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Title: Expressional Study of Permeability glycoprotein (P-gp) and Multidrug Resistance Protein 1 (MRP-1) in Drug-Resistant Mesial Temporal Lobe Epilepsy

Running Title: Drug-resistance in mesial temporal lobe epilepsy

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Abstract

About 30% of epileptic patients do not react 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). The major drawback so far is the expressional analysis of transporters in equal numbers of drug-resistant epileptic tissue and age-matched non-epileptic tissue. We have studied these two transporters in the sclerotic hippocampal tissues resected from the epilepsy surgery (n=15) and compared their expression profile with the tissues resected from non-epileptic autopsy cases (n=15). 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. The study demonstrated 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 is availability of more than 20 types of anti-epileptic drugs (AEDs) 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). Drug-resistant epileptic patients are at an increased risks of premature death, injuries, psychosocial dysfunction, and a reduced quality of life (Löscher & Friedman, 2020; Zavala-Tecuapetla, Cuellar-Herrera, & Luna-Munguia, 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). There is lack of understanding regarding the mechanism of drug-resistance in MTLE; the pathogenesis is therefore, considered 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 & 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 elimination of AEDs from diseased 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 BBB. It prevents adequate drug entry to the target location in brain by actively expelling the drugs (Sisodiya, Lin, Harding, Squier, & Thom, 2002).

Two ATP-binding cassette (ABC) drug-efflux transporters are thought to be chief players in resistant MTLE – multidrug-resistance protein 1 (MDR-1) gene encoding 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 BBB (Xiong, Mao, & Liu, 2015). MRP-1 is an organic ion-transporter; it shares 15% of its amino acid sequence with P-gp. Unlike P-gp, MRP1 is found in the choroid plexus epithelium and ependymal epithelium cells for the protection of 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 (Rizzi et al., 2002; H. Volk, Potschka, & Löscher, 2005; H. A. Volk, Potschka, & Löscher, 2004). 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., 2018). 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, Bullock, Faull, Hladky, & Barrand, 2010). Therefore, inclusion of control tissue from brains without known neurological disease is necessary to validate the results.

The current study was designed to analyze the expression 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. Moreover, immuno-histochemical analysis and confocal fluorescence microscopy were used to determine cellular localization of these transporters.

Materials and Methods

Subjects

The hippocampal tissue was obtained from the drug-resistant MTLE patients (n=15) underwent surgery for MTLE in the Neurosurgery department of Post Graduate Institute of Medical Education and Research, Chandigarh, India. The clinical data of the patients is given in the Table 1. The tissue from the patients with pathologically proven hippocampal sclerosis only was taken. The patients with seizures' types other than partial seizures were not included in the study. The tissues showing any structural intracranial insults, for example, gliomas, meningitis, and neurofibromatosis were also excluded.

Controls

The hippocampal tissue taken from the autopsies (n=15) performed in the Forensic Medicine department of Post Graduate Institute of Medical Education and Research, was used as control tissue. 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 patients with any severe injury of head, haemorrhage, etc., not included in the study. The clinical data has been provided in the Table 2.

Tissue was collected only after informed written consent from the relatives of patients and controls.

Ethical approval

The study was conducted after getting sanction from the Institute Ethics Committee of Post Graduate Institute of Medical Education and Research; vide no. INT/IEC/000931 dated: 25/06/2018.

Processing of hippocampal tissue

Under sterile conditions, the collected tissue was put immediately in the saline. After that, the collected tissue was distributed into sections – one section in RNA later at -20°C, one section in frozen at -80°C and another section fixed in 10% formalin overnight at room temperature.

A part of the fixed tissue was processed for histology in each case. Using standard procedure, hematoxylin and Eosin (H&E) staining was performed and the slides were then assessed by histopathologist for the identification of hippocampal sclerosis.

Real-time PCR

Trizol method (*Ambion*) was used to isolate total RNA by taking an amount of 0.5-1 g of tissue kept in the RNA later. Spectrophotometric measurements (*Biotek Epoch*) were performed for the assessment of quality and quantity of the RNA. The absorbance taken for measurements were at A260/A280. cDNA was synthesized using *BioradiScript cDNA synthesis kit* as per instructions after this. For real-time PCR, primers at 250 nM concentration were used – human MDR-1 forward 5'-TCAGCTGTTGTCTTTGGTGC-3'; reverse 5'-GGTCGGGTGGGATAGTTGAA-3', human MRP-1 forward 5'-TTGGTGATATTCGCCATTGA-3'; reverse 5'-ACGCATAGTGGATGGCTTTC-3', and human (Glyceraldehyde 3-phosphate dehydrogenase) GAPDH forward 5'-TGAACGGGAAGCTCACTGG-3', reverse 5'-TCCACCACCCTGTTGCTGTA-3'. Evaluation of gene expression was done using SYBR Green chemistry on ABi Step One Plus RT-PCR system. The data was standardized to GAPDH, the internal control. The data was again standardized to the non-epileptic control. The data was then presented using $2^{-\Delta\Delta C_t}$ formula (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). The data was represented in the form of plots and standard criterion was followed to describe gene's status whether up regulated or down regulated.

Western Blot (WB)

Hippocampal tissue was properly thawed and washed with phosphate buffered saline (PBS). Total proteins were extracted ice cold 1X RIPA lysis buffer. 1ml of 1X RIPA lysis buffer was added along with 2 μ l protease inhibitor (*Sigma-Aldrich*). Bicinchoninic acid (BCA) method was used for the analysis of protein concentration. using BCA kit (*BioRad*). The samples were resolved after heating on sodium-dodecyl sulphate polyacrylamide gel (SDS-PAGE) using 4% stacking and 8% separating gel. Polyvinylidene difluoride (PVDF) membrane was used to blot the resolved proteins. The blocking was performed in the BSA at 25°C. Blotted PVDF membranes were incubated with specific primary antibodies overnight at 4°C – (a) mouse monoclonal MDR-1 at a concentration of 1:200 (*Santa-Cruz Biotech.*, *sc-71557*), (b) mouse monoclonal MRP-1 at a concentration of 1:50 (*Abcam*, *ab24102*) and (c) mouse monoclonal β -actin (an internal control) at a concentration of 1:5000 (*Thermo fisher*, *M5A-15739*). After proper washing with TBST buffer, blotted membranes were incubated with respective secondary antibodies at room temperature – rabbit anti-mouse (1:10,000, *Abcam*, *ab97046*). Enhanced chemiluminescence (ECL) kit (*BioRad*) was used for the visualization

of blotted membranes. For data interpretation, ImageJ Plus software was used for the densitometry analysis.

Immuno-histochemistry (IHC)

Immuno-histochemical analysis was done for the confirmation of real-time PCR and western blot results by evaluating the protein localization and expression. After processing with standard protocol, 5µm thick sections were taken on poly-L-lysine coated slides. Deparaffinization was done in xylene (three 10 minutes changes) followed by hydration of sections through graded alcohols (Absolute or 100% ethanol, 70% ethanol, 50% ethanol and distilled water for 5 minutes each). Hydrogen peroxide and de-ionised water in the ratio of 1:9 was used for endogenous blocking by incubating sections for 10-15 minutes. Antigen retrieval was done to increase the immunogenicity using Tris-EDTA buffer at pH 9.0. The sections were placed in incubator at 55°C for 4-8 hours. 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°C. Primary antibody was omitted in the negative control. HRP-conjugated rabbit anti-mouse secondary antibody (1:2000, *Abcam*, *ab97046*) was used at room temperature. 0.5% 3, 3'-diaminobenzidine was used to develop peroxidase activity. Hematoxylin was used as a counterstain. The slides were properly cleaned and cover slipped after mounting with di-n-butyl phthalate (DPX). Observation was done 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 scale bars 100 µm for the detailed analysis. Histo-score (H-score) was calculated using formula $H\text{-score} = [1 \times (\% \text{ cells with } 1+) + 2 \times (\% \text{ cells with } 2+) + 3 \times (\% \text{ cells with } 3+)]$, where 1+, 2+ and 3+ are the intensity scores of immunostaining according to the appearance (Cheon, Lee, Parhar, & Kang, 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 taken on poly-L-lysine coated slides. Deparaffinization was done in xylene (three 10 minutes changes) followed by hydration of sections through graded alcohols (Absolute or 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 retrieval was done to increase the immunogenicity using Tris-EDTA buffer at pH 6.1 in oven for 12 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 immunofluorescence microscope (*Evos FL auto BX53*). 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 scale bars 100 µm 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 $CTCF = \text{integrated density} - (\text{measured area of the selected cell} \times \text{mean fluorescence of background})$. Background signals were measured in areas without signal.

Statistical analysis

Softwares used for data analysis and graph preparation were - GraphPad Prism version 8.0 and SPSS version 23.0. 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 data

Clinical and demographic data of the patients and controls is documented in Table 1 and Table 2. 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 is 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.

Expression of transcripts of P-gp and MRP-1 genes in hippocampal tissue

Relative mRNA levels of P-gp and MRP-1 genes of drug-resistant MTLE patients and non-epileptic controls are shown in the Figure 1 (a-d). Both P-gp and MRP-1 were detected in the hippocampal tissue. It has been observed that mRNA levels of P-gp and MRP-1 were higher in the patient tissues in comparison to the control tissues (Fig. 1b and 1d). The mean $\pm$ SD (Δ Ct value) of patient group was 10.12 ± 5.47 and for control group was 0.51 ± 0.73 for P-gp. And mean $\pm$ SD (Δ Ct) of patient group was 2.99 ± 2.40 and for control group was 0.97 ± 0.69 for MRP-1. The fold change was calculated for each patient (n=15), 66% of the patients showed overexpression of P-gp. 11 out of 15 patients showed overexpression of MRP-1 (Fig. 1a and 1c). Elevated levels of P-gp and MRP-1 in the patients was ranging from 4-6 folds than the controls. The results were statistically significant for P-gp (p value < 0.0001) and MRP-1 (p value 0.01). Fold change seen for P-gp was more pronounced than MRP-1 in the MTLE patients.

Expression and localization of P-gp and MRP-1 proteins in hippocampal tissue

Western blot analysis further revealed that there was increased protein expression of both P-gp and MRP-1 with an apparent molecular weight of 170 and 172 kDa respectively in the patients and controls using sc-71557 and ab24102 antibodies (Fig. 1e). The densitometric

analysis showed that the results were statistically significant using β -actin as standard (Fig. 1f). 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 for P-gp (p value 0.006) and 0.67 ± 0.04 in the patients and 0.36 ± 0.04 in the controls for MRP-1 (p value <0.001).

The cellular localization of P-gp and MRP-1 was determined by immunohistochemistry using the same antibodies. The results demonstrated that these proteins were present in both diseased and control tissues. P-gp showed an increased immunostaining in the MTLE patients as compared to the controls (Fig.2). In the diseased patients, the immunoreactivity (IR) was moderate to strong. Maximum staining was observed at the BBB (Fig.2b and 2c). 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.2c). 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 BBB. Very mild cytoplasmic IR was also observed in the neurons (Fig. 2a). No glial cells were found immunostained in the control cases.

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.3). In the MTLE patients, IR was cytoplasmic in glial cells. Some of the neurons were also showing immunopositivity (Fig.3a). In the controls, mild IR in neurons and glial cells was seen (Fig.3b). In the vessel, stained endothelial cells were seen neither in patients nor in controls (Fig.3c and 3d). Semi-quantitative analysis by H-score also confirmed the IHC results (Table 3).

Further, immunofluorescence of P-gp and MRP-1 was checked in the MTLE and non-epileptic control tissue. Immunofluorescence staining confirmed that the expression of P-gp and MRP-1 was seen in the hilar region of the dentate gyrus. The immunofluorescence images labeled with P-gp (red) and MRP-1 (red) only described the presence or absence of particular protein along with nuclear dye DAPI (blue) in patients and controls. There was increased immunofluorescence seen in the MTLE cases for both the efflux transporters (Fig.4). The results were statistically significant for P-gp. Mean and standard error was 116293 ± 11698 in the MTLE cases and was 38887 ± 2583 in the controls with p value 0.0002. Mean and standard error for MRP-1 was 79172 ± 5032 in the MTLE cases and 49942 ± 6304 in

the controls with p value 0.006 which was statistically significant. The number of positive cells for P-gp in diseased and controls cases was showing a significant difference whereas positive cells in case of MRP-1 was significant but not much varied among the diseased and controls cases.

Correlation of disease parameters with P-gp and MRP-1

There might be a possibility of link between drug-efflux transporters and disease parameters of epilepsy. We tried to find any relation between increased expression of drug-efflux transporters and different disease parameters. We correlated the expression pattern of P-gp and MRP-1 with - duration of epilepsy, duration of drug-resistance and frequency of seizures using Spearman's rank correlation. P-gp or MRP-1 showed no correlation with any of the described parameter (Table 4).

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. One of the most resistant forms of epilepsy in humans is MTLE with HS (Pohlen, Jin, Tobias, & Maheshwari, 2017). Therefore, we only incorporated patients with confirmed hippocampal sclerosis in the present study. About 30–50% of the epileptic patients are resistant to the present treatments, which is associated with five-fold death rate in comparison to the general population (Sharma, Rani, Waheed, & Rajput, 2015). The removal of epileptic tissue with surgery remains the only solution for drug-resistant patients (Bergey, 2013; Burtcher & Schwarzer, 2017), but even then seizure freedom was attained for at least 1 year in only 50-80% of cases (Spencer & 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. 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., 2002; Sisodiya et al., 2006; Tishler et al., 1995; Weidner et al., 2018). 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 (Potschka, Fedrowitz, & Löscher, 2001). This is a single stranded transmembrane glycoprotein composed of 1280 amino acids. Various barrier and excretory tissues express P-gp. P-gp delivers hydrophobic and amphipathic molecules of cells or membranes inside to outside by active transport. This physiological function of expelling xenobiotics of extensive molecular variability is considered as the critical defense mechanism which protects internal brain milieu. Under physiological conditions, P-gp is expressed in the luminal membrane of the brain capillary endothelial cells and in the astrocyte foot processes surrounding the capillaries which constitute the blood-brain barrier (BBB). Its expression is found to be slightly evident in neurons or glial cells. 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, Bauer, & Hartz, 2008). In diseased state, P-gp is not only highly expressed in endothelial cells, but also in brain parenchyma (D'GIANO, 1997). 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.2b and 2c). 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, P-gp is also expressed on the astrocytes and neurons in the parenchyma. 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 (H. 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 in comparison to the non-epileptic 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 distributed in the choroid plexus epithelium and ependymal epithelium cells of BCB. These cells prevent the entry of harmful substances or drugs into brain tissue (Sodani, Patel, Kathawala, & Chen, 2012). 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. In the normal brain, the expression of MRP-1 in the neurons and glial cells has not been constantly stated in the literature (Ashraf, Kao, & Bendayan, 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 (Van Vliet, Redeker, Aronica, Edelbroek, & Gorter, 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.3 a-d). 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 (Chen, Wang, Xiao, Wei, & Xu, 2013; Kubota et al., 2006; Yi, 2012). 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 over expression of both efflux transporter i.e. P-gp and MRP-1, in the epileptic brain tissue (Kuteykin-Teplyakov, Brandt, Hoffmann, & Löscher, 2009; Rizzi et al., 2002; Seegers, Potschka, & Löscher, 2002; H. Volk et al., 2005; H. A. Volk et al., 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, Gang, Yunxia, Shuangshuang, & Rongrong, 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. 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). 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 up-regulation 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 parameters with P-gp or MRP-1.

Conclusions

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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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 ($\Delta\Delta Ct$) between MTLE patients and non-epileptic controls. Similarly, protein expression is measured (e) P-gp and MRP-1 proteins are overexpressed in MTLE patients in comparison to the non-epileptic controls with β -actin as standard (f) relative density of the western blot data by densitometric analysis using ImageJ software.

Unpaired student t tests results are shown by symbols as follows - * ($p < 0.05$) *** ($p < 0.001$) **** ($p < 0.0001$) represents significant difference among the study groups. Error bars are showing the standard error of mean.

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

Figure 2 – Immuno-histochemical staining of hippocampal tissue in the hilar region of the dentate gyrus using P-gp monoclonal antibody. (a) In the control cases mild cytoplasmic IR was observed at the BBB in the capillary endothelial cells (asterisks) and in neurons (arrowheads). (b) and (c) In the MTLE cases 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. (d) Negative control. All scale bars 100 μm

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

Figure 3 – These are the representative images of immunoreactivity (IR) to MRP-1 monoclonal antibody in the hilar region of the dentate gyrus. (a) Increased cytoplasmic IR of MRP-1 can be seen in the neurons (arrowheads) and glial cells (arrows) of patients in comparison to the controls (b). (c) and (d) MRP-1 was not observed in the endothelial cells

(asterisks) of capillaries in both patients and controls. (e) Negative control. All scale bars 100 μ m

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

Figure 4 - Immunofluorescence of P-gp and MRP-1 using confocal fluorescence microscopy in the controls and MTLE cases. The panels (a), (b), (c) and (d) are depicting DAPI stained neuronal nuclei (blue). Red immunofluorescence, for P-gp is seen in (e) and (f), and for MRP-1 is seen in (g) and (h) panels respectively. The merged images of DAPI and P-gp are seen in panels (i) and (j), and the merged images of DAPI and MRP-1 are seen in panels (k) and (l). The area depicted in the images is the dentate hilar region of the hippocampus. Increased immunofluorescence of both the efflux transporters is seen in the MTLE cases as compared to the controls. The densitometric graphs with corrected total cell fluorescence (CTCF) are showing statistically significant increase of P-gp (p value 0.0002) and MRP-1 (p value 0.006) proteins in MTLE. All scale bars 100 μ m

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

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

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

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 3: Semi quantitative analysis of IHC of P-gp and MRP-1 using H score in Mesial temporal lobe epileptic patients and non-epileptic controls.

Drug-efflux transporters	Total cells/stained cells in 10 HPFs at 40x		H-score		
	Control	Diseased	Control	Diseased	p-value
P-gp	10722/293	15181/1175	57	268	<0.0001
MRP-1	16104 /374	14487/701	49	183	0.0004

Table 4: 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

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	$\rho = 0.02$	$\rho = -0.03$	$\rho = -0.39$
	$p = 0.92$	$p = 0.90$	$p = 0.14$

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