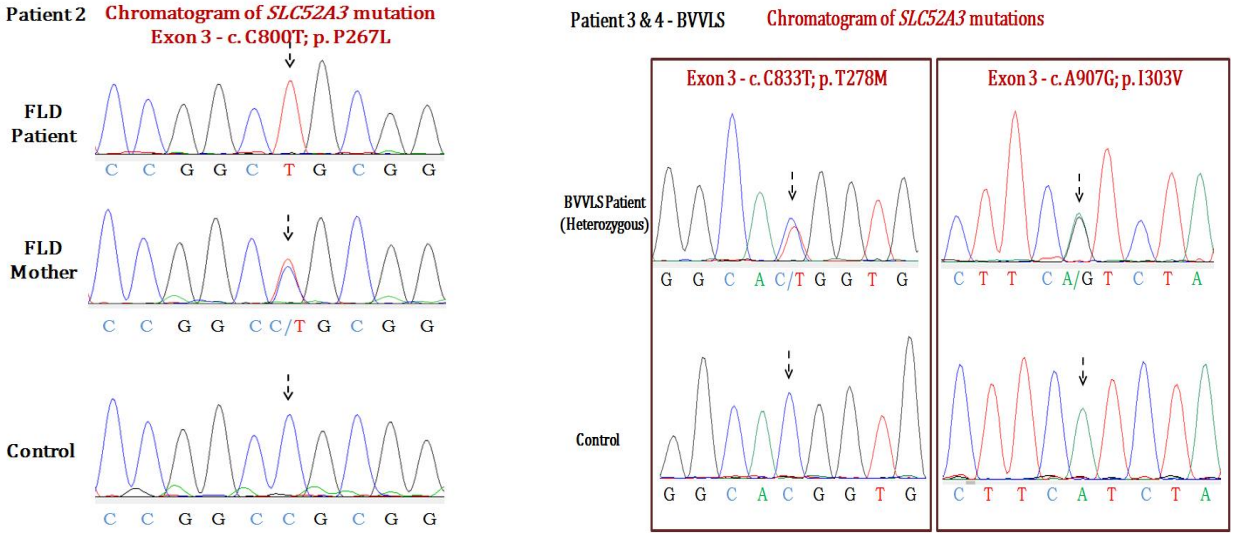


Fig. 1. Chromatogram showing mutations in the hRFVT-3 in the patients and their parents.

Confocal microscopy imaging of hRFVT-3 expression

To assess the defect of identified mutations in the membranous of expression, recombinant constructs of wildtype and mutant hRFVT-3 (p.T278M, p.P267 and p.I330V) were generated in eGFP-C3 vector and transfected in to HeLa cells and imaged under confocal microscopy. The results evidenced that the clinical variant hRFVT-3 proteins are expressed fully in the membrane as observed for wildtype hRFVT-3 (Fig. 2). These findings suggest that the defect in riboflavin transport by this *SLC52A3* mutation from the patients is not because of impaired trafficking of hRFVT-3 protein to the cell membrane; rather the defect appears to be in transport function due to impairment of riboflavin transporter.

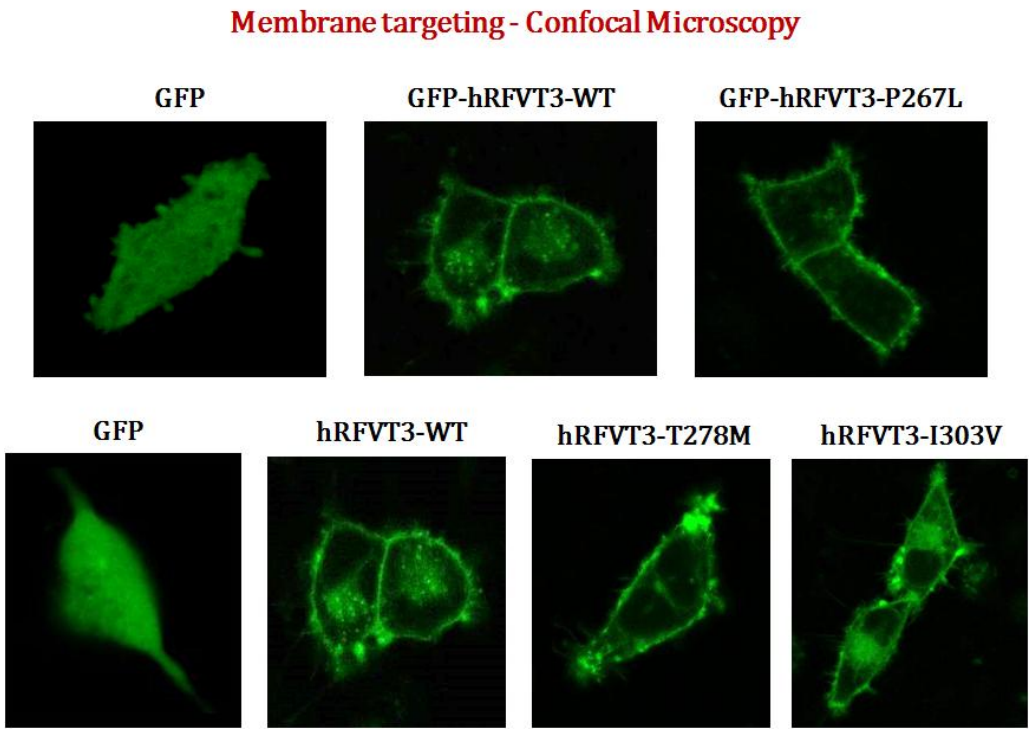


Fig. 2. Cellular distribution of wildtype and mutant hRFVT-3 in HeLa cells.

In another study, we have used computational tools to predict amino acids that are present in hRFVT-3 involved in substrate recognition and transport, which resulted in identification of amino acids at the position of R422K and R426K in hRFVT-3 as crucial for substrate binding. Hence, we generated recombinant constructs of wildtype and mutant hRFVT-3 (p. R422K and p.R426K) in eGFP-C3 vector and transfected in to HeLa cells for imaging by confocal microscopy. Mutants generated based on computational prediction analysis such as R422K and R426K were failed to localize in the membrane and internalized in the ER compartments (Fig. 3), which suggest that these two amino acids at the position of R422K and R426K in hRFVT-3are essential for its expression in the membrane.

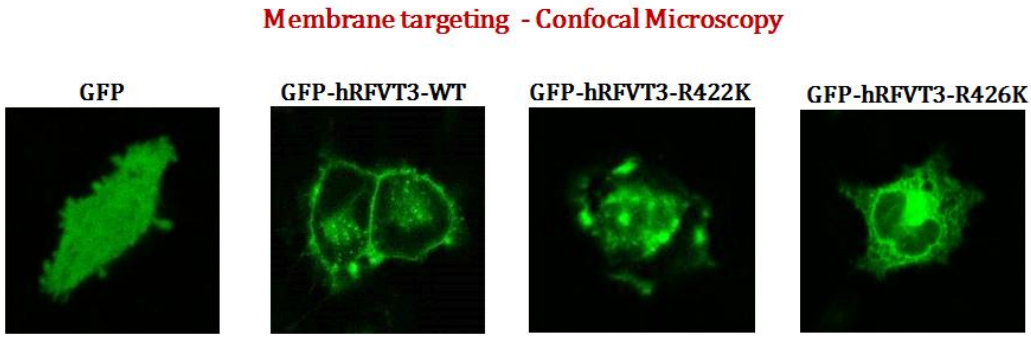


Fig. 3. Cellular distribution of wildtype and mutant hRFVT-3 in HeLa cells.

Generation of a novel *Drosophila* model of BVVLS

Mitochondrial dysfunction has previously been implicated in an array of neurodegenerative disorders. Since riboflavin metabolites in the form of FAD and FMN are critical components of the mitochondrial electron transport chain (ETC), we hypothesized that reduced riboflavin transport would result in impaired mitochondrial activity. For this, we used *Drosophila* as an *in vivo* model and confirmed that global knockdown of the single *Drosophila* RFVT homologue revealed reduced levels of riboflavin, downstream metabolites, and ETC complex I activity. RFVT knockdown in *Drosophila* also resulted in severely impaired locomotor activity and reduced lifespan, mirroring patient pathology, and these phenotypes could be partially rescued using a novel esterified derivative of riboflavin. Our findings suggest a role for mitochondrial dysfunction in BVVLS and validate riboflavin esters as a potential therapeutic strategy.

Exome data analysis of MMND patients of Indian ethnicity

In addition, I was involved in the exome data analysis of previously exome sequenced data of a total of 35 Madras Motor Neuron disease patients (MMND) from Indian origin and our analysis has identified several novel candidate genes with rare variants that are all seemed to be highly associated with the disease phenotypes and are currently being investigated for their functional implications in the disease progression.

Publications:

1. Manole A, Jaunmuktane Z, Hargreaves I, Pandraud A, Salpietro V, Pope S, Ludtmann MH, Horga A, Scalco RS, Li A, **Ashokkumar B**, Lourenço CM, Horvath R, Chinnery PF, Toro C, Singleton AB, Abramov AY, Muntoni F, Hanna MG, Reilly MM, Revesz T, Kullmann DM, Jepson JEC, and Houlden H. Mitochondrial impairment in a treatable childhood neuropathy. Brain (Submitted, Ref-2017-00099).

2. Udhayabanu T, Varalakshmi P, Manole A, Houlden H and **Ashokkumar B**. Molecular mechanisms of riboflavin transport in heart: an insight into the implications of riboflavin deficiency in cardiovascular pathogenesis. Cardiovascular Research (Under submission, 2017).

3. Udhayabanu T, Manole A, Varalakshmi P, Houlden H and **Ashokkumar B**. Riboflavin Responsive Mitochondrial Dysfunction in Neurodegenerative Diseases. Special Issue "Current Strategies for the Biochemical Diagnosis and Monitoring of Mitochondrial Disease". Journal of Clinical Medicine (ISSN 2077-0383)-Invited Review (Under submission, 2017).

Conferences and Seminar:

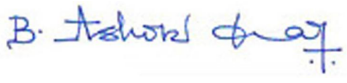
1. Participated in International Symposium on Synaptopathies Symposium' organized by UCL Institute of Neurology, University College, London (October 06, 2016).
2. Attended a special Conference on Festival of Genomics, London organized by The Front Line Genomics Team (January 31 and February 01, 2017).

iii) Proposed utilization of the experience in India:

- ✓ Knowledge obtained by this training on whole exome sequencing and data analysis could be applied on considering exome sequencing as one of the diagnostic tool to identify rare variants of genes that are defective in disease conditions. The implications of whole exome sequencing in disease diagnosis could be shared and passed on to other fellow researchers, clinicians and students in the home country.

- ✓ Knowledge gained in whole exome sequencing for identifying disease variants has provided an opportunity to establish exome sequencing as a diagnostic tool in the neonatal care in India.

- ✓ This training at University College London has facilitated in establishing an INDO-UK collaborative research projects with Prof. Henry Houlden, Institute of Neurology, UCL that range from molecular genetics and exome sequencing to function analysis, which would help in developing this field further for the betterment of mankind and society of both countries.



Signature of ICMR-IF

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