Gene expression changes in an animal model of in utero irradiation-induced cortical dysplasia

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ABSTRACT – Purpose. Cortical Dysplasia (CD) is the histopathological substrate in almost half of all drug-resistant focal epilepsies. Little is known about the gene expression profile of CD. As such information may help target therapeutics more effectively, our aim was to perform a gene expression analysis of an animal model of cortical dysplasia induced by in utero irradiation. Methods. Nine offspring from irradiated animals, and nine age-matched controls were sacrificed at post-natal day 60. Cortical and hippocampal regions were separated, and total ribonucleic acid (RNA) was extracted using a commercially available kit (Qiagen®). RNA was then subjected to a gene expression analysis using an oligonucleotide microarray platform (Illumina®). After statistical analysis, genes were considered differentially expressed when a p value less than 0.001 was observed. Real-time, quantitative polymerase chain reaction (RT-qPCR) was used to confirm microarray results for three genes via the Livak method. Results. Twenty three genes from cortical tissue met criteria for altered gene expression. Six genes from cortex seemed relevant to the pathogenesis of CD. Two genes that promoted cell survival (connective tissue growth factor and peroxiredoxin) were upregulated. One gene that promoted excitotoxic neurodegeneration (latrophilin-2) was downregulated. Two genes involved in glutamate (protein kinase C-α) and AMPA receptor recycling (NEEP-21) were downregulated. One gene, (Shank-1) involved in the control of dendritic maturation, was downregulated. Conclusion. Gene expression analysis in this animal model revealed some of the potential mechanisms by which CD may lead to the phenotype of intractable epilepsy. The downregulation of genes that are involved in glutamate and AMPA receptor recycling may lead to increased excitability. Disinhibition of aberrant dendritic branching, resulting from a downregulation of Shank-1, may also result in an increase in sprouting, excitation and/or hypersynchrony. Finally, genes promoting cell survival, either directly (connective tissue growth factor, peroxiredoxin) or indirectly (latrophilin-2) may allow CD tissue to survive the excitotoxic injury that it produces, thus allowing it to perpetuate the epileptic condition over time.

Key words: cortical dysplasia, in utero radiation, animal model, microarray, gene
In 1971, Taylor first described the histopathological entity of cortical dysplasia (CD) after examining a series of 10 biopsies from patients undergoing surgical resection of lesions thought to have been causing intractable epilepsy. CD has been extensively studied since then, and is now known to range from diffuse malformations involving the whole brain to more focal abnormalities, also known as focal cortical dysplasia (CD).

Various types of CD are characterized by the presence of molecular, cellular, architectural and electrophysiological changes that confer a lowered threshold for the development of induced and spontaneous seizures (Baraban et al., 1995; Möddel et al., 2005; Roper et al., 1995). In fact, CD is now recognized as the histopathological substrate of almost half of all the drug-resistant focal epilepsies. Despite advances in imaging and localization techniques, intractable epilepsy resulting from CD is especially difficult to manage because surgical resection of CD lesions leads to, in general, lower rates of seizure freedom when compared to other etiologies (Hirabayashi et al., 1995; Kloss et al., 2002; Palmini et al., 1991; Palmini et al., 1995; Siegel et al., 1996). In addition, reoperation does not seem to improve outcomes substantially (Gonzalez-Martinez et al., 2007).

**Current understanding of CD pathophysiology**

A large body of knowledge regarding the pathophysiology of CD has accrued since Taylor’s initial description, but our current understanding remains incomplete, which likely accounts for the poor clinical outcome in CD-related epilepsy. What we presently understand relates to certain basic histopathological, electrophysiological and molecular findings. There are excellent reviews dealing with these findings (Bautista et al., 2003; Najm et al., 2004; Palmini et al., 2004; Raymond et al., 1995; Rickert, 2006). In brief, CD likely results from varying degrees of migration abnormalities, which result in histopathological changes such as the persistence of cells in the cortical molecular layer, heterotopic clustering of dysplastic cells, disorganization of the cortical laminar architecture, including loss of the perpendicular (columnar) orientation of neurons relative to the cortical surface (Prayson and Estes, 1995). Patients harboring CD were found to have three key intrinsic electrocorticographic findings-repetitive electrographic seizures, repetitive bursting discharges, and rhythmic discharges (Palmini et al., 1995; Widdess-Walsh et al., 2007). At the molecular level, various receptor abnormalities, including potentiation of the NMDA and AMPA receptors (Mikuni et al., 1999; Najm et al., 2004; Najm et al., 2000; Ying et al., 1999), in addition to a decrease in inhibitory interneurons can be observed.

More recently, microarray studies of human tissue with CD have illustrated various alterations in the expression profile of multiple genes which may be involved in the pathogenesis of epileptogenicity including the potential of neocortical excitatory connections via the differential in situ upregulation of NMDA receptor subunits such as NR2B and NR2C, as well as a downregulation of certain inhibitory receptors such as γ-amino-butyric acidA (GABA_A) (Najm et al., 2000).

**Rationale for gene expression analysis**

While the above findings are no doubt important, we do not yet understand how CD tissue continues to produce epilepsy over the lifetime of the patient, yet not succumb to the same excitotoxicity it produces. Some preliminary answers may lie in comparisons between CD and the tuberous sclerosis complex (TSC). Both entities share certain striking histopathological, molecular as well as genotypic similarities (Becker et al., 2002a; Becker et al., 2002b), in addition to the loss of cell growth and size control mechanisms albeit in different ways. Neurotrophins were shown to play a significant role in the pathogenesis of TSC (Kyin et al., 2001). Indeed, Kyin et al. reported upregulation of certain genes that may confer survival advantage to cells that sustain the epileptogenic condition in CD. Likewise, Kim et al. found upregulation of other pro-survival genes in surgical CD specimen (Kim et al., 2003).

In this study, we report on the changes in gene expression in an animal model of in utero irradiation-induced cortical dysplasia. We hypothesized that multiple genes, involving distinct pathways are differentially upregulated or downregulated in CD. Roper et al. (1995) showed that in utero radiation in rats leads to the expression of diffuse neocortical and hippocampal CD and is associated with a decreased threshold for the development of seizures. We have previously used this animal model for the study of CD and spontaneous epileptogenicity in vivo (Kondo et al., 2001; Kellinghaus et al., 2004; Oghlakian et al., 2009).

These animals have previously been shown to develop spontaneous electrographic seizures, but more recently, were found to develop spontaneous seizures only after the delivery of a pro-epileptic agent.

**Methods**

**Animal model**

The procedure for the induction of CD in fetal Sprague-Dawley rats has been described before. Briefly, two pregnant, out bred Sprague-Dawley rats were irradiated with...
145cGy of γ irradiation using a Cesium-137 source by means of a Shepherd irradiator (JL Shepherd and Associates, San Fernando, CA) on the 17th day of gestation (E17). The duration of exposure was approximately 35 seconds. Two other pregnant Sprague-Dawley rats were not irradiated, and their pups were used as age-matched controls. Day of insemination was designated as E0, which was confirmed by noting the presence of a vaginal plug. All pups were born on E22, which was also designated as post-natal day 0 (PND 0). At PND 21, pups were weaned from their dams, and separated into male and female litters. All animals were housed in our animal facility with 12 hour light/dark cycles and had access to food and water ad libitum. The use of animals was approved by the Institutional Animal Care and Use Committee at Cleveland Clinic.

Animals were sacrificed via decapitation and their brains were cut and quickly isolated in either RNAlater® RNAse free solution (Ambion, Austin, TX), and stored in - 80°C until RNA isolation. Additionally, two brains from each experimental group were saved in 4% paraformaldehyde solution until histopathological studies are performed.

Histopathology

Brain slices were maintained for two days in 4% paraformaldehyde in PBS, and then were transferred to 30% sucrose in PBS for at least 48 hrs before processing. Thereafter, brains were frozen and serially sectioned in the coronal plane into 30 μm slices using a cryostat (Leica CM-1850, Heidelberg, Germany). Sections were then saved in cryostorage solution and stored at - 20°C until further processing. One set of sections representative of various anterior-posterior brain regions were thaw-mounted onto silane-coated slides and stained with Cresyl Violet (CV) for histological examination. Stained sections from animals of both groups were blindly analyzed according to the same criteria as previously described in order to establish the presence of dysplasia in various brain areas (Kondo et al., 2001, Kellinghaus et al., 2004, Oghlakian et al., 2009).

These criteria included the following:
- presence of cortical neuronal clustering;
- persistence of neurons in the molecular layer;
- lack of columnar and layering organization;
- and/or presence of neuronal dispersion in any of the hippocampal subfields.

Brain slices from all in utero radiated rats showed histological changes as defined above and illustrated in figure 1. None of these changes were observed in any of the control (non radiated) rats.

RNA Isolation

Frozen brain slices stored in RNAlater were thawed on ice, and dorsal motor cortex and hippocampal regions from treated and control animals were separated. As the main purpose of the study was to assess gene expression differences between CD and normal neocortical tissues, only cortex was used for this analysis, and tissue was homogenized using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA extraction was then performed using a commercially available kit followed by a DNAsé digestion step (Qiagen RNEasy Mini Kit, and Qiagen RNAsé Free DNAsé set, respectively, Valencia, CA). The quality of total RNA was confirmed by running all RNA samples on a 1% agarose gel containing ethidium bromide. Quantity was ascertained using a UV/Vis spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). RNA was stored in -80°C until ready for microarray analysis.

Microarray analysis

250 ng of RNA was reverse transcribed into cRNA and biotin-UTP labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX). cRNA was quantified using a nanodrop spectrophotometer and the cRNA
quality (size distribution) was further analyzed on a 1% agarose gel. cRNA was hybridized to the Illumina RatRef-12 v1 Expression BeadChip using standard protocols (provided by Illumina, San Diego, CA). Individual hybridizations were performed on each sample, and no pooling of samples took place.

Statistical analyses
Gene expression analyses were performed over the total of 18 cortex samples with 9 samples in each of the control and the CD groups. The summarized data from the raw microarray data were log2 transformed and processed with background correction, quantile normalization and variance stabilization (Bolstad et al., 2003). The log2 transformation facilitates the downstream linear model-based comparative analysis. The variance stabilization method removes the mean-variance dependency in the data. These pre-processing steps generally reduce bias and produce robust summary expression measurements (Dunning et al., 2008). Quality control analyses were applied to detect the outlier samples. Expression signals with an Illumina detection threshold < 0.05 across all the samples were used. Linear models and empirical Bayes method in Limma were used to identify differential expression in our experiment between the control and CD groups (Smyth, 2004). The moderated-t approach was applied, in which the posterior residual standard deviations were used in place of ordinary standard deviations. The empirical Bayes approach in the Limma method is equivalent to shrinkage of the estimated sample variances towards a pooled estimate, resulting in far more stable inference. The top genes with significant p value less than 0.001 were exported for the downstream pathway and functional analysis. This P value cutoff is less stringent than the usual FDR adjusted p value cutoff of 0.05 and we chose this method in order to better facilitate downstream pathway and functional analyses. In addition, biologically low abundance genes with only moderate expression changes may still be of substantial significance under pathological conditions, and specifically, in our context.

Pathway analyses methodology
Functional annotation of the genes were obtained using publicly available genome annotation tools and pathway databases such as NCBI, FatiGO2, Rat Genome Database, and KEGG. The commercial pathway analysis tools, Metacore (Metacore™, www.genego.com) and Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com) were also used as they provided more comprehensive functional information for these genes not only in the context of physiological processes and pathways but also their molecular interactions, drug targets, diseases, and other vital information. For genes that did not map to any known pathways, a map of proteins interacting upstream or downstream of these genes were obtained from these commercially available pathway analysis tools.

Polymerase chain reaction assays
A number of factors were involved in choosing genes for confirmation with quantitative real-time polymerase chain reaction (qRT-PCR). Three genes, connective tissue growth factor (CTGF), latrophilin-2 (LPHN-2), and neuronal enriched endosomal protein of 21kDa (NEEP-21, also known as NSG-1) were chosen due to robustness of differential expression based on microarray fold change data; in addition, these genes represented significant elements of pathways that may have a potential role in epileptogenesis in the setting of CD.
Quantitative, real-time polymerase chain reaction (qRT-PCR) was performed using RNA samples from the above three genes noted to be differentially expressed based on the statistical analyses, and standardized to β-actin (reference gene). β-actin was chosen as a reference gene after confirming that its expression was unchanged across all samples. 10 μL samples containing 50 ng of RNA from each of six animals (three control and three CD) were used to fill 90 wells on a 96 well optical PCR plate (Applied Biosystems, Foster City, CA), and six were left blank. A mixture containing buffers, nucleotides, primer, nuclease-free water, and reverse transcriptase was prepared according to manufacturer’s recommendations (High Capacity cDNA archive kit, Applied Biosystems, Foster City, CA). 10μl of this mixture was added to each of the 90 wells containing RNA. The PCR plate was then run on a Bio-Rad i-Cycler thermal cycler with real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) and RNA was reverse-transcribed into cDNA.
For the hybridization step, pre-designed Taqman Gene Expression Assays (Applied Biosystems) primer-probe sets specific for each target gene and the reference gene were used. Taqman probes were selected based on sequences that contained intron-exon boundaries in order to minimize the effects of contamination with genomic DNA. In addition, probe sets containing sequences complementary and in proximity to the 3’ end of the mRNA were chosen because the poly-A tail at the 3’ end lends some protection to the mRNA from damage resulting from RNAses.
Taqman probes, which were FAM-dye labeled with a non-fluorescent quencher, were diluted in a master mix (Applied Biosystems) and were applied to the cDNA in the PCR plate. PCR was then run using this plate on the Bio-Rad i-Cycler beginning with an initial denaturing step, followed by 50 cycles of melting and elongation per the manufacturer’s recommended settings (Applied Biosystems). Fluorescence was measured after each cycle. Relative gene expression was then calculated from resulting threshold cycle (Ct) values, and fold change in gene
expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), where fold change = $2^{-\Delta\Delta CT}$, $\Delta CT=CT$ (target gene) – $CT$ (reference gene), and $\Delta\Delta CT=\Delta CT$ (CD rat) – $\Delta CT$ (control rat). Pearson correlation coefficient was calculated to assess the strength of the correlation between microarray and RT-PCR fold change values, and significance was assessed ($< 0.01$) using the t-test for correlation (figure 2).

Figure 2. Compartmentalized Network Diagram involving genes of interest.
- Green arrows indicate activation. Red arrows indicate inhibition. Black arrows indicate interaction that is not confirmed to be either one of activation or inhibition.
- CTGF: transforming growth factor β (TGF-B) induces the formation of CTGF (Grotendorst 1997). CTGF binds and inhibits vascular endothelial growth factor (VEGF) and inhibits angiogenesis (Inoki et al., 2002). Conversely, VEGF may induce the expression of CTGF in some cell types (Suzuma et al., 2000). NF-Kappa-β may downregulate the expression of CTGF (Tian et al., 2005).
- PKCA: PKCA can be activated by intermediates such as VEGF to induce migration in certain tumor cell types (Podar et al., 2002) as well as by certain Rho-dependent GTPases such as cdc42 in the regulation of cell motility (Slater et al., 2001). PKCA interacts with the excitatory amino acid transporter-type 3 (EAAT-3) and likely induces its recruitment to the plasma membrane in neurons (Davis et al., 1998). PKCA also activates AMPA receptor via phosphorylation of the subunit GLUR1 (Roche et al., 1996).
- SHANK-1: connects glutamatergic receptors to subcellular machinery via interaction with intermediates such as GRIP (Lim et al., 2001). It also may serve as an intermediate between the receptor for α-latrotoxin (LPHN-2) and downstream mediators (Krienkamp et al., 2000). In addition, it inhibits Densin-180 mediated dendritic branching (Quitsch et al., 2005).
- NEEP-21: also interacts through intermediates such as GRIP in the regulation of AMPA receptor subunit (GLUR2) recycling (Steiner et al., 2002, 2005)
- PRDX2: Synaptic activation via NMDA receptors activates PRDX and promotes protective, anti-oxidant responses (Papadia et al., 2007). PRDX2 scavenges peroxide (Low et al., 2007) before the latter can activate the pro-inflammatory NF-KB pathway (Byun et al., 2002). However, PRDX-2’s action may be inhibited through phosphorylation by cdk5 (Qu et al., 2007).
- CDK5: may potentiate the degradation of NR2B subunit of the NMDA receptor, and inhibit learning and synaptic plasticity (Hawasli et al., 2007).
- RICS: is associated with the NR2B subunit of the NMDA receptor (Hayashi et al., 2007), and inhibits an important downstream mediator of neurite outgrowth, cdc42 (Nasu-Nishimura et al., 2006).
Results

Microarray gene expression results in dysplastic and non-dysplastic brains

As shown in Table 1, using the statistical criteria detailed above, 23 genes were found to be differentially expressed in the cortex: 19 genes were downregulated, and 4 genes were upregulated in the dysplastic tissue. Of the 23 genes, 7 were predicted genes for which homology exists between human and rat sequences. Nine of the 23 genes were loci (LOC) that indicated predicted genes or exons that need to be further validated. This left us with seven genes that had been definitively validated.

Of these 7 genes, 6 were felt to be associated with pathways that may be relevant to the pathophysiology and epileptogenesis of CD. These pathways included:
- regulation of cell growth/anti-apoptosis;
- regulation of neurite outgrowth;
- regulation of neurotransmitter/receptor function.

Differentially expressed genes and their corresponding pathways (Table 2)

Genes involved in the regulation of cell growth and survival
- CTGF: The connective tissue growth factor gene is over expressed by about 1.4 x the control state among animals with in utero induced CD. RT-PCR analysis of CTGF expression showed a 1.4-fold increase when compared with the reference gene, and thus correlated well with our microarray findings. Upon pathway analysis, we found that the CTGF gene may be activated via multiple mechanisms, including via the nuclear factor (NF)-kappa B pathway, in response to various stimuli as well as in pathological conditions such as cancer (Metacore). Once activated, CTGF may enhance cell survival pathways by acting through other anti-apoptotic intermediates, such as the defender against cell death (DAD-1) protein, BCL-2, as well as the Akt family of protein kinases (Metacore, data not shown).
- PRDX-2: Peroxiredoxin-2 (Prx2) was found to be slightly over expressed in CD when compared with the normal state approximately 5% higher in CD rats compared with the normal state. Although this fold change was low, the gene’s differential expression was significant as compared to controls (p < 0.001). Pathway analysis suggests that Prx2 may indirectly inhibit the activation of the NF-kappa-B pathway by eliminating reactive oxygen species (ROS), thus preventing on the one hand, NF-kappa-B’s positive effects on cell proliferation (Kang et al., 1998; Schreck et al., 1991). On the other hand, Prx2 may also enhance cell survival pathways by preventing the accumulation of ROS.
- LPHN-2: Latrophilin-2 (Lphn-2) is a presynaptic receptor that is activated by a neurotoxin known as latrotoxin (Ichtchenko et al., 1999). Our analysis revealed a downregulation of Lphn-2 by ~20%. RT-PCR analysis revealed a downregulation by almost 50% of the control state. Pathway analysis suggests that LPHN-2 may act via a scaffolding protein, Shank-1, to increase presynaptic exocytosis of neurotransmitter, in addition to activating transcriptional factors such as Akt1 (Metacore, data not shown). Other loci, whose expression were found to be significantly altered, namely, FK-506 binding protein, cdk5, and ubiquitin specific peptidase 45 may all enhance cell survival pathways, but a lack of validation in the species used in this study, precludes definitive assignment of roles in these pathways.

Genes involved in the regulation of neurite outgrowth
Shank-1: Sh-3 and Ankyrin repeat-1 (SHANK-1) is a sub-membranous scaffolding protein that has various cellular functions, including regulation of dendritogenesis (Ehlers, 2002). SHANK-1 was down regulated by about 15% in our microarray analysis. SHANK-1 may play a role in multiple pathways that may be related to the pathobiology of CD tissue, including acting with LPHN-2 in order to enhance the activity of transcriptional factors such as Akt. In addition, SHANK-1 is active in regulation of dendritogenesis, and synaptic plasticity by acting through intermediates such as HOMER and F-actin, in addition to Densin-180 (Ehlers, 2002; Quitsch et al., 2005).

Genes involved in neurotransmitter and receptor function
- PKCA: The gene for Protein Kinase C-alpha (PKCA) was found to be down regulated in our analysis to approximately 87% of the normal state (p < 0.001). PKCA may be involved in multiple signaling pathways, including neural differentiation (Marín-Vicente et al., 2008), synaptic long-term depression, potentiation, and Calcium-mediated signaling. It is difficult to establish the putative impact of down-regulation of PKCA on its myriad pathways in the setting of CD. Likely, multiple pathways are affected by such down-regulation. Another potentially interesting role for PKCA is in its involvement in glutamate recycling as PKCA may increase the concentration of certain types of glutamate transporters, thereby decreasing the concentration of neurotransmitter in the synapse (Davis et al., 1998).
- NEEP-21: Neuronal-Enriched Endosomal Protein of 21 kDa (NEEP-21) was down regulated to 75% of its normal state. RT-PCR showed downregulation to approximately 79% of the normal state, and thus correlated well with our microarray findings. NEEP-21 may be involved in neuronal alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptor subunit recycling pathways. NEEP-21, an endosomal protein, acts through other intermediates such as GRIP in order to remove certain AMPA receptor subunits following neuronal activation (Steiner et al., 2002). NEEP-21 then likely determines the fate of these endocytosed subunits depending on the method of activation, as well as the specific type of subunit (Lee et al., 2004).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Symbol</th>
<th>Function</th>
<th>Up/ Down</th>
<th>Fold Change (log2)</th>
<th>p value</th>
<th>Accession ID</th>
<th>Probe sequence</th>
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<td>Zinc finger protein 688</td>
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<td>Ribonuclease-MRP 38 subunit (pred)**</td>
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<td>I15</td>
<td>Cytokine; T, NK cell activation, proliferation</td>
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<td>Down</td>
<td>0.419</td>
<td>0.0009147</td>
<td>NM_024128.3</td>
<td>GGCGAGGAGGCTGAGGAGGAGGAGGAGGAG</td>
</tr>
<tr>
<td>Peroxiredoxin-2</td>
<td>Prd2</td>
<td>Antioxidant, anti-apoptotic</td>
<td>Up</td>
<td>0.072</td>
<td>0.0009339</td>
<td>NM_017169.1</td>
<td>ATCTCACCTTGGCCCTTTACCTG-GATGTCTTGGTCGCTGCAAGGAGGAG</td>
</tr>
<tr>
<td>Protein Kinase C-alpha</td>
<td>Prkca</td>
<td>Involved in regulation of glial glutamate transporters</td>
<td>Down</td>
<td>0.200</td>
<td>0.0009859</td>
<td>XM_343975.2</td>
<td>GTTCGCTGCTGCTGCTGCAAGGAGGAGGAGGAG</td>
</tr>
</tbody>
</table>

Gene expression changes in an animal model of in utero irradiation-induced cortical dysplasia

RT-PCR results: as stated above, quantitative RT-PCR was used to confirm microarray results of differential expression for selected genes that included CTGF, LPHN-2 and NEEP-21 using the Livak method and β-actin as the reference gene. These genes had robust expression changes on microarray analyses, in addition to a possible role in the pathophysiology of CD-related epilepsy, leading us to choose them as our candidate genes for RT-PCR confirmation. All fold change values obtained via RT-PCR correlated well with fold change values obtained during the microarray analysis. Pearson correlation coefficient was \( r = 0.92 \), and the coefficient of determination \( r^2 \) was 0.85. This correlation was found to be significant using t-test for correlation (p < 0.01).

### Discussion

Our results showed altered gene expression related to three main pathways that may be involved in the pathophysiology of epilepsy in an animal model of CD. These pathways include those involved in cell survival, regulation of neurite outgrowth as well as in the recycling of neurotransmitters and their receptors.

#### The expression of genes associated with cell growth and survival in CD

Two genes, namely, connective tissue growth factor (CTGF) and peroxiredoxin-2 were up regulated, and one gene, latrophilin-2, was down regulated. In addition, there were other, as yet not validated gene loci that may be important in this regard as well.

CTGF gene produces a secreted, extracellular matrix protein involved in the regulation of cellular functions such as mitogenesis, cell adhesion, differentiation and survival (Bradham et al., 1991; Brigstock, 1999). It is associated with advanced tumor grade, and has been observed as an angiogenic (Pan et al., 2002) and invasive marker (Demuth et al., 2008) in high grade gliomas, allowing neoplastic cells to migrate. In the setting of CD, overexpression of CTGF may aberrantly promote excessive migration, leading to the abnormal cytoarchitecture

<table>
<thead>
<tr>
<th>Table 2. Distribution of gene function.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
</tr>
<tr>
<td>Latrophilin 2</td>
</tr>
<tr>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>Peroxiredoxin-2</td>
</tr>
<tr>
<td>Cyclin-dependent kinase 5, regulatory subunit 2 (LOC)*</td>
</tr>
<tr>
<td>FK-506 binding protein 10 (LOC)</td>
</tr>
<tr>
<td>Ubiquitin specific peptidase 45 (pred)**</td>
</tr>
<tr>
<td>SH3/ankyrin domain gene 1 (SHANK-1)</td>
</tr>
<tr>
<td>Rho-GTPase activating protein (pred)</td>
</tr>
<tr>
<td>Neuronal enriched endosomal protein of 21kDa (NEEP-21)</td>
</tr>
<tr>
<td>Protein Kinase C-alpha</td>
</tr>
<tr>
<td>Cyclic AMP-regulated phosphoprotein (LOC)</td>
</tr>
<tr>
<td>Interleukin 15</td>
</tr>
<tr>
<td>CD84 Antigen (LOC)</td>
</tr>
<tr>
<td>Absent, small of homeotic (pred)</td>
</tr>
<tr>
<td>Retrotansposon gag domain 4 (pred)</td>
</tr>
<tr>
<td>Ribonuclease-MRP 38 subunit (pred)</td>
</tr>
<tr>
<td>Ribulose-5-phosphate-3-epimerase (pred)</td>
</tr>
<tr>
<td>Epithelial ankyrin 3 (pred)</td>
</tr>
<tr>
<td>Hypothetical protein LOC310926</td>
</tr>
<tr>
<td>Zinc finger protein 688 (pred)</td>
</tr>
<tr>
<td>Similar to Camello-like 2 (pred)</td>
</tr>
<tr>
<td>piggybac transposable element derived 5 (pred)</td>
</tr>
<tr>
<td>Similar to RCK (pred)</td>
</tr>
</tbody>
</table>

* LOC: indicates predicted genes/exons that are not yet validated.
** Pred: indicates predicted gene based on homology between human and rat sequences. This sequence would correspond to a specific, known human gene.
classically seen, as well as its subsequently deranged electrophysiological characteristics, lowering the threshold for the development of seizures.

Peroxiredoxin-2 (Prx2) is an anti-oxidant enzyme important in clearing potentially harmful reactive oxygen species (ROS) from cells. Importantly, the peroxiredoxin family (I-VI) was found to be highly expressed in pilocytic astrocytomas (Nordfors et al., 2007), and may be an important mediator of the cytotoxic drug and radiation resistance of neoplasms (Kinnula and Crapo, 2004). Our analysis showed only a slight up regulation of this gene, and it remains to be seen with further protein work whether this up regulation is enough to confer characteristics of cell survival in the face of seizure-induced hyperexcitability.

Latrophilin-2 (Lphn) is the receptor for the black widow spider venom neurotoxin-$\alpha$-latrotoxin. Lphn assumes one of three forms (1-3) and, when stimulated, causes a massive release of neurotransmitter from the presynaptic terminal, resulting in nerve terminal degeneration (Ichtchenko et al., 2007). Such down regulation may indirectly protect CD from excitotoxic injury mediated by Lphn-2.

Other gene expression results that remain not validated, but which may also promote cell survival include FK-506 binding protein 10 (up regulated), cyclin-dependent kinase 5-cdk5-(down regulated) (Brecht et al., 2003; Qu et al., 2007). Cdk5 down regulation may result in a protective response in the face of neurodegenerative influences related to seizures.

Taken together, our gene and pathways analyses results suggest that CD may possess multiple mechanisms that allows for cell survival in the setting of excitotoxic conditions. In addition, an abnormal potentiation of cell migration may also play a role in the pathophysiology of CD. Similarly, another microarray study of human CD has shown that an abnormality in programmed cell death may lead to the evolution of CD (Kim et al., 2003). Kim et al. (2003) showed that genes coding for trophic factors such as transforming growth factor B-1 were highly expressed, but could not conclude the causal relationship of such expression in the setting of CD.

The expression of genes involved in the regulation of neurite outgrowth in CD

In this microarray study, we found that the scaffolding protein and regulator of neurite outgrowth, SHANK-1, was down regulated. Such down regulation may increase dendritic branching through a loss of inhibition.

These observations lead us to propose that in CD, certain genes involved in the inhibition of neurite outgrowth are down regulated. This loss of inhibition may indirectly increase dendritic branching, potentiating the effects of excitatory influences, and therefore may decrease the threshold for seizure development.

The expression of genes associated with neurotransmitter uptake and receptor function in CD

PKCA is involved in numerous pathways. Of interest in the setting of CD, however, we found that the cell surface expression of one of the five subtypes of glutamate transporters, excitatory amino acid carrier-1 (EAAC-1) also known as excitatory amino acid transporter-3 (EAAT-3), was regulated by PKCA (González et al., 2002). It is possible, then, that a down regulation of PKCA may result in a decrease in the level of reabsorption of synaptic glutamate, possibly allowing more glutamate to activate neurons, resulting in hyperexcitability and seizure expression. However, these results are complicated by the finding that PKCA may redistribute other, predominantly glial glutamate transporters, such as GLT-1 (EAAT-2), into the cytosol (González et al., 2005). It is unclear, therefore, given the downregulation of PKCA in CD, what the balance of receptor expression is during activation in the setting of CD, and its implications on epileptogenesis and epileptogenicity.

Neuron-enriched endosomal protein of 21kDa (NEEP-21) is involved in the proper recycling of specific AMPA receptors subunits such as GLU-2 upon NMDA receptor activation (Steiner et al., 2002). While the process of AMPA receptor recycling may be intricate, the observation of down regulation of NEEP-21 leads to questions regarding the number of AMPA receptors that remain at the membrane after activation, compared to the normal state. If a higher number of active AMPA receptors exist in CD compared to normal, we may infer that the threshold for excitation is lowered, providing a mechanism for the expression of epileptogenicity.

Taken together, the combined effect of down regulation of both PKCA as well as NEEP-21 may have far-reaching implications on the pathophysiology of CD. Although both proteins likely are involved in multifaceted roles in receptor and neurotransmitter recycling, the changes we observed may lead to a faulty lowering of the threshold required for neuronal activation, producing seizures. Future cell culture work using freshly resected dysplastic tissue should confirm whether PKCA and NEEP-21 are indeed down regulated in human CD — if so, gene “knockout” studies may ultimately lead to a better understanding of the functional implications of such differential gene expression.

Other studies

Multiple groups have previously reported gene changes in the setting of CD. Crino et al., in 2001 found an up regulation of certain excitatory glutamatergic receptor subunits, such as AMPA and NMDA receptors, as well as a down-regulation of certain GABA receptor subunits (Taylor et al., 2001). Their techniques did not involve microarrays; rather, gene expression analyses were per-
formed by hybridizing mRNA with an array which comprised a preselected sample of various excitatory and inhibitory receptor subunit cDNA. In addition, the mRNA used was isolated from neurons using laser microdissection techniques. We did not find any of the same genes encoding for receptor subunits to be differentially expressed. These differences in observed changes may be partially due to mRNA isolation techniques: we used whole tissue preparations, which included not only neurons, but also glial elements as well. In addition, our analysis was through unsupervised class discovery at the genomic level via microarray; on the other hand, Crino et al. used a more restrictive set of genes. The same group also showed an over expression of intermediate filaments in balloon cells of CD using similar techniques (Taylor et al., 2001). Our animal model of in utero radiation induced CD is not associated with balloon cells. Another group evaluated gene expression in CD via microarray using single cytomagalic neurons, and found an up regulation of the mammalian target of rapamycin (mTOR) cascade to be up regulated (Ljungberg et al., 2006). Ljungberg et al. (2006) concluded that since the mTOR cascade resulted in cell growth and survival, an over expression of elements involved in this cascade likely leads to a loss of control of cell size, resulted in cytomagalic neurons. Although the merits of using whole tissue versus single neuron isolation for gene expression analysis is beyond the scope of this article, the development of whole tissue analysis techniques may lead to a more seamless practicability of use in the clinical setting. In addition, the use of whole tissue may give a better representation of the in vivo condition. And while microarray technology has revolutionized the study of differential gene expression, and its implications on various forms of neurological disease (Ginsberg et al., 2000; Lewohl et al., 2000; Mirnics et al., 2000; Park et al., 2003; Rich et al., 2005; Rorive et al., 2006; Suzuki et al., 2004; van den Boom et al., 2003; Whitney et al., 1999), standardization across different platforms is still in the developing stages, and only recently have techniques attempted to increase the inter- and intra-rater reliability across different platforms (Shi et al., 2006).

**Conclusion**

In a rat model of in utero irradiation, resulting in histopathologically and electrophysiologically confirmed CD, gene expression analysis using a microarray platform was performed. Our analysis showed that genes involved in cell survival are up regulated, leading us to believe that CD tissue may possess the machinery that allows it to adapt to and survive the excitotoxicity induced by chronic, recurrent seizures. In addition, our analysis further revealed a loss of control over certain pathways involved in neurite outgrowth, potentiating the effects of excitatory influences via spatial summation. Lastly, faulty receptor and neurotransmitter recycling may also be at play in CD. Our study is limited because although these gene expression changes are noted, and some were confirmed with RT-PCR, we cannot definitively conclude which are causally related to epileptogenesis or epileptogenicity. The findings at the protein level remain unknown, and therefore further work exploring the downstream intermediates of such differential gene expression is a necessary next step in realizing the importance of these findings. Further protein, cell culture and electrophysiological studies are urgently needed to study these changes in further detail in order to find potential therapeutic targets for patients suffering from intractable epilepsy as a result of CD.

**Disclosures.**

None.

**References**


Davis KE, Straff DJ, Weinstein EA, et al. Multiple signaling pathways regulate cell surface expression and activity of the excitatory


