

Microsatellite instability and copy number variation in mitochondrial Displacement loop region in Juvenile myoclonic epilepsy

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Abstract

Introduction: Microsatellites undergo mutations at a very high rate ranging from 10⁻⁶ to 10⁻² per generation, called as Microsatellite Instability (MSI). Such mutations are commonly caused by the loss of DNA mismatch repair (MMR). In the present study we analyzed the frequency of Microsatellite Instability in the Hypervariable regions (HVR1 and HVR2) of Mitochondrial D loop segments which the noncoding control region of human mitochondrial DNA (mtDNA) in Juvenile Myoclonic Epilepsy (JME) patients from South Indian population. Mitochondrial genetic changes at hotspots are considered as a core molecular step of mutations in various types of epilepsy and cancers.

Objective: To analyze hypervariable region 1 and 2 (HV1 and HV2) of mtDNA D-loop region and to establish a new mutation database of mtDNA in South Indian population.

Materials and Method: Thirty five Juvenile Myoclonic Epilepsy patients were investigated from three states of south India. Genomic DNA was extracted and Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) was performed by using standard primers to identify microsatellite instability.

Result: We observed 29 different mutations in HVR-1 and found 13 mutations in HVR-2 including MSI, Sequence mismatch (SMM), deletion and insertions in the mtDNA D-loop region. The most frequent deletion involved was a dinucleotide repeat of CA.

Conclusions: MtDNA mutations are associated with the mitochondria microsatellite instability (mtMSI), may play a specific role in the genetic based epilepsy. Further investigations should focus on the tRNAs and rRNAs loci in the mtDNA mitochondrial genome to understand the mechanism of mtDNA and nDNA.

Keywords: MSI, D-loop, CNVs, mtDNA, JME, Hypervariable region

Introduction

Human mtDNA is a double-stranded, closed circular molecule composed of 16,569 base pairs. It contains only 37 genes: 13 of these genes encode proteins, and the remaining 24 are consisting of 22 tRNAs and 2 rRNAs that are necessary for the synthesis of those 13 polypeptides. Mitochondrial dysfunction caused by mtDNA mutations, such as point mutations, insertions and deletions, has been implicated in aging.^(1,2) Mitochondria produce energy, and their genome is responsible for regulating OXPHOS function and aberrations in mtDNA may interrupt this process and ultimately leading to abnormal function of the cell. The displacement loop (D-loop), located between nucleotides 16,450 and 576, which contains essential transcription and replication elements.⁽³⁾ Microsatellites, or simple sequence repeats (SSRs), are polymorphic loci present in mtDNA that consist of repeating units of one to six base pairs in length. The repeated sequence is consisting of di-, tri- and tetra-nucleotide repeats. Insertions and deletion type of alterations in the microsatellite regions may result in changed expression of associated genes and finally changing the phenotype of the organism. These genomic alterations are named microsatellite instability.

MtDNA intrinsic characteristics represents a critical cellular target for damage. Due to the lack of protective histones, DNA-binding proteins and DNA repair systems, mtDNA has a much higher rate of mutation compared to nuclear DNA.⁽⁴⁻⁶⁾ The mtDNA mutations are detected in various forms of cancers and tumor cell lines (Guerra et al., 2012; Guo et al., 2013). In the present study, we investigated the changes in mtDNA D-loop structure that affects the mitochondrial and neuronal function.

Maniyar. R.Z, Doshi M.A. et, al (2017) revealed that missense mutation changes that codon for one amino acid is replaced by a codon for another amino acid and mutation is a change in one DNA base pair.⁽⁷⁾

The findings of the present study may be helpful in understanding the mechanism of altered neurotransmitter function in JME.

To determine the effect of damaged mtDNA on mitochondrial function, a Clark oxygen electrode was used to measure changes in the mitochondrial respiratory control rate (RCR) and the phosphorus/oxygen (P/O) value.⁽⁸⁾

Materials and Method

Ethics approval and consent to participate: As the study involve human participants approved by the Research Ethics Committee for JME patients from Krishna Institute of Medical Sciences University (KIMSU), Karad, (M.H), and India was obtained. Written informed consent was taken from all participants and in the case of minors, from their parents or legal guardians.

Patient recruitment: Peripheral blood was collected in EDTA tubes from 35 unrelated JME cases (20 males and 15 females) from three states of Telangana, Andhra Pradesh and Hyderabad-Karnataka region of Karnataka in South India. The processing of the samples was done at Sandor proteomics Private Ltd, Banjara Hills, Hyderabad (T.S) and stored at 4°C till further use. Their age ranged from 12 to 34 years with an average of 26.5± SD years at the time of diagnosis.

Study design and methods: The present study was carried out from 2003-2015 in the department of Anatomy, K.M.S.U, Karad (M.H) to conduct the research work in collaboration with Bioserve biotechnologies (India) private Ltd. Mallapur, R.R (Dist); Hyderabad (T.S) and Sandor proteomics Private Ltd, Banjara Hills, Hyderabad (TS).

DNA extraction: Genomic DNA was extracted from blood (n=50) using standard proteinase-K digestion and phenol-chloroform extraction protocols. PCR was performed to amplify the microsatellite using published primers and Single Strand Conformation Polymorphism (PCR-SSCP) was done to identify the sequence variation.

Amplification of microsatellite in the mitochondrial genome: The most common way to detect microsatellites is to design PCR primers that are unique to either side of the repeated portion. PCR was performed in a total volume of 25 (μ)L, and the hot-start technique (D'Aquila et al. 1991) was applied to the reactions of both segments. The PCR was performed in a 50 μl reaction volume mix containing 40 ng of template DNA, 6.25 pmol of each primer, 200 IM of dNTPs, 1.5 mM MgCl₂, 1· reaction buffer and 0.3 U of Taq pol enzyme (Bangalore Genei, India). Primers used PCR conditions followed are given in Table 1 &2. The PCR products are separated either by gel electrophoresis in an automated sequencer and performed with 40ng of genomic DNA. Forward and reverse primers were designed and conditions were optimized to amplify and sequence the template mtDNA by Big Dye terminator cycle sequencing. The result was compared with the Cambridge reference sequence.

Table 1: Primer Sequences for mtDNA D-loop region used in the PCR amplification and sequence analysis

mtDNA region	D - loop position	Sequence stream	Annealing(°C)	Primer (5'-3')
16070-16360	HVR-I	Forward	56	TCC ACA CAG ACA TCA ATA ACA
		Reverse		AAA GTG CAT ACC GCC AAA AG
200-550	HVR-II	Forward	58	CCC ATA CTA CTA ATC TCA TCA
		Reverse		TTT GGT TGG TTC GGG GTA TG

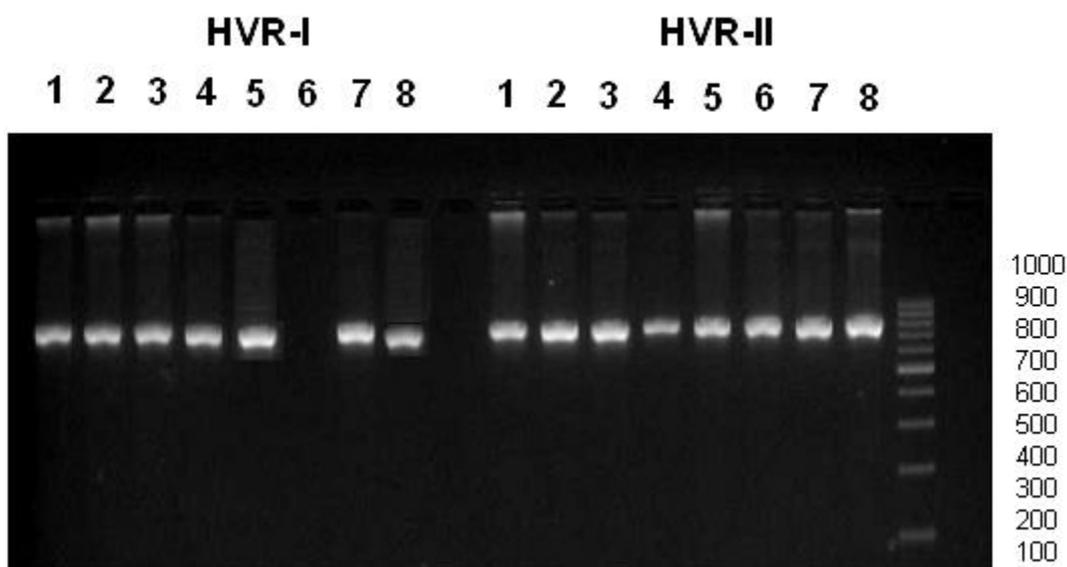


Fig. 1: PCR amplification and sequence analysis of mtDNA D-loop regions (HVR-1 and HVR-2). Molecular weight of the PCR product is about 487 bp for HVR-I and 472 bp for HVR-II, respectively

Table 2: Parameters of PCR conditions (35 cycles) for amplifying HV-segments of mtDNA D-loop region

Cycling condition	Temperature (°C)	Duration
Initial denaturation	94	5 minutes
Denaturation	94	1 minute
Annealing	56 (HV1), 58 (HV2)	1 minute
Extension	72	1 minute
Final extension step	72	5 minutes

Agarose Gel Electrophoresis: Agarose gel electrophoresis was used to examine the quality and quantity of extracted DNA. The amplified PCR products were checked by electrophoresis using 2% agarose gel with ultraviolet illumination run at 110V for 15 minutes after ethidium bromide staining. The PCR product of gel plate placed in alpha imager through UV rays and the image was visualized on monitor for analysis of the quality of the primers.

Statistical analysis: Participant characteristics, mitochondrial D-loop variants and copy number were described as mean \pm SD by Microsoft software for windows (version 7). Chi-square tests were used to compare frequency of mitochondrial D-loop variants in JME patients, and D-loop variants numbers. $P < 0.05$ was considered statistically significant.

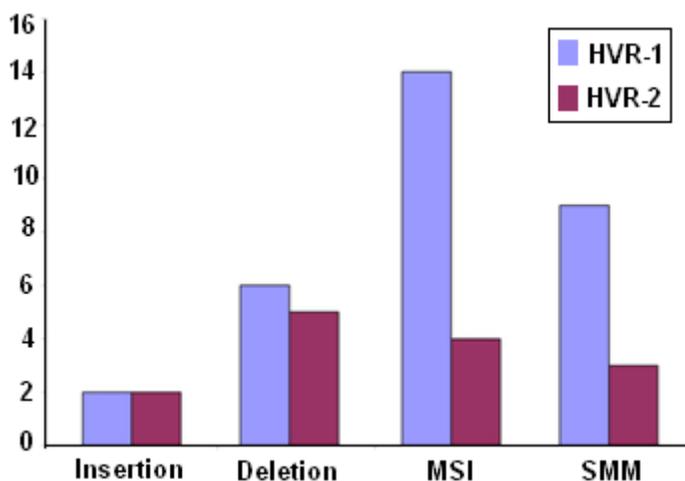
mtDNA D-loop polymorphism: Restriction fragment length polymorphisms (RFLPs) were used for analysis of mtDNA D-loop sequence polymorphisms. The mtDNA HVR1 and HVR2 sequences of each individual were compared with the revisited Cambridge Reference Sequence (rCSR) by using the Applied Biosystems Seq Scape ver. 2.5 Software. Sequence analysis was performed using an ABI PRISM 377 genetic analyzer sequences instrument. Mutations were confirmed by repeated analyses from both H and L strands.

Result

MSI and mutation in mtDNA: To our knowledge, this is the first study to identify microsatellite instability in the mitochondrial genome of JME patients.

In the present study, we used mtDNA sequencing to investigate polymorphisms in two of the hypervariable segments (HVR1 and HVR2). However we found within the non-coding region of the mitochondrial genome among 35 JME patients blood samples. Alignments were made with the Cambridge Reference Sequence (CRS) for human mitochondrial DNA (mtDNA).

We evaluated mitochondrial microsatellite instability (MSI) in the non-coding D-loop region because any alterations in the coding region can adversely affect on mitochondrial function. We designed primers to amplify two hypervariable segments of the control region of mtDNA and mutations were detected by polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) analysis. Both are hotspots for base pair substitutions (Wallace et al, 1995). Because sequencing would provide the highest resolution for screening for suitable polymorphisms, and we sequenced the amplicons using automated DNA sequencing. The genomic distribution of microsatellite instability (MSI) is determined by the repeat nucleotide (mono-, di-, tri-, etc.) type are over represented in coding sequences. Gains or losses of single repeats would destroy the reading frame unless the mutation encompassed a codon triplet.

**Fig. 2: Variation of MSI, Insertion and deletion in HV1 and HV2 segments of D-loop region in JME patients**

The majority of sequence variations were identified MSI and SMM in HV1 segment (Table 3). The mutations of nucleotide insertion equally presents both HV1 and HV2 regions. A large scale of six deletions was found in the HV1 segment and five deletions in HV2 region (Table 3). The deletions could cause a complete defect of ATP production and increase abnormal ROS generation.^(9,10)

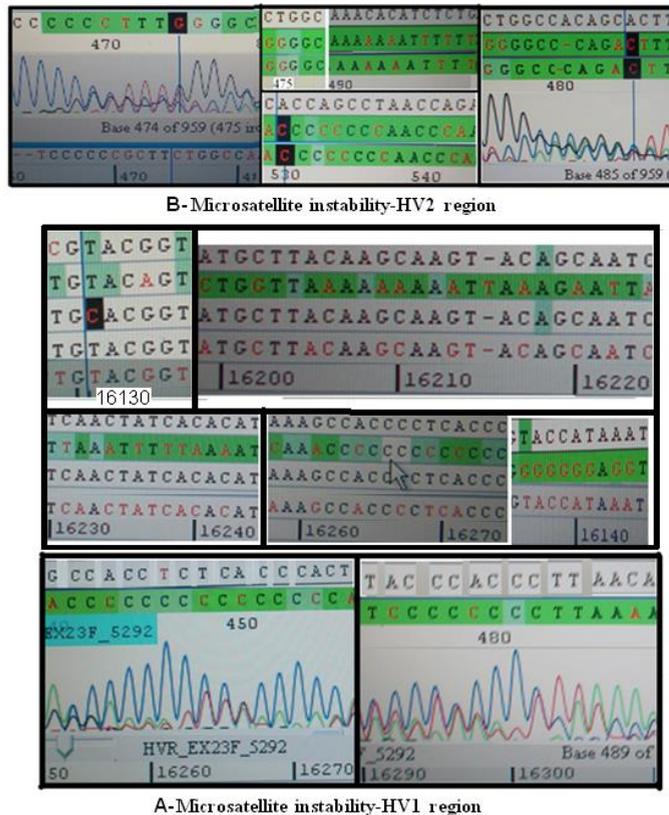


Fig. 3: Electropherogram displaying mtMSI in direct sequencing of base pair in hypervariable regions (HV1 and HV2)

A high frequency of mitochondrial microsatellite instability (mtMSI) was detected in the D-loop region. Microsatellite alleles differ in their number of repeats in different nucleotide position in two hypervariable segments of the D-loop region of HV1 and HV2 segments in JME phenotype reflecting the insertions, deletions, and sequence mismatch were encountered. Mismatch repair (MMR) system is known to be responsible for correction of any error arising during DNA replication. To maintain the genomic fidelity, the MMR system has to be efficient in correcting these mutations. A high degree of length heteroplasmy (HV1 and HV2) segments was found in the regions of poly-C/A/G/T tracts (Fig. 3). The mtDNA copy number alterations have been first described in oncocyctic glioblastoma multiform GBM samples by Marucci *et al.*⁽¹¹⁾

We detected MSI in HV1 region in various base pair position as follows

C stretch interrupted by A in the sequence of $\begin{matrix} \text{CCCACC} \\ \text{CCCCCC} \end{matrix}$ at nucleotide positions 16295-16298

T/A in the sequence of $\begin{matrix} \text{CCCCCTCTCACCCA} \\ \text{CCCCCCCCCCCCCC} \end{matrix}$ at nucleotide positions 16254-16268.

C stretch interrupted by A/G/T in the sequence of $\begin{matrix} \text{ACAGCCCT} \\ \text{CCCCCCCC} \end{matrix}$ at the nucleotide position from 16205-16213

We found MSI in HV2 region in various base pair position as follows

T stretch is interrupted by C $\begin{matrix} \text{CTT} \\ \text{TTT} \end{matrix}$ at nucleotide position (472-474),

CTGG
 C stretch is interrupted by G GGGG at nucleotide position 465-470,
 CTGG
 C stretch is interrupted by C/T GGGG at nucleotide position (475-478),
 ACCAGTCT
 C stretch is interrupted by A/G/T CCCCCCCC at nucleotide position (530-538),

We were unable to draw any correlations between the number of mutations and clinical characteristics. One attractive hypothesis is that damage to the mitochondrial genes could increase production of reactive oxygen species, not only leading to further mutations within the mtDNA but also increasing the instability of nuclear DNA.

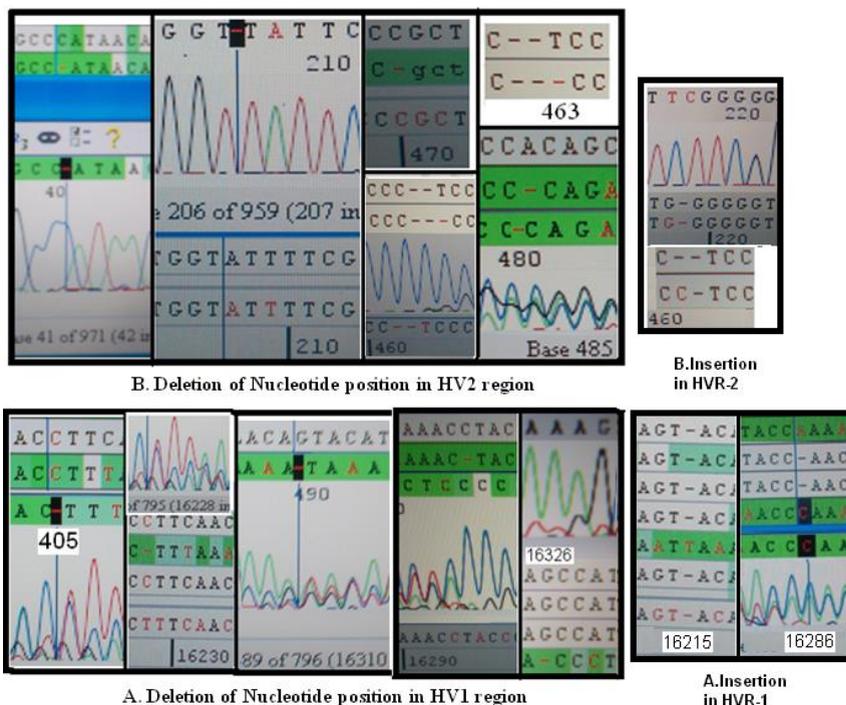


Fig. 4: Copy number variations including deletions and insertion detected in variable nucleotide sites in HV1 and HV2 segments

A large scale of deletions was found in the two hypervariable regions and in poly-C/A tracts and rarely poly-T tract identified in various positions in these regions (Fig. 4).

Nucleotide insertions observed equally in both these regions (HV1 and HV2) and in poly- C/T detected in these regions (Fig. 4).

Discussion

The present study showed that most of the mtMSI, MSS and CNVs were commonly detected in the D-loop region. The MSI at the non-coding D-loop region disrupting mitochondrion-induced apoptosis (Yin et al., 2004; Mambo et al., 2005). The segment 1,120 bp between position 16,024 and position 575 of the D-loop is often called the controlled zone, which is responsible for regulating the replication and transcription of the whole mtDNA molecule (Wang et al., 2007). Deletions, point mutations, insertions, duplications, and microsatellite instability (MSI) have been detected

throughout the genome, and certain mtDNA mutations are associated with specific cancers and genetic based epilepsy.

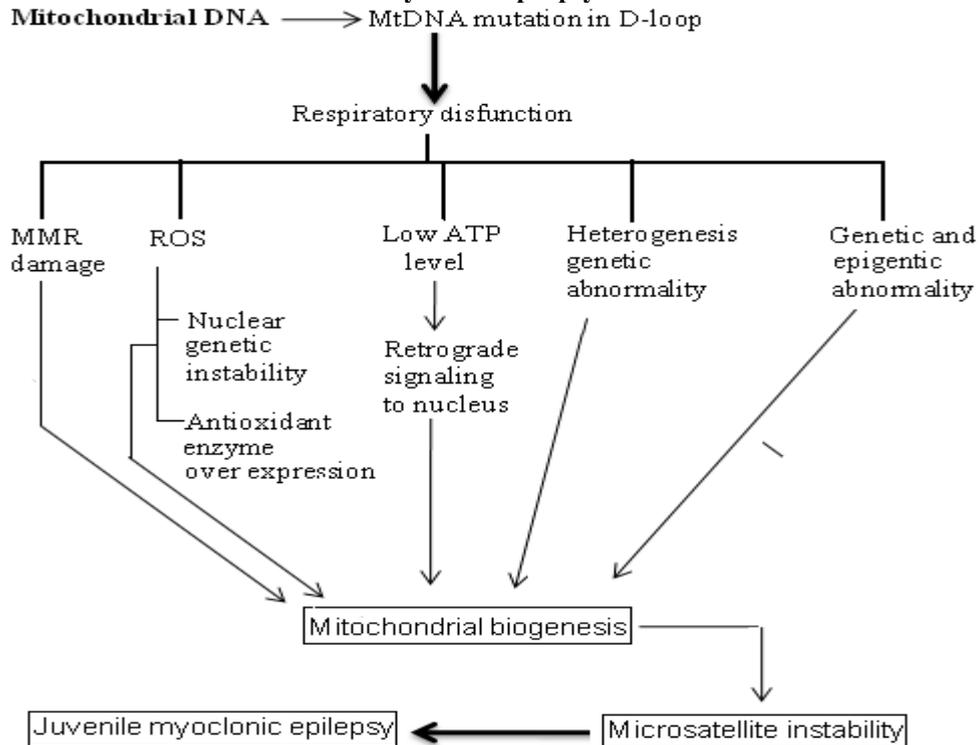
The D-loop mutations affect the binding activity of mitochondrial transcription factor A (TFAM), and thereby resulting in reduced replication and transcription, as well as a reduction in mtDNA copy number in JME. The mtDNA mutations are seen in various forms of cancer and tumor cell lines (Guerra et al., 2012; Guo et al., 2013).

Microsatellite instability has found in mtDNA of colorectal and gastric carcinomas.^(12,13) The production of reactive oxygen species can also be synergistically enhanced by a positive feedback mechanism and produce further mtDNA mutations.⁽¹⁴⁾ However another possibility is that mtDNA mutations affect mitochondrial functions during the apoptotic process in tumor cells. Mitochondrial genome somatic point mutations were most frequently found in the D-loop region, especially in a poly-cytosine (poly-C) mononucleotide repeat tract

located between 303 and 315 nucleotides known as D310. This nucleotide base position identified as a hot spot region for somatic mtDNA mutations in various human cancers, including brain disorder. MtDNA

instability found in 45 colorectal carcinomas in the D-loop region. Burgart et al. (1995) demonstrated that mtDNA alterations in the D-loop region are found in gastric cancers (12.5%).

Flow Chart 1: Schematic diagram of mtDNA dysfunction leads to MSI and CNVs and formation of Juvenile myoclonic epilepsy



Detection of microsatellite instability: The most common microsatellite in humans is a dinucleotide repeat of the nucleotides C and A. MSI is regarded as one of the phenotypes of defective DNA mismatch repair system and high risk for neurological disorder and cancer. Malfunctions of mismatch repair genes have been associated with nuclear MSI. The mtMSI within the mononucleotide repeat at nucleotide position 303–309 has been frequently observed in endometrial tumors.⁽¹⁵⁾ However, there is a significantly higher frequency of the CA-repeat instability in breast cancer (42.5%).⁽¹⁶⁾

Maniyar Roshan. Z; et, al.⁽¹⁷⁾ analyzed the nuclear DNA in LGI4 gene and detected a nonsense mutation in N-terminus, may change to a single base pair to the alteration of a large region of chromosome loci on 19q13.11. MtDNA point mutations are usually maternally inherited and may occur within protein, and recently reported with levels of mutation at < 25% in clinically affected tissues.⁽¹⁸⁾

Tamura et, al.⁽¹⁹⁾ analyzed the tumor types studied and found mutations of the mtDNA D-loop region including HV1 and HV2 in 45 Japanese patients with gastric cancer, and found mutations in 4% of the tumors. Conversely, Alonso et al.⁽²⁰⁾ detected mutations

in the mtDNA D-loop region in 23% colorectal tumors and 37% gastric tumors.

The elevated rates of basal ROS production by catecholamine metabolism might explain the high loads of mtDNA deletions observed in aged substantia nigra neurons.^(21,22) Astroglial cells are considered to contain much more glutathione than neurons,⁽²³⁾ so that the additional ROS producers of astroglial cells might be in general less relevant.

In 1999, Kirches *et al.* revealed high mtDNA sequence variants in 12 astrocytic tumors.⁽²⁴⁾

Conclusion

MSI will provide the analyzing for applicability of molecular characterization of epilepsy and based on available evidence mtDNA may play a key role in the seizures and multiple stages in the process of JME. It could be suggested that mtDNA mutations could be a genetic aberration target in JME development. Further investigations should focus on the tRNAs and rRNAs loci in mtDNA mitochondrial genome to understand the mechanism of genetic based study of JME. These findings highlight a new approach in Epilepsy research.

Declaration of conflicting interests

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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