Role of Permeability glycoprotein (P-gp) and Multidrug resistance protein 1 (MRP-1) in drug-resistance in mesial temporal lobe epilepsy

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Short Title: Drug-resistance in mesial temporal lobe epilepsy

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Abstract

About 30% of patients with epilepsy do not respond to anti-epileptic drugs leading to refractory seizures. The pathogenesis of drug-resistance in Mesial Temporal Lobe Epilepsy (MTLE) is not completely understood. Increased activity of drug-efflux transporters might be involved, resulting in subclinical concentrations of the drug at the target site. The major drug-efflux transporters are permeability glycoprotein (P-gp) and multidrug-resistance associated protein-1 (MRP-1). We have studied these two transporters in the sclerotic hippocampal tissues resected from the epilepsy surgery and compared their expression profile with the tissues resected from non-epileptic autopsy cases. Statistically significant over expression of both P-gp (p-value<0.0001) and MRP-1 (p-value 0.01) at gene and protein levels was found in the MTLE cases. The fold change of P-gp was more pronounced than MRP-1. Immunohistochemistry of patient group showed increased immunoreactivity of P-gp at blood brain barrier and increased reactivity of MRP-1 in parenchyma. The results were confirmed by confocal immunofluorescence microscopy. This suggested that P-gp in association with MRP-1 might be responsible for the multi-drug resistance in epilepsy.

Keywords: Drug-efflux transporters; Drug resistant epilepsy; Mesial temporal lobe epilepsy; P-gp; MDR1; MRP1

Introduction

Epilepsy is the most prevalent neurological condition affecting approximately 69 million people worldwide (Rawat et al., 2020). Mesial temporal lobe epilepsy (MTLE) is the common form of partial epilepsy. There are more than 20 types of anti-epileptic drugs (AEDs) available but around 20-40% of the MTLE cases are multi drug resistant. Uncontrolled seizures have been associated with the sudden unexpected death in epilepsy (SUDEP) in 6 per 1000 epilepsy patients per year (Laxer et al., 2014). Patients with refractory epilepsy have increased risks of premature death, injuries, psychosocial dysfunction, and a reduced quality of life (Löscher and Friedman, 2020; Zavala-Tecuapetla et al., 2020). Therefore, understanding drug resistance in MTLE is an urgent clinical need.

The International League Against Epilepsy (ILAE) defines drug-resistant epilepsy as "failure of adequate trials of two tolerated, appropriately chosen and used antiepileptic drug schedules (whether as mono therapies or in combination) to achieve sustained seizure freedom" (Kwan et al., 2010). Drug-resistance in MTLE is not fully understood; the pathogenesis is thought to be multifactorial. Resistance is usually against multiple drugs having different modes of action. This suggests that pathophysiological basis for developing drug resistance is non-specific (Kwan and Brodie, 2005). The principal factors which are thought to be involved in pharmacoresistant MTLE include etiology of disease, seizure activity progression under drug treatment, the complex temporal patterns, abnormalities in neuronal networks, changes in drug uptake across blood brain barrier (BBB), changes in AEDs targets, and removal of AEDs from epileptogenic tissue through drug-efflux transporters (Lachos et al., 2011).

This is further explained well by two widely accepted hypothesis – target and transporter hypothesis. The most accepted explanation is the transporter hypothesis. It proposes that drug resistance occurs due to up regulation of efflux transporters in the brain capillaries of the BBB. It prevents adequate drug entry to the target location in brain by actively expelling the drugs (Sisodiya et al., 2002).

Two ATP-binding cassette (ABC) drug-efflux transporters are thought to be chief players in resistant MTLE – permeability glycoprotein (P-gp) and multidrug resistance associated protein 1 (MRP-1). P-gp is a transmembrane glycoprotein which is primarily distributed in the organs which are linked to absorption, metabolism, and excretion. In brain it is found at the blood brain barrier (BBB) (Xiong et al., 2015). MRP-1 is an organic ion-transporter; it shares 15% of its amino acid sequence with P-gp. Unlike P-gp, MRP1 is mainly distributed in

the choroid plexus epithelium and ependymal epithelium cells, which are involved in preserving the blood cerebrospinal fluid barrier (BCB) (Keppler, 2011). Under physiological conditions both the transporters expel the harmful substance to maintain equilibrium of internal environment of brain.

Various animal trials have shown that there is an increase in the expression of the P-gp in the refractory epilepsy. A significant increase in the drug concentration has been seen in knockout mice lacking *p-gp* protein (Dombrowski et al., 2001; Rizzi et al., 2002; Volk et al., 2005). Similarly MRP-1 was found to be over expressed in animal models of refractory MTLE (Feldmann et al., 2013; Sisodiya et al., 2002). A study conducted on blood samples collected from epileptic patients found increased expression of these efflux transporters in drug resistant epileptics in comparison to patients controlled by AEDs. Most of the available efflux transporters studies on MTLE patient samples lack suitable control tissue to compare the data which is essential for result interpretation (Dombrowski et al., 2001; Kubota et al., 2006; Sisodiya et al., 2002; Tishler et al., 1995; Weidner et al., 2019). For control most studies have used either disease adjacent tissue or tissue obtained from surgeries done for other pathologies like for A-V malformation, aneurysm etc. P-gp and MRP-1 dysfunction has also been reported in many neurological diseases including tumors (de Klerk et al., 2010; Ginguené et al., 2010; Jablonski et al., 2014; Kortekaas et al., 2005; Lam et al., 2001; Sakata et al., 2011; Vogelgesang et al., 2006; Wijesuriya et al., 2010). Therefore, inclusion of control tissue from brains without known neurological disease is necessary to validate the results.

The current study was designed to evaluate the role of P-gp and MRP-1 in detail and to find out the relative importance of these two in promoting drug resistance in MTLE. The study was done using hippocampal tissues obtained from, the drug-resistant MTLE patients undergoing surgery and age matched control tissue obtained from autopsy. We have studied P-gp and MRP-1 at both gene and protein level. To understand the mechanism of their action, immuno-histochemical analysis and confocal fluorescence microscopy was used for cellular localization of these transporters.

Materials and Methods

Subjects

The hippocampal tissue was obtained from the patients of refractory MTLE (n=15) who had undergone surgery for MTLE in the department of Neurosurgery, PGIMER, Chandigarh,

India. The clinical data of the patients is given in the Table 1. Only patients with pathologically proven hippocampal sclerosis were selected. Cases with any other type of seizures like focal cortical dysplasia, absence seizures, tonic-clonic seizures, atonic seizures, clonic seizures, or tonic or myoclonic seizures were not included in the study. Tissues with structural intracranial lesion like gliomas, meningitis, and neurofibromatosis were also excluded.

Controls

The control hippocampal tissue was taken from the autopsies (n=15) being done in the department of Forensic Medicine, PGIMER, Chandigarh, India. Samples were collected from the autopsies done within 4 hours after death with only non-neurological causes. Death due to non-neurological causes was considered for the study, while cases with head injury, brain haemorrhage, or history of neurological disease were excluded. The clinical data has been provided in the Table 2.

Tissue was collected after written and informed consent from patients and families of controls.

Ethical approval

The study was conducted after approval from the Institute Ethics Committee of PGIMER, Chandigarh, India; vide no. INT/IEC/000931 dated: 25/06/2018.

Tissue Processing

The tissue samples were collected under sterile conditions and stored immediately in the isotonic saline solution and kept in ice. Tissue was divided in three parts one part was stored in RNA later at -20^{O}C , the second part was frozen at -80^{O}C and the third part was fixed in 10% formalin overnight at room temperature.

A part of the fixed tissue was processed for histology in each case. Hematoxylin and Eosin (H&E) staining was done and the slides were evaluated by histopathologist to diagnose hippocampal sclerosis.

Quantitative real time (qPCR)

Around 0.5 g of tissue frozen in RNA later was processed. Total RNA was isolated by using Trizol method (Ambion). The yield and purity of the RNA was assessed by

spectrophotometric measurements (Biotek Epoch) by measuring absorbance at A260/A280 using microplate reading. Synthesis of cDNA was done using a commercially available kit (BioradiScript cDNA synthesis kit) according to the manufacturer's instructions. To find out the relative gene expression, quantitative real time PCR was performed using specific primers using SYBR Green chemistry (ABi SYBR Select Master Mix) on ABi Step One Plus RT-PCR system (Applied Biosystems). Primers were used at 250 nM concentration– human MDR-1 left 5'-TCAGCTGTTGTCTTTGGTGC-3'; right 5'-GGTCGGGTGGGATAGTTGAA-3', human MRP-1 left 5'-TTGGTGATATTCGCCATTGA-3'; right 5'-ACGCATAGTGGATGGCTTTC-3', and human (Glyceraldehyde 3-phosphate dehydrogenase) GAPDH left 5'-TGAACGGGAAGCTCACTGG-3', right 5'-TCCACCACCCTGTTGCTGTA-3'. Following cycling parameters were used - 7 minutes for 95°C followed by 40 cycles of 10 seconds at 95°C and 30 seconds at 62°C. Reaction volume was 10 µl. Quantitative PCR was done in duplicate to quantify the mRNA expression. The data was standardized to the housekeeping gene, GAPDH which aided as an internal control. The data was again standardized to control which was followed by final data presented in the form of relative fold change by $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Representative plots of relative expression for each gene were plotted. The standard criterion was followed to define the up regulation (fold change >1.5) and down-regulation (fold change <0.5) of all the genes.

Western Blot

Tissue was washed with cold PBS buffer and total protein was extracted using 1ml RIPA lysis buffer and 2µl protease inhibitor (Sigma). The concentration for all samples was analysed by bicinchoninic acid (BCA) method using BCA kit (BioRad) and albumin (conc. 2mg/ml) as standard. Each sample was heated for 3–5min and resolved. The total protein was separated on sodium-dodecyl sulphate polyacrylamide gel (SDS-PAGE) with 4% stacking gel and 8% separating gel. Proteins were blotted onto polyvinylidene difluoride (PVDF) membranes. After blocking with bovine serum albumin at room temperature for 2 hours, membranes were incubated with primary antibodies: mouse monoclonal MDR-1 (1:200, Santa-Cruz Biotech., sc-71557), mouse monoclonal MRP-1 (1:50, Abcam, ab24102) and mouse monoclonal β-actin (1:5000, Thermo fisher) overnight at $4^{O}C$. The membranes were incubated with respective secondary antibodies: rabbit anti-mouse (1:10,000, Abcam) at room temperature for 1 hour. β-actin was used as an internal control. Visualization of the membrane was done using enhanced chemiluminescence (ECL) kit (BioRad). Densitometry

analysis was performed using ImageJ Plus software. Width and intensity of the band were measured and quantification was performed. The ratio of area of sample/β-actin of the band intensities was taken for quantification.

Immuno-histochemical analysis (IHC)

Immuno-histochemical analysis was done for the confirmation of QPCR and western blot results by evaluating the protein localization and expression. After processing with standard protocol, 5µm thick sections were applied to pre-coated slides with poly-L-lysine. The paraffin sections were deparaffinized in xylene using three changes of 10 minutes each. Sections were hydrated gradually through graded alcohols by washing in 100% ethanol, 70% ethanol and 50% ethanol for 5 minutes each. For endogenous blocking, hydrogen peroxide and de-ionised water (1:9) was used to quench endogenous peroxidase activity for 20 minutes. Antigen was retrieved in Tris-EDTA buffer at pH 9.0 in incubator at 55^{O}C for four hours to increase immunogenicity followed by washing with 0.1 M phosphate-buffered saline, pH 7.2. Incubation with primary antibodies - mouse monoclonal MDR-1 (1:200, Santa-Cruz Biotech., sc-71557) and mouse monoclonal MRP-1 (1:20, Abcam, ab24102) was performed by an overnight incubation at 4^{O}C. Negative control was used by omitting the primary antibody. The slides were incubated with respective HRP-conjugated rabbit anti-mouse secondary antibody (1:2000, Abcam, ab97046) at room temperature for 1 hour. Peroxidase activity was developed in 0.5% 3, 3'-diaminobenzidine till the desired stain intensity developed. Counter staining was done with hematoxylin. Sections were cleared in xylene & mounting medium i.e. di-n-butyl phthalate in xylene (DPX) was added. Slides were covered with cover slip and observed under light microscope by two unbiased observers.

Semi quantitative analysis of IHC using H score:

The cells with clearly outlined nuclei were observed and counted in 10 random fields of view in each section of patient (n=15) and non-epileptic control (n=15) at 40X magnification for the detailed analysis. Histo-score (H-score) was calculated using formula H-score = [1 × (% cells with 1+) + 2 × (% cells with 2+) + 3 × (% cells with 3+)], where 1+, 2+ and 3+ are the intensity scores of immunostaining according to the appearance (Cheon et al., 2001). Each section was examined under a light microscope by two unbiased observers in blinded manner. It was calculated in the 10 high power fields in the section and the average was taken as H score. On the basis of H score the IR was categorized as mild (<50), moderate (50-100), or

strong (>150). The selected sections were photographed using Olympus microscope with Prog Res Capture Pro 2.9.01 software.

Immunofluorescence

For immuno-labelling, tissues were processed with standard protocol. 5µm thick sections were applied to pre-coated slides with poly-L-lysine. The paraffin sections were deparaffinized in xylene using three changes of 10 minutes each. Sections were hydrated gradually through graded alcohols by washing in 100% ethanol, 70% ethanol and 50% ethanol for 5 minutes each. The tissue sections were then washed in the running water (de-ionised) for one minute. After PBS rinsing, antigen was retrieved in Tris-EDTA buffer at pH 6.1 in oven for 12 minutes to increase immunogenicity and kept at room temperature for 30 minutes. The slides were incubated with two different primary antibodies (used in IHC) mixed in the immunofluorescence (IF) buffer and incubated at room temperature for one hour. Antibody binding was seen by incubation with appropriate Alexa Flour (647-red) conjugated secondary antibodies Anti-mouse (Thermo-fisher Scientific A21235, dilution 2µ/ml) diluted in the immunofluorescence buffer in dark for one hour. After giving 2-3 washes with PBS, the slides were counterstained with 60 µl DAPI (4′,6-diamidino-2-phenylindole) and mounted using glycerol. Slides were stored in the dark by covering with aluminium foil at 4˚C prior to analysis using confocal microscope. All the samples were processed on the same day in order not to confound with the quantification. Fluorescence of cells and images were determined at 40X magnification using the ImageJ plus software. The color channels were split by selecting the image. Images were stacked after making composite and splitting the different channels. An image montage was created as displayed images. For densitometric analysis, z-stacks of the photographed images were converted to grayscale. Signal intensity was quantified as integrated density by simply marking a circle around the cell. For further analysis - area, mean gray value and integrated density were calculated. Corrected total cell fluorescence (CTCF) was interpreted by using formula as CTCF = integrated density – (measured area of the selected cell× mean fluorescence of background). Background signals were measured in areas without signal.

Statistical analysis

GraphPad Prism version 8.0 and SPSS version 23.0 were used for data analysis and graph preparation. Statistical significance was determined using Student's t-test comparing control versus patients. Mean, standard deviation (SD), standard error of mean (SEM) and range was

calculated for age, onset age of seizure, epilepsy duration and seizure frequency. The drug-efflux transporters – P-gp and MRP-1 were correlated with duration of epilepsy, duration of drug-resistance and seizure frequency. Correlation was determined by calculating Spearman's rank correlation coefficient, ρ. A p-value of ≤0.05 was considered as significant. Unpaired student's t-test was applied to determine any significant difference of H score and of immunofluorescence (CTCF values), between MTLE cases and non-epileptic controls.

Results

Clinical and demographic data of the patients and controls was documented. In the patient group (9 males and 6 females) - mean age of the patients was 29.27±10.12 years, onset age of seizures was 12.13±6.26 years, duration of epilepsy was 15.13±10.70 years and seizure frequency was 8.60±5.86/month. The whole data of patients was expressed in comparison with the age matched controls. In the non-epileptic control group (13 males and 2 females) - mean age of the controls was 40.47±13.68 years.

P-gp -The quantitative PCR results showed an overexpression of P-gp (Fig.1a and 1b). The mean±SD (ΔCt value) of patient group was 10.12±5.47 and for control group was 0.51±0.73. The results were statistically highly significant (p-value <0.0001).The mean±SD of the ΔΔCt values was 4.63±4.94. The p-value was 0.008 which is statistically significant. On analyzing fold change for each MTLE patient, 66% of the patients showed overexpression.

The gene expression was validated by protein expression. Western blot results showed that protein expression of P-gp was also increased in the MTLE patients in comparison to the normal controls (Fig.2a and 2b). Taking the reference value of control cases as 1, the mean value for P-gp was 1.49±0.25 in patients. The mean and SD of the ratio of area was 0.60±0.02 in the patients and 0.42±0.08 in the controls. The results were statistically significant with p-value 0.006.

P-gp also showed an increased immunostaining in the MTLE patients as compared to the controls (Fig.3). In the diseased patients, the immunoreactivity (IR) was moderate to strong. Maximum staining was observed at the blood brain barrier (Fig.3c). The endothelial cells in blood vessels demonstrated cytoplasmic as well as membranous staining. The neuropil surrounding the capillaries depicted moderate immunostaining; this would include foot processes of astrocytes. Moderate to severe intensity cytoplasmic IR was also observed in the glial cells (Fig.3d). In addition moderate IR was displayed by neurons; it was punctate in

pattern and cytoplasmic as well as membranous in location. In the controls, mild IR was seen in endothelial cells of the capillaries at the blood brain barrier. Very mild cytoplasmic IR was also observed in the neurons. (Fig. 3a and 3b). No glial cells were found immunostained in the control cases.

Semi-quantitative analysis by H-scoring also showed increased expression of P-gp. Among total 15,181 studied cells in the MTLE patients, 1175 cells were showing immunopositivity which was the 7.7% of the total cells. Increased IR was seen in 80% of the MTLE cases in comparison to the normal controls. In controls, a total of 10,722 cells were counted and only 293 cells were IR which was 2.7% of the total cells. Average H-score was 268 in MTLE cases and 57 in non-epileptic controls. The results were statistically highly significant with p-value <0.0001.

MRP-1 -Similarly, the qPCR data was statistically significant for MRP-1, with p-value 0.01 (Fig.1c and 1d). There was an overexpression of MRP-1 in eleven out of fifteen MTLE patients. The mean±SD (ΔCt) of patient group was 2.99±2.40 and for control group was 0.97±0.69. The mean±SD of the $\Delta\Delta$Ct values was 3.54±6.45 with p-value 0.13 which was not significant. The fold change was calculated and the overexpression was statistically significant (p-value 0.02).

Western blot results also showed that MRP-1 protein was increased in all patient samples as compared to normal (Fig.2a and 2b). Taking the reference value of control cases as 1, the mean value for MRP-1 was 1.20±0.14 in patients. The mean and SD of the ratio of area was 0.67±0.04 in the patients and 0.36±0.04 in the controls. The results were highly significant with p-value <0.001.

Further, immuno-histochemical analysis of MRP-1 was done and increased expression was observed in the MTLE cases in comparison to the non-epileptic controls (Fig.4). In the MTLE patients, IR was cytoplasmic in glial cells. Some of the neurons were also showing immunopositivity (Fig.4d and 4e). In the controls, mild IR in neurons and glial cells was seen (Fig.4a and 4b). In the vessel, stained endothelial cells were seen neither in patients nor in controls (Fig. 4c and 4f).

Semi-quantitative analysis by H-score also confirmed the IHC results. A total of 14,487 cells were counted in the MTLE patients out of which 701 cells showed immunopositivity. This was only 4.8% of the total studied cells. Among 16,104 cells studied in the controls, 374 cells

were showing immunopositivity which was approximately 2.3% of the total cells. Increased IR was seen in majority of the MTLE cases. The average H-score was 183 in the MTLE cases and 49 in the non-epileptic controls. The results were statistically significant with p-value 0.0004.

To further verify the IHC results, immunofluorescence of P-gp and MRP-1 was performed in the MTLE and non-epileptic control tissue. There was increased immunofluorescence seen in the MTLE cases for both the efflux transporters (Fig.5). The results were statistically significant for P-gp (p value 0.0002) and MRP-1 (p value 0.006).

No correlation of either P-gp or MRP-1 was found with duration of epilepsy, duration of drug-resistance or frequency of seizures (Table 3).

Discussion

Drug efflux transporters are important for maintenance of homeostasis in the brain in physiological state. Pathological conditions like epilepsy seem to increase their activity of xenobiotic efflux many fold. Thus efflux transporters become an important determinant of drug distribution within the CNS. We have investigated the expression pattern of drug efflux transporters P-gp and MRP-1 in the tissue resected from multi drug resistant MTLE patients and compared it with that in non-epileptic controls. We have found statistically increased gene expression profiles for both the efflux transporters in diseased. Similar results were obtained in the protein analysis. Immuno-histochemical and immuno fluorescent studies have provided histological as well as cellular localization of these increased efflux transporters in hippocampal tissue in drug resistant MTLE.

Drug resistance has been seen in about 1/3rd of the total patients who are treated with different AEDs. MTLE with hippocampal sclerosis represents one of the most refractory forms of human epilepsy (Asadi-pooya et al., 2016). Therefore, we only incorporated patients with confirmed hippocampal sclerosis in the present study. About 30–50% of the patients are refractory to the available pharmacological treatments, which is associated with five-fold death rate in comparison to the general population (Laxer et al., 2014; Sharma et al., 2015). Surgical resection of the epileptogenic focus remains the only solution for patients whose seizures cannot be efficiently controlled by AEDs (Bergey, 2013; Burtscher and Schwarzer, 2017), but even then only about 50–80% achieve seizure freedom for at least 1 year (Spencer and Huh, 2008). Over expression of the drug-efflux transporters was first described for multi-

drug resistant cancer cells. Overexpression of transporters was also found in capillary endothelial cells of brain tissue obtained from patients with resistant epilepsy (Brandt et al., 2006). There are various studies which explain the involvement of ABC efflux transporters in drug-resistance MTLE; the most important transporters are P-gp and MRP-1 (Aronica et al., 2004; Dombrowski et al., 2001; Kubota et al., 2006; Sisodiya et al., 2006, 2002; Tishler et al., 1995; Weidner et al., 2019). Both efflux transporters maintain the stability of internal environment by extruding the xenobiotics in physiological conditions. The expression pattern of these transporters changes from physiological to pathological states. In pathological conditions drug efflux lead to suboptimal concentrations of drugs at the target site, causing multidrug resistance.

P-gp or MDR-1 is an ATP-binding cassette subfamily B member 1 (ABCB1). It is encoded by the MDR1 (ABCB1) gene in humans located at chromosomal region 7q21 (Löscher and Potschka, 2005). This is a single stranded transmembrane glycoprotein composed of 1280 amino acids. P-gp protein is expressed in various barrier and excretory tissues such as intestine, liver, and kidney. It actively exports hydrophobic and amphipathic molecules of cells or membranes inside to outside. This physiological function of expelling xenobiotics of extensive molecular variability is considered as the critical defense mechanism which protects internal brain milieu. In the normal human brain, P-gp is expressed in the luminal plasma membrane of the brain capillary endothelial cells and in the astrocyte foot processes surrounding the capillaries which constitute the blood-brain barrier (BBB). P-gp expression is found to be only slightly detectable in neurons or glial cells under physiological conditions. In pathological conditions like epilepsy, stroke etc. biochemical cues from the variations in the internal environment of brain lead to increased expression of P-gp (Miller et al., 2008). In diseased state, P-gp is not only highly expressed in endothelial cells, but also in brain parenchyma (Lazarowski et al., 2007). In our results, we have found over expression of P-gp in the endothelial cells of the blood vessels as well as in the hippocampal neurons (Fig.3c and 3d). In animal tissue it has been found that under physiological condition, the MDR1 gene expression is limited to capillary endothelial cells and astrocytes around capillaries. But after epileptic seizures, it is also expressed on the parenchymal astrocytes as well as on neurons. So, there is not only an increase in the expression level of the P-gp but also the number of sites which express these transporters (Dombrowski et al., 2001; Volk et al., 2005). Our results are in accordance as the m-RNA expression of the gene (MDR-1) was increased five

folds in MTLE patients as compared to controls in the present study. Similarly on western blot P-gp levels were also found to be significantly raised in diseased group.

MRP-1 is specific organic anion transporter. This is composed of 1531 amino acids and is encoded by ABCC gene located at chromosomal region 16p31.1. It is distributed in the kidneys, liver, lungs, testes and peripheral blood mononuclear cells. In the brain, MRP-1 is mainly distributed in the choroid plexus epithelium and ependymal epithelium cells. These cells are involved in preserving the blood cerebrospinal fluid barrier (BCB) to prevent the entry of harmful substances or drugs into brain tissue (Tang et al., 2017). Sisodiya et al., 2002 found increased expression of MRP-1 in samples collected from epileptic patients (Sisodiya et al., 2002). In the present study we have also found that MRP-1 gene was over expressed in the epileptic brains and also translated into much higher MRP proteins. Over expression of MRP-1 have been reported but there are conflicting reports on its location in the nervous system. Neuronal or glial MRP1 expression in the normal brain has not been consistently reported in the literature (Ashraf et al., 2014). However, in the present study, MRP-1 IR was detected in both neurons and glial cells in control tissue, though it was mild in comparison to the IR seen in the epileptic tissue. Vliet et al, 2005, have reported MRP-1 expression in endothelial cells of the capillaries in hippocampal tissue from chronic epilepsy (Vliet et al., 2005). Contrary to this in the present study MRP-1 immuno-positivity was not seen in endothelial cells of capillaries in either control or diseased tissues (Fig.4 a-f). Similar to our results, a consistent MRP1 over expression in parenchyma (neurons and astrocytes); not in endothelial cells has been reported in epileptic tissue by various studies (Kubota et al., 2006; Wang et al, 2001; Han et al, 2012; Chen et al, 2013). Decleves et al., 2000 have also reported higher expression of MRP-1 in cultured astrocytes as compared to cultured endothelial cells (Decleves et al., 2000). This suggests that efflux mechanism is also at work within the brain parenchyma, in neurons and glial cells, and this becomes overactive in the resistant cases, preventing the AEDs to achieve clinically effective concentration at target site.

Various animal trials have shown genetic as well as protein up regulation of the P-gp and MRP-1 in the brain tissue in refractory epilepsy (Kuteykin-Teplyakov et al., 2009; Rizzi et al., 2002; Seegers et al., 2002; Volk et al., 2005, 2004). In our study, we have observed the similar up regulation at genetic as well as protein level for both the transporters. Jinming et al., 2018 investigated the expression levels of P-gp and MRP-1 in peripheral blood of patients with epilepsy and found them increased in the drug-resistant patients as compared to the patients responding to AEDs (Jinming et al., 2018). More interestingly, it has been reported

in a PET study that increased P-gp function in the temporal lobe of patients with drug-resistant TLE reverted back to normal after epilepsy surgery; this was seen only in patients who responded to the surgery but not in patients having non-satisfactory surgical outcome (Bauer et al., 2014). It has been suggested that over expression and increased activity of active efflux mechanisms in epileptic brain play a significant role in therapeutic failure of pharmacological treatment of AEDs (Clinckers et al., 2008). Our results are in accordance with these studies. In the present study the quantum of increase was more for P-gp than MRP-1, which might indicate that P-gp has bigger role to play in causing drug resistance in MTLE.

There are conflicting reports in the literature regarding the status of P-gp and MDR-1 after acute epilepsy. Few studies have reported increase in mRNA expression for P-gp after 3-48 hours of seizure episode, returning to normal within 72 hours to 2 weeks (Rizzi et al., 2002; Tang et al., 2017). On the other hand, Kuteykin-Teplyakov et al 2009, found that after 6-24 hours of status epilepticus in rats, P-gp and MRP-1 mRNA expression was decreased in the hippocampus, followed by increased expression after two days (Kuteykin-Teplyakov et al., 2009). The findings of these studies suggests that overexpression of P-gp and MRP-1 is a temporary phenomenon in early stage of epilepsy. We have used the hippocampal tissues from long-standing drug-resistant MTLE patients and have found consistently raised levels of both efflux transporters at genetic and protein level. Therefore, recurrent seizures in the long run might lead to permanent genetic up regulation of the efflux transporters. We correlated P-gp and MRP1 expression profiles with duration of epilepsy, duration of drug-resistance and frequency of seizures of all the patients, in order to detect any trend which describe the drug-resistance pattern. We did not find any correlation between any of these factors with P-gp or MRP-1.

Conclusion

Drug-resistance in MTLE is common and results in severe morbidity and mortality. We studied the expression pattern of P-gp and MRP-1 in MTLE cases in comparison to age matched non-epileptic controls. Tissue location for these transporters was found using monoclonal antibodies. These results show that P-gp and MRP-1 are important determinants of bio availability and tissue distribution of anti-epileptic drugs in the brain. As per our study, P-gp and MRP-1 are found to be over expressed in most of the MTLE cases. This was confirmed at both genetic as well as protein level. In MTLE patients, over expression of P-gp

was more pronounced than that of MRP-1. Drug efflux transporters act at brain barriers as well as within the brain parenchyma. The increase in efflux transporters levels can be pharmacologically inhibited to achieve optimal drug penetration to target site in refractory mesial temporal lobe epilepsy and avoid surgery for seizure control.

Limitations

It is still not clear whether overexpression of efflux transporters is intrinsic (present before the onset of epilepsy) or acquired i.e. it is a result, of epilepsy, of uncontrolled epileptic seizures, of treatment with different AEDs, or of combination these factors. Moreover, only P-gp and MRP-1 were studied in resistance mechanism; other drug efflux transporters should also be studied. We only focused on the theory of multidrug resistance due to transporters' over expression; transporter and target mechanisms may overlap in some patients.

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and material

The information regarding the resources, reagents and data availability that support the findings of this study should be directed to the corresponding author and will be considered upon reasonable request.

Author Contributions

MK collected the tissue, carried out the experimentation and drafted the manuscript. TG designed the study, analyzed the data, prepared the figures and contributed to the manuscript. MG helped in carrying out the experimentation. NS and YSB were involved in conception and acquisition of material. BDR helped in the histological analysis and PSK has contributed in data interpretation. SKG has significantly contributed in conception of the work and supervised the work. All authors have read and approved the final manuscript. Each author has agreed to be accountable for all aspects of the work ensuring that questions related to

the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Figure legends

Figure 1 – The figure is depicting the mRNA expression data of P-gp and MRP-1 in MTLE patients in comparison to the non-epileptic controls. Both the efflux transporters show up regulation in the patients. (a) and (c) are depicting fold change in P-gp and MRP-1 respectively in individual MTLE patients. (b) and (d) shows relative expression (ΔΔCt) between MTLE patients and non-epileptic controls.

P-gp – permeability glycoprotein; MRP-1 – multidrug resistance protein 1; MTLE – mesial temporal lobe epilepsy; *** statistically highly significant; * statistically significant

Figure 2 - The figure is showing protein expression data of P-gp and MRP-1 (a) P-gp and MRP-1 proteins are overexpressed in MTLE patients in comparison to the non-epileptic controls with β-actin as standard (b) relative density of the western blot data by densitometric analysis using ImageJ software.

P-gp – permeability glycoprotein; MRP-1 – multidrug resistance protein 1; MTLE – mesial temporal lobe epilepsy; *** statistically highly significant; * statistically significant

Figure 3 – Immuno-histochemical staining of hippocampal tissue using P-gp monoclonal antibody. In the control cases (a) and (b) mild cytoplasmic IR was observed at the BBB in the capillary endothelial cells (asterisks) and in neurons (arrowheads). In the MTLE cases (c) and (d) capillary endothelial cells (asterisks) show moderately positive IR for P-gp. Outer to the capillary, IR can also be appreciated in glial elements (which would be foot processes of astrocytes forming BBB); immuno-stained glial nuclei are depicted by arrows. Neurons (arrowheads) show moderate cytoplasmic as well as membranous staining. 40X magnification.

P-gp – permeability glycoprotein; BBB – blood brain barrier; MTLE – mesial temporal lobe epilepsy; IR – immunoreactivity

Figure 4 – These are the representative images of IR of MRP-1. Increased cytoplasmic IR of MRP-1 can be seen in the neurons (arrowheads) and glial cells (arrows) of patients (d) and (e)

in comparison to the controls (a) and (b). (c) and (f) No IR was observed in the endothelial cells (asterisks) of capillaries in both patients and controls. 40X magnification.

MRP-1 – multidrug resistance protein 1; IR – immunoreactivity; MTLE – mesial temporal lobe epilepsy

Figure 5 - Immunofluorescence of P-gp and MRP-1 using confocal fluorescence microscopy in the controls and MTLE cases. The images are depicting DAPI stained nuclei (blue), P-gp & MRP-1 (red) and their merged images respectively. Increased immunofluorescence of both the efflux transporters is seen in the MTLE cases at 40X magnification. The densitometric graphs are showing statistically significant increase of P-gp and MRP-1 proteins in MTLE. 40X magnification.

P-gp – permeability glycoprotein; MRP-1 – multidrug resistance protein 1; MTLE - Mesial temporal lobe epilepsy, *** - statistically significant ** - statistically significant.

S.N.	Age/Sex	Onset age (years)	Surgery age (years)	Epilepsy duration (years)	Seizure frequency (episodes/month)	AEDs before surgery	Side of surgery
1.	31/F	13	31	18	8-9	LV,TG,FR	Right
2.	54/M	11	54	43	8-9	LP,TG,FR	Right
3.	29/M	15	29	14	3-4	LP,TG,FR	Right
4.	10/M	5	10	5	9-10	LP,TG,CL	Right
5.	33/F	12	33	2	1-2	LV,CB,CL	Left
6.	23/F	5	23	10	20-21	CB,LP,LV, CL,LA	Right
7.	21/M	5	21	14	4-5	CB,CL,LV, LP,TG	Right
8.	27/F	19	27	8	12-13	LV,GD	Right
9.	29/M	20	29	9	2-3	CB,CL,LV, VP	Right
10.	32/M	2	32	30	3-4	TG,FR,LC	Left
11.	26/M	7	26	19	8-10	LV,CL,CB, VP	Left
12.	35/M	15	35	20	7-8	LV,CL,CB, VP	Left
13.	28/F	21	28	7	8-9	CL,CB,VP, LV	Right
14.	42/F	20	41	21	15-20	ZX,LP,FR, LC	Left
15.	19/M	12	19	7	1-2	LP,TG,FR	Right

Table 1: Clinical characteristics of 15 cases of Mesial temporal lobe epilepsy (MTLE) with hippocampal sclerosis (HS) - age, sex (Male – M; Female – F), onset age, age at surgery, epilepsy duration, seizure frequency, antiepileptic drugs (AEDs) taken before surgery – Levetiracetam (LV), Tegretol (TG) , Frisium (FR), Levipil (LP), Lamotrigine (LA), Carbamazepine (CB), Clobazam (CL), Gardenal (GD), Valproate (VP), Zenoxa (ZX) and side of surgical resection.

S.N.	Age	Sex	Etiology diagnosis	PMI (in hours)	Pathology
1.	23	Male	Trauma	2	None
2.	24	Male	Trauma	4	None
3.	52	Male	Trauma	4	None
4.	45	Female	Trauma	4	None
5.	26	Male	Trauma	4	None
6.	28	Male	Trauma	4	None
7.	50	Male	Trauma	2	None
8.	60	Male	Trauma	2	None
9.	45	Male	Trauma	2	None
10.	59	Male	Trauma	3	None
11.	26	Male	Trauma	2	None
12.	45	Female	Trauma	4	None
13.	52	Male	Trauma	4	None
14.	48	Male	Trauma	2	None
15.	24	Male	Trauma	3	None

Table 2: Clinical characteristics of 15 non-epileptic controls - age, sex, etiology diagnosis, post-mortem interval (PMI) and pathology.

Drug efflux transporter	Duration of epilepsy	Duration of drug-resistance	Frequency of seizures
MDR-1	$\rho = 0.03$	$\rho = -0.30$	$\rho = -0.27$
	$p = 0.90$	$p = 0.27$	$p = 0.31$
MRP-1	$\rho = 0.02$	$\rho = -0.03$	$\rho = - 0.39$
	$p = 0.92$	$p = 0.90$	$p = 0.14$

Table 3: Spearman's rank correlation coefficient, ρ, and p-value was calculated to find out the correlation of P-gp and MRP-1 with duration of epilepsy, duration of drug-resistance and seizure frequency of Mesial temporal lobe epileptic patients.