Growth-associated protein 43 and progressive epilepsy in cortical dysplasia

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Abstract

Objective: To investigate growth-associated protein 43 (GAP-43), a marker for axonal growth and synaptic plasticity, as potential substrate for progressive epilepsy and potential predictor of postsurgical seizure outcome in patients with focal cortical dysplasia (FCD). Methods: GAP-43 immunohistochemistry was performed on cortical specimens from 21 patients with FCD: 12 with FCD type II (IIA or IIB) and nine with FCD type IA. Twenty normal anterior temporal lobe specimens from patients with mesial temporal lobe epilepsy due to hippocampal sclerosis (mTLE/HS) were used as controls. Semiquantitative analysis of GAP-43 staining patterns was performed. Additionally, GAP-43 immunoblotting was performed on resected tissue from three patients with FCD type IIA/B; GAP-43 protein levels in electroencephalography-verified epileptic, and distal nonepileptic, areas were compared within each patient. Two outcome categories were used: completely seizure free (Engel IA) versus not seizure free. We examined the relationship of GAP-43 scores with epilepsy duration and seizure-free outcome for each of the three pathologies. Results: Within-patient GAP-43 expression is selectively increased in the epileptic as compared to nonepileptic cortex. GAP-43 immunoreactivity (IRs) patterns were seen on the cell surface and tubular punctate structures intercellularly only in FCD cortex. Higher GAP-43 scores were correlated (P < 0.0001) with longer epilepsy duration only in FCD IIA/B. Lower GAP-43 scores were associated with better surgical outcome in the same group. No such relationship was observed in FCD IA. Interpretation: GAP-43 proteins are not only associated with intrinsic epileptogenicity but may be markers of progressive epilepsy and predictors of postoperative seizure outcome in patients with pharmacoresistant epilepsy due to FCD IIA/B.

Introduction

Focal cortical dysplasias (FCDs) are the most common pathologic substrates in both adults and children with pharmacoresistant focal neocortical epilepsy and postoperative seizure outcome has been less successful as compared to patients with mesial temporal lobe epilepsy due to hippocampal sclerosis (mTLE/HS). Previous studies suggest that the most important predictor of success following epilepsy surgery is the complete resection of the epileptic focus.¹⁻⁵ There has been increasing awareness that epileptogenicity in FCDs encompasses a more complex network extending beyond the lesion.⁶⁻⁷ Moreover, epilepsy associated with FCDs is a progressive disease which is supported by compelling evidence of seizure worsening over time, change in electroencephalography (EEG) patterns, and improved outcomes with early surgical resection.¹⁻⁵,⁸⁻¹¹ The histopathology of FCDs has been classified into two main categories: type I FCD refers to cortical dyslamination and type II is characterized by the presence of dysmorphic neurons without (type IIA) or with balloon...
cells (type IIB). The recently ILAE classification of FCDs made modifications to the Palmini classification by adding type III FCD, in which FCD occurs in combination to additional pathology.

Currently, little is known about the epileptogenic substrate that underlies the evolution of epileptogenic networks in FCDs. We speculate that synaptic remodeling contributes, in part, to the structural changes underlying the development of epileptogenic networks. Growth-associated protein 43 (GAP-43) has been known as a marker for axonal growth and synaptic plasticity. GAP-43 is maximally expressed during brain development. Once growing axons reach their targets, and synaptogenesis is established, GAP-43 levels rapidly decline. Re-expression of GAP-43 in human adult brain occurs during axonal spouting following stroke, mossy fiber sprouting in sclerotic hippocampi, and axonal regeneration in multiple sclerosis and posttraumatic brain injury lesions.

To explore potential substrates that contribute to local epileptogenic networks and progressive epileptogenesis, we investigated GAP-43 protein levels in electrophysiologically defined epileptic versus nonepileptic brain samples from patients with FCD-associated intractable epilepsy. We then studied the expression of GAP-43 in epileptic brain samples characterized by different FCD subtypes. We used specimens that were pathologically characterized as FCD IIA or FCD II (IIA and IIB) based on the Palmini classification. Furthermore, we correlated the expression of GAP-43 with duration of epilepsy and surgical outcome.

**Patients and Methods**

**Patients and pathological subgroups**

Human neocortical samples were obtained from patients who underwent surgical resection at the Cleveland Clinic Epilepsy Center. The study was approved by the Cleveland Clinic Institutional Review Board. Since the aim is to identify the epileptogenesis substrate in FCDs, we excluded specimens from patients undergoing hemispherectomy or patients with additional pathologies (type III FCDs). The following specimens collected for research from 2000 to 2012 were available for analysis: 12 nontemporal lobe specimens with pathologically verified FCD type II (IIA or IIB) and nine nontemporal lobe specimens with FCD type IA. Additionally, 20 histologically normal neocortical temporal lobe specimens from patients of mTLE/HS were used as “epilepsy” control samples. The detailed patient demographic data are shown in Table 1.

**Table 1. Demographics of patients with FCD IIA/B and FCD IA.**

| Pt ID | Age at surgery (years) | Sex | Epilepsy duration (years) | Type epilepsy | MRI lesion | Second GTC seizure frequency | Type of surgery | FCD | Invasive EEG | Seizure free | Duration follow-up (months) |
|-------|------------------------|-----|---------------------------|---------------|------------|-------------------------------|----------------|-----|-------------|--------------|-----------------------------|
| 1     | 2.8                    | F   | 0.29                      | Focal N       | No         | TPO                           | IIB            | No  | Yes         | 27            |
| 2     | 4.1                    | F   | 3.60                      | Focal Y       | Daily      | F + P                         | IIB            | EcoG| Yes         | 63            |
| 3     | 5.3                    | M   | 4.80                      | Focal Y       | No         | TPO                           | IIA            | EcoG| Yes         | 11            |
| 4     | 1.5                    | F   | 1.5                       | Focal Y       | No         | TPO                           | IIA            | SDG | Yes         | 117           |
| 5     | 7.5                    | M   | 5.03                      | Focal Y       | No         | F                             | IIA            | EcoG| Yes         | 115           |
| 6     | 22                     | F   | 20.08                     | Focal N       | No         | F                             | IIB            | SDG | No          | 132           |
| 7     | 17                     | F   | 14.07                     | Focal Y       | 2/year     | F + T                         | IIA            | SDG | No          | 14            |
| 8     | 13.5                   | F   | 12.56                     | Focal Y       | 2/year     | F                             | IIB            | SDG | No          | 115           |
| 9     | 13.1                   | F   | 11.09                     | Focal Y       | No         | TPO                           | IIB            | EcoG| No          | 49            |
| 10    | 37.6                   | M   | 27.599                    | Focal Y       | 1/15 years | P                             | IIA            | No  | Yes         | 15            |
| 11    | 19.2                   | M   | 16.76                     | Focal Y       | 1/12 years | F                             | IIB            | EcoG| No          | 133           |
| 12    | 50.2                   | M   | 45.18                     | Focal Y       | No         | F                             | IIB            | SDG | Yes         | 35            |
| 13    | 6.2                    | M   | 6.17                      | Focal N       | No         | F                             | IA             | SDG | No          | 20            |
| 14    | 18.9                   | M   | 13.93                     | Focal N       | No         | F                             | IA             | SDG | Yes         | 6             |
| 15    | 12.2                   | F   | 5.3                       | Focal N       | Daily      | F                             | IA             | SDG | No          | 140           |
| 16    | 25.1                   | M   | 13.07                     | Focal N       | No         | F                             | IA             | SDG | Yes         | 144           |
| 17    | 9.3                    | F   | 5.34                      | Focal Y       | No         | F                             | IA             | No  | Yes         | 102           |
| 18    | 25.3                   | M   | 20.36                     | Focal Y       | No         | F                             | IA             | SDG | Yes         | 120           |
| 19    | 4.7                    | F   | 2.74                      | Focal N       | 2/month    | F                             | IA             | No  | No          | 30            |
| 20    | 12.5                   | F   | 5.47                      | Focal N       | No         | F                             | IA             | No  | No          | 76            |
| 21    | 30.9                   | M   | 18.93                     | Focal N       | 3/year     | P                             | IA             | Yes | Yes         | 25            |

Pt, patient; MRI, magnetic resonance imaging; FCD, focal cortical dysplasia; GTC, secondarily generalized tonic clonic seizure; F, frontal lobe; T, temporal lobe; P, parietal lobe; TPO, temporo-parieto-occipital lobes; SDG, subdural grids; EcoG, Intraoperative electrocorticography.
**Tissue preparation and GAP-43 Immunohistochemistry**

We performed simultaneous immunostaining of tissue sections from all three pathology groups (FCD IA, FCD IIA/B, and normal-appearing temporal neocortex). Immunohistochemistry (IHC) staining was done on free-floating sections (30-μm) as described previously. Specifically, we used GAP-43 antibody (1:1000, rabbit polyclonal; Sigma-Aldrich, Inc., St Louise, MO). No specific ICC staining was seen in the absence of GAP-43 antibody.

**Semiquantitative analyses of GAP-43 IHC labeling**

GAP-43 has been shown not to be accumulated in the somata after being synthesized because GAP-43 is packed on vesicles and rapidly transported down axonal processes. Since somata are unstained with GAP-43 antibody in human cortex, we studied GAP-43 stained elements around cell surface (Rim) and intercellular tubular punctate structures. The quantitative analysis of IHC staining was developed based on our previously published methodology. GAP-43 staining was qualitatively analyzed by two blinded investigators (Z. Y., A. N.). We graded the patterns of cell surface (Rim) staining and intercellular tubular punctate staining according to the following system (also see Fig. 3):

- **Percentage of neurons with Rim staining:**
  - 4 = more than 50% of neurons in the section have GAP-43 stained Rim pattern
  - 3 = less than 50% of neurons in the section have GAP-43 stained Rim pattern
  - 2 = about 5–10% of neurons in the section have GAP-43 stained Rim pattern
  - 1 = few neurons in the section have GAP-43 stained Rim pattern

- **Intensity of tubular punctate staining:**
  - 4 = intensely dark
  - 3 = moderately dark
  - 2 = some staining
  - 1 = no staining

The final grade was obtained by adding scores for both Rim and tubular staining (maximum grade of 8 and minimum of 2).

**Western immunoblotting and protein quantification**

Three separate patients who had intractable focal epilepsy and MRI-identifiable FCD lesions (two patients with frontal and one with occipital lesion) underwent prolonged video-EEG evaluation with intracranial (subdural) electrodes to identify the ictal onset zone and guide surgical resection. Two types of tissue (epileptic area and nonepileptic area) were collected from each patient guided by findings of intracranial EEG. As previously described, the epileptic area was determined by the ictal onset pattern on subdural electrodes. A nonepileptic area was defined as the area at the margin of the resected cortex showing minimal (<1 spike/h) or no interictal activity, and no ictal seizure pattern, during prolonged subdural EEG recordings. Resection of nonepileptic regions is dictated by the surgical approach. In these patients we were able to collect both epileptic and nonepileptic blocks that were frozen fresh with dry ice for immunoblotting. No extra specimens were available to be fixed in 4% paraformaldehyde, therefore GAP-43 IHC staining was not performed. Two patients had FCD IIB and one had FCD type IIA.

For immunoblotting, the gray matter from specimens was dissolved and homogenized. Following centrifugation of the lysate, 20 μg of protein were dissolved in Laemmli buffer containing β-mercaptoethanol, boiled for 5 min, run on a 10–20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. After blocking, the blots were incubated overnight with GAP-43 antibody (rabbit polyclonal antibody, 1:3000 dilution, AB 7462; Abcam, Cambridge, MA) at 4°C, followed by incubation with anti-rabbit IgG conjugated with horseradish peroxidase (Jackson Immunoresearch, West Grove, PA). Protein–antibody complexes were then visualized with enhanced chemiluminescence reagents (ECL-PLUS; Amersham, Arlington Heights, IL).

To quantify the optical densities of GAP-43 bands on western blot, the film was scanned by a flatbed scanner (Microtek, Scanmaker 1000XL; Microtek Lab, Inc, Santa Fe Springs, CA). Digital images containing the gel bands were imported into the Gel-Pro Analyzer (Media Cybernetics, Rockville, MD). Band densities were analyzed with Image Pro Plus v6.1 Analyzer (Media Cybernetics, Rockville, MD). The digitized gray value of each band was imported into a Microsoft Excel spreadsheet.

**Outcome definition**

Two outcome categories were used: completely seizure free (Engel class IA) and not seizure free. Only patients with Engel class IA outcome at their last follow-up are considered seizure free. The rest are considered not seizure free.

**Statistical methods**

Ordinal logistic regression was used to examine the relationship between GAP-43 scores and epilepsy duration in the three patient groups (pathology FCD IIA/B, FCD IA,
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and mTLE/HS). Pearson chi-square was used to compare high versus low GAP-43 scores with epilepsy outcome. Significance level of all statistical tests was set at 5%. Statistical analysis was performed using JMP software (JMP Pro 9.0.0; SAS Institute, Cary, NC).

Results

Patient characteristics

Samples from 12 patients with FCD IIA/B and nine with FCD IA were analyzed. For the FCD IIA/B group, mean age of seizure onset was 2.69 years, mean epilepsy duration 13.55 years, and mean age at time of surgery 16.22 years. For the FCD IA group, mean age of seizure onset was 5.99 years, mean epilepsy duration 10.15 years, and mean age at time of surgery 16.14 years. We also analyzed 20 histologically normal neocortical samples patients with mTLE/HS who underwent standard anterior temporal lobe resection with removal of mesial structures. In the mTLE/HS group, mean age of seizure onset was 13.38, mean epilepsy duration 21.98, and mean age at time of surgery 36.15 years. Regarding age at seizure onset, the FCD IIA/B group had seizure-onset at a younger age, as compared to FCD IA ($P = 0.037$) and mTLE/HS ($P = 0.025$) groups. There was no significant difference in seizure onset age between FCD IA and mTLE/HS groups. As for epilepsy duration, there was no significant difference between FCD IIA/B versus FCD IA groups or FCD IIA/B versus mTLE/HS groups. However, mTLE/HS patients had longer duration of epilepsy as compared to the FCD IA group. There was no significant difference in duration of postoperative follow-up between FCD IIA/B and FCD IA groups ($P = 0.73$).

GAP-43 and epileptogenicity

To determine whether GAP-43 is increased in the same patient in the dysplastic epileptic cortex as compared to nonepileptic cortex, we examined GAP-43 protein levels in three patients using western blot analysis. As shown in Figure 1, the epileptic samples from all three patients exhibited clear within-patient increase in GAP-43 protein, as compared to the corresponding nonepileptic areas. The optical densities of GAP-43 protein bands demonstrated that, in each of the three patients, the epileptic area clearly showed higher gray values compared to nonepileptic cortex.

Characterization of GAP-43 IHC in human epileptic cortex

The features of GAP-43 IHC in normal and dysplastic cortex are shown in Figure 2. The normal-appearing cortex is defined by well-preserved columnar organization and horizontal lamination with no dysmorphic neurons. In normal-appearing neocortex, there was absence of GAP-43 immunoreactivity (IR) in the neuronal somata and cell surface, and lack of punctate tubular elements. Rather, GAP-43 IHC showed a homogenous, nonspecific background staining. In the dysplastic cortex, GAP-43 IR was seen within the neuronal cell surface resulting in a rim staining appearance, and was also present in between neurons giving the appearance of punctate tubular structures. This punctate tubular staining can have an intensely “clumped” appearance at higher magnification. Neuronal somata were negative for GAP-43 IR. The somata of balloon cells were uniformly, but faintly stained. The semi-quantitative grading scales for GAP-43 stained rims and punctated tubular structures, described in the Patients and Methods section, are illustrated in Figure 3. Statistical analysis (unpaired, two-tailed $t$-test) showed that FCD II group had significantly higher GAP-43 score than either FCD I group ($P = 0.016$) or control group ($P = 0.0001$). The FCD I group did not have significantly higher GAP-43 score than control group ($P = 0.29$).
absence of significant difference in epilepsy duration between FCD IIA/B and FCD IA groups, GAP-43 protein scores in patients with FCD IA were not correlated with epilepsy duration (Fig. 4B). We should mention that FCD IA group had a small sample size. Similarly GAP-43 scores did not correlate with duration of epilepsy in the mTLE/HS group even though these patients had longer epilepsy duration (Fig. 4C).

Figure 2. Characterization of growth-associated protein 43 (GAP-43) immunohistochemistry (IHC) in normal and dysplastic cortex. Photomicrograph cresyl echt violet (CV) and GAP-43 (IHC) staining from normal-appearing cortex (A, C, and E) and dysplastic cortex (B, D, and F–I). In the normal-appearing cortex (A, C, and E): CV-stained section (A) shows well-laminated cortical pyramidal cells with their dendrites appropriately positioned toward the pial surface. The adjacent section with GAP-43 IHC (C) shows only background staining. At higher magnification (E), no specific GAP-43 immunostaining can be seen in cell bodies or intercellular space. By contrast, in the dysplastic cortex (B, D, and F–I): CV-stained section (B) show that the vertical and horizontal laminations are disrupted and dysmorphic cells are darkly stained. In this area, GAP-43 (D) shows increased immunoreactivity. At higher magnification (F), it reveals that GAP-43 stained cell surface as rim appearance and also stained punctate clumps or tubular structures. Those GAP-43 stained patterns in dysplastic cortex are illustrated at higher magnification (I). The balloon cells are strikingly large opalescent cytoplasm with eccentric nuclei (G). Some of these balloon cells are faintly stained with GAP-43 in the cytoplasm (H). Scale bars: 200 μm in (A, B, C, and D); 100 μm in (E–H); 50 μm in (I).
No correlation between GAP-43 expression with age of seizure onset, frequency of seizures, or frequency of interictal spikes was seen in any of the three patient groups studied. There were no correlations between GAP-43 scores with age at time of surgery in mTLE/HS or FCD IA group; only in FCD II group, GAP-43 scores correlated better with age at time of surgery ($R = 0.76$).

**GAP-43 and outcomes**

In the FCD IA group, postoperative seizure outcomes were variable and showed no association with GAP-43 scores. Notably, in the FCD IIA/B group patients with lower GAP-43 scores were more likely to have a seizure-free outcome ($P = 0.038$; Pearson chi-square). However, no difference was observed in the proportion of patients who were or were not seizure free among patients with higher GAP-43 scores. In addition, the preoperative seizure frequency did not affect seizure outcome in either group.

**Discussion**

**GAP-43 protein expression and epileptogenicity in FCDs**

GAP-43 is differentially upregulated in FCD II epileptic cortex as compared to adjacent nonepileptic cortex within the same patient indicating that GAP-43 expression may contribute to epileptogenic mechanisms. Despite no associations found between GAP-43 and frequency of seizures or interictal spikes in three groups of patients studied, it is possible that epileptic discharges may enhance GAP-43 expression. The dynamic interplay between GAP-43 and epileptic discharges may manifest as a positive feedback mechanism.

The GAP-43 IRs are higher in the epileptic dysplastic cortex (FCD type II) compared to FCD IA and normal-appearing cortex which suggests that GAP-43 may be specifically upregulated in type II FCDs. However, lack of impressive GAP-43 expression in FCD type IA may relate to small sample size.
Previous research showed increased GAP-43 mRNA in dysplastic neurons without corresponding increased protein. It is not surprising that no GAP-43 IR is present in the somata of mature neurons (showed in their study and ours). We carefully examined GAP-43 immunostaining patterns and demonstrated that in dysplastic cortex, GAP-43 IRs are concentrated in the cell surface giving rise to a rim-like expression pattern. Additionally, GAP-43 IRs are observed in between cells forming punctate clumps or tubular structure. Future studies with electron microscope are needed to characterize the subcellular localization of GAP-43 and to examine synaptophysin and its parallel relationship with GAP-43 in the dysplastic neurons. The balloon cells are known to have no synaptic connectivity and presence of GAP-43 in the somata of balloon cells merely indicates these dysplastic cells have intrinsic altered ability to upregulate GAP-43 expression.

Another recent study demonstrated increased Connexin-43, the Gap junction channel proteins in the epileptic cortex of FCD type IIB.30 The synergistic effects of increased expression of GAP-43, which is the marker for synaptogenesis, along with Connexin-43 which enables rapid propagation of electrical activities, may lead to intrinsic epileptogenicity in FCD.

GAP-43 protein and progressive epileptogenesis in FCDs

Our study shows that higher GAP-43 IRs correlate with longer epilepsy duration in patients with FCD type II. Prior electrophysiological studies with direct cortical recordings have demonstrated the intrinsic epileptogenicity of such FCD lesions.29,31,32 Among many plausible epileptogenic mechanisms, we hypothesize that dysplastic neurons in FCD II retain their ability to continue upregulating GAP-43 expression which may associate with synaptogenesis and presence of positive feedback interplay between intrinsic epileptic discharges with GAP-43. Furthermore, the plasticity of GAP-43 associated synaptogenesis and upmodulated Gap junction channels may underlie the functional and structural network connectivity and progressive epileptogenicity.

The mechanisms for increased expression of GAP-43 protein are likely multifactorial such as an intrinsic program-driven increased expression of GAP-43 mRNA in dysmorphic neurons.26,33 Second, the increase in GAP-43 proteins could be modulated by NMDA receptor activation.34,35 Previous studies have demonstrated upregulation of the NMDA receptor complex in dysplastic neurons.23,27,28,36–38 Such hypothesis can be tested by immunostaining for both GAP-43 and NMDA receptors and the protein levels for both in relation to epilepsy duration in larger patient sample. Put together, the upregulation...
of NMDA subunits could provide a molecular-functional underpinning for seizure- (and time-) dependent selective expression of GAP-43 in FCD type II lesions. In turn, the plastic changes GAP-43 associated synaptogenesis and rearrangement of Gap junction channels could account for the expanding epileptic network that may underlie the temporal progression of epilepsy in patients with type II FCDs.

It should be noted that less impressive correlations between GAP-43 and type IA may be related to small sample size. In addition, patients in the control group are older than FCD II. It could be argued that age may affect expression of GAP-43, as aged rats lose the ability to upregulate GAP-43 after seizures. However, we found no association between GAP-43 protein with age at time of surgery in control group, whereas in FCD II group patients had similar age span at surgery, GAP-43 was found positively associated with older age at time of surgery. Nonetheless, future prospective studies should be designed with age matching and large samples.

Our study has limitations: retrospective design, data collection at a tertiary center, and referral bias may limit the overall generalizability of our findings. With respect to GAP-43 significant differences were detected only within the FCD type II group, we cannot exclude that additional differences in FCD IA might have been missed due to the relatively small sample size. Furthermore, the optimal minimum follow-up period should be 24 months to allow dynamic expression of surgical outcome. The preliminary findings from the current study need to be confirmed and validated by future prospective studies with larger patient samples, age matching, and longer follow-up duration.

Our finding that longer epilepsy duration is associated with higher GAP-43 level may have clinical implications. We recently showed in a cohort of intractable frontal lobe epilepsies that patients with shorter duration of epilepsy are more likely to become seizure free after surgery. Assuming that GAP-43 expression is a biological correlate (biomarker) of disease progression, and considering our current findings – which indicate an increase in GAP-43 protein expression in relationship with longer epilepsy duration in patients with FCD type II – our observations could be clinically relevant and aligned at least in part with results of surgical outcome studies. It should be noted, however, that the tissue samples studied here were not necessarily obtained from the same patients who had been included in our surgical outcome series.

It is possible that focal epileptic networks continue to expand in epilepsy due to FCD type II, therefore, early timing of resection of FCD type II lesions may halt the progression of epileptogenesis. In our FCD II group, patients whose samples exhibited lower expression of GAP-43 were more likely to have had a seizure-free outcome after focal surgical resection. However, higher scores of GAP-43 expression were associated with variable postoperative outcomes. This may be due to several factors including the relatively small sample size and the variable durations of postoperative follow-up. Importantly, seizure-free outcomes are also determined by the extent of the epileptic network and the completeness of its resection. These hypotheses should be further explored in future prospective studies.

**Authorship and Contributions**

All authors have made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content; and final approval of the version to be published.

**Conflict of Interest**

Imad Najm has potential conflict interest of receiving research grant from U.S. Department of Defense and speaking fees from UCB Pharm.

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