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Detection of microchromosomal aberrations in refractory epilepsy: a pilot study

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ABSTRACT – Seizures often occur in patients with microchromosomal aberrations responsible for moderate to severe intellectual disability. We hypothesised that epilepsy alone could be caused by microdeletions or microduplications, which might also relate to epilepsy refractory to medication. Chromosomes from 20 subjects with epilepsy and repeated failure of antiepileptic medication were examined using molecular methods. Firstly, the 41 subtelomeric regions were scanned using fluorescence *in situ* hybridization and multiplex ligation-dependent probe amplification. Secondly, a genome-wide scan was carried out using oligonucleotide-array comparative genome hybridisation on two platforms: Nimblegen and Agilent. Two aberrations (2/20) were identified: a recurrent microdeletion at 15q13.3 previously characterised in patients with seizures that generally respond to medication, and a novel 1.15 Mb microchromosomal duplication at 10q21.2 also present in the unaffected mother. We conclude that gene content of microchromosomal aberrations is not a major cause of refractory seizures, but that microchromosomal anomalies are found in an appreciable fraction of such cases.

Key words: array-CGH, epilepsy, FISH, microchromosomal abnormality, MLPA, subtelomere

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High resolution molecular approaches are now replacing light microscopy in cytogenetics. Small structural chromosomal aberrations are detected by genome-wide oligonucleotide-array comparative genome hybridization (CGH), which interrogates the entire human genome (Friedman

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et al., 2006). This approach simultaneously detects benign copy number variants (CNVs) comprising part of the normal differences between individuals, making the interpretation of low frequency and novel CNVs as benign or pathogenic challenging (Sharp *et al.*, 2008). One recurrent 1.5 megabase (Mb) interstitial deletion at 15q13.3 accounts for one percent of cases with idiopathic generalised epilepsy (IGE) (Dibbens *et al.*, 2009; Helbig *et al.*, 2009). The same microdeletion has been detected in individuals with additional or alternative manifestations including intellectual disability (ID), autism spectrum disorders, schizophrenia (Mulley and Dibbens, 2009) and sometimes without any apparent disease phenotype (Dibbens *et al.*, 2009; van Bon *et al.*, 2009). Why the pathogenic CNVs can be so variable in their expressivity, and even nonpenetrant in some carriers, remains to be determined. Apart from targeted and anecdotal reports (de Kovel *et al.*, 2010; Dibbens *et al.*, 2009; Helbig *et al.*, 2009; Heron *et al.*, 2007; Marini *et al.*, 2009; Mulley *et al.*, 2006) there are no published genome-wide surveys in epilepsy cohorts to ascertain the overall rate of microchromosomal abnormalities associated with seizures, either treatable or refractory.

Extensive studies focussed on rearrangements of the subtelomere regions have identified pathogenic changes in patients who have both seizures and ID (Baker *et al.*, 2002; Colleaux *et al.*, 2001; Davies *et al.*, 2003; Kleczkowska *et al.*, 1993; Knight-Jones *et al.*, 2000; Martin *et al.*, 2002; Meinecke and Vogtel, 1987; Popp *et al.*, 2002; Rio *et al.*, 2002; Rossi *et al.*, 2001; Slavotinek *et al.*, 1999). For example, a study by Anderlid *et al.* (2002) found that four of their ten patients with ID and subtelomeric rearrangements also had seizures. Where described, predominantly generalised seizures have been identified in these patients, including myoclonic, absence, clonic (Knight-Jones *et al.*, 2000) and generalised tonic-clonic seizures (Baker *et al.*, 2002; Knight-Jones *et al.*, 2000; Slavotinek *et al.*, 1999), infantile spasms were the only other seizure type noted (Knight-Jones *et al.*, 2000; Rossi *et al.*, 2001).

Thirty per cent of idiopathic epilepsies have an inadequate response to antiepileptic drugs (Kwan and Brodie, 2000). Most cases with treatment failure have no known aetiology and have normal structural brain imaging. Refractory epilepsy can occur in the setting of normal intellect, but it can also be associated with varying degrees of ID. This association with ID may be due to a shared underlying aetiology, or in some cases an epileptic encephalopathy may cause the ID.

The 15q13.3 microdeletion is one example of a phenotype initially identified in individuals with ID that was subsequently extended to a pure IGE phenotype (Helbig *et al.*, 2009). We know that the larger chromosomal abnormalities visible by light microscopy are often associated with seizures which in many cases are refractory (Singh *et al.*, 2002). Here we posit that the smaller molecularly defined structural variations detectable by emerging technologies may also cause seizures. We examined cases with refractory seizures for the presence of microchromosomal abnormalities. Specific microchromosomal aberrations may lead to the identification of candidate genes for refractory epilepsy.

Patients and methods

Patients

We studied 20 subjects ascertained from the hospital and private epileptology practices of the authors and by referral to our epilepsy genetics research group. We selected cases with refractory epilepsy and no major structural lesions based on neuroimaging. The epilepsy syndrome for each patient was established, including data from EEG and MRI brain scans, where available. Intellectual status, whilst not a selection criterion, was determined by neuropsychological assessment, or when unavailable, clinical observation. The results of other investigations such as cytogenetics and fragile X molecular testing were also obtained. Cases of Dravet syndrome were excluded.

Patient 1 was included in the first phase of this study with subtelomere FISH and MLPA (see below), and was subsequently diagnosed with epilepsy and mental retardation limited to females (EFMR) with a known *PCDH19* mutation (Dibbens *et al.*, 2008). Since EFMR may be responsive to treatment (Scheffer *et al.*, 2008) and her affected sister was not refractory, this subject was kept within the test cohort to determine if additional structural variation might account for the refractory nature of the seizures. Patient 15 was known to have a balanced translocation t(3;16)(p21.32;p11.1) which is also carried by his unaffected father and therefore thought to be unrelated to either the epilepsy or its refractory nature.

Subtelomere FISH

Fluorescence *in situ* hybridization (FISH) was carried out as previously described (Baker *et al.*, 2002). This involved scanning all 41 of the subtelomeric regions from the human chromosome complement for deletions using a standard set of FISH probes.

Multiplex ligation-dependent probe amplification

Multiplex ligation-dependent probe amplification (MLPA) analysis (Schouten *et al.*, 2002) expanded the sensitivity of the subtelomeric scan to detect duplications and smaller deletions, neither of which would be detectable by FISH. Genomic DNA of each patient was isolated using firstly a Qiagen Blood DNA mini kit and then the DNA was re-extracted using a Qiagen QIAquick PCR purification kit. MLPA testing was carried out using the SALSA

PO69 and PO36B human telomere test kits (MRC-Holland, Amsterdam, The Netherlands). Both SALSA PO36B and PO69 kits contain one unique probe per subtelomeric region for all chromosomes except the short arms of acrocentric chromosomes. The two probe sets have no target sequence in common, but are generally within half a Mb of the telomere, have exact and known locations on the DNA sequence map and generally detect duplications and smaller deletions missed by multiprobe FISH (Northrop *et al.*, 2005). In addition, SALSA P036B contains probes in the pericentromeric long-arm regions of the acrocentric chromosomes (referred as to 13*, 14*, 15*, 21*, 22*). MLPA analysis was carried out according to the manufacturer's instructions. PCR products were separated and quantified by capillary electrophoresis on an ABI 3100 Avant DNA analyzer (Applied Biosystem), using GeneMapper analysis software (version 3.7). Interpretation of output was done as described by (Mulley *et al.*, 2006).

Array-CGH

Array comparative genomic hybridisation (array-CGH) was performed initially on a Nimblegen 135K 12 plex whole genome array (HG18 cat no 080310) with a same sex dye reversal. The hybridisation data was analysed using SignalMap software (v1.9 Roche NimbleGen; Madison, Wisconsin). Positive results were confirmed with a second array platform; SurePrint G3 human CGH 8X60K oligonucleotide microarray (Agilent Technologies, Santa Clara CA cat no.G4450A). These results were analysed using DNA Analytics (v 4.0 Agilent Technologies, Santa Clara CA).

The 10q21.2 microduplication detected by array CGH was further confirmed by dye swap and its gene content determined by Ensembl. The 15q13.3 microduplication was confirmed by quantitative PCR as described previously (Dibbens *et al.*, 2009) and its gene content determined by Ensembl to verify that it was identical to the recurrent 15q13.3 microdeletion syndrome associated with a range of previously defined syndromes, including IGE (Mulley and Dibbens, 2009).

The Austin Health Human Research Ethics Committee approved this study and informed consent was obtained for all subjects.

Results

The subjects with refractory seizures had a variety of epilepsy syndromes. Average age of seizure onset was 5.07 years (range: six weeks-16 years). Thirteen of the 20 patients had generalised epilepsy, with generalised spike or polyspike and wave discharges based on EEG recording. Six patients had refractory focal epilepsy and one patient had both focal and generalised epilepsy syndromes. Their clinical details are summarised in *table 1*.

Neuropsychological testing had been conducted on 14 of the 20 patients. Intellectual disability was present in 10/20 patients, with the majority (8) falling in the mild ID range *(table 1)*. Two had borderline intellect and one had normal development with later cognitive decline. MRI brain scans had been performed on 17 patients, with 14 reported as normal and three with non-specific findings. None of the subjects had a documented family history of ID. Eight of the subjects had a family history of epilepsy, including patient 1 with EFMR. Standard cytogenetic analysis had been performed on 14 of the 20 patients. Patient 15 was found to have a balanced translocation t(3;16)(p21.32;p11.1) by standard cytogenetics. The other 13 subjects had normal chromosomes by standard cytogenetics. All 20 patients had standard molecular testing for fragile X syndrome and all were negative.

FISH and MLPA analyses of the 20 participants found no abnormalities in the subtelomere regions. High resolution oligonucleotide array CGH showed no detectable molecular abnormality at or near either of the translocation breakpoints 3p21.32 or 16p11.1 for the translocation in patient 15, whose status remained balanced at the molecular level. Patient 14 had the common recurrent IGE associated 15q13.3 microdeletion of approximately 1.5 Mb detected by both array platforms and confirmed by quantitative PCR. His mother was negative for the microdeletion. DNA from his deceased father was extracted from a paraffin embedded tumour tissue biopsy but was too degraded to obtain a result, thus it was not possible to determine whether the deletion was sporadic or familial. Patient 19 had a novel 10q21.2 microduplication of approximately 1 Mb, inherited from his unaffected mother. The 15q13.3 microdeletion and the 10q21.2 microduplication as detected by both the Nimblegen and Agilent oligonucleotide-array CGH platforms are shown in *figure 1*.

Discussion

Many of the epilepsies have been inferred or demonstrated to have a genetic basis (Helbig *et al.*, 2008; Heron *et al.*, 2007). Chromosomal imbalances have also been described in conditions involving seizures or EEG abnormalities at either the macro- (Singh *et al.*, 2002) or micro-level (de Kovel *et al.*, 2010; Helbig *et al.*, 2009). Unbalanced translocations with deletions of the subtelomeres are known to lead to several severe syndromes involving ID and seizures, including Wolf-Hirschhorn and Miller-Dieker syndromes. Of particular note is ring 20, which is known to have a strong association with intractable epilepsy. Ring 20 formation may affect expression of neuronal genes within the p and/or q arm subtelomeric regions of chromosome 20.

Table 1. Patients with refractory epilepsy.

Patient	Age (y)/ gender	Age at seizure onset (y)	Seizure types	Number of AEDs	EEG	MRI	Intellect	Diagnosis
1	19 / F	2	CPS, SPS, H, SE	6	Right fronto-temporal ictal rhythm	Ventriculomegaly	Mild ID	Refractory focal epilepsy
2	22 / M	4	M, T	5	GSW, GPFA	Normal	Severe ID	Refractory SGE
3	22 / F	14	GTCS, M, Ab	5	GSW, PSW	ND	Normal	Refractory JME
4	16 / M	5.5	FS, GTCS, Ab	3	3Hz GSW	ND	Normal	Refractory generalised epilepsy
5	26 / F	10	GTCS, Ab, At	12	PSW	Normal	Normal	Refractory generalised epilepsy
6	4 / F	0.7	GTCS, CPS, SPS, Ab	3	Normal	Normal	Mild ID	Refractory focal epilepsy
7	41 / F	16	GTCS, M, Ab, NCS	7	2.5-3Hz GSW, PSW	Normal	Normal	Refractory generalised epilepsy
8	36 / F	4	GTCS, M, Ab, At, T, SE, NCS	8	2-3Hz GSW, GPFA with brief seizure	Normal	Normal with later decline	Refractory generalised epilepsy
9	20 / F	6	AEM, M	2	4-5Hz GSW, GPFA	ND	Learning difficulties	Absences with eyelid myoclonia
10	13 / F	2.5	FS, GTCS, CPS, M, H	7	Left temporal discharges	Normal	Borderline	Refractory focal epilepsy
11	20 / F	0.1	GTCS, CPS, SPS, T, SE	5	DS	Normal	Mild ID	Refractory focal epilepsy, ataxia
12	43 / F	10	GTCS, Ab, NCS	15	GSW, PSW	Normal	Normal	Refractory absence epilepsy
13	44 / M	5	GTCS, Ab	7	2-3Hz GSW, PSW	Normal	Mild ID	Refractory SGE
14	14 / M	3	Ab, single T	6	3Hz GSW	Normal	Borderline	Refractory CAE
15	19 / M	4	GTCS, Ab, M, SPS	12	GSW	Normal	Mild ID	Refractory generalised epilepsy
16	16 / F	1.5	Ab, CPS	6	GSW, PSW DS, MFID	Ventriculomegaly	Mild ID	Refractory TLE and CAE
17	14 / M	5	Ab, single T	3	GSW, PSW, MFID	Normal	Mild ID	Refractory CAE
18	19 / F	0.5	TCS, CPS, T	10	Slow spike-wave with GPFA	Normal	Moderate ID with decline	Refractory SGE
19	13 / M	5.5	SGTCS, CPS, SE	2	Irregular GSW	Hippocampal asymmetry	Normal	Refractory focal epilepsy
20	7 / M	2	TCS, Ab, At, M	4	Right temporo-occipital discharges	Normal	Mild ID	Refractory focal epilepsy

ND: not done; ID: intellectual disability; Ab: absences; AEM: absences with eyelid myoclonia; At: atonic; CPS: complex partial seizures; FS: febrile seizures; GTCS: generalised tonic-clonic seizures; H: hemiclonic; M: myoclonic seizures; SE: status epilepticus; SGTCS: secondary generalised tonic-clonic seizures; SPS: simple partial seizures; T: tonic; GSW: generalised spike and wave; PSW: polyspike and wave; GPFA: generalised paroxysmal fast activity; MFID: multifocal interictal discharges; DS: diffuse slowing; CAE: childhood absence epilepsy; IGE: idiopathic generalised epilepsy; JME: juvenile myoclonic epilepsy; SGE: symptomatic generalized epilepsy; TLE: temporal lobe epilepsy.

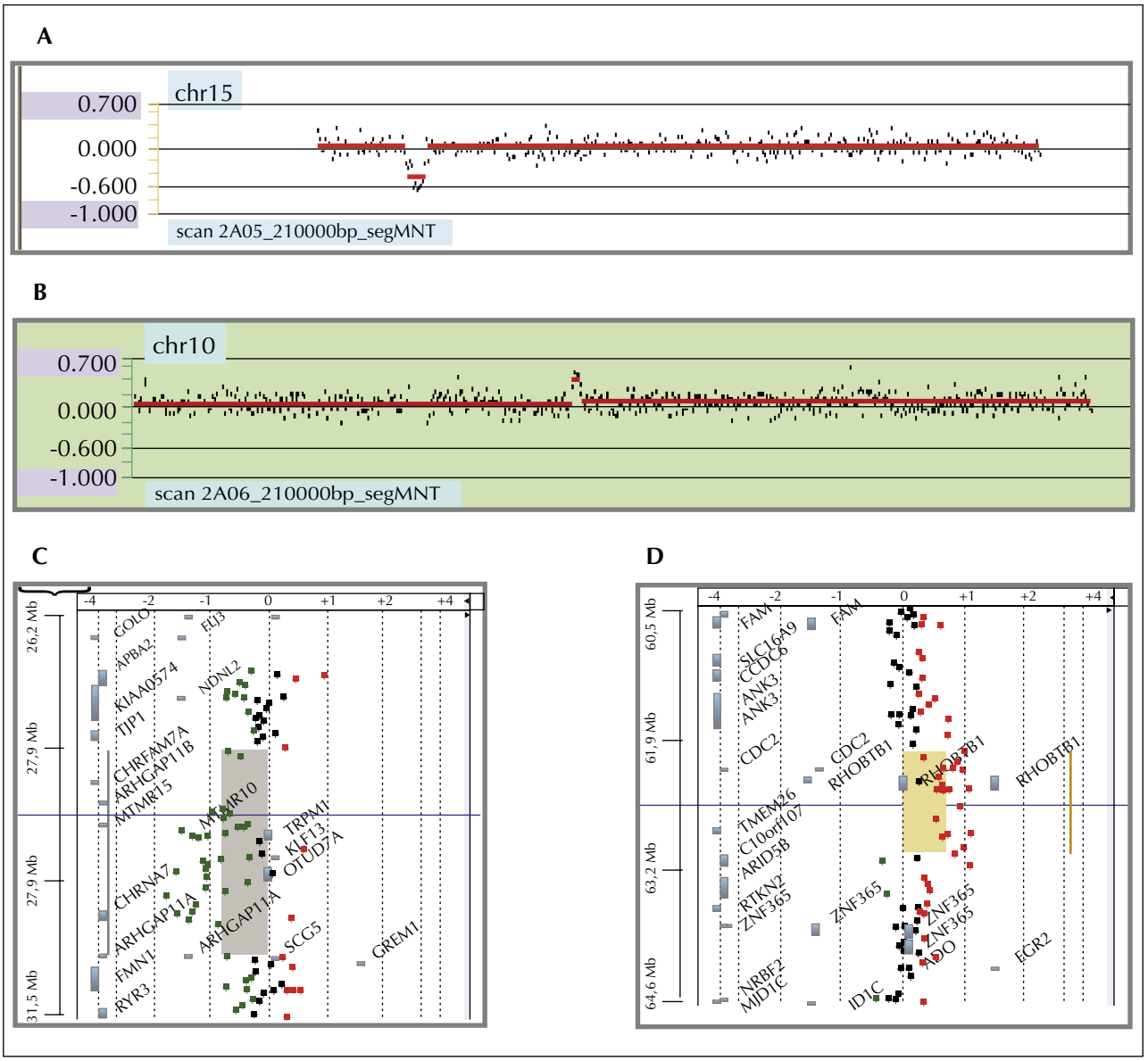

Figure 1. Nimblegen array CGH platform showing: **A**) 15q13.3 microdeletion and **B**) 10q21.2 microduplication; Agilent array CGH platform confirming (**C**) 15q13.3 microdeletion and (**D**) 10q21.2 microduplication.

In the previous studies that have identified subtelomeric changes in subjects with seizures, the nature of the seizures was predominantly generalised (Baker *et al.*, 2002; Knight-Jones *et al.*, 2000; Rossi *et al.*, 2001; Slavotinek *et al.*, 1999). Similarly, the majority of the refractory patients in this study have generalised epilepsies. Subtelomeric aberrations have been found in at least two patients with focal findings based on EEG recording (Knight-Jones *et al.*, 2000). Unfortunately, most reports do not provide details of the seizures experienced by their subjects. We therefore included five patients with definite refractory focal epilepsy and three others with generalised and focal features based either on EEG or clinical features.

For our patients with refractory seizures we failed to find any changes in the subtelomeric regions at the resolution of either the FISH or MLPA molecular probes. Thus, we were not able to demonstrate that refractory seizures were commonly related to any specific subtelomeric microchromosomal rearrangement. The next question was whether interstitial sub-microscopic deletions or duplications anywhere in the remainder of the genome might trigger refractory seizures. The recently reported 15q13.3 microdeletion of approximately 1.5 Mb encompasses the genes *MTMR15, MTMR10, TRPM1, KLF13, OTUD7A, CHRNA7* and *ARHGAP11B* and is associated with ID, autism, schizophrenia and IGE (Mulley and Dibbens,

2009). Based on published data from our group and others (de Kovel *et al.*, 2010; Dibbens *et al.*, 2009) and our own clinical experience, patients with the 15q13.3 microdeletion have seizures that are generally responsive to treatment. This microdeletion, whilst likely to contribute to the epilepsy phenotype seen in our patient, is not likely to be the cause of the refractory nature of his seizures.

The 10q21.2 microduplication spans 1.15 Mb and creates additional intact genomic copies of three genes: cell division control protein 2 (*CDC2*), a Rho GTPase (*RHOBTB1*) and an uncharacterised transmembrane protein 26 gene (*TMEM26*). It also duplicates the sequence encoding an antisense RNA *(BC041470)* and the 5′ end of the Ankyrin 3 gene (*ANK3*). Both *CDC2* and *TMEM26* are expressed in human brain (UCSC Genome Browser v215) making it possible that they contribute to the pathogenesis of seizures. Regulators of Rho proteins such as *RHOBTB* are known to regulate signal transduction and organization of the cytoskeleton. *RHOBTB1* shows ubiquitous expression with high expression in foetal brain and regions of the adult brain including the hypothalamus, thalamus and prefrontal cortex; making it also feasible that duplication of this gene may contribute to the seizure phenotype. The *Ankyrin 3* gene is also a good candidate epilepsy gene, showing high expression in foetal and adult brain as well as being involved in the clustering of sodium channels, which are well known to be involved in epilepsy, in axons. The microduplication overlaps the 5′ end of the normal copy of the *ANK3* gene and therefore possibly disrupts the promoter region and thus expression of the normal gene copy. Thus, the mechanism by which the 10q21.2 microduplication contributes to the occurrence of refractory seizures in this patient is not yet understood.

There are no reports of this 10q21.2 microduplication in DECIPHER (Database of Chromosomal Imbalance and Phenotype in Humans) or in the Database of Genomic Variants (http://projects.tcag.ca/variation). Seven much smaller nonpathogenic CNVs (five deletions and two duplications) have been reported within the 10q21.2 region in the Database of Normal Variation (http://cnv.chop.edu). Whilst the size and novelty of the 10q21.2 duplication is highly suggestive of a role contributing to the seizures in this patient, incomplete penetrance would need to be invoked in order to explain the absence of symptoms in the mother with an affected child. Incomplete penetrance is frequently observed for other pathogenic CNVs in epilepsy and other disorders (Dibbens *et al.*, 2009; Sharp *et al.*, 2008; van Bon *et al.*, 2009).

Eighteen of our 20 subjects did not have microchromosomal lesions. This pilot study suggests that this mechanism is not a frequent cause of epilepsy refractory to therapeutic treatments. The 15q13.3 deletion is known to act as a rare variant with high effect and thus requires additional genetic factors for expression. It is not normally associated with refractory seizures, thus, in our patient, other factors are likely responsible for refractoriness (de Kovel *et al.*, 2010; Dibbens *et al.*, 2009). The 10q21.2 microduplication is novel, but whether it can be excluded as causative for refractory seizures, since it was detected in the mother who did not have seizures, is difficult to assess. A range of other microdeletions are reported not to be associated with refractory seizures (de Kovel *et al.*, 2010), consistent with the precedent established by the 15q13.3 deletion. Furthermore, no relationship has been confirmed between genetic variation and multidrug transporter proteins (Szoeke *et al.*, 2006; Tan and Berkovic, 2006). Why one third of subjects with idiopathic epilepsy are refractory remains unsolved. □

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