Original article

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Sensitive quantitative detection of somatic mosaic mutation in "double cortex" syndrome

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ABSTRACT – *Aims.* Somatic mutation of the lissencephaly-1 gene is a cause of subcortical band heterotopia ("double cortex"). The severity of the phenotype depends on the level of mutation in brain tissue. Detecting and quantifying low-level somatic mosaic mutations is challenging. Here, we utilized droplet digital PCR, a sensitive method to detect low-level mutation.

Methods. Droplet digital PCR was used in concert with classic genotyping techniques (SNaPshot assays and pyrosequencing) to detect and characterize the tissue mosaicism of a somatic mutation (*LIS1* c.190A>T; p.K64X) in a patient with posterior bilateral SBH and refractory epilepsy.

Results. The high sensitivity of droplet digital PCR and the ability to target individual DNA molecules allowed us to detect the mutation at low level in the brain, despite the low quality of the DNA sample derived from formalin-fixed paraffin-embedded tissue. This low mutation frequency in the brain was consistent with the relatively subtle malformation resolved by magnetic resonance imaging. The presence of the mutation in other tissues from the patient permitted us to predict the timing of mutagenesis.

Conclusion. This sensitive methodology will have utility for a variety of other brain malformation syndromes associated with epilepsy for which historical pathological specimens are available and specific somatic mosaic mutations are predicted.

Key words: *LIS1* gene, somatic mosaic mutation, subcortical band heterotopia, Double Cortex

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The most frequent recognized cause of subcortical band heterotopia (SBH) is mutation of the doublecortin gene (*DCX*), which gives rise to an X-linked dominant disorder presenting in females (Gleeson *et al*., 1998). Rarely, somatic mosaic mutations in the lissencephaly-1 gene (*LIS1*; also known as *PAFAH1B1*), affecting only a portion of migrating neurons in the brain, manifest in a "double cortex" pattern of SBH (Jamuar *et al*., 2014; Mineyko *et al*., 2010; Pilz *et al*., 1999; Sicca *et al*., 2003; Uyanik *et al*., 2007). Such mutations are estimated to account for 5 to 10% of lissencephaly cases (reviewed in Poduri *et al*. 2013).

Here, we utilized a sensitive assay to investigate a *LIS1* mutation (p.K64X) in a patient with SBH and detect it for the first time in brain tissue, confirming the diagnosis. Using the droplet digital PCR (ddPCR) technique, and two other classic molecular approaches, we also predicted the timing of mutagenesis to be very early during development based on detection of the mosaic mutation in other tissues, a finding consistent with the "double cortex" pattern. We show that the ddPCR technique is powerful for identifying low-level somatic mosaic mutations that cause brain malformations when historical pathological specimens are available.

Materials and methods

Clinical subject

We studied a patient with refractory focal epilepsy associated with mild intellectual disability and delayed speech. Genomic DNA was extracted from blood using the Qiagen QIAamp DNA Maxi Kit (Hilden, Germany). Saliva was obtained using the Oragene kit and genomic DNA was extracted using the prepIT■L2P kit (DNA Genotek Inc, Ontario, Canada). For formalin-fixed paraffin-embedded (FFPE) brain tissue, phenol-chloroform extraction or the Qiagen FFPE Tissue Kit was used. The Human Research Ethics Committee of Austin Health, Melbourne, Australia, approved this study (Project No. H2007/02961). Informed consent was obtained from the participant for involvement in the study and the use of clinical information and images. All experiments were performed in accordance with the relevant guidelines and regulations of this committee.

Droplet digital PCR

We designed custom probes (WT: VIC-ATTACAAAAG**A**AGGTAACTAA-MGB-NFQ and K64X: FAM-ATTACAAAAG**T**AGGTAACTAA-MGB-NFQ) and primers (LIS1.2 FWD-BIOT 5′-TGGAAAAAAAATGGACATCTGTTA and LIS1.2 REV 5′-TGCA GAAGAATGTTATTTTCAGAA) to detect the *LIS1* c.190A>T (p.K64X) mutation and wild-type allele. Droplet generation, PCR cycling, and droplet reading were performed according to the manufacturer's recommendations (Bio-Rad, Hercules, CA). Briefly, probes and primers were mixed with 2x ddPCR Supermix for probe (Bio-Rad) at 217 nM and 435 nM final concentrations for each probe and each of the primers, respectively, and mixed with 10 ng of DNA sample to a final volume of 23 µl. In total, 20 µl of reactions were loaded in an 8-channel droplet generator cartridge (Bio-Rad) and droplets were generated with 70 µl of droplet generation oil (Bio-Rad) by using the manual QX200 Droplet Generator. Following droplet generation, samples were manually transferred to a 96-well PCR plate, heat-sealed, and amplified on a C1000 Touch thermal cycler using the following cycling conditions: 95°C for 10 minutes for one cycle, followed by 40 cycles at 94°C for 30 seconds and 55°C for 60 seconds, one cycle at 98°C for 10 minutes, and 12°C for an unlimited period. Post-PCR products were read on the QX200 droplet reader (Bio-Rad) and analysed using the QuantaSoft software.

SNaPshot and pyrosequencing assays

The p.K64X mutation was PCR amplified using specific primers to the third coding exon of the *LIS1* gene using the reference human gene transcript (RefSeq transcript NM_000430). The standard protocol on a Veriti thermal cycler (Applied Biosystems, Carlsbad, CA) was used for PCR amplification. PCR products were used for SNaPshot and pyrosequencing assays as follows.

For SNaPshot, PCR products were purified using exonuclease I (2 units) and shrimp alkaline phosphatase (5 units) (EXOSAP) treatment. Reactions were then set up using Multiplex Ready Reaction Mix and pooled control and sample PCR products and primers, and thermal cycling completed on a Veriti Thermal Cycler according to the manufacturer's instructions. SNaPshot products were then subjected to post-extension EXOSAP and resolved on a 3730xl DNA Analyzer (Applied Biosystems).

For pyrosequencing, PyroMark Gold Q96 SQA Reagents (Qiagen) and custom biotin-5′ labelled forward primer (LIS1.2 FWD-BIOT 5′-TGGAAAAAAAATGGACATCTGT TA), reverse primer (LIS1.2 REV 5′-TGCAGAAGAATGTTATTTTCAGAA), and sequencing primer (5′-AAAGAAAAAAGACTTAGTTA) were used on a PyroMark Q96 instrument (Qiagen) according to the manufacturer's instructions. Data were analysed with Pyro Q-CpG software (Qiagen, version 1.0.9), as previously described (Lim *et al*., 2014).

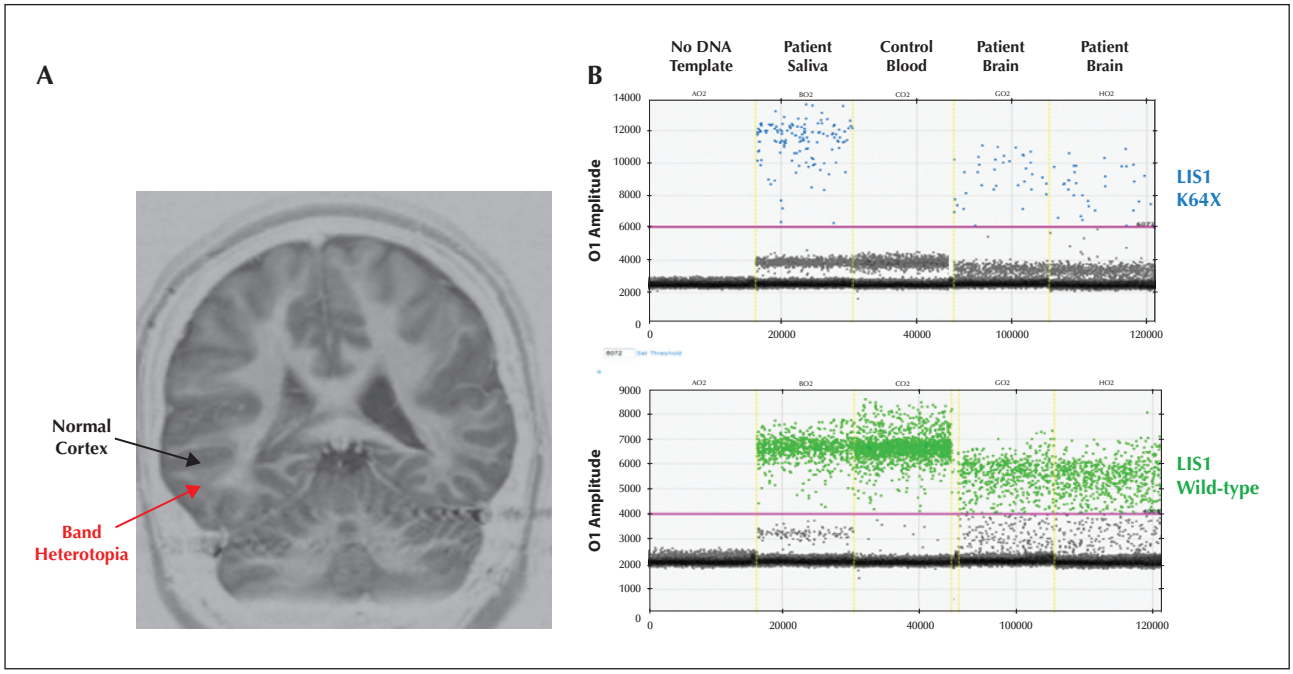

Figure 1. *LIS1* mutation detected by ddPCR in an Australian patient with "double cortex" syndrome. (A) Brain MRI of the Australian patient showing a coronal view of normal cortex (black arrow) and bilateral posterior subcortical band heterotopia (red arrow). (B) ddPCR read-out showing droplets positive (blue) for K64X mutant probe in brain- and saliva-derived DNA from the patient. Droplets positive for wild-type probe are green and droplets without DNA template are grey.
NTC: no template control; 7354-5: saliva; ANON34: healthy control blood-derived DNA; 7354-12: temporal lobe-derived brain DNA run in duplicate.

Results

Clinical report

The 40-year-old patient had multiple febrile seizures between 2 and 4 years of age. She had delayed speech and mild intellectual impairment. At age 7 years, focal seizures began, characterized by an aura of fear, followed by loss of awareness and bilateral dystonic posturing. Seizures occurred in clusters and sometimes evolved to bilateral convulsive seizures. She did not respond to antiepileptic drugs and video-EEG monitoring at age 14, in 1991, suggested a left temporal onset. MRI at that time, using a 0.3T instrument, was regarded as normal and she underwent a standard left anterior temporal lobectomy. Histopathological examination of the left temporal lobe, hippocampus, and uncus specimens collected at surgery revealed cerebral neocortex, white matter, and periventricular grey matter. There was mild molecular layer gliosis but no evidence of hippocampal sclerosis or dysplastic cortex. Seizures did not improve post-surgery. Reinvestigation revealed independent bilateral posterior quadrant seizures and 1.5T MRI (*figure 1A*) revealed bilateral posterior SBH. Clusters of focal seizures continued into her forties despite multiple combinations of antiepileptic drugs.

Mutation detection by droplet digital PCR

ddPCR uses microfluidics and surfactant chemistries to emulsify input DNA into thousands of uniformly-sized droplets, and then amplify them with fluorescently labelled TaqMan probes before measuring fluorescence on a droplet reader, as we and others have previously described (Oxnard *et al.*, 2014; Tsao *et al.*, 2015). Based on fluorescence intensity, the number of mutation positive and wild-type templates is quantified in order to calculate the frequency of a mutant allele. While this approach is "site-specific", relying on prior knowledge of the precise mutation from pre-screening, it has the advantage of being highly sensitive (down to 0.1% frequency) making it 10-fold more sensitive than sequencing (Abyzov *et al.*, 2017). The approach is highly suitable for old, degraded DNA from FFPE specimens because individual DNA molecules can be investigated. For these reasons, it was the method of choice for the patient studied here. The patient's formalin-fixed and paraffin-embedded (FFPE) surgical sample from 1991 was retrieved and analysed along with a saliva DNA sample. The brain-derived DNA sample, extracted using the Qiagen FFPE Tissue Kit, was of poor quality (260/280 ratio: 1.95; by Nanodrop), produced a low yield (50 µl at 28 ng/µl by Nanodrop), and was highly degraded given it was

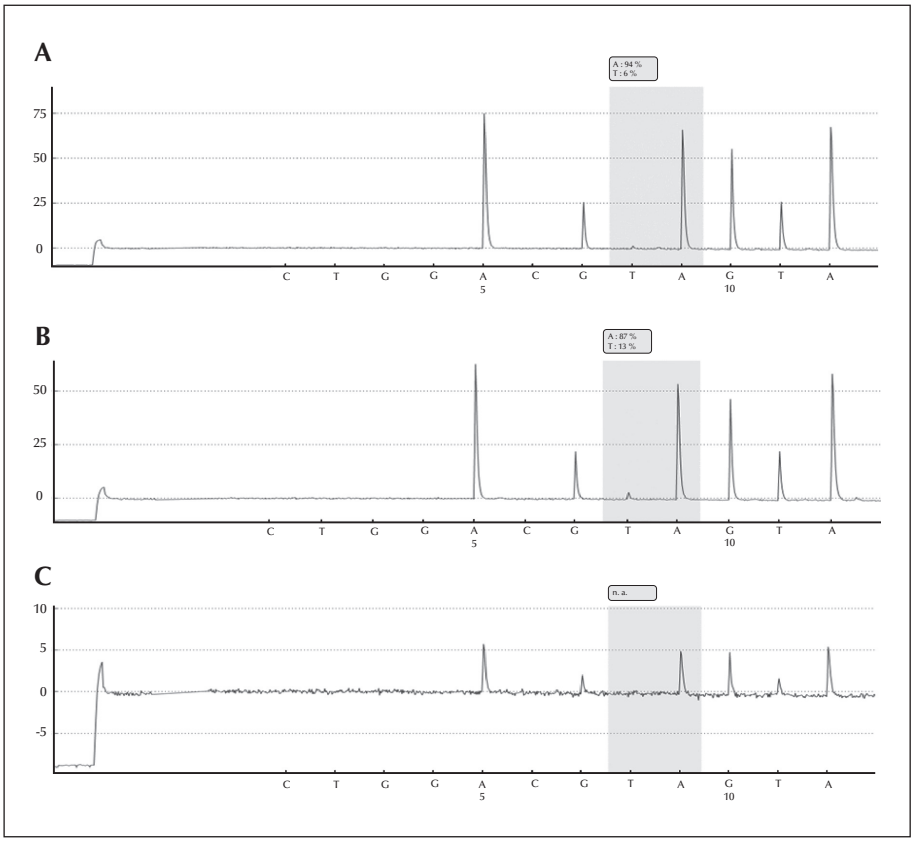

Figure 2. *LIS1* mutation detected by pyrosequencing using blood- and saliva-derived DNA. Pyrosequencing assay results show detection of the mutant p.K64X allele in blood (A) and saliva (B), but not in brain (C). The c.190 wild-type (A) and mutant (T) allele are shown in the shaded box. N.a.=no call.

obtained and fixed over 25 years ago. Using sensitive ddPCR, the *LIS1* p.K64X mutant allele was detected in DNA extracted from FFPE brain tissue using the Qiagen FFPE Tissue Kit (~5% mutant allele frequency) and saliva using the prepIT■L2P kit kit (~13% mutant allele frequency) (*figure 1B*). No copies of mutant template were detected in the healthy control blood-derived DNA extracted using the Qiagen QIAamp Maxi Kit (*figure 1B*).

Molecular analyses of other tissues

We previously reported the LIS1 p.K64X mutation in blood-derived DNA of the patient by subcloning (Jamuar *et al*., 2014). This finding was confirmed here by SNaPshot and pyrosequencing assays (~6% mutant allele frequency) (*figure 2*A). We were also able to detect mutant allele in saliva (~13% mutant allele frequency) at the same level as that achieved by ddPCR, providing confirmation by an orthogonal method (*figure 2*B). However, we failed to identify the allele in phenol-chloroform-extracted brain-derived DNA by pyrosequencing (*figure 2*C). The presence of the mutation in multiple tissues from different lineages suggests that it arose very early post-zygotically, a phenomenon we have previously described for another epilepsy syndrome (Vadlamudi *et al*., 2010).

Discussion

Our findings confirm that somatic *LIS1* mutation is an important cause of SBH and that such mutations can be present at very low levels in brain tissue due to mosaicism. They may arise very early during development, as suggested by their presence in two distinct cellular lineages. Furthermore, these low-level somatic mosaic mutations have been challenging to detect in FFPE tissue because the DNA quality is generally lower than that from other tissue sources, and the sample often contains more impurities due to the fixing and embedding process which we have shown can interfere with mutation detection methods (Do

and Dobrovic, 2012, 2015). As such, the novelty of this report is not the discovery of the mutation itself, in light of our previously published work (Jamuar *et al*., 2014), but rather the confirmation of the presence of the mutation in the brain by application of a new and highly sensitive technique for low-frequency mutation detection, suitable for old and degraded brain tissue DNA. This is important and timely for patients with brain malformations and epilepsy because much of the biobank tissue available at most clinical research centres is still historical formalin-fixed, paraffin-embedded brain specimens, similar to that studied here. That these historical specimens can be successfully investigated for mutation, even when traditional approaches have failed, is important for future investigation of somatic mutation.

The mutation was not detectable in the archival brain tissue by conventional approaches, such as SNaPshot or pyrosequencing using traditional phenol-chloroform extraction. A ~5% frequency for the former approach is below the threshold of detection, and for the latter, the poor quality of the FFPE-derived brain DNA sample likely impeded detection. Despite these significant limitations, ddPCR was sensitive enough to detect the low level mutant allele in the brain sample. The use of the Qiagen FFPE Tissue Kit is also likely to have helped overcome these challenges. Together, these approaches are important tools to maximise detection of mutant allele signal from poor quality DNA samples.

The low-level somatic mosaic *LIS1* mutation reported here in brain is consistent with the clinical presentation and imaging findings of the patient. Patients heterozygous for *LIS1* mutation have the much more severe defect of lissencephaly, presumably a consequence of all neurons expressing the mutant allele, leading to a more diffuse and severe neuronal migration defect (Gleeson *et al*., 1998). This methodology is likely to have significant utility for a variety of other brain malformation syndromes associated with epilepsy for which there is prior knowledge of the somatic mosaic mutations involved from pre-screening, such as focal cortical dysplasias, or for which there is a known recurrent mutation, such as in Sturge-Weber syndrome (Shirley *et al*., 2013). In addition, this methodology may be used for important applications in situations where available DNA templates for neurological diagnosis are present at low levels or are of poor quality, such as from FFPE tissue. □

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TEST YOURSELF

(1) Are somatic mutations inherited?

(2) Can droplet digital PCR be used to detect low-level mutations?

(3) Is it possible to deduce the timing of mutagenesis based on the detection of mutations in tissues of different origin?

Note: Reading the manuscript provides an answer to all questions. Correct answers may be accessed on the website, www.epilepticdisorders.com, under the section "The EpiCentre".