Supplementary Online Content


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Corrected online January 5, 2011.

This supplementary material has been provided by the authors to give readers additional information about their work.
HRM and DNA sequencing

**DICER1** gDNA analysis

The PCR reactions for High Resolution Melt (HRM) analysis were done in 96 well plates from Bio Rad (Ontario, Canada) using the mastermix and the LCGreen Plus from Transition Technologies (Ontario, Canada). The plates were then transferred to the LightScanner instrument and the melt curves were analyzed by the software provided by Idaho Technologies. This technique was used as a presequencing selection for amplicons harboring variants. Samples with altered melt curves were sequenced and these sequences were analyzed using the SeqMan software (by DNAsstar, Madison, WI). The Sorting Intolerant from Tolerant (SIFT) and Polyphen-2 algorithms were used to predict whether the amino acid changes caused by novel DNA sequence variants might affect protein function.

**DICER1** cDNA analysis

Oligo(dT)-primed cDNAs were synthesized using the SuperScriptTM Pre-Amplification System for First Strand cDNA Synthesis Kit (Invitrogen). Primers were designed in exons 17 and 19 to amplify a 550bp fragment in presence of exon 18 and a 370bp fragment when exon 18 is spliced out due to the c.2805-1G>T mutation (see eTable 2). Absence of exon 18 was confirmed by direct sequencing.

Family E splice site confirmation

Two primers pairs were designed in exons 17 and 19 to amplify 573 bp and 501 bp fragments by PCR in the presence of exon 18 and 390bp and 318 bp fragments, respectively, when the 183 bp exon 18 is spliced out due to the c.2805-1G>T mutation (see eTable2 for all PCR primer sequences and annealing temperatures). All PCR amplifications were performed using the QIAGEN HotStarTaq PCR system (Qiagen, Mississauga, Ontario).

RNA analysis

**mRNA analyses**

Total RNA was extracted from 5 million cells from independent lymphoblastoid cell line (LCL) cultures. 2 µg of mRNA was retrotranscribed using an oligo-dT and Superscript III reverse transcriptase (Invitrogen). Expression of DICER1 mRNA was measured by quantitative real-time PCR using an ABI Prism 7900 HT Real-time PCR system (Applied Biosystems, CA). Reactions were performed in a final volume of 20 µl containing TaqMan Universal PCR Master Mix (Applied Biosystems, CA) and 80 ng of retrotranscribed mRNA. Pre-designed TaqMan assays were used to specifically amplify cDNA derived from DICER1 (Applied Biosystems, Hs00229023_m1) and GAPDH (Applied Biosystems, 433376F) mRNAs.

Inhibition of Nonsense Mediated Decay (NMD)

NMD was blocked by the addition of 28 µg/ml of cycloheximide (Sigma, Oakville, Ontario) within cell culture flasks containing 5 million LCLs at a density of 500,000 cells/ml. After 4 hours of treatment, non-treated and treated cells were pelleted and RNA was extracted using the RNeasy kit (Qiagen, Mississauga, Ontario).
miRNA profiling of lymphoblastoid-derived miRNA

RNA was quantified with the NanoDrop 2000 and the quality of total RNA samples was confirmed with the Agilent 2100 bioanalyzer. 100ng of all RNA samples were end-labeled with Cyanine-3 and hybridized to Unrestricted Human miRNA Microarrays Release 12.0 (Design ID 021827) with the Agilent miRNA Microarray System with miRNA Complete Labeling and Hyb Kit V2 (Agilent, Mississauga, Ontario). Microarrays were scanned with the Agilent DNA Microarray Scanner and quantified with Agilent Feature Extraction 10.5.1.1. Green processed signal from the Feature Extraction output was entered into Agilent GeneSpring 7.3.1 for analysis.

miRNA studies on thyroid tissue

Total RNA was prepared using the mirVana® RNA isolation kit (Ambion Inc., TX) according to the manufacturer’s instructions. Frozen tissue samples were homogenized in lysis buffer using a Polytron tissue homogenizer (Kinematica Inc., NY). RNA from formalin fixed sections was prepared using the 50 ng of total RNA were reverse transcribed using the Megaplex primer Pools (Human Pools A v2.1, Applied Biosystems, CA) and the TaqMan MicroRNA Reverse transcription kit. The cDNA was then applied on a TaqMan low-density array (Human MicroRNA A Card v2.0) and data acquired using an Applied Biosystems 7900HT Fast Real-time PCR system. Data was analysis was performed using the R analysis software (Applied Biosystems, CA). Individual miRNA expression was validated using Taqman miRNA Assays (Applied Biosystems, CA), and values were normalized to U6 snRNA. Fold change was calculated using the DDCT method3.

Loss of Heterozygosity (LOH) Analyses

LOH in FFPE tumors and/or affected tissue was tested using two distinct methods based on the nature of the mutation. Mutations involving insertions or deletions of nucleotides at the DNA level were amplified by PCR using primers end-labeled with γ-P33 using T4 polynucleotide kinase (Invitrogen Life Technologies, Burlington, Ontario) in a forward labeling reaction. The products were then separated by electrophoresis on a 5% denaturing acrylamide gel where the relative intensity of the two bands (wild-type and mutant) could be visually compared in blood and in the affected tissue. In the case of point mutations involving a single nucleotide change at the DNA level, the region surrounding the mutation was amplified by PCR and directly sequenced. The relative intensity of the peaks at the heterozygous mutation site was visually compared between blood and affected tissue.
References

<table>
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<tr>
<th>Study</th>
<th>Age at diagnosis of SLCT</th>
<th>Thyroid Goitre</th>
<th>Familial MNG</th>
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<tr>
<td>Javert and Finn, 1951&lt;sup&gt;4&lt;/sup&gt;</td>
<td>daughter 17 yrs</td>
<td>mother, not stated</td>
<td>not stated</td>
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<td>Goldstein and Lamb, 1970&lt;sup&gt;5&lt;/sup&gt;</td>
<td>cousin (1) 17 yrs</td>
<td>MNG (at autopsy), 53 yrs</td>
<td>not stated</td>
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<td>Jensen &lt;i&gt;et al.&lt;/i&gt;, 1974&lt;sup&gt;6&lt;/sup&gt;</td>
<td>sister (1), 18 yrs</td>
<td>not stated</td>
<td>4 cases in all</td>
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<td>Benfield &lt;i&gt;et al.&lt;/i&gt;, 1982&lt;sup&gt;7&lt;/sup&gt;</td>
<td>16 yrs</td>
<td>MNG, 14 yrs</td>
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<td>O’Brien and Wilansky, 1981&lt;sup&gt;8&lt;/sup&gt; (Family A in this study)</td>
<td>18 yrs</td>
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<td>Zaloudek and Norris, 1984&lt;sup&gt;9&lt;/sup&gt;</td>
<td>sister (2), 14 yrs</td>
<td>not stated</td>
<td>not stated</td>
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<td>not stated</td>
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<td>Gheorghisan-Galateanu &lt;i&gt;et al.&lt;/i&gt;, 2003&lt;sup&gt;11&lt;/sup&gt;</td>
<td>63 yrs</td>
<td>MNG, 44 yrs</td>
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<td>Niedziela, 2008&lt;sup&gt;12&lt;/sup&gt; (Family B in this study)</td>
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<td>MNG, 46 yrs</td>
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<td>This study (Family C)</td>
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<td>MNG 18 yrs</td>
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MNG = multinodular goiter  
SLCT = Sertoli-Leydig cell tumor of the ovary
### eTable 2. PCR Primers

<table>
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<tr>
<th>Amplicon</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Product Size (bp)</th>
<th>Annealing T°C</th>
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<td>Family A: LOH</td>
<td>GGGCTTTATGAAAGACTGCTG</td>
<td>tgcttacCTGTTTCGAAATTTAAA</td>
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<td>Family B: LOH</td>
<td>gaaagcatcattctgttctgaag</td>
<td>TTCAACTCAATGGATATGGTAACCT</td>
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<td>Family C: LOH</td>
<td>TGTTTGATCATCCAGATGCAG</td>
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<td>Family D: Expression</td>
<td>ACGCTCTGGAGAGGTTACCA</td>
<td>AAGAGGTAAGACACAGTATGCTGAA</td>
<td>184</td>
<td>60</td>
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<tr>
<td>Family E: Splicing</td>
<td>ttcggctgaaaaacctg</td>
<td>cacagtgatgtggaattgg</td>
<td>573</td>
<td>60</td>
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<tr>
<td>Family E: Splicing</td>
<td>ctaagctgctagagctt</td>
<td>getecctgcatgaaagg</td>
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<td>60</td>
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<tr>
<td>Family E: LOH</td>
<td>tctttgattttaatcatatcttcc</td>
<td>acataaaaatcgatgaggctga</td>
<td>143</td>
<td>60</td>
</tr>
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</table>

Letters in upper case represent DNA sequences within exons and letters in lower case represent DNA sequences within introns.
eFigure 1A-E. Electrophoreograms of wild-type sequences and mutations in probands with DICER1 mutations and familial MNG/SLCT (A-C) or familial MNG (D-E). Nucleotides shown as “N” indicate sites where dual peaks are present. Arrows indicate mutation site. Family A: the c.871_874delAAAG mutation was found in four individuals and is protein truncating in nature with no specific predicted effect on the PAZ domain. Family B (sequence from cDNA): the c.2457C>G mutation creates a de novo splice site leading to an in-frame deletion of 21 base pairs r.2437_2457del21 (p.Ile813_Tyr819del) resulting in a predicted conformational change of the PAZ domain. Family C: the c.5018_5021delTCAA mutation was found in three individuals and is protein truncating in nature with no specific predicted effect on the PAZ domain. Family D: the c.2516C>T (p.Ser839Phe) missense mutation was found in 20 individuals and is predicted to result in a conformational change in the PAZ domain. Family E (sequence from cDNA): r.2805_2987del183 (c.2805-1G>T, p.Tyr936_Arg996del) mutation was found in seven individuals and is caused by a G to T change which destroys the splice acceptor site upstream of exon 18, leading to skipping of exon 18 and the resulting in-frame deletion of 61 of the 123 amino acids comprising the PAZ domain.
eFigure 2. **DICER1** mutations and their effect on mRNA

A: mutant mRNAs from families A and C are targeted and degraded by nonsense-mediated mRNA decay (NMD). Lymphoblastoid cell lines (LCLs) from carriers of the c.871_874delAAAG (family A, individual II-2) and c.5018-5021delITCAA (family C, individual III-1) mutations respectively, and from a non-carrier were treated or not treated with cycloheximide (CHX), a potent inhibitor of the NMD. Amplification of the 7-8 exon junction (left) or part of exon 23 (right) in treated and non-treated LCLs indicated that in presence of CHX the amount of mutant mRNA was increased in both carriers. No mutant mRNA was detected in the controls.

B: amplification of the exon 15-16 junction of mRNA derived from LCLs from family B, individual II-2 indicated that mutant mRNA produced is insensitive to NMD (left). The upper band seen in this individual is a non-specific heteroduplex. Sequencing of the PCR products revealed that the c.2457C>G mutation leads to the creation of a de novo splice site at position 22 of exon 16 and the in-frame deletion of the first 21 bases of exon 16 (right). Numbered rectangles and intervening bold lines represent exons and introns of **DICER1**, respectively. Red vertical lines indicate the location of the C>G mutation at the DNA level. The splicing pattern is indicated by the pairs of gray connecting lines above exons and introns. The acceptor splicing site of exon 16 is modified due to the presence of the mutation. Depicted exons and introns are not to scale.

C: Splice mutation in family E. Primers in **DICER1** exons 17 and 19 amplify a heterozygous 183bp deletion in a c.2805-1G>T mutation carrier (individual II-4) cDNA (lane 1 = heterozygous mutation carrier; lane 2 = non-carrier). Sequence analysis of the two amplified products from the mutation carrier (right) shows perfect excision of exon 18 in the smaller fragment (middle trace) and wild-type sequence in the larger fragment (upper and lower traces).
**eFigure 3.** Electrophoretogram of the c.2516C>T substitution found in exon 16 of *DICER1* in family D and conservation of the resultant amino acid change p. Ser839Phe.
eFigure 4. Schematic diagram of the partial structure of DICER1 protein. The effect of the splice mutation found in family E is shown. Direct sequencing of the 390 bp and 318 bp PCR fragments revealed an in-frame excision of exon 18. The schematic diagram of DICER1 highlights the functional domains of the protein. The amino acid sequence shown represents the entire PAZ domain, with red amino acids indicating those lost as a result of the splice mutation. Underlined amino acids represent those in the PAZ domain which are part of the DICER1-specific loop.
Figure 5. A) LOH analysis - family A, individual II-2 (panel I), family C, individual III-1 (panel II) and family E, individuals III-3 (panel III) and II-3 (panel IV): no LOH. For panels I and II, Lane 1 = negative control gDNA; lanes 2-3 = proband gDNA (lane 2 blood, lane 3 SLCT); Lane 4 = graphic representation of heterozygous mutation carrier: black = wild-type allele, red = mutant allele. Panel III = reference gDNA showing the mutation from saliva (family E, individual III-3 was used as no germline DNA was available from II-3). Panel IV = DNA from the RMS in individual II-3. B) Immunohistochemistry of the SLCT from family B, individual II-2 (panel I) and the RMS from family E, individual II-3 (panel II, histology and panel III, IHC). Panel I shows increased expression of DICER1 in the Sertoli cells (SC), but less intense staining in Leydig cells (LC). Panels II and III show the the rhabomyosarcoma arising in individual II-3 from family E. The morphological appearance of the tumor suggests the alveolar sub-type (Panel II), but molecular confirmation could not be carried out (hematoxylin, phyloxine and saffron staining, image at 40x magnification). Immunohistochemical staining with anti-DICER1 antibody revealed diffuse cytoplasmic staining of the tumor (Panel III), with endothelial cells within a capillary (top right, white arrow) also staining positively (ab14601, Abcam, MA, 1:50 dilution, labeled with polymer-HRP anti-mouse, stained with DAB+ and counterstained with hematoxylin, image at 40x magnification).
**eFigure 6.** Schematic diagram of the position of reported germ-line *DICER1* mutations. The exons are represented by vertical bars and the introns are represented by the intervening horizontal thin lines. Mutations are shown by their position along the gene, with exons shown approximately to scale. Mutations from this study are shown above the *DICER1* gene structure in green type and previously reported mutations are shown below it in black type.15,16 (* indicates mutation from Reference 16; other mutations from Reference 15). Notations were adjusted to reflect the NM_001195573 coding sequence (Ensembl no.: ENST00000393063). SLCT = Sertoli-Leydig cell tumor of the ovary; FMNG = familial multimodal goitre; PPB = pleuropulmonary blastoma; LC = lung cysts; CN = cystic nephroma; FCN = familial cystic nephroma; ERMS = embryonic rhabdomyosarcoma; RMS = rhabdomyosarcoma.