

Functional characterization of multiple *DICER1* mutations in an adolescent

Dear Editor,

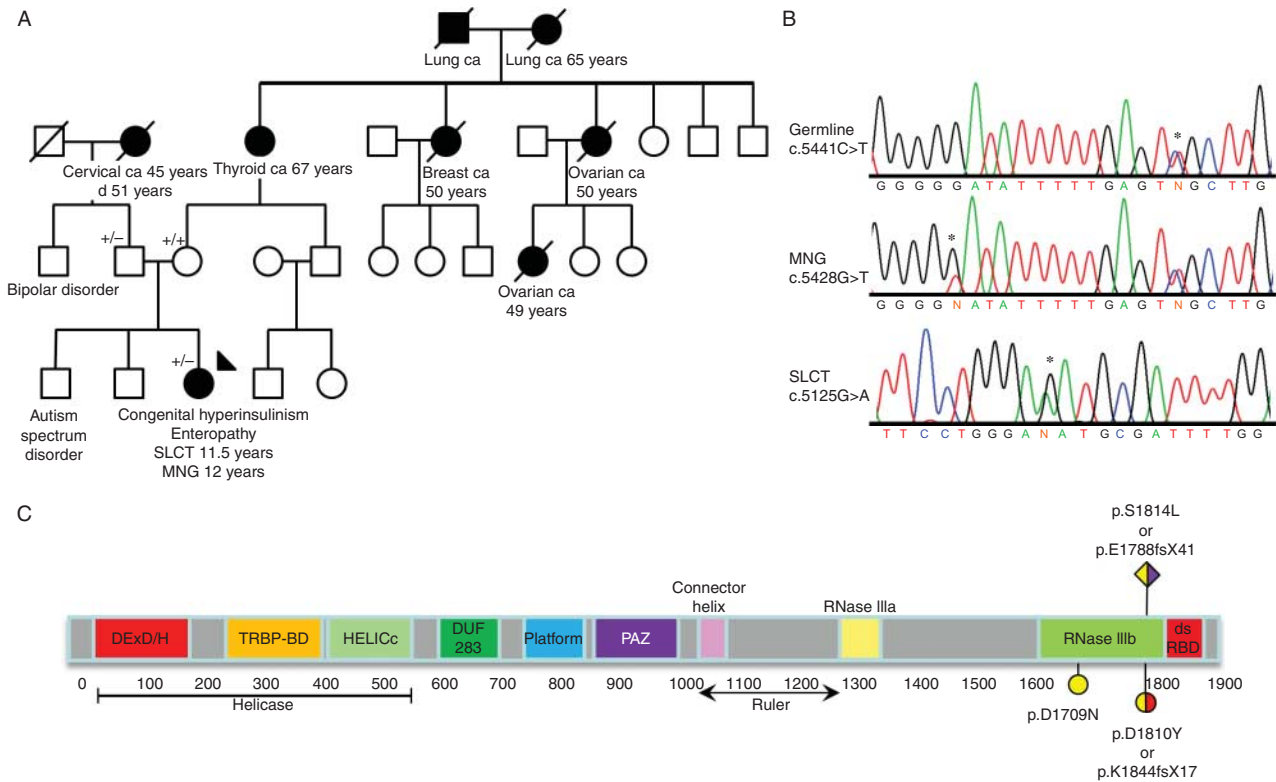
We report the case of an adolescent Caucasian female diagnosed with both multinodular goiter (MNG) and a Sertoli-Leydig cell tumor (SLCT) of the ovary (Fig. 1A). She had, at the age of 11.5 years, lower abdominal pain, but tests were not ordered at this point. At 12 years of age bilateral thyroid nodules were detected and 1 year later a thyroidectomy was performed due to pain and progressive enlargement of nodules. Histopathology showed MNG (i.e., nodular hyperplasia) (Supplementary Figure 1, see section on supplementary data given at the end of this article). Pelvic ultrasound and abdominal computed tomography, undertaken at age 12.5 years because of increasing abdominal discomfort, revealed an ovarian tumor measuring 44×39×56 mm with solid and cystic components. Alpha-fetoprotein (AFP) was elevated at 43 µg/l (normal values: <12 µg/l). Right salpingo-oophorectomy was performed. At this time, the histopathological diagnosis was an adult granulosa cell tumor (GCT) of the ovary (like SLCT, a subtype of sex cord-stromal tumor) with no capsular involvement (Supplementary Figure 2). There was no evidence of lymphadenopathy and no lymph node or omental sampling was performed. Currently, the patient is well, is euthyroid on thyroxine supplementation, and menses are regular.

The patient has a complex past medical history consisting of congenital hyperinsulinism which began shortly after birth and required medical treatment with diazoxide until 24 months of age. She had failure to thrive with recurrent diarrhea. At 25 weeks of age, a jejunostomy Roux-en-Y with button was performed for enteral nutrition. Histology of the small intestinal biopsy demonstrated partial villous atrophy to subtotal villous atrophy (not shown) and she was diagnosed with multiple protein allergies. A low allergen diet was instituted under the supervision of a pediatric immunologist and dietician resulting in improved growth. By 22 months of age, gastrointestinal symptoms had resolved and duodenal and

jejunal biopsies were normal with no villous atrophy or abnormal inflammatory cell component. A skin rash distributed over the lower abdomen and legs was noted at 10 months and persisted through childhood. Skin biopsy was consistent with atopic dermatitis.

The reported maternal family history included papillary thyroid cancer ($n=1$), breast cancer ($n=1$), and ovarian cancer ($n=2$) affecting second degree relatives, while a paternal second degree relative died at the age of 51 due to cervical cancer. These diagnoses remain unconfirmed by pathology reports. Autism spectrum disorder has been diagnosed in the patient's brother and the mother and father are well and asymptomatic (Fig. 1A).

Constitutional genetic analyses performed included a comparative genomic hybridization array, which revealed a novel paternally inherited small (~1.0 Mb) terminal deletion of 7p22.3 of unknown significance. In view of the congenital hyperinsulinism, testing of *ABCC8* was undertaken. This identified a paternally inherited novel mutation that mutates a highly conserved residue: c.946G>A; COSM687051; p.G316R, which likely explains this aspect of her medical history (Nessa et al. 2015). Germline genetic testing for *PTEN* mutations (MPLA and both gDNA and cDNA sequencing of entire coding region, including five common mutations located in the *PTEN* promoter, PathWest Diagnostic Genomics Laboratory, Nedlands, Western Australia) did not identify any gene variants of clinical significance. The patient was referred for *DICER1* genetic analysis. Sanger sequencing of the proband's blood lymphocyte gDNA revealed a novel paternally inherited (Fig. 1 and Supplementary Figure 3) germ-line *DICER1* mutation, c.5441C>T. The mutation c.5441C>T is predicted to encode p.S1814L (Fig. 1B and C). The best described function of *DICER1* protein is to remove the terminal loop from pre-miRNA to produce mature microRNAs (miRNAs). Mature miRNAs are called

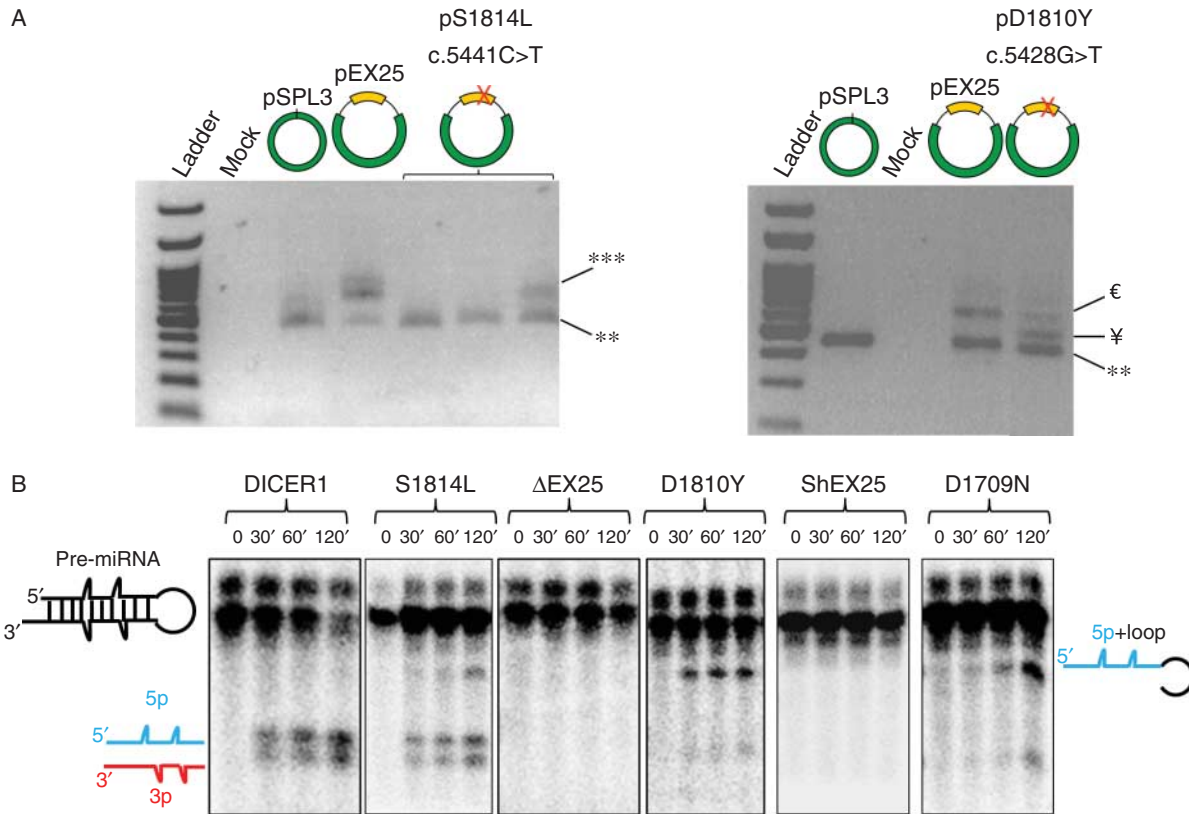
**Figure 1**

The proband has germ-line DICER1 mutation c.5441C>T, somatic mutation c.5428G>T in her MNG, and somatic mutation c.5125G>A in her SLCT. (A) Pedigree showing the germ-line mutation was paternally inherited (see Supplementary Figure 3); +/- indicates heterozygosity for the c.5441C>T DICER1 mutation; although the DICER1 mutation was inherited paternally, we note the possibility of a BRCA1 or BRCA2 mutation in the maternal relatives. (B) Sanger sequencing traces showing mutations as marked with *: germ-line mutation c.5441C>T, SLCT somatic mutation c.5125G>A, and MNG somatic mutation c.5428G>T. (C) predicted position of the germ-line

and somatic mutations on a linear representation of DICER1 protein; circles represent somatic mutations, the diamond represents a germline mutation, yellow color (left half of divided shapes and undivided circle) represents missense mutation, purple color (right half of divided diamond) represents deletion created by exon 25 skip, and red color (right half of divided circle) represents the deletion created by the cryptic splice site in exon 25. SLCT, Sertoli-Leydig cell tumor; MNG, multinodular goiter. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-15-0460>.

5p miRNAs if they originate from the 5 prime end or 3p miRNAs if they originate from the 3 prime end of the pre-miRNA. Typically, germ-line mutations identified in DICER1 are truncating mutations occurring outside of the RNase III domains and are predicted to completely inactivate one copy of the gene (Foulkes et al. 2014). The c.5441C>T missense mutation is doubly unusual in that it occurs within the RNase IIIb domain (Fig. 1C and Supplementary Figure 4) and is not predicted to be truncating. Moreover, S1814 is not one of the critical catalytic (1702 or 1729 (Heravi-Moussavi et al. 2012)) or metal ion binding residues within this domain (these are located at positions 1705, 1709, 1713, 1810, and 1813 (Takeshita et al. 2007)) known to affect processing of 5p microRNAs (miRNAs). We, therefore, set out to establish the effect of the c.5441C>T mutation on miRNA processing.

In silico analysis of c.5441C>T predicted that this single base substitution has two likely protein products. One is the aforementioned missense mutation p.S1814L and the other is p.E1788fsX41 (abbreviated as p.ΔEX25), resulting from exon 25 skipping in the transcript (EX-SKIP (Raponi et al. 2011) see ** in Fig. 2A, left panel). Treatment of lymphoblastoid cells from the father with cycloheximide (to inhibit nonsense mediated decay) did not reveal DICER1 transcript lacking exon 25 (Supplementary Figure 5). We employed an *in vitro* cleavage assay (Supplementary Materials and methods) using radioactively labeled precursor microRNA-122 (pre-miR-122) and FLAG immunoprecipitated p.S1814L, p.ΔEX25, WT, and known 3p and 5p controls to evaluate the functional significance of these two alternate protein products with respect to miRNA biogenesis (see Supplementary Figure 6). Additional variants within the RNaseIIIa/b domains and

**Figure 2**

Analysis of the effect of DICER1 mutations c.5441C>T and c.5428G>T on exon skipping and pre-miR122 cleavage. (A) Exon cassette experiments demonstrate that c.5441C>T can cause exon skipping and c.5428G>T and can create a cryptic splice site. **represents skipped exon 25, ***represents

exon 25 bearing c.5441C>T, € represents exon 25 with 5428G>T, and ¥ represents cryptic splice site. (B) *In vitro* cleavage time course demonstrate the effect of missense mutations on pre-miR122 processing. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-15-0460>.

elsewhere have also been evaluated using our *in vitro* cleavage assay. The variant p.S1814L has reduced 5p and 3p miRNA generation and produces some 5p miRNA + loop (5p+loop), as compared to a normal DICER1 protein (Fig. 2B). This decrease in both 5p and 3p miRNAs is somewhat unexpected given that the mutation is situated in the RNase IIIb domain. This particular substitution could compromise access to the RNase IIIa/b domain by altering protein folding (Supplementary Figure 4). We postulate that the effect of incomplete miRNA generation on transcript silencing is dependent on what species of miRNA is loaded into the RNA induced silencing complex (RISC). For example, off target silencing could occur if either 5p+loop or 3p is loaded instead of 5p. Alternatively, no silencing might result if neither 5p+loop nor 3p is loaded. In contrast, the predicted protein p.ΔEX25 does not produce either 5p or 3p miRNAs (Fig. 2B).

Sanger sequencing of the region encoding the DICER1 RNase III domains in gDNA extracted from the formalin

fixed paraffin embedded (FFPE) ovarian tumor revealed the somatic mutation, c.5125G>A, which is predicted to encode p.D1709N (Fig. 1B and C). We were unable to determine phase of germ-line and somatic mutation in the ovarian tumor. We repeated the *in vitro* cleavage assay using this mutation. As previously reported (Heravi-Moussavi *et al.* 2012), this mutation renders the resulting DICER1 protein incapable of producing 5p miRNAs and causes the accumulation of a 5p+loop species (Fig. 2B). Given the finding of a somatic DICER1 mutation in the ovarian tumor, the initial diagnosis of adult GCT was questioned, since these neoplasms do not appear to be part of the DICER1 syndrome (Foulkes *et al.* 2014). Following pathological review, the histopathological diagnosis was revised to an SLCT of intermediate grade with a minor heterologous component of hepatoid cells (Supplementary Figure 2A, B, C, D, E and F). The hepatoid areas showed focal positivity for AFP by immunohistochemistry (not shown) which may explain the proband's elevated

AFP levels. The tumor tested negative for the presence of the c.402C>G *FOXL2* mutation known to be present in almost all adult GCTs (Shah *et al.* 2009), further supporting the diagnosis of SLCT over adult GCT.

Sanger sequencing of the RNase III-encoding exons in gDNA extracted from a FFPE MNG sample revealed a somatic *DICER1* mutation, c.5428G>T, which is predicted to encode p.D1810Y (Fig. 1B-C). To our knowledge, this is the first report of a somatic *DICER1* mutation in an MNG. Moreover, we were able to show that the germ-line (c.5441C>T) and somatic (c.5428G>T) mutations were *in trans* (Supplementary Figure 7, see section on supplementary data given at the end of this article) and thus the ‘two-hit’ paradigm is fulfilled for MNG pathogenesis. *In silico* analysis of the c.5428G>T mutation predicted an increased chance of exon 25 skipping (EX-SKIP (Raponi *et al.* 2011) denoted by € in Fig. 2A, right panel) and the creation of a cryptic splice site (BDGP (Reese *et al.* 1997) ¥ in Fig. 2A, right panel, and Supplementary Figure 8). *In vitro* exon cassette assay suggests that c.5428G>T does create a cryptic splice site (Fig. 2A, ¥), which is predicted to be translated to a shorter protein, p.K1844fsX17, here abbreviated to ShEX25. The ShEX25 *DICER1* protein cannot produce 5p or 3p miRNAs (Fig. 2B). However, c.5428G>T may also behave as a missense mutation (p.1810Y). In this case, it has a defect in 5p miRNA generation (Fig. 2B).

The most common mechanism by which tumorigenesis occurs in the *DICER1* syndrome (OMIM 601200) is germ-line inactivation of one *DICER1* allele followed by one somatically acquired mutation, occurring on the remaining allele, which affects exons encoding the RNase IIIb domain. This combination results in a decrease in 5p miRNA generation in susceptible initiating cells at a critical time during tissue development, which promotes lesion formation (Foulkes *et al.* 2014). The two hits we observed in the SLCT are consistent with these findings (Fig. 2B). It is notable that we observed the same two-hit phenomenon in the MNG (Supplementary Figure 7), as goiter can be either monoclonal or polyclonal in nature (Kopp *et al.* 1994). Moreover, MNG is not classified by the World Health Organization as a neoplasm, but as a benign hyperplasia (Horowitz *et al.* 2004). Further studies of *DICER1* related goiters are warranted.

This report documents the occurrence of a novel germ-line *DICER1* single base pair substitution (c.5441C>T) localized to within the RNase IIIb domain in a patient with two conditions clearly associated with the *DICER1* syndrome. We consider this mutation deleterious because i) it has not been reported before (ExAC, EVS, ClinVar),

ii) it lies one residue away from the metal ion binding residue 1813, and iii) has a defect in miRNA processing. The clinical phenotype, however, associated with this mutation appears to be somewhat milder than the very severe phenotypes that are associated with either germline or mosaic RNase IIIb mutations that directly impinge on metal ion binding residues (de Kock *et al.* 2014, 2015, Klein *et al.* 2014), emphasizing the need for full characterization of previously unreported *DICER1* variants. This requires a combined approach involving pathology, genetics, biochemistry, and molecular modeling. After the accepted preprint of this paper was published on-line, Rutter *et al.* (2015) reported a family with 6 members with differentiated thyroid cancer, MNG, and SLCT and complete segregation of the same c.5441C>T; p.S1814L germline mutation we report here. This finding strongly supports our conclusion that this mutation is pathogenic.

Using diverse methods, we have demonstrated that the three *DICER1* missense mutations studied here can have distinct effects on miRNA processing, which may be mediated by differing effects of the single base pair changes on the *DICER1* mRNA and protein. Thus, c.5125G>A functions as a missense mutation, leading to loss of 5p miRNA, whereas c.5441C>T, functioning as a missense mutation, affects both 5p and 3p miRNA generation *in vitro* and c.5428G>T can generate a cryptic splice site. We also report the first instance of biallelic *DICER1* mutations in a MNG. Further, the identification of a somatic *DICER1* mutation in the ovarian tumor prompted review of the original pathology, further illustrating the use of adjunct molecular analysis in the diagnosis of uncommon and histologically problematic ovarian neoplasms. Future studies to characterize individual novel missense mutations will need to include cell and/or animal models.

M K Wu¹

L de Kock¹

L S Conwell^{2,3,4}

C J R Stewart⁵

B R King^{6,7}

C S Choong^{8,9}

K Hussain¹⁰

N Sabbaghian¹

I J MacRae¹¹

M R Fabian¹²

W D Foulkes^{1,13}

¹Department of Medical Genetics, Lady Davis Institute Jewish General Hospital, McGill University, Montréal, Quebec, Canada

²Department Endocrinology and Diabetes, Lady Cilento Children's Hospital, Brisbane, Queensland, Australia

³School of Medicine, University of Queensland, Brisbane, Queensland, Australia

⁴Queensland Children's Medical Research Institute, Brisbane, Queensland, Australia

⁵Department of Histopathology, King Edward Memorial Hospital, Perth, Australia

⁶John Hunter Children's Hospital, Lookout Road, Newcastle, New South Wales, Australia

⁷School of Medicine and Public Health, Hunter Medical Research Institute, University of Newcastle, Rankin Park, New South Wales, Australia

⁸School of Paediatrics and Child Health, University of Western Australia, Crawley, Western Australia, Australia

⁹Department of Paediatric Endocrinology and Diabetes, Princess Margaret Hospital for Children, Child and Adolescent Health Service, Subiaco, Western Australia, Australia

¹⁰Genetics and Epigenetics in Health and Disease Genetics and Genomic Medicine Programme UCL Institute of Child Health Great Ormond Street Hospital for Children, London, UK

¹¹The Scripps Research Institute, 3215 Merryfield Row, San Diego, California, USA

¹²Departments of Oncology and Experimental Medicine, McGill University, Montréal, Quebec, Canada

¹³Program in Cancer Genetics, Departments of Oncology and Human Genetics, McGill University, Montréal, Quebec, Canada, H3T 1E2

(Correspondence should be addressed to W D Foulkes; email: william.foulkes@mcgill.ca)

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-15-0460>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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