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ORIGINAL ARTICLE

# Regulation of Keap-Nrf2 axis in temporal lobe epilepsy—hippocampal sclerosis patients may limit the seizure outcomes

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## Abstract

**Background** Accumulation of reactive oxygen species (ROS) exacerbates neuronal loss during seizure-induced excitotoxicity. Keap1 (Kelch-like ECH-associated protein1)-nuclear factor erythroid 2–related factor 2 (Nrf2) axis is one of the known active antioxidant response mechanisms. Our study focused on finding the factors influencing Keap1-Nrf2 axis regulation in temporal lobe epilepsy (TLE) associated with hippocampal sclerosis (HS) patients.

**Methods** Based on post-surgical follow-up data, patient samples ($n = 26$) were categorized into class 1 (completely seizure-free) and class 2 (only focal-aware seizures/auras), as suggested by International League Against Epilepsy (ILAE). For molecular analyses, double immunofluorescence assay and Western blot analysis were employed.

**Results** A significant decrease in expression of Nrf2 ($p < 0.005$), HO-1; $p < 0.02$) and NADPH Quinone oxidoreductase1 (NQO1; $p < 0.02$) was observed in ILAE class 2. Keap1 ($p < 0.02$) and histone methyltransferases (HMTs) like SetD7 (SET7/9; SET domain-containing 7 histone lysine methyltransferase) ($p < 0.009$) and enhancer of zeste homolog 2 (EZH2; $p < 0.02$) and methylated histones viz., H3K4me1 ($p < 0.001$), H3K9me3 ($p < 0.001$), and H3K27me3 ($p < 0.001$) was upregulated in ILAE class 2. Nrf2-interacting proteins viz., p21 ($p < 0.001$) and heat shock protein 90 (HSP90; $p < 0.03$) increased in class 1 compared to class 2 patients.

**Conclusion** Upregulation of HMTs and methylated histones can limit phase II antioxidant enzyme expression. Also, HSP90 and p21 that interfere with Keap1-Nrf2 interaction could contribute to a marginal increase in HO-1 and NQO1 expression despite histone methylation and Keap1. Based on our findings, we conclude that TLE-HS patients prone to seizure recurrence were found to have dysfunctional antioxidant response, in part, owing to Keap1-Nrf2 axis.

**Keywords** Temporal lobe epilepsy (TLE) · Hippocampal sclerosis (HS) · Oxidative stress · Epigenetic regulation · Histone methylation · Antioxidant response

## Introduction

Temporal lobe epilepsy (TLE) with hippocampal sclerosis (HS) is one of the most common causes of epilepsy, with nearly one-third of patient population remaining drug-resistant. Patients experiencing seizures develop a new and random neuronal network, which induces significant changes in brain electrical activity [1]. This significantly impacts the quality of life and cognitive changes, thereby necessitating surgical resection of epileptic foci [2]. Standard temporal lobe resection surgery remains the mainstay treatment in drug-refractory cases, with 60–70% remaining seizure-free over long term [3]. Existing anti-seizure medications predominantly target seizure propagating mechanisms and act by suppressing seizures. The reasons for the persistence of seizures in a significant number of patients after surgical treatment are yet unclear. An irregular neuronal network arising from recurrent seizures further promotes random neuronal firing and excitotoxicity, which is often attributed to dopamine and glutamate signaling [4]. The

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resultant effect includes disturbance in cellular homeostasis, lipid peroxidation, and reactive oxygen species (ROS) accumulation which leads to oxidative stress (OS) [5, 6]. Also, ROS upregulation aggravates neuroglial damage, thereby reducing the seizure threshold [6–8]. It is considered one of the significant underlying causes of epilepsy progression [9]. Endogenous antioxidant defense mechanisms counteract excessive ROS-induced cellular damage [10]. This system includes phase I antioxidant enzymes like catalase, peroxidase, superoxide dismutase (SOD), glutathione S-transferase M1 (GSTM1), NAD(P)H quinone oxidoreductase (NQO1), and heme oxygenase-1 (HO-1) that can effectively detoxify excessive ROS formed in the tissues [11–15].

Nuclear factor erythroid 2-related factor2 (Nrf2 encoded by NFE2L2), a Cap'n Collar basic leucine zipper transcription factor, regulates transcription of phase II antioxidant enzymes such as NAD(P)H quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1) [12, 16]. Kelch-like ECH-associated protein 1 (Keap1) negatively regulates Nrf2 by forming a complex with Cul3 (cullin 3, an E3 ubiquitin ligase) and subjecting Nrf2 to proteasomal degradation, thereby maintaining low steady-state levels of Nrf2 [17, 18]. In connection with the regulation of cellular antioxidant response, Nrf2 upregulates NQO1 and HO-1 [6]. Also, upregulation of HO-1 and NQO1 has been shown to confer protection on neurons from an increasing OS [13–15, 19, 20].

During oxidative stress (OS), electrophiles react with Cysteine (Cys) sensors within Keap1 and alter its conformation [21]. Therein, Nrf2 escapes Keap1 regulation and translocates into the nucleus, where it binds to ARE (antioxidant response elements) by forming a dimer with small Maf proteins and elicits an antioxidant response [22, 23]. Keap1 and Nrf2 expression and interactions are regulated at the transcriptional and post-translational levels by several factors [24]. At gene level, Keap1 and Nrf2 expression is regulated by histone methylation (mono (me1), di (me2), or tri (me3)) at arginine (Arg)/lysine (Lys)/histidine (His), which is critical in the compaction of chromatin and resultant gene repression [25]. Depending on the Lys residue methylated and extent of methylation, the corresponding gene can be activated or suppressed. Lysine residues of histone 3 (H3Ks) play an important role in modifying histones by methylation that has a transcriptional consequence. Studies indicated that H3K27me3 negatively regulates Nrf2 expression and downregulates HO-1 and NQO1 gene expression [26, 27]. EZH2 (Enhancer of zeste homolog 2), the catalytic subunit of multimeric complex viz., polycomb repressive complex 2 (PRC2), is a histone Lys methyl transferase that induces transcriptional repression by trimethylating H3K27 and also influencing H3K9 methylation at *Nrf2* gene promoter to repress its transcription [26, 27]. Another histone methyl transferase known as SetD7 (SET7/9; SET domain-containing 7 histone lysine methyltransferase), specifically monomethylates H3K4, facilitating the binding of stimulating protein-1 (Sp-1) at *Keap1* gene promoter and upregulates Keap1 protein expression [28]. Also, post-translational modifications modulate Keap1-Nrf2 interaction during oxidative stress, allowing Nrf2 translocation into the nucleus and binding to ARE elements of HO-1 and NQO1 [29, 30]. Besides histone methylation, SetD7 also methylates Lys372 of p53, thereby increasing protein stability, which upregulates target gene p21 [31, 32]. The C-terminal region of p21 can interact with DLG (ASP-21, Leu-25, and Gly-31) and ETGE ($^{76}$LDEETGEFL$^{84}$) motifs of Nrf2 [33] and compete with Keap1-Nrf2 complex formation [34], thereby facilitating the Nrf2 stabilization. Another potent Nrf2 stabilizers are the autophagic adopter protein, sequestosome-1 (SQSTM1/p62) [35]. It binds to the DC (Double glycine repeat domain and C-terminal region) pocket of Keap1 like Nrf2 and facilitates Nrf2 to escape from ubiquitin-dependent degradation [36, 37].

In view of the criticality of the Keap1-Nrf2 axis for antioxidant response, as mentioned above, against an exacerbated oxidative stress in an epileptogenic setting, we attempted to understand the molecular underpinnings of antioxidant response comprising of above-mentioned components in the current study.

## Materials and methods

### Sample collection and ethical guidelines

The brain tissue (TLE/HS) samples ($n = 26$) were frozen with liquid nitrogen and then stored at −80 °C, immediately after surgery, at the Krishna Institute of Medical Sciences (KIMS), a tertiary care center. Control (post-mortem) brain samples ($n = 4$) were obtained from the National Institute of Mental Health and Neuro Sciences (NIMHANS), brain bank, Bangalore, India. The KIMS ethical committee and the KIMS Foundation and research center (KFRC) approved all the procedures, including experimental and sample collection processes. The patient or relative's prior consent was taken for this study. Institutional ethical committee (IEC) guidelines were followed, and study sets were anonymized. Postoperatively, patients were followed up for at least 3 years for outcome assessment categorized as per International League against Epilepsy (ILAE) classification [38]. ILAE Commission Report. Proposal for a new classification of outcome with respect to epileptic seizures following epilepsy surgery [38]. Cases with ILAE class 1 and 2 were included for unequivocal analysis, as people with uncontrolled seizures (ILAE class 3 and above) could have

**Table 1** List of TLE-HS patients with clinical data representing the type of epilepsy, gender, onset age, duration, age at the time of surgery, and outcome class according to International League Against Epilepsy (ILAE). We can observe outcome independent of onset age or age of patient at the time of surgery

| Case | Characteristic | Gender | Onset age | Age of surgery | Duration | Localization | | Seizures/Y before surgery | Post-surgery outcome (3 years) | ILAE outcome class |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | TLE-HS | M | | 10 Y | 40 Y | 30 Y | HS | 09 | 90% decrease | Class 2 |
| 2 | TLE-HS | M | | 9 Y | 13 Y | 4 Y | HS | 08 | Seizures free | Class 1 |
| 3 | TLE-HS | M | | 5 Y | 15 Y | 10Y | HS | 10 | 90% decrease | Class 2 |
| 4 | TLE-HS | | F | 3 Y | 4.6 Y | 1.6Y | HS | 08 | Seizures free | Class 1 |
| 5 | TLE-HS | | F | 16 Y | 27 Y | 11 Y | HS | 07 | Seizures free | Class 1 |
| 6 | TLE-HS | M | | 10 Y | 13 Y | 3 Y | HS | 09 | 90% decrease | Class 2 |
| 7 | TLE-HS | | F | 4 Y | 12 Y | 8 Y | HS | 10 | Seizures free | Class 1 |
| 8 | TLE-HS | M | | 7 Y | 12 Y | 5 Y | HS | 09 | 90% decrease | Class 2 |
| 9 | TLE-HS | | F | 6 Y | 14 Y | 8 Y | HS | 24 | Seizures free | Class 1 |
| 10 | TLE-HS | | F | 5 Y | 18 Y | 13 Y | HS | 10 | 90% decrease | Class 2 |
| 11 | TLE-HS | M | | 9 Y | 10 Y | 1 Y | HS | 11 | Seizures free | Class 1 |
| 12 | TLE-HS | | F | 2 Y | 14 Y | 12 Y | HS | 08 | 90% decrease | Class 2 |
| 13 | TLE-HS | M | | 5 Y | 8 Y | 3 Y | HS | 09 | Seizures free | Class 1 |
| 14 | TLE-HS | M | | 34 Y | 35 Y | 1 Y | HS | 10 | 90% decrease | Class 2 |
| 15 | TLE-HS | M | | 2 Y | 25 Y | 23 Y | HS | 08 | Seizures free | Class 1 |
| 16 | TLE-HS | M | | 11 Y | 21 Y | 10 Y | HS | 09 | Seizures free | Class 1 |
| 17 | TLE-HS | M | | 6 Y | 13 Y | 7 Y | HS | 09 | 90% decrease | Class 2 |
| 18 | TLE-HS | | F | 1 Y | 3 Y | 2 Y | HS | 10 | Seizures free | Class 1 |
| 19 | TLE-HS | M | | 11 Y | 14 Y | 3 Y | HS | 09 | 90% decrease | Class 2 |
| 20 | TLE-HS | M | | 15 Y | 25 Y | 10 Y | HS | 12 | Seizures free | Class 1 |
| 21 | TLE-HS | M | | 3 Y | 11 Y | 8Y | HS | 09 | 90% decrease | Class 2 |
| 22 | TLE-HS | M | | 4 Y | 10 Y | 6Y | HS | 09 | 90% decrease | Class 2 |
| 23 | TLE-HS | | F | 1 Y | 4 Y | 3Y | HS | 10 | Seizures free | Class 1 |
| 24 | TLE-HS | | F | 3 Y | 5 Y | 2Y | HS | 09 | Seizures free | Class 1 |
| 25 | TLE-HS | M | | 13 Y | 25 Y | 12Y | HS | 10 | 90% decrease | Class 2 |
| 26 | TLE-HS | M | | 15 Y | 27 Y | 12 Y | HS | 10 | 90% decrease | Class 2 |

**Table 2** Control samples collected from NIMHANS's Human brain repository with details including age, gender, the reason for death, neurological infections, HIV/HBsAg, and brain region. Control samples without any chronic neurological condition were selected

| S. No. | Gender | Age | Cause of death | Post-mortem interval | Significant neuro pathology | Disease | HIV/HBsAg | Brain region |
|---|---|---|---|---|---|---|---|---|
| 1 | Male | 25 years | Suicide | 30 h | Absent | No | Negative | Temporal |
| 2 | Female | 27 years | Drowning | 28 h | Absent | Diabetes | Negative | Temporal |
| 3 | Male | 37 years | Heart attack | 40 h | Absent | No | Negative | Temporal |
| 4 | Male | 27 years | Accident | 35 h | Cerebral injury | No | Negative | Temporal |
| 5 | Female | 25 years | Homicide | 37 h | Absent | No | Negative | Temporal |

extensive epileptogenic networks or pathways interfering with outcomes (Tables 1 and 2).

### Study design

The tissue samples were subjected to Thioflavin T (ThT) assay to quantify the protein aggregates, which was reported in our earlier studies, and the data was correlated with clinical outcomes [39]. Based on this information, patient samples were categorized into ILAE outcome classes. For the current study, patients who were completely seizure free ($n = 13$) and patients experiencing auras ($n = 13$) were pooled into 4 samples in each group based on ThT assay for further analyses.

### Preparation of tissue lysate and immunoblotting

Approximately 100 mg of the tissue sample was homogenized in Dounce homogenizer. Radioimmunoprecipitation assay buffer (RIPA) was used to isolate the whole-cell proteins. The protease inhibitor cocktail (Sigma-Aldrich, cat no: P8340) and phosphatase inhibitors (Sigma-Aldrich, cat no: P5726) were added to the tissues before homogenizing, and proteins were isolated and quantified. Forty micrograms of protein from all tissue samples were subjected to SDS-PAGE. A wet transfer of proteins onto nitrocellulose membrane (Bio-Rad, USA) overnight was done in Towbin buffer at 25 V. The non-fat skimmed milk powder (Sigma-Aldrich, cat no: M7409) 5% in tris buffer saline tween 20 (TBST) was used to block the nonspecific binding of the antibody. Blots were then incubated with primary antibodies Nrf2 (Sc-722), Keap-1 (Sc-15246), Ezh2 (CST-5246), H3K27Me3 (CST-9733), H3K4Me1 (CST-9723), H3K9Me3 (CST-13969), SetD7 (CST-2813), p21 (Novus bio, AF1047), p62 (Sc-28359), NQO1 (Novus bio, MAB7567), and HO-1 (Novus bio, AF3776) diluted to 1:1000 in TBST buffer overnight at 4 °C after subsequent washing with TBS and TBST the blots were incubated at room temperature for 1 h with secondary IgG antibodies anti-mouse (CST-G21040), anti-rabbit (CST-7074P2), and anti-goat (Thermo fisher, A16005) conjugated with HRP with 1:15,000 dilution. Later, blots were washed with TBS and TBST and developed with a chemiluminescent reagent (Clarity™ western ECL substrate 1705060) using Bio-Rad, USA, molecular imager.

### Double immunofluorescence

Five-micrometer-thick tissue sections ($n = 4$) were used to perform a double immunofluorescence assay. The paraffin-embedded tissue blocks were prepared using the tissue stored in a 10% formalin solution. The control tissues collected from the brain bank were not suitable for formalin fixation because they were held at −80 °C. Slides were subjected to deparaffinization with xylene and rehydrated with ethanol (100%, 95%, and 70%). Then citrate buffer 10 mM, pH 6.0, was boiled, and slides were kept in the buffer to retrieve the antigen. The blocking buffer was added to the section to prevent nonspecific binding. Buffer was provided with 5% normal goat serum (CST-5425) and 1% BSA dissolved in 1× PBS containing 0.3% Triton X-100. The slides were kept in a humid chamber at room temperature for 1 h. Following the incubation, the blocking buffer was replaced with primary antibodies and incubated for 16 h at 4 °C to check Keap1 and Nrf2 in TLE-HS brain sections. Later, primary antibody was washed with PBS three times. To this 1:1000 dilution fluorescent conjugated anti-rabbit IgG (4412S Alexa fluor® 488) from CST and anti-goat IgG (A32816, Alexa fluor® 555) from Thermo Fisher Scientific, secondary antibodies were added and incubated at room temperature in the dark. After 1 h, sections were washed with 1× PBS and mounted with a mounting agent (CST-8961S) Prolong® Gold antifade reagent with DAPI. The images were obtained using Carl Zeiss LSM 710 with Zen Blue software, and the fluorescent intensity was quantified with Image J software.

## Data analysis

The statistical analysis between groups was performed using a Student *t*-test by the Newman-Keuls method for post hoc analysis. Data were expressed as mean ± SEM. A *p*-value of $< 0.05$ was considered statistically significant and was determined by Sigma Plot 2000 software for the Windows version.

## Results

### Nrf2 protein expression was decreased in TLE-HS patients with seizure recurrence

Nrf2 plays a central role in regulating the expression of phase II detoxifying enzymes to neutralize ROS. In our study, it was observed that Nrf2 (Fig. 1c) protein expression was higher in ILAE class 1 ($p < 0.005$; 2.58 ± 0.43) compared to class 2 (0.86 ± 0.071) and controls ($p < 0.02$; 1.65 ± 0.30). Therefore, we focused on the Nrf2 negative regulator Keap1, which directs Nrf2 for ubiquitination in the cytosol. It was observed that Keap1 (Fig. 1b) protein expression was significantly upregulated in TLE-HS patients belonging to ILAE class 2 ($p < 0.02$; 1.57 ± 0.13) compared to class 1 (1.43 ± 0.24) and controls (1.05 ± 0.06).

Furthermore, a double immunofluorescence assay was performed for Keap1 and Nrf2 in ILAE class 1 and class 2 TLE-HS patient samples (Fig. 2a). It was observed that the fluorescence intensity of Keap1 was higher in class 2 ($p < 0.001$; 3.93 ± 0.34) and class 1 ($p < 0.001$; 3.29 ± 0.34 when compared with Nrf2. This data correlated with Keap1 (Fig. 1b) and Nrf2 (Fig. 1c) immunoblot assay findings. This finding suggests that Nrf2 is significantly negatively regulated in class 2 patients compared to completely seizure-free patients.

### Histone methyltransferases regulate Keap1 and Nrf2 expression

Previous studies reported that HMTs SetD7 and EZH2 are known to methylate H3K4 and H3K27 residues in Keap1 and Nrf2 promoter regions, respectively, and control their expression by modifying the methylation status of histones [26, 27, 28]. Based on these reports, we evaluated the protein

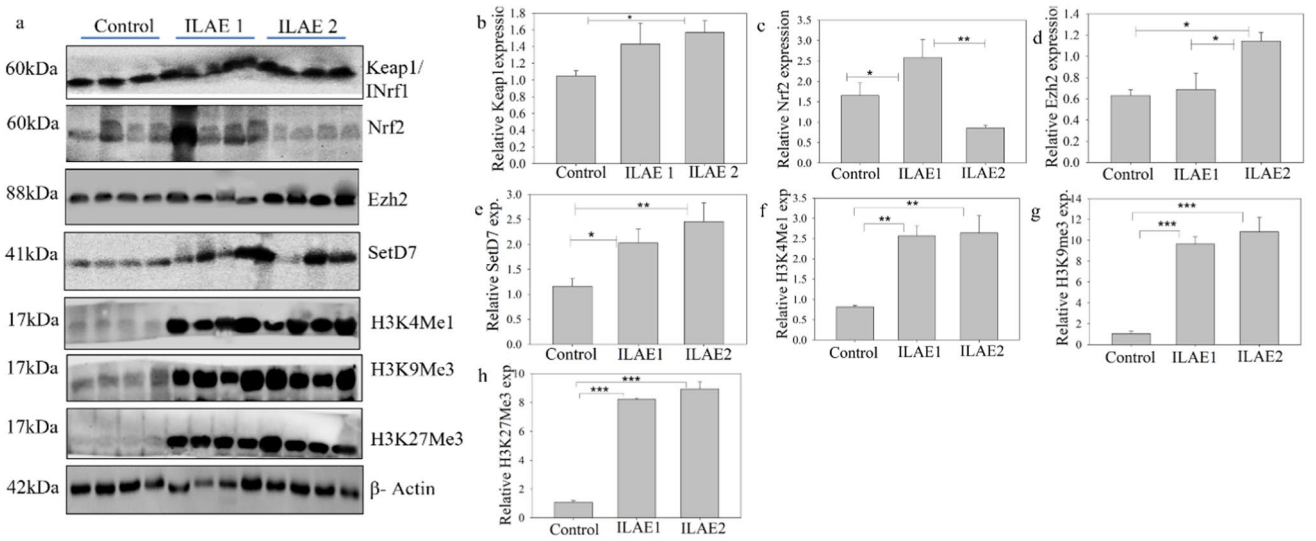


**Fig. 1** **a** Immunoblot analysis for Keap1 (**b**), Nrf2 (**c**), Ezh2 (**d**), SetD7 (**e**), H3K4Me1 (**f**), H3K9Me3 (**g**), H3K27Me3 (**h**) and β-actin was done for the controls and resected epileptic patient brain samples and protein quantification of corresponding immunoblot results represented by bar graphs. Beta-actin is used as internal control represented by the base panel. Data representing mean ± SEM, $n = 4$. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$

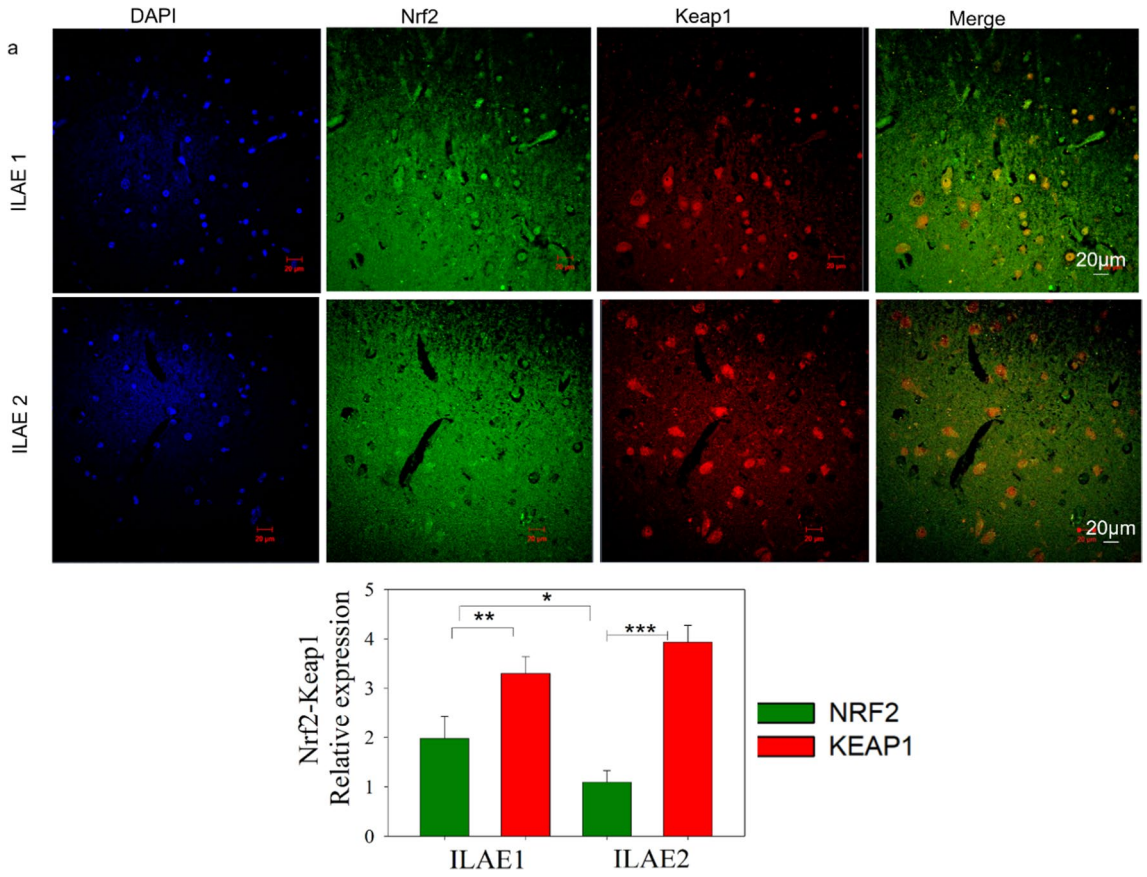


**Fig. 2** Double immunofluorescence analyses to estimate the relative fluorescence intensity of Keap1 (red) and Nrf2 (green), DAPI (blue) in TLE-HS clinical samples. The scale bar represents 20 μm, $n = 3$. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$

expression of SetD7 and EZH2 in TLE-HS patient samples. EZH2 (Fig. 1d) expression significantly increased in ILAE class 2 patients ($p < 0.02$; $1.14 \pm 0.08$) as compared to class 1 ($0.68 \pm 0.15$) and controls ($0.63 \pm 0.05$). SetD7 (Fig. 1e) expression was found to be increased in ILAE class 2 ($p < 0.009$; $2.45 \pm 0.091$) and class 1 ($p < 0.03$, $1.91 \pm 0.30$) compared to controls ($1.19 \pm 0.21$). This indicates that significant negative regulation over Nrf2 expression is imposed by histone methylation.

## EZH2 and SetD7 could methylate histones responsible for Nrf2 and Keap1 expression in TLE-HS patients

EZH2 trimethylates H3K27 and influences H3K9 trimethylation [40]. H3K27me3 and H3K9me3 histone marks in promoters transcriptionally repress Nrf2 gene expression. In our study, it was observed that H3K27me3 (Fig. 1h) expression significantly increased in ILAE class 1 and 2 categories ($p < 0.001$; $8.22 \pm 0.08$, $p < 0.001$; $8.93 \pm 0.51$), respectively, compared to controls ($1.09 \pm 0.11$). H3K9me3 (Fig. 1g) band intensity also increased in ILAE class 1 and 2 ($p < 0.001$; $9.64 \pm 0.70$, $p < 0.001$; $10.80 \pm 1.39$) respectively than controls ($1.06 \pm 0.22$). Furthermore, SetD7 monomethylates H3K4 (Fig. 1f). H3K4me1 positively regulates Keap1 expression, leading to additional negative control over Nrf2 availability in the cytoplasm. In TLE-HS patient samples, we found a significant increase of H3K4me1 expression in classes 1 and 2 ($p < 0.005$; $2.56 \pm 0.25$, $p < 0.007$; $2.63 \pm 0.43$), respectively, compared to autopsy control samples ($0.81 \pm 0.03$). Therein, upregulation of histone methylation could contribute to significant suppression of Nrf2 and could also support Keap1 expression.

## p62 and p21 proteins which can bind to Keap1 and Nrf2 were upregulated in TLE-HS patients

Looking into the interactor proteins of Nrf2, autophagy adapter protein p62, and apoptosis inhibitor protein p21 can interact directly with Keap1 and Nrf2, respectively. In TLE-HS patients, we found increased expression of p62 (Fig. 3b) in ILAE class 1 ($p < 0.001$; $2.05 \pm 0.24$) and class 2 ($p < 0.001$; $2.65 \pm 0.27$) as compared to controls ($0.36 \pm 0.15$). Nrf2-interacting protein p21 (Fig. 3c) band intensity was also significantly high in TLE-HS samples belonging to ILAE 1 ($p < 0.001$, $3.28 \pm 0.20$) compared to controls ($1.95 \pm 0.15$) and ILAE 2 ($p < 0.03$, $2.32 \pm 0.12$). The keap1 interacting protein HSP90 [41] (Fig. 3d) was found to be increased in class 1 ($p < 0.03$; $5.89 \pm 0.37$) as compared to class 2 ($4.52 \pm 0.27$) and controls ($1.26 \pm 0.15$). This could suggest that p21 and p62 interactor proteins interfere

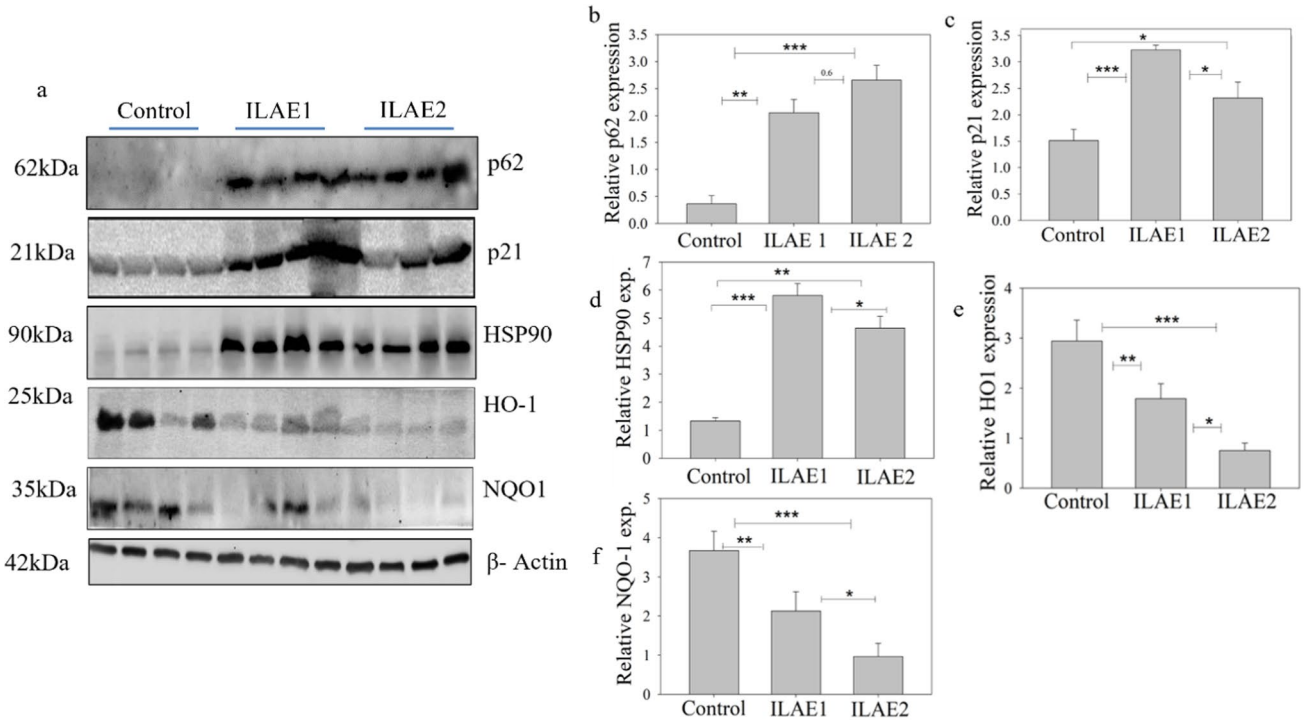


**Fig. 3** **a** Immunoblot analysis for p62 (**b**), p21 (**c**), HSP90 (**d**), HO-1 (**e**), NQO1 (**f**), and β-actin were probed for the control and resected epileptic patient brain samples and protein quantification of corresponding immunoblot results represented by bar graphs. Beta-actin is used as internal control, represented by the base panel. Data representing mean ± SEM, $n = 4$. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

with Nrf2-Keap 1 interaction, thereby preventing ubiquitin-mediated Nrf2 degradation.

### Expression of phase II detoxifying enzymes is compromised in TLE-HS patients

Phase II detoxifying enzymes are essential to clear excessive ROS produced during excitotoxicity-induced oxidative stress as in epileptic setting. Therefore, expression patterns of phase II detoxifying enzymes such as HO-1 and NQO1 were investigated. We found an increased expression of HO-1 (Fig. 3e) in ILAE class 1 ($p < 0.02$; $1.81 \pm 0.21$) compared to ILAE class 2 ($0.62 \pm 0.09$) but not with respect to controls ($p < 0.001$; $2.97 \pm 0.32$). Similarly, NQO1 (Fig. 3f) band intensity increased significantly in class 1 ($p < 0.02$; $2.12 \pm 0.35$) compared to ILAE class 2 patient samples ($1.06 \pm 0.2$). However, controls ($3.67 \pm 0.49$) showed a significant increase compared to class 1 ($p < 0.009$) and class 2 ($p < 0.001$). This indicates that phase II antioxidant response is reduced in class 2 TLE-HS patients.

## Discussion

Antioxidant activity is pivotal in protecting neurons and other cells in the seizure microenvironment. A defective antioxidant response can lead to ROS-induced membrane lipid oxidation and cell death [42]. Excessive free radicals generated during excitotoxicity and frequent seizures have been reported in TLE-HS patients [43]. This could lead to a reduction of seizure threshold resulting in spontaneous, recurrent seizures. Since cell death due to excitotoxicity is apparent in TLE-HS patients [44], it is critical to understand the molecular basis governing the antioxidant response signaling mechanisms and their impact on inherent epileptogenesis in TLE-HS patients. Keap1-Nrf2 axis is an important antioxidant response mechanism involved in neutralizing the free radicals by inducing phase II detoxifying enzyme expression [10].

The current study aimed at understanding the epigenetic underpinnings of Keap1-Nrf2 signaling and the downstream antioxidant response [28, 45]. Histone methylation is one of the gene regulation mechanisms which can either repress or promote transcription, and hence gene expression depending on the methylation sites and extent of methylation on Lys residues in the respective histones. It has been reported that H3K27me3 negatively regulates Nrf2 expression and downregulates HO-1 and NQO1 gene expression [26, 27]. The catalytic component of PRC2, viz., EZH2 trimethylates H3K27 and influences H3K9 methylation so as to repress Nrf2 transcription [46–48]. In line with this, earlier studies reported that EZH2 that trimethylates H3K27 promotes cell death by reducing Nrf2-dependent drug resistance in lung cancer tissues [45]. In the current study, TLE-HS patients showed a significant increase in EZH2 and SetD7 expression in ILAE class 2 compared to class 1. This indicates that there could be a decrease in Nrf2-mediated antioxidant response by promoting Keap1 expression. In line with this, we observed a significant increase in Keap 1 expression and a corresponding decrease in Nrf2 protein in class 2 patient samples. In our study, we observed that histone trimethylation status of H3K27 and H3K9 corresponded with reduced Nrf2 expression in class 2 samples compared to class 1. Alternatively, SetD7 monomethylates H3K4 and has been reported to positively regulate Keap1 expression in diabetic retinopathy [28]. A similar increase in SetD7 expression was observed in class 2 patient samples in our study.

It has been well documented that Keap1-Nrf2-ARE pathway regulates NQO1 and HO-1 expression in the central nervous system [13–15, 19, 20, 46]. Nrf2 is a transcription factor that binds to ARE of phase II antioxidant enzyme genes during oxidative stress [47, 48]. In the current study, NQO1 and HO-1 protein expression was elevated in class 1 compared with class 2 patient samples. Based on the significant contribution of Nrf2-Keap, it could be suggested that Keap1-Nrf2 dysregulation could possibly aggravate epileptic seizures and cognitive impairments [9].

The N-terminus of the Nrf2 protein has ETGE and DLG sequence motifs that bind explicitly with the two Kelch domains of Keap1 homodimer and is targeted by cullin3-based ubiquitin E3 ligase [49–51]. Since the half-life of Nrf2 is significantly less (20 min), and sequestered by Keap1 in the cytoplasm before it enters the nucleus [52], Nrf2 needs to overcome Keap1 regulation. Earlier studies reported that the Keap1-Nrf2 interaction is destabilized by p62 and p21 [33, 53]. Komatsu M et al. suggested that p62, an autophagy adapter protein, can also interact with the Kelch motif of Keap1 and increase Nrf2 half-life [36, 53]. The Kelch motif is critical for interaction with Nrf2's ETGE and DLG motif for the purpose of ubiquitination of Nrf2 in the cytosol [48]. Also, p21 stabilizes Nrf2 against Keap1 and facilitates its nuclear translocation. The KKR motif of p21 interacts with ETGE and DLG motifs in Nrf2 and compromises the Keap1-Nrf2 ubiquitination [33]. In an attempt to understand the role of p62 and p21 in relation with Nrf2-Keap 1 axis in an epileptogenic setting, we examined their corresponding expression patterns in our study population. We found a significant upregulation of p62 in TLE-HS patient samples than controls due to the accumulation of protein aggregates and chronic ER stress and resulting cell death during seizures in TLE-HS patients. With respect to p21, there was a significant increase in its expression in class 1 compared to class 2. Given the regulatory effect of p21 and p62 on the stabilization/destabilization of Keap1-Nrf2 complex, it could be suggested to play a role in mediating the phase II antioxidant enzyme-mediated detoxifying response in TLE-HS patients.

Our study indicated that TLE-HS patients did not exhibit an increase in Nrf2 expression despite seizure-induced oxidative stress. It indicates that Nrf2 is strongly and negatively regulated in TLE-HS patients prone to seizure recurrence. Commenting on the significance of the results in the current study, with respect to epigenetic underpinnings of Keap1-Nrf2 axis, a significant variation in the expression of histone methylases (as discussed above) and their target antioxidant response components was observed in our study population. Compromised functioning of Keap1-Nrf2 axis could lead to excessive ROS accumulation, cytotoxicity, and cell death in an epileptic brain that could lead to the reduction of seizure threshold [6–8]. All these observations point towards a relationship between the downregulation of antioxidant response that in turn has implications for the development of random neural networks that propagate seizure microenvironment in patients and, eventually seizure recurrence [54].

## Conclusion

In the current study, TLE-HS patients with seizure recurrence displayed significantly reduced antioxidant enzyme expression that could be attributed to excitotoxicity. There was an upregulation of histone methyltransferases and methylated histones that, in turn, affected Nrf2 transcription. Also, Keap1, a negative regulator of Nrf2, was upregulated, particularly in ILAE class 2, compared to class 1. Therein, our study findings conclude that antioxidant response might be critical in alleviating seizure recurrence in TLE-HS patients. In view of significant tissue metabolic stress in an epileptogenic setting, inquiring into the basis of antioxidant response as in the current study would enable the understanding of molecular basis and development of possible novel therapeutic targets for mitigating epileptic seizure recurrence.

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## Declarations

**Ethical approval** This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the University of Hyderabad IAEC/UH/151/2017/05/PPB/P13.

**Conflict of interest** The authors declare no competing interests.

**Informed consent** Written informed consent was obtained from all individual participants included in the study.

**Consent to publish** For the current study, consent to publish has been received from all the patients.

**Disclaimer** The funding body had no role in the design, collection of the sample, management, conduct of the study, interpretation of data, preparation, review, or approval of the manuscript, or decision to submit the manuscript for publication.

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