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miRNA expression analysis in cortical dysplasia: Regulation of mTOR and LIS1 pathway

Ji Yeoun Lee[a,1], Ae-Kyung Park[b,1], Eun-Sun Lee[a], Woong-Yang Park[c,d], Sung-Hye Park[e], Jung Won Choi[a], Ji Hoon Phi[a], Kyu-Chang Wang[a], Seung-Ki Kim[a,*]

[a] *Division of Pediatric Neurosurgery, Seoul National University Children's Hospital, Seoul National University College of Medicine, Seoul, Republic of Korea*
[b] *College of Pharmacy, Sunchon National University, Jeonnam, Republic of Korea*
[c] *Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Suwon, Republic of Korea*
[d] *Translational Genomics Laboratory, Samsung Genome Institute, Samsung Medical Center, Seoul, Republic of Korea*
[e] *Department of Pathology, Seoul National University Hospital, Seoul National University College of Medicine, Seoul, Republic of Korea*

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Summary Cortical dysplasia (CD) is a common cause of epilepsy in children and is characterized by focal regions of malformed cerebral cortex. The pathogenesis and epileptogenesis of CD have not been fully elucidated, and in particular, the potential role of epigenetics has not been examined.

miRNA microarray was performed on surgical specimens from CD ($n = 8$) and normal control ($n = 2$) children. A total of 10 differentially expressed miRNAs (DEmiRs) that were up-regulated in CD were identified including hsa-miR-21 and hsa-miR-155. The microarray results were validated using quantitative real-time PCR. After searching for the putative target genes of the DEmiRs, their biological significance was further evaluated by exploring the pathways in which the genes were enriched. The mammalian target of rapamycin (mTOR) signaling pathway was the most significantly associated, and the pathway of lissencephaly gene in neuronal migration and development was also noted.

* Corresponding author at: Division of Pediatric Neurosurgery, Seoul National University Children's Hospital, Seoul National University College of Medicine, 101 Daehak-ro, Jongno-gu, Seoul 110-744, Republic of Korea. Tel.: +82 2 2072 3084; fax: +82 2 744 8459.
E-mail address: nsthomas@snu.ac.kr (S.-K. Kim).
[1] These authors contributed equally to the manuscript.

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This study suggests a possible role for miRNAs in the pathogenesis of CD, especially in relation to the mTOR signaling pathway. Future studies on the epigenetic mechanisms underlying CD pathogenesis and epileptogenesis are needed.

Introduction

Cortical dysplasia (CD) is a subgroup of malformations in cortical development (MCD) characterized by anatomic disorganization of the cellular layers and the presence of morphologically abnormal cells (Bentivoglio et al., 2003; Blumcke et al., 2009). With the advancement of magnetic resonance imaging (MRI) of the brain, the ease of diagnosis of MCD has increased greatly, and CD is the most frequently detected MCD (Bronen et al., 1997; Cotter et al., 1999; Gomez-Anson et al., 2000; Palmini et al., 1994). Hence, epilepsies previously thought to be cryptogenic are now recognized to be secondary to CD, which is one of the most common causes of medically intractable epilepsy in children (Krsek et al., 2008; Palmini et al., 1994). Surgical resection is the treatment of choice for these lesions, but the outcome is not always satisfactory. There is a great clinical need for research on CD pathogenesis and the mechanisms underlying its epileptogenicity.

In search for the pathogenesis of CD, studies on its genetic etiology have been performed with a focus on a neurodevelopmental origin. Additionally, the discovery of the causal gene in other subtypes of MCD such as tuberous sclerosis (TSC) or the link between hemimegalencephaly (HME) and genetic syndromes have supported the possibility of a molecular genetic etiology of CD (Crino, 2009). However, most of these studies have focused on genetic mutations (Becker et al., 2002; Fassunke et al., 2004) or aberrant gene expression (Kim et al., 2003), and no studies examining epigenetics are available. This study aimed to evaluate the miRNA expression in CD and to compare that to normal brain cortex miRNA expression to look for a post-translational explanation of CD pathogenesis and epileptogenicity.

Methods

Patients

Surgical specimens were obtained during epilepsy surgery from patients with medically intractable epilepsy. The patients ($n = 15$; mean age at surgery: 8 years) were diagnosed with CD (type I: 8, type II: 7; Table 1). The normal cortex was obtained during surgery for deep seated lesions (Table 2). The diagnoses were confirmed by a pathologist (PSH) according to the recent CD classification scheme (Blumcke et al., 2011). Four cases (mean age at surgery: 6 years) with a histologically normal cortex were included as normal controls. For the miRNA microarray, 8 CD samples (mean age at surgery: 9 years) and 2 normal controls (ages: 7 and 13 years) were used. Then, to validate the microarray results, the expression levels of the DEmiRs were confirmed by quantitative real-time PCR (qRT-PCR) using a different set of 8 CD samples (mean age at surgery: 7 years) and 3 normal controls (mean age at surgery: 6 years). CD case #13 and normal control #2 were used for both the microarray and qRT-PCR (Tables 1 and 2). All the samples were snap-frozen immediately after the resection and were stored at $-80\,^{\circ}C$ until use. Informed consent was obtained from the patients' parents or guardians for the study, which was approved by the Institutional Review Board of the Seoul National University Hospital.

miRNA microarray chip processing and analysis of the miRNA expression data

Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion, Austin, TX) with a small RNA enrichment procedure. An Agilent Human miRNA Microarray Kit (V3) was used as the miRNA microarray chip for the hybridization. The RNA labeling and hybridization were performed according to the manufacturer's instructions. The microarray images were scanned with an Agilent microarray scanner. The total gene signals were extracted using the Agilent Feature Extraction software and were further $\log_2$-transformed. To identify the differentially expressed miRNAs (DEmiRs) between the normal brain tissue and the CD, Bayesian moderated t-statistics were computed (Smyth, 2004), and the analysis was confined to the miRNAs whose expression signals were defined as ''detected'' by the Agilent Feature Extraction software in all the samples. The miRNAs were defined as significantly up- or down-regulated if their t-test p values were less than 0.05 and their fold changes were greater than 2-fold. An unsupervised hierarchical clustering analysis was performed with the standardized expression values using the ''Manhattan'' distance metric and the ''Ward'' linkage algorithm.

Validation of the DEmiR by quantitative Real-Time PCR

The DEmiRs identified by the microarray were validated using TaqMan probes (Applied Biosystems, Carlsbad, CA) using the Applied Biosystems 7500 Real-time PCR system. The cDNAs were prepared with the High-Capacity cDNA Synthesis Kit (Applied Biosystems) using 10 ng of the total RNA as a template. The miR sequence-specific reverse transcription-PCR (RT-PCR) primers for hsa-miR-21, 130b, 155, 193b, 199b and the endogenous control RNU6B were used (Ambion). The reactions were performed in triplicate according to the TaqMan Gene Quantitation assay protocol.

Fischer's exact test was performed for statistical evaluation. P-value less than 0.05 was considered significant.

Pathway analysis with experimentally verified target genes

Using Tarbase 6.0, we identified the experimentally confirmed human target genes for the up- and down-regulated miRNAs. To exclude the genes expressed at low levels in