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Mutational and expression analysis of the reelin pathway components CDK5 and doublecortin in gangliogliomas

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Abstract Gangliogliomas represent highly differentiated glioneuronal tumors frequently occurring in young patients with focal epilepsies. Dysplastic neurons are a neuropathological hallmark of this neoplasm. Here, we have analyzed two major components of the reelin pathway associated with neuronal migration and cortical cytoarchitecture in gangliogliomas, i.e., cyclin-dependent kinase 5 (CDK5) and doublecortin (DCX). The genomic structure of human CDK5 was identified by an "*in silico*" cloning approach using the "high throughput genomic sequencing" (htgs) databank, NCBI BLAST 2.1. DNA sequence analysis of CDK5 and DCX was carried out in tissue samples obtained from 23 patients and compared with control DNA from non-affected individuals (n=100). For gene expression analysis of CDK5 and DCX, a quantitative real time reverse transcription-PCR TaqMan assay was used with mRNA from gangliogliomas (n=22) and non-lesional central nervous tissue control tissue (n=7). The human CDK5 gene is located on chromosome 7q36 and contains 12 exons. Its coding sequence reveals 90.1% homology to the mouse counterpart. A novel pseudogene of CDK5 was found on chromosome 8. While the mutational analysis of CDK5 and DCX did not reveal any sequence alterations in gangliogliomas, a lower expression was observed for both genes in tumor compared to control tissue samples. The present data indicate that mutations of CDK5 and DCX genes are not involved in the development of gangliogliomas. A novel pseudogene on chromosome 8 has to be taken into account for future studies on CDK5.

Keywords Cyclin-dependent kinase 5 · Doublecortin · Pseudogene · Reelin-pathway · Ganglioglioma

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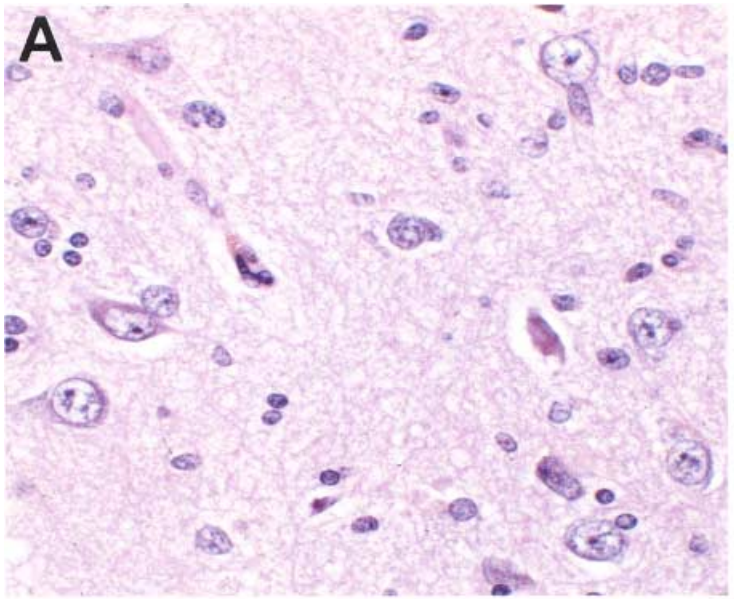
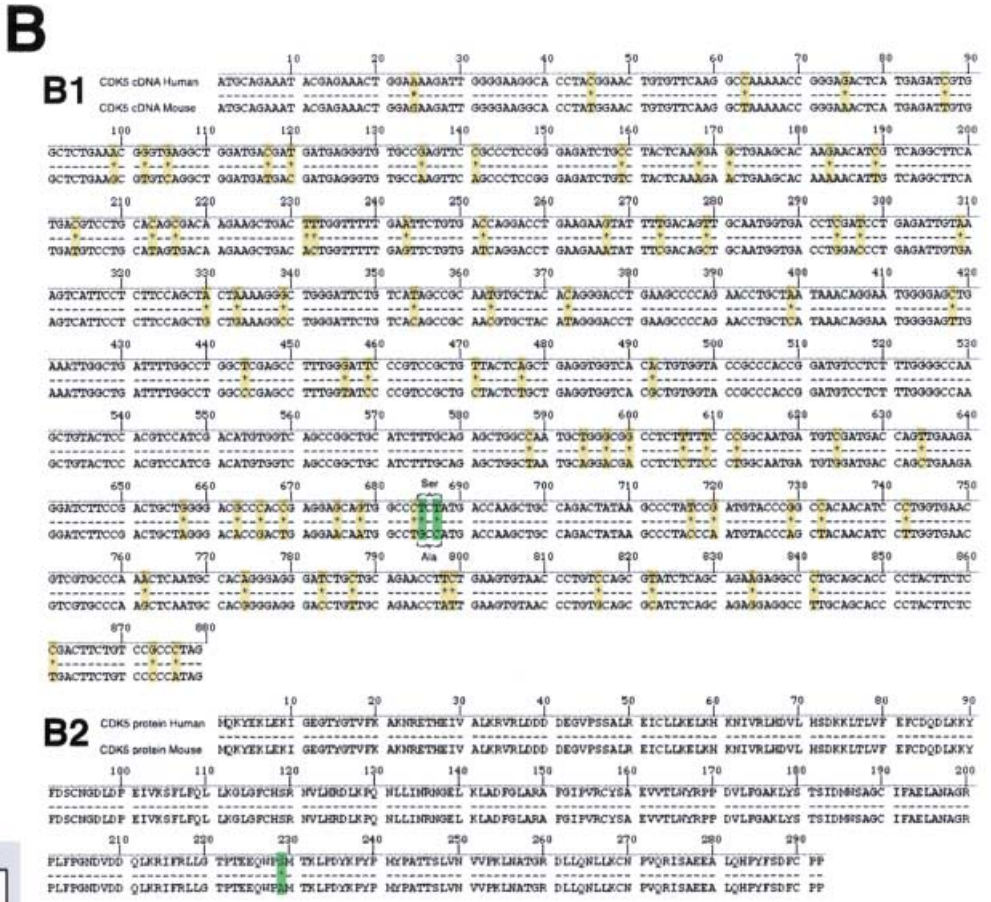
Introduction

Gangliogliomas represent a common neoplasm in young patients suffering from chronic, intractable focal epilepsy, i.e. temporal lobe epilepsy [7]. Histopathologically, these tumors are characterized by an admixture of neoplastic glial and dysplastic neuronal elements (Fig. 1A). Due to the focal nature of gangliogliomas, the differentiated glioneuronal phenotype and the benign clinical course of these neoplasms, gangliogliomas may evolve from a developmentally compromised or dysplastic precursor lesion [7]. Histological similarities with non-neoplastic glioneuronal lesions, i.e., focal cortical dysplasia or cortical tubers associated with tuberous sclerosis (TSC) point towards common pathogenetic mechanisms in gangliogliomas and hamartomatous lesions [34, 35]. Recently, the stem cell epitope CD34 has been characterized in the majority of gangliogliomas, which is compatible with an maldevelopmental origin [8]. Molecular genetic studies have characterized only few genes potentially involved in ganglioglioma pathogenesis, i.e., the tumor suppressor gene TSC2 (tuberin) appears to associate with neoplastically transformed glial cells in gangliogliomas [4].

The reelin signal transduction cascade plays a major role for neuronal development, modification of the cytoskeleton and cellular migration processes (for review see [12, 18, 32]). Key effector components of the reelin pathway are doublecortin (DCX; Xq22.3-Xq23) as well as cyclin-dependent kinase 5 (CDK5; 7q36, see below). DCX is a microtubule-associated phosphoprotein maintaining cytoskeletal plasticity during axonal outgrowth, neuronal maturation and cell migration [19]. Impaired DCX function





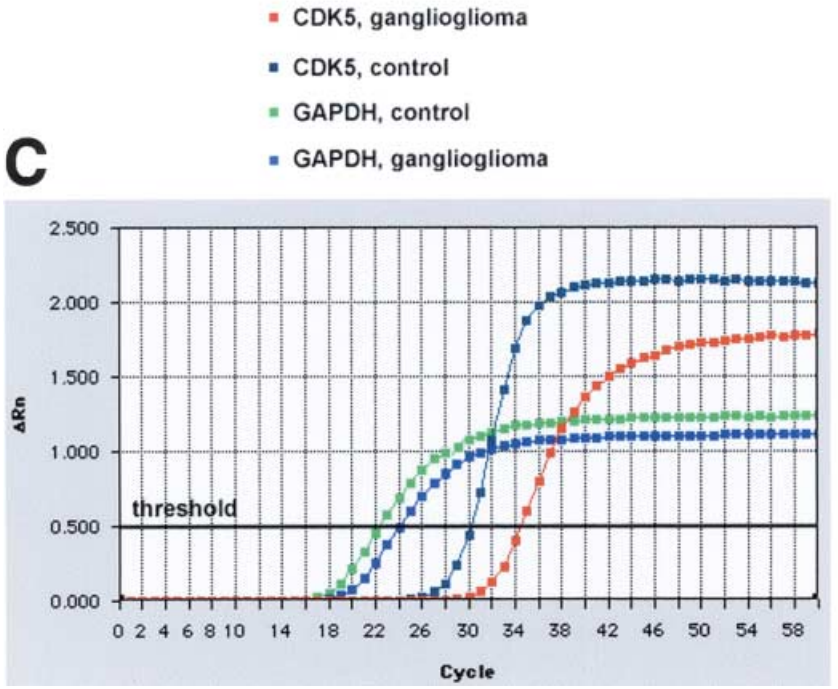


Fig. 1 **A** Typical histopathological appearance of a ganglioglioma characterized by an admixture of dysplastic neuronal and highly differentiated glial cell elements; hematoxylin and eosin staining, ×40. **B(1, 2)** Sequence homologies of the human and mouse CDK5 cDNA and protein. Note the high degree of homology of the human and mouse CDK5 cDNA (90.1%, **B1**) and proteins (99.7%, **B2**). The proteins differ by only one amino acid. The serine is exchanged by alanine at amino acid 229. **C** Quantitative determination of CDK5 and DCX mRNA was carried out by real time quantitative reverse transcription-PCR. The increase of fluorescence intensity per PCR cycle reflects the specific amplification as shown in an exemplary fashion for the amplification of CDK5 and GAPDH (reference gene) mRNA from ganglioglioma compared with control tissue samples. For data analysis, the fluorescent signals are normalized to an internal passive reference dye, yielding a normalized value ΔR_n. At the indicated threshold fluorescent signal levels, the threshold cycles (C_t) for CDK5 and GAPDH in gangliogliomas and controls are determined. Note that GAPDH expression is rather similar in ganglioglioma and control, but mRNA levels for CDK5 are considerably decreased in ganglioglioma (*CDK5* cyclin-dependent kinase 5, *DCX* doublecortin)

in vitro results in interruption of microtubuli. Thereby, the cellular shape and cytoskeletal function as well as cell migration are compromised in vivo [15, 16, 31]. The double cortex syndrome represents a specific neuronal migration disorder caused by (inactivating) mutations of the DCX gene [14] with clinically manifest seizures, cognitive dysfunction and neurological deficits. Missense mutations in the DCX gene have been associated with a X-linked double cortex syndrome [1]. Neuronal migration defects appear to be responsible for heterotopic, subcortical gray matter localization in these patients [28].

Similar to DCX, the CDK5 gene is involved in appropriate neuronal maturation. CDK5 is specifically expressed in postmitotic neurons and muscle cells. It interacts with cyclin D (D1, D2, D3) and is functionally related to G-/S-phase cell cycle transition [11]. CDK5 ablation in mice results in severe alterations of CNS architecture by migration defects [13, 25]. Functional loss of CDK5 results in impairment not only of neuronal movement but also differentiation [9, 25]. CDK5 interaction with its activator-protein p35 is essential for proper microtubulus assembly [17, 26]. By complex formation with p25, the active form of p35, CDK5 induces cytoskeletal interruption and apoptosis [3, 27]. A recent study shows that CDK5 interacts with the *N*-methyl-D-aspartate class of glutamate receptors (NMDAR) known to be critically involved in CNS development, synaptic transmission and memory formation [22].

DCX and CDK5 may thus represent genes potentially involved in the pathogenesis of glioneuronal lesions with a highly differentiated, although dysplastic, neuronal component. Here, we present a systematic mutational and expression analysis of both genes in patients suffering from ganglioglioma associated intractable chronic epilepsy.

Materials and methods

Surgical specimens

Biopsy samples were obtained from patients with chronic pharmaco-resistant epilepsy who underwent surgical treatment in the Epilepsy Surgery Program at the University of Bonn Medical Center. In all patients (n=23), surgical removal of the tumor was necessary to achieve seizure control. In 15 patients, additional blood samples were available for DNA extraction. Informed and written consent was obtained from all patients and studies were conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the University of Bonn Medical Center. Surgical specimens were directly snap frozen for DNA/RNA isolation in liquid nitrogen. All tumors were reviewed by the same neuropathologists and graded according to the guidelines of the World Health Organization [21]. DNA obtained from blood samples of 100 Caucasians without a history of epilepsy or other known CNS diseases served as controls.

DNA/RNA isolation, cDNA synthesis

Nucleic acid samples from tumor cells were isolated from snap frozen tissue (n=23). Care was taken to prepare DNA/RNA only from neoplastic tissue by microscopic evaluation of respective cryostat sections stained for hematoxylin and eosin. Areas with non-lesional brain tissue were avoided by microscopic dissection. Brain tissue samples (n=7) without histopathological alterations served as controls for mRNA expression analysis. Care was taken to provide equal gray/white matter CNS tissue components for RNA extraction. DNA was extracted with chloroform and phenol according to standard procedures as described elsewhere [30]. Total RNA was isolated by TRIZOL reagent (Life Technologies) according to the manufacturer's protocol. RNA isolation was followed by DNase treatment. For cDNA synthesis the first-strand cDNA-synthesis kit from Gibco BRL was used according to the manufacturer's guidelines.

Genomic structure analysis of human CDK5

The genomic structure of the human CDK5 gene was determined by an "*in silico*" cloning approach using the human genome databank, NCBI BLAST 2.1 [2]. A previously cloned human CDK5 mRNA (GenBank AY049778) was used as hybridization probe for virtual hybridization. Exon-intron junctions were compared to the genomic organization of the mouse CDK5 gene [23].

Single-strand conformation polymorphism and sequence analysis of PCR products

Single-strand conformation polymorphism (SSCP) analysis of DCX was performed according to previously published protocols [1]. Our mutational analysis of CDK5 was based on the published cDNA sequence as well as on "*in silico*" cloning of the human genomic structure (see above). All PCR products were separated on non-denaturing gels (12% polyacrylamide, 5% glycerol, TBE buffer) by electrophoresis (20 W, 15°C) and visualized by a silver staining procedure [6]. Direct sequencing of PCR products was performed on semiautomated sequencers (373, 377; PE Biosystems) after sample preparation according to the manufacturer's protocol by a Taq cycle sequencing kit (ABI PRISM, PE Biosystems) using the primers outlined in Table 1.

Quantification of mRNA

A Perkin Elmer Biosystems PRISM 7700 Sequence Detection System (TaqMan) was used for mRNA quantification. The quantitative real time reverse transcription (RT)-PCR approach takes advantage of the 5'-nuclease activity of Taq-polymerase for cleavage of a dual-labeled fluorogenic hybridization probe and consecutive monitoring of the emission increase at dye specific wavelengths during PCR [10, 29]. After normalization to an internal reference (ΔR_n), the threshold was set in the exponential phase of the PCR. The threshold cycle (C_t) was used for quantification of the input copy number of the target mRNA [5]. Relative quantification ($\Delta\Delta C_t$) was applied for CDK5 and DCX, where the number of target mRNA copies was normalized to GAPDH. Oligonucleotides for real-time RT-PCR were specifically designed for the human DCX and CDK5 cDNA using primer express software (PE Biosystems, Foster City) as presented in Table 2. The TaqMan EZ RT-PCR Kit (PE Biosystems) was used for a one-tube, single-enzyme RT-PCR according to the manufacturer's protocol in a reaction volume of 12.5 µl. Total RNA (50 ng) was used as template for each reaction. Reaction conditions were 300 µM each dNTP, 1× TaqMan EZ Buffer, 0.1 U/µl rTth DNA polymerase, 0.01 U/µl AmpErase UNG, 5.0 mM $Mn(OAc)_2$, 100 nM fluorogenic probe and primer concentrations as in Table 2. GAPDH oligonucleotides and conditions were used as described by the manufacturer. Cycling conditions were 50°C for 2 min, 60°C for 20 min RT step, followed by 95°C for 5 min, and 60 cycles of 94°C for 15 s and 59°C for 60 s.

Results

No mutations of the CDK5 and DCX genes were observed in the present series of gangliogliomas. By virtual hybrid-

Table 1 Primers and annealing temperatures for CDK5 PCRs. Two approaches for mutation analysis were performed. The first was carried out on cDNA-, the later on genomic level. For exons 6–8 after a first long range PCR nested PCR assays for the individual exons followed (*CDK5* cyclin-dependent kinase 5)

	Forward primer	Reverse primer	Annealing temperature
cDNA fragment			
1	5'-CCGCGATGCAGAAATACGAG-3'	5'-CCAAAGTCAGCTTCTTGTCG-3'	60°C
2	5'-TCAGGCTTCATGACGTCCTG-3'	5'-CAGCCAATTTCAGCTCCCCA-3'	67°C
3	5'-CAATGTGCTACACAGGGACC-3'	5'-TGACCACATGTCGATGGACG-3'	65°C
4	5'-TCTTTGGGGCCAAGCTGTAC-3'	5'-GTTCACCAGGGATGTTGTGG-3'	60°C
5	5'-AAGCCCTATCCGATGTACCC-3'	5'-CTGTCTCACCCTCTCAAGAG-3'	55°C
Exon			
1	5'-GGCCAGAGTCTTAAAACCGAG-3'	5'-TGGGAACGCTAAGGTTGGAG-3'	60°C
2	5'-GGCATTTCCTGGCTTAGGGA-3'	5'-GGACGTCATGAAGCCTAGGG-3'	60°C
3	5'-TTGAGGGCCTGGGCTGG-3'	5'-GCCACACCGGCAAGGAG-3'	60°C
4	5'-GCTCCTTGCCGGTGTGG-3'	5'-GAGTTTAACCTCAATCTGGGCC-3'	60°C
5	5'-GGTTAAACTCTGTCCCATTCCC-3'	5'-TGCTTACACCGAATGCAGACTC-3'	60°C
Exs 6–8	5'-TGTCACTCTGAACCAGGTGCTG-3'	5'-CTCTGCAACACCCCAGCAC-3'	60°C
6	5'-TGTCACTCTGAACCAGGTGCTG-3'	5'-AGTCCTCTTCCCTCTGTGCTC-3'	60°C
7	5'-GGGAATGGAGAGGCTGGGG-3'	5'-TGGGTCCTGGGGTTGGAG-3'	60°C
8	5'-CCTCTCCTCTCTGAGCCTCCT-3'	5'-CTCTGCAACACCCCAGCAC-3'	60°C
9	5'-GACTGGAGCTGGAAGGTCAGG-3'	5'-CCCATTGTGCTCAAAAGTCCATGGA-3'	60°C
10	5'-GGCGGTGGGTATGAGGAATC-3'	5'-TACATCGGATAGGGCTGTGGAG-3'	60°C
11	5'-CCCTACCTCTAGTCTGACCCT-3'	5'-GTGTCCTGACCCACCCTCT-3'	60°C
12	5'-CCTGCCACCTCCTTTCCC-3'	5'-TGCTGGAGCACAGCGCAC-3'	60°C

Table 2 Primers, fluorescent oligonucleotides and primer concentrations used for quantitative real-time reverse transcription-PCR of DCX and CDK5 (*For* forward primer, *Rev* reverse primer, *Fluor* fluorescent oligonucleotide, *FAM* reporter dye, *TAMRA* quencher dye, *DCX* doublecortin)

Gene	Oligonucleotide	Concentration
DCX	For 5'-CAAGAGCCCTGGTCCTATGC-3'	50 nM
	Rev 5'-GGCTGCTGGAGGTTCCGT-3'	300 nM
	Flour 5'-FAM-CGAAGCAAGTCTCCAGCTGACTCAGCA-TAMRA-3'	
CDK5	For 5'-GGCCAAGCTGTACTCCACGT-3'	300 nM
	Rev 5'-CATTGGCCAGCTCTGCAAA-3'	900 nM
	Fluor 5'-FAM-CATCGACATGTGGTCAGCCGGC-TAMRA-3'	

Table 3 Genomic organization of the human CDK5 gene located on chromosome 7, which is based on the 100% blast hit result Hs7_7861 (GenBank NT_007704) using the human CDK5 mRNA sequence as "*in silico*" hybridization probe (GenBank AY049778). The third triplet of exon 9, the only sequence difference between mouse and human CDK5, which results in an amino acid exchange, is given in bold [mouse TCT (Ser) to human GCC (Ala)]

Exon	Exon size (bp)	5' sequence	...	3' sequence	Genomic position (bp)
1	38	ATG CAG	...	GGG GAA G	1342866–1342828
2	89	GC ACC	...	GAT GAG	1342178–1342089
3	68	GGT GTG	...	ATC GTC AG	1341993–1341925
4	61	G CTT CAT	...	TGT GAC CAG	1341814–1341753
5	57	GAC CTG	...	GTA AAG	1341654–1341597
6	96	TCA TTC	...	AAC AGG	1340866–1340770
7	75	AAT GGG	...	GCT GAG	1340624–1340549
8	97	GTG GTC	...	TTT GCA G	1340391–1340294
9	70	AG CTG **GCC**	...	ATC TTC CG	1340114–1340044
10	61	A CTG CTG	...	TAT AAG	1339483–1339422
11	81	CCC TAC CCG	...	CTG TTG CAG	1339310–1339229
12	88	AAC CTT	...	CCC TAG	1339113–1339025

ization of the human genome databank with human CDK5 cDNA (GenBank AY049778), a 100% hit was found with Hs7_7861 located on human chromosome 7 (working draft sequence, GenBank NT_007704). It contains the human CDK5 genomic sequence. The human CDK5 gene is located on 7q36. Similar to the corresponding mouse gene, the human CDK5 gene contains 12 exons (Table 3). The human CDK5 mRNA and protein exhibit considerable homology to the mouse CDK5 mRNA and protein (90.1% and 99.7%, respectively) (Fig. 1B). A novel pseudogene

of CDK5 was identified on chromosome 8 (GenBank NT_008157, 92% homology to CDK5 mRNA). This pseudogene contains several stop codons in the 5' region. The first stop codon is found within the fourth triplet (TAA).

For expression analysis of CDK5 and DCX in gangliogliomas, a real time RT-PCR was applied using a relative quantification protocol (Fig. 1C). A significantly lower expression of the CDK5 (55% in gangliogliomas vs controls; *t*-test $P<0.05$) and DCX genes (30% in gangliogliomas vs controls; *t*-test $P<0.005$) was observed in surgical tumor tissue. As control, normal CNS tissue (temporal lobe) with equivalent parts of gray and white matter was obtained from epilepsy patients not suffering from neoplastic or maldevelopmental lesions.

Discussion

In the present study, we have tested the hypothesis that molecular genetic alterations of the DCX and CDK5 genes occur in highly differentiated glioneuronal tumors. Considering their important role for neuronal development, migration and also apoptosis, these genes appeared as interesting candidates involved in the pathogenesis of gangliogliomas. Vulnerable gene loci previously shown to be associated with malignant gliomas have been found to be unaffected in this glioneuronal neoplasm [33]. SSCP analysis for DCX and CDK5 in a series of 23 tumors failed to detect mutations in the present study. We conclude therefore, that mutational inactivation of the DCX and CDK5 genes does not play a role in the formation of these neoplasms. "*In silico*" hybridization and sequencing analysis identified a CDK5 pseudogene which could be allocated to chromosome 8. This pseudogene appears to be silent since stop codons are present at the 5' region. For mutational studies, the CDK5 pseudogene represents a potential pitfall. Only analysis using primers specific for the genomic CDK5 sequence is suitable to avoid false-positive results.

Levels of DCX and CDK5 transcripts in gangliogliomas were also analyzed here. For this, a highly sensitive real time RT-PCR protocol had to be applied on sufficient amounts of starting mRNA, since CDK5 and DCX are expressed at rather low levels in normal as well as neoplastic CNS tissue (Fig. 1C). Significantly lower expression levels of both CDK5 and DCX transcripts were observed in gangliogliomas compared to mRNA of normal brain from corresponding anatomical sites. Taking transcriptional differences due to individual genetic background into account, non-affected peritumoral CNS tissue could serve as a reasonable control for expression analysis in gangliogliomas as it has been used before in highly differentiated glial neoplasms [20]. This aspect must be addressed in future studies. Molecular alterations may differ between the glial and dysplastic neuronal components of gangliogliomas as we have shown previously for a mutation of the TSC2 tumor suppressor gene, which exclusively affected the glial component [4]. Generally, the low levels of expression of both genes do not allow a cellular resolution by real time RT-PCR starting from laser microdissected samples. Similarly, in situ hybridization did not achieve sufficient sensitivity. Additional experiments will be required to determine the biological significance of this finding.

At this point, it remains unclear whether down-regulation of DCX and CDK5 mRNA underlies the observed lower expression of both genes in gangliogliomas compared to controls. Transcriptional silencing by promoter methylation may constitute a possibility. A second potential mechanism for reduction of CDK5 and DCX transcript levels in gangliogliomas would involve lack of reelin pathway signaling. A recent study shows that impaired function of more than a single component in the reelin signaling cascade results in a synergistically negative effect on positioning of cortical neurons in the developing mouse brain [24]. Although mutational analysis of CDK5 and DCX revealed no sequence alterations of these genes in gangliogliomas, impaired reelin signaling nevertheless represents a potential mechanism for glioneuronal tumor development. Additional reelin pathway components such as reelin itself and its receptors, fyn as well as mDab1 and p35 constitute interesting candidates to be studied by expression and gene sequence analysis in gangliogliomas. Neoplastic transformation of the glial component may be driven by other neurodevelopmental tumor suppressor genes, such as tuberin and/or hamartin [4]. Work focusing on these aspects is in progress.

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