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Biallelic *DICER1* mutations occur in Wilms tumours

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Running title: *DICER1* mutations in Wilms Tumour

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ABSTRACT

DICER1 is an endoribonuclease central to the generation of microRNAs (miRNAs) and short interfering RNAs (siRNAs). Germ-line mutations in *DICER1* have been associated with a pleiotropic tumour predisposition syndrome and Wilms tumour (WT) is a rare manifestation of this syndrome. Three WTs, each in a child with a deleterious germ-line *DICER1* mutation, were screened for somatic *DICER1* mutations and were found to bear specific mutations in either the RNase IIIa (n = 1) or RNase IIIb domain (n = 2). In the two latter cases, we demonstrate that the germline and somatic *DICER1* mutations were *in trans*, suggesting that the two-hit hypothesis of tumour formation applies for these examples of WT. Among 191 apparently sporadic WT, we identified 5 different missense or deletion somatic *DICER1* mutations (2.6%) in 4 individual WTs; one tumour has two very likely deleterious somatic mutations *in trans* in the RNase IIIb domain (c.5438A>G and c.5452G>A). *In vitro* studies of two somatic single base substitutions (c.5429A>G and c.5438A>G) demonstrate exon 25 skipping from the transcript, a phenomenon not previously reported in *DICER1*. Further we show that *DICER1* transcripts lacking exon 25 can be translated *in vitro*. This study has demonstrated that a subset of WT exhibit two “hits” in *DICER1*, suggesting that these mutations could be key events in the pathogenesis of these tumours.

Key words: miRNA, pediatric tumours, exon splicing silencer

Introduction

Wilms tumour (WT) (OMIM 194070) is the most common pediatric renal malignancy occurring in ~1/10000 live births [1, 2]. WTs are considered to be an excellent model on which to study the proposed relationship between malignancy and aberrant differentiation [3]. Most WTs are sporadic, but at least 5% are familial [1]. Syndromes associated with WT include Wilms tumour, aniridia, genitourinary anomalies, and mental retardation; WAGR (OMIM 194072), Wilms tumour, aniridia, genitourinary anomalies, mental retardation syndrome, and obesity; WAGRO (OMIM 612469), Beckwith-Wiedemann (OMIM 130650), Denys-Drash (OMIM 194080), and Perlman (OMIM 267000) [4-6]. WT has also recently been reported to occur in the *DICER1* mutation-associated familial pleuropulmonary blastoma (PPB) syndrome (OMIM 601200) [7, 8]. Several genes have been identified as being somatically mutated in WT including *WT1*, *CTNNB1*, *IGF2*, *TP53*, *WTX*, *DIS3L2*, *FBXW7*, and *MYCN* [5, 9]. There are overlapping distributions of molecular abnormalities at 11p15, *WTX*, *WT1* and *CTNNB1* in WT [9, 10], but other genes could be contributing to the etiology of WT [3, 11-13]. WT histopathology is heterogeneous and tumours associated with different types of nephrogenic rests and histologies tend to show different underlying genetic changes [14, 15]. A revised model for WT ontogeny takes into account genetic data (*WT1* status, 11p15 imprint control region 1 methylation status, gene expression patterns of other genes) and histological data [14]. Nevertheless, each WT is thought to be of monoclonal origin [16].

Forty years ago, it was hypothesized that the “two-hit” model for cancer could be a mechanism for WT [17]. According to this model, sporadic tumours develop following two independent

events occurring in both copies of the same gene, while hereditary cases involve germ-line transmission of one altered gene and acquisition of a somatic mutation on the other allele.

Although several studies in the 1980s reported WT1-affected WTs behaving in similar manner to retinoblastoma [18-22], subsequently, the “two-hit” model for WT has not been as strongly supported as it has been for retinoblastoma [12], perhaps due to genetic heterogeneity.

DICER1 (GenBank accession no: NG_016311.1; ENSEMBL: ENST000000343455) is an RNase endoribonuclease which catalyzes the cleavage of double-stranded RNA (dsRNA) substrates to produce both mature microRNAs (miRNAs) and short interfering RNAs (siRNAs) [23]. In the case of miRNAs, DICER1 cleavage produces 5' (5p) and 3' (3p) miRNAs form hairpin precursors. MiRNAs are, in turn, estimated to post-transcriptionally regulate the expression of >30% of genes [24]. DICER1 is also involved in regulation of chromatin, alternative splicing, DNA replication timing, genome stability, and cellular senescence [25, 26]. *DICER1* is mutated in the *DICER1* mutation-associated familial PPB syndrome which includes PPB, cystic nephroma, ovarian Sertoli-Leydig cell tumours (SLCT), intraocular medulloepithelioma, pituitary blastoma, thyroid non-toxic goitres/cysts and other phenotypes [7, 8, 27-29].

DICER1 protein is organized in a series of domains of which the RNase IIIa and RNase IIIb domains are thought to dimerize intramolecularly with magnesium to form the active site of the enzyme [30-35] (Figure 1). Interestingly, *DICER1* mutations in the RNase IIIb domain (c.4996-5535; p.1681-1845) were identified in ~60% of nonepithelial ovarian cancers, especially SLCT [36, 37]. The RNase IIIb domain was considered to be a “hotspot” for mutation. As WT and ovarian SLCT are both part of the spectrum of neoplasms present in the *DICER1*-related

pleiotropic tumour predisposition syndrome, we set out to investigate sporadic and familial WT's to determine whether a) somatic *DICER1* mutations were present in WT (with a focus on the RNase IIIb and/or RNase IIIa domains) and b) the “two-hit” hypothesis is supported in WT's with two *DICER1* mutations.

Materials and Methods

Hereditary cases

Case 1 is a male diagnosed with WT at age 2 years (2 yrs) (Figure 1a). The WT was described as a triphasic nephroblastoma with a prominent blastemal component; scattered areas were dominated by immature tubules with primitive to more mature epithelium. There was no evidence of anaplasia, no evidence of nephrogenic rests, and no involvement of one regional lymph node. The adjacent uninvolved kidney was histologically normal.

Case 2 is a female diagnosed with WT at 5 yrs (Figure 1b). This lesion was described as a triphasic nephroblastoma with no evidence of anaplasia. Two of 7 regional lymph nodes were involved by tumour. There was no rhabdomyoblastic differentiation and no evidence of nephrogenic rests. Case 2 also developed a multinodular goiter (MNG) at 10 yrs.

Case 3 is a male diagnosed with WT at 4 yrs (Figure 1c). This tumour was described as a triphasic nephroblastoma with predominant primitive blastemal component comprising two-thirds of the tumour with primitive tubular structures and focal spindle cells indicating the presence of epithelial and stromal components respectively. There was diffuse anaplasia, mostly involving blastema but focally mesenchymal tumour cells, with markedly enlarged

hyperchromatic nuclei and multipolar mitotic figures. There was no evidence of nephroblastomatosis.

Sporadic WTs

191 sporadic WTs were assembled of which 120 were derived from fresh frozen WT samples and 71 were formalin-fixed paraffin-embedded (FFPE) sporadic WT samples (Table 1). The fresh frozen WT DNA samples were obtained from North America and Western Australia. The FFPE WT samples were obtained from Canada and New Zealand. Sex ratio, age at diagnosis, and ethnic distribution of samples is shown in Table 1.

Screening DICER1 in hereditary WT

The three hereditary WT cases (Figure 1a-c) contain germline mutations that have previously been reported [8]. Somatic mutations were determined by PCR from DNA derived from each WT [38] followed by Sanger sequencing (Genome Quebec).

Screening DICER1 in sporadic WT

DNA from 120 fresh frozen WT samples was screened for the entire *DICER1* gene and the 71 FFPE sporadic WT samples were screened for *DICER1* RNase IIIb and RNase IIIa domains. Screening the entire *DICER1* gene was determined by PCR of region of interest (as in [38] with the exceptions for exon 3, 7, 10, and 22 (Table S1)). Exon 7 was amplified as described in [38] and exons 3, 10, and 22 were amplified using a Touchdown PCR program [39] with annealing temperature decreasing by 0.5°C from 65°C to 60°C followed by Sanger sequencing (Genome Quebec). DNA from FFPE tissue was extracted (QIAmp DNA FFPE Tissue kit or REPLI-g FFPE kit) and

only RNase IIIb and RNase IIIa domains were screened (Table S2) amplified using the program above followed by Sanger sequencing (Genome Quebec).

Determining phase of DICER1 mutations

To determine the phase of mutations for cases 1 and 2, fresh frozen WTs were obtained, RNA was collected (TriReagent, Molecular Research Center, Inc.), cDNA was constructed (QuantiTect Reverse Transcription Kit, Qiagen), and PCR amplified (using primers 5'-TAGTGGCATTTCACCAAAC-3' and 5'-ACTGCACACACGGAGAGATG-3' for case 1 and 5'-TTCAACTCTTTATCTGCCAATTAATC-3' and 5'-ACTGCACACACGGAGAGATG-3' for case 2). To determine phase of *DICER1* mutations in somatic case N, a PCR fragment encompassing both mutations was amplified using primers 5'-TGGACTGCCTGTAAAAGTGG-3' and 5'-TGAACCTTTCCCTTTGATG-3'. PCR fragments were TA cloned into pCR-XL-TOPO (Invitrogen). DNA from 10-40 clones was collected (QIAprep Spin Miniprep Kit, Qiagen) and sent for Sanger sequencing (Genome Quebec) using the primers described above. Institutional review board approval was granted to the study by at the relevant institutions.

Exonic Splicing Silencer (ESS) in vitro splicing experiment

DNA from case 2 and case N was amplified using primers 5'-ACTGCTCGAGGTTAGTGCTGTTCAAAAC-3' and 5'-ACTGGGATCCCTGCAATCAAAATGAAAG-3' and cloned in pSPL3 (Invitrogen). DNA was extracted from clones (QIAprep Spin Miniprep Kit, Qiagen) and verified to contain mutation of interest by Sanger sequencing (Genome Quebec) (data not shown). 2.5 µg of each construct was transiently transfected into COS-1 cells (Lipofectamine, Invitrogen) for 24 hours. RNA was

collected (TriReagent, Molecular Research Center, Inc.), cDNA was constructed (QuantiTect Reverse Transcription Kit, Qiagen), and PCR amplified using primers described [40]. PCR products were sequenced using the primers previously described [40] (Genome Quebec) as were the upper band seen for the case N (Figure S3) that were gel purified (QIAquick gel extraction kit, Qiagen).

Western blot analysis of DICER1 lacking exon 25

The pCAGGS-FLAG-DICER1 construct was a gift from A. Gurtan [41]. A control pCAGGS-FLAG vector was created by excising *DICER1* from pCAGGS-FLAG-DICER1 using HindIII (New England Biolabs). A pCAGGS-FLAG-DICERdeltaExon25 construct was made by PCR-amplifying the truncated portion from pTC1 (see Figure 3a) with 5'-CGCTTAGAATTCCTGGGAGA-3'/5'-CAGTGGTACCTCAGCTATTGGGAAC-3', restricting with SacI/KpnI (New England Biolabs) and cloning into SacI/KpnI restricted pCAGGS-FLAG-DICER1. 15 µg of each construct was transiently transfected into COS-1 cells (Lipofectamine, Invitrogen) for 24 hours. Protein lysates were collected as described previously [42]. 50 µg of each protein lysate was loaded onto a Criterion XT precast 4-12% Bis-Tris Gel (BioRad), transferred to nitrocellulose, and probed with 1:1000 dilution of anti-FLAG M2 antibody (Agilent Technologies), 1:2000 dilution anti-Dicer antibody (D38E7, Cell Signaling Technology, Inc.), and 1:2000 dilution of anti-actin antibody (I-19, Santa Cruz Biotechnology).

Results

Somatic DICER1 mutations in three WT cases associated with germline DICER1 mutations

The three hereditary WT cases are summarized in Figure 1a-c [8]. All germ-line mutations cause frameshifts which create premature termination codons (PTCs) (Figure 1, Figures S1-S3). The somatic mutations seen in cases 1 and 2 affect the RNase IIIb domain while the somatic mutation in case 3 affects the RNase IIIa domain (Figure 1d and e). The somatic mutations for both case 1 and 3 are predicted to be probably damaging by

Polyphen2(<http://genetics.bwh.harvard.edu/pph2/index.shtml>) [43] and to affect protein function by SiftBlink(http://sift.jcvi.org/www/SIFT_BLink_submit.html) [44] (Figure 1f).

The germline and somatic mutations for cases 1 and 2 were shown to be *in trans* (Figures S1 and S2). We were unable to determine phase in case 3. Notably, the sister of case 3 was diagnosed with a cystic nephroma, but a search for mutations in the RNase IIIa or RNase IIIb domains did not reveal any variants (data not shown).

While attempting to determine the phase of mutations in case 2, we noted that the somatic mutation resulted in loss of exon 25, formally c.5429A>G;

r.5365_5527delCTTAGGAGATCTGAGGAGGATGAAGAGAAAGAAGAGGATATTGAAG
TTCCAAAGGCCATGGGGGATATTTTTGAGTCGCTTGCTGGTGCCATTACATGGATA
GTGGGATGTCACTGGAGACAGTCTGGCAGGTGTACTATCCCATGATGCGGCCACTAA
TAG (Figures S2). We postulate that c.5429A>G has resulted in a net gain of one additional
exonic silencing site (ESS) in exon 25 (Figure S6, <http://genes.mit.edu/fas-ess/> [45]).

Furthermore, the EX-SKIP program (<http://ex-skip.img.cas.cz/> [46]) predicts that the c.5429A>G variant has a higher chance of exon skipping than the wild type allele. A minigene experiment

comparing splicing efficiency of exon 25 with and without c.5429A>G supports the notion that c.5429A>G promotes exon 25 exclusion (Figure 3). Using RT-PCR, we show that the exon 25-depleted transcript is tumour-specific (Figure S2d). Sequencing of RT-PCR products in the WT of case 2 confirmed the exon 25 skip in the lower species while the upper species showed some evidence of c.5429A>G, suggesting that its ability to promote exon 25 exclusion is not 100% efficient (Figure S2d).

To assess whether or not an exon-25 depleted *DICER1* transcript could be translated, we performed Western blots against FLAG and DICER1 in exogenously over-expressed a FLAG-tagged version of this construct (pCAGGS-FLAG-DICERdeltaexon25) along with full-length DICER1 (pCAGGS-FLAG-DICER) (Figure 4). The exon-25 depleted construct is expressed albeit to a lower degree than full-length DICER1.

Screening sporadic WT samples for DICER1 mutations

Several intronic mutations were observed (Table S3). Twelve different coding mutations were observed (6.1%) of which 5 (2.6%) were either missense or deletion mutations (Table 1, Figure 2), and the remaining 7 (3.6%) were silent mutations (Figure S7). Missense/deletion mutations were seen in four individual WTs (cases C, M, N, and X, Figure 2). With the exception of case C, all of the missense/deletion mutations resided in either the RNase IIIb or RNase IIIa domain (Figure 2). Only one missense mutation (c.2614G>A = rs149242330) was previously assigned an rs number which suggests that all other missense/deletion mutations reported here are novel *DICER1* variants.

Case N was found to have 2 mutations (c. 5438A>G and c.5452G>A, see Figure 2). These mutations were *in trans* (see Figure S4) and due to the presence of clones bearing neither mutation, we reason that both of the mutations were acquired somatically. To prove this point, we obtained constitutional DNA from this case, which was found not to bear either c.5438A>G or c.5452G>A (data not shown). The mutation (c.5452G>A; p.A1818T) is predicted to be probably damaging by Polyphen2 and to affect protein function by SIFTBLink (Table 2). *In silico* analysis showed that exon 25 with c.5438A>G had a net gain of two additional ESSs (Figure S5). Other *in silico* analyses of exon 25 with or without c.5438A>G suggests that c.5438A>G would result in exon 25 exclusion and a minigene experiment supports this hypothesis (Figure 3).

Cases C, M, and X each possessed a single *DICER1* mutation (Figure 2). The mutation in case C was near the PAZ domain (c.2614G>A = rs149242330; p.A872T) and is predicted to be probably damaging by Polyphen2 and tolerated by SIFTBLink (Table 2). Constitutional DNA for case C determined that it was a germline mutation (data not shown). The mutation in case M was in the RNase IIIb domain (c.5330T>A; p.L1777H) is predicted to be possibly damaging by Polyphen2 and to be tolerated by SIFTBLink (Table 2) and is a germline mutation (data not shown). No constitutional DNA was available for case X, therefore we cannot determine if the mutation was of germline or somatic origin. The mutation in case X was between the PAZ and RNase IIIa domains (c.3329A>G; p.I1110S) is predicted to be benign by Polyphen2 and tolerated by SIFTBLink (Table 2).

Discussion

Our identification of three WT cases with two *DICER1* mutations *in trans* support a “two-hit” hypothesis of tumour formation. The germline frameshift mutations in each of the hereditary WT cases are probably subject to nonsense mediated decay (NMD) due to the presence of PTCs. As was demonstrated for other germ-line *DICER1* mutations [47], cloning results for cases 1 and 2 show a lack of PTC-bearing clones which supports the notion of NMD of these transcripts (Figures S1 and S2). In addition, we identified one sporadic WT with two somatic mutations *in trans*, both of which were located in the RNase IIIb domain.

While residues E1705, E1709, D1713, D1810, and E1813 were identified as being important for Mg^{2+} binding and intramolecular dimerization [34], Heravi-Moussavi et al. (2012) suggest that metal binding sites reside at residues 1705, 1709, 1810 and 1813 and active site residues are positioned at 1702 and 1729 [37]. Therefore, the somatic mutation observed in case 1 (c.5138A>C; p.D1713A, Figure 1f) is likely to negatively affect the miRNA-generating ability of *DICER1* in this WT. In case 2, *DICER1* transcript lacking exon 25 missing, would lack the majority of its RNase IIIb domain (we predict p.E1788fsX41) including active site residues 1810 and 1813 [34, 37]. An *in vitro* over-expression of a FLAG-tagged *DICER1* construct lacking exon 25 showed that this construct can be translated albeit not as efficiently as intact *DICER1* (Figure 4). *In vitro* cleavage assays of synthesized *DICER1* RNase IIIb domain bearing either D1810A or E1813A showed a reduced ability to cleave dsRNA substrate [34] which would suggest that miRNA generation may be compromised in case 2. To our knowledge, c.5429A>G is the first example of a single base substitution in *DICER1* leading to exon skipping. The idea

that ESSs can contribute to human disease is an emerging concept [46, 48, 49] which seems to apply in this particular case of *DICER1*-related WT.

Since the data for cases 1 and 2 show that these tumours have two *in trans* *DICER1* mutations, we suggest that kidney cells bearing defective DICER1 are predisposed to develop WT (Figure 5A). In this way, the “two-hit” model [17] may still apply for *DICER1*- affected WTs. We further suggest that it is the aberrant miRNA-generating function of the defective DICER1 that is responsible for the deleterious effects. The same model (Figure 4A) may apply to the SLCT samples with two *in trans* *DICER1* mutations SLCT-19 (germline c.5018_5021delTCAA + somatic c.5125G>A) and SLCT-20 (germline c.2528delC + somatic c.5125G>A), reported previously [37].

Since case N had two *in trans* somatically acquired mutations (c.5438A>G or c.5452G>A, Figures 2, S4) and had no mutations in *WT1* or *CTNNB1* (data not shown), we suggest that this tumour may be an example where two somatic hits to the RNase IIIb domain were sufficient for pathogenesis (Figure 5B). Furthermore, as we have demonstrated that c.5438A>G results in exon 25 exclusion in *DICER1* transcripts (Figure 3), and like case 2, we suggest that miRNA generation in case N would be compromised due reduced levels of DICER1 protein lacking key residues 1810 and 1813. We note that a number of papers using *Dicer1*-null mouse embryonic stem cells were derived from a construct with loxP sites located around the RNase IIIb domain. We suggest, in light of our protein expression work here, that these *Dicer1*-null cells may in fact produce low levels of DICER1 protein lacking the RNase IIIb domain [41, 50, 51].

Twenty of the 95 samples provided by the Children's Oncology Group (COG) (Table 1) possessed somatic *WT1* mutations (data not shown). Of the three WTs with *DICER1* mutations identified from the Whole *DICER1* Screening set, neither of the two cases (M and N) that have available data possessed *WT1* mutations (data not shown). Cases 1, 2, and 3 also did not have *WT1* defects and Case 2 also does not have any abnormalities at 11p13 or 11p15 by cytogenetic or SNP array analysis [52]. Given that the *DICER1*-related tumors (Table S4) do not have a remarkably distinct pathological appearance, it is possible that alternate initiating genetic lesions in *WT1*-mutated and *DICER1*-mutated WTs converge on the same genetic pathway to give rise to lesion formation.

For WTs where only one *DICER1* mutation was identified (cases C, M, and X, Figure 2), there remains a small possibility that second hit occurred in regions not screened with our methodology (e.g. promoter, deep intronic regions, UTRs for all cases; mutations outside of RNase IIIa or IIIb for cases M and X). Case C (c.2614G>A = rs149242330; p.A872T) probably developed a WT due to WAGR syndrome since the child carries a genomic deletion coupled with a somatically acquired insertion in *WT1* (c.1135_1138dupTACG), which is not accompanied by any other *DICER1* or *CTNNB1* mutations (data not shown). The position of the mutated *DICER1* residues in cases C, M, and X suggest that they could influence the miRNA landscape. For example, mutation of the PAZ domain (Y971A, Y972A, E1036A) results in global reduction of miRNA processing [41], therefore case C (mutation just outside the PAZ domain) and possibly case X (mutation between PAZ and RNase IIIa domain) may be associated with a reduced number of miRNAs. Case M (c.5330T>A; p.L1777H) may have some impact on the miRNA generating ability of *DICER1* since residue 1777 is within the RNase IIIb domain

and may affect active site architecture and/or activity and/or protein-protein interactions.

However, the clinical relevance of each of these mutations is as yet unexplored.

Now that a growing list of genes have been associated with initiation and progression of WT, it may be possible to consider stratification of WTs into groups: Scott et al (2012) have selected three groups on the basis of somatic mutations at *WT1*, *CTNNB1*, *WTX* and *TP53*, and imprinting at 11p15 [10]. Another stratification schema, based on gene expression, has also been proposed [14]. It is plausible that *DICER1* mutation status and/or miRNA profiles may allow for further stratification of some of these sub-groups. Perhaps different WT genes may regulate a critical set of miRNAs needed for normal differentiation of renal progenitor cells.

RNase IIIb appears to be a “hotspot” for mutations in our set of WTs (e.g. cases 1, 2, M, and N; Table 1 and Figure 2). This phenomenon was first reported in non-epithelial ovarian cancers [37]. More recently, one such mutation was seen in a testicular germ cell tumour [53], but the pathogenicity of the observed variant is questionable. We have identified two familial WT cases (cases 1 and 2) and one sporadic WT case (case N) which all support the “two-hit” model of WT with respect to *DICER1* (Figure 5A for cases 1 and 2, Figure 5B for case N). Moreover, WT arising in children with germline *DICER1* mutations (Table S4) that have not been investigated genetically may bear a “second hit” in *DICER1*. We postulate that in some WTs, *DICER1* mutations act as key “driver-type” mutations [54] and that these two hits maybe sufficient for tumorigenesis: cells with only RNase IIIb-defective *DICER1* may have an altered variety and/or quality of miRNAs, particularly of the 5p variety [41]. This selective loss of 5p-miRNAs could underpin the oncogenic process by promoting proliferation while at the same time inhibiting

differentiation. Notably, human cancers bearing mutant *DICER1* RNase IIIb mutations at its metal binding sites have a dramatic loss in processing of mature 5p-miRNAs [51]. Two of the single base substitutions (c.5429A>G and c.5438A>G) appear to promote exon 25 exclusion from *DICER1* transcripts. We have shown that exon 25 depleted transcripts can be translated (Figure 4), but whether these mutant proteins alter miRNA production in the same way as a full-length transcript carrying a disease-associated missense mutation in RNase IIIb remains to be determined. The concept of miRNA [55-57] and *DICER1* [58] being critical in normal kidney development and development of kidney disease has been previously discussed [59]. Identifying the specific miRNAs that are missing from *DICER1*-affected WTs and comparing and contrasting them with those miRNAs that are altered in *WT1*-affected WTs may provide clues to the nature of other genes whose over-expression contributes to WT formation. This could lead to further understanding of all the processes that link WT pathogenesis to normal kidney development.

Author contributions

MKW conceived and carried out cloning and ESS experiments, created figures, and wrote the manuscript. NS and MKW carried out whole gene screening. SA-K assisted MWK in conducting RNase IIIa/b screening of FFPE WT. BX, CB, DZ, AER, ME, CC, CSC, AC, TYT, DMI, and PRG provided samples and detailed discussions. WDF led the project. All authors were involved in writing the paper and had final approval of the submitted version.

List of abbreviations

WT= Wilms tumour

DUF= domain of unknown function

dsRBD= double stranded RNA binding domain

COG-RTS= Children's Oncology Group Renal Tumours Study

FFPE= formalin-fixed paraffin embedded

ESS= exonic splicing silencer

MNG= multinodular goitre

UTR= untranslated region

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Table 1. Prevalence of exonic mutations in *DICER1* in sporadic WTs.

Tumour set (N)	Sex ratio (M:F)	Mean age at diagnosis in months (min, max)	Ethnicities of samples	No. of exonic mutations (Frequency)	No. of different exonic mutations (Frequency)	Changes in coding sequence	Predicted amino acid change	Mutation type	No. of tumours with that/those mutation(s)	No. missense /deletion mutations
Whole <i>DICER1</i> screening (N=120)	1:1.4	35 (2, 162)	1 Australian Aboriginal 15 African American 8 Asian 84 Caucasian 8 Hispanic 1 Native American 1 Other 1 Unknown 1 no information	14 (7.3%)	10 (5.2%)	c.2614G>A (rs149242330)	p.A872T	missense	1	
						c.5330T>A	p.L1777H	missense	1	
						c.5438A>G c.5452G>A	p.E1788fsX41 p.A1818T	deletion missense	1	
						c.1935G>A (rs61751177)	p.P645P	silent	2	
						c.2997T>G (rs12018992)	p.L999L	silent	3	
						c.3213A>G (rs148959399)	p.R1071R	silent	1	
						c.3033G>A (rs8019857)	p.A1011A	silent		
						c.4515T>C (rs141308332)	p.S1505S	silent	1	
						c.4680G>A (rs61729797)	p.A1560A	silent		
						c.4680G>A (rs61729797)	p.A1560A	silent	1	
RNase IIIa/b screening (N=71)	1:1.6	38 (3, 159)	1 African 1 Asian 55 Caucasian 14 no information	3 (1.6%)	2 (1.0%)	c.3972G>A (rs45562437)	p.K1324K	silent	1	
						c.3328A>G	p.I1110V	missense	1	
						c.3972G>A (rs45562437)	p.K1324K	silent	1	
Total (N=191)	1:1.5	36 (2, 162)		17 (8.9%)	12 (6.2%)				14 (7.3%)	5 (2.6%)

Table 2. Polyphen2 and SIFTBLink predictions for exonic variants seen in sporadic WTs.

Case	Somatic mutation and predicted protein change	Protein domain affected	Polyphen2 ^I	SIFTBLink ^{II}
C	c.2614G>A=rs149242330; p.A872T	before PAZ	PROBABLY DAMAGING with a score of 0.999 (sensitivity: 0.14; specificity: 0.99)	TOLERATED with a score of 0.33. Median sequence conservation: 2.04 Sequences represented at this position:72
M*	c.5330T>A;p.L1777H	RNase IIIb	POSSIBLY DAMAGING with a score of 0.809 (sensitivity: 0.84; specificity: 0.93)	TOLERATED with a score of 0.10. Median sequence conservation: 1.92 Sequences represented at this position:62
	c.5438A>G; p.E1788fsX41	RNase IIIb	-	-
N	c.5452G>A;p.A1818T	RNase IIIb	PROBABLY DAMAGING with a score of 0.997 (sensitivity: 0.40; specificity: 0.98)	AFFECT PROTEIN FUNCTION with a score of 0.00. Median sequence conservation: 1.93 Sequences represented at this position:68
X	c.3328A>G; p.I1110V	Between PAZ and RNase IIIa	BENIGN with a score of 0.000 (sensitivity: 1.00; specificity: 0.00)	TOLERATED with a score of 0.30. Median sequence conservation: 2.28 Sequences represented at this position:57

^I<http://genetics.bwh.harvard.edu/pph2/index.shtml> [43]

^{II}http://sift.jcvi.org/www/SIFT_BLink_submit.html [44]

Figure legends

Figure 1. Hereditary cases of WT (see [8]; case 1 = family 5; case 2 = family 3; case 3 = family 4) . (a) Pedigree for familial case 1 (proband indicated with arrow). (b) Pedigree for familial case 2. (c) Pedigree for familial case 3. (d) Schematic drawing of DICER1 domains showing amino acid positions and position of acquired mutations. (e) Germline and somatic mutations observed in the 3 familial cases of WT. ^IPhase of mutations in case 3 was not possible because RNA was not available for this tumour. ^{II}DNA and other materials for this case provided by A. Charles and C.S. Choong. DUF= domain of unknown function; dsRBD= double stranded RNA binding domain

Figure 2. Missense/deletion mutations in *DICER1* in sporadic WTs. ⁱⁱⁱsample was FFPE

Figure 3. Cloning strategy to demonstrate c.5429A>G and c.5438A>G act as exonic splicing silencer sites. (a) diagram of experimental strategy; (b) agarose gel showing RT-PCR products of introduced vectors and sequencing results demonstrating that c.5429A>G and c.5438A>G both promote exon 25 exclusion. pSPL3 vector = p; pEX25= pSPL3 containing wildtype exon 25; pTC1 = pSPL3 containing exon 25 with c.5429A>G; p.TC2= pSPL3 containing exon 25 with c.5438A>G.

Figure 4. Western blot of FLAG-tagged DICER constructs. 1= pCAGGS-FLAG; 2= mock; 3= pCAGGS-FLAG-DICER1; 4= pCAGGS-FLAG-deltaexon25 (a) Anti-FLAG blot; (b) anti-Dicer blot.

Figure 5. Models for *DICER1*-related WT formation. (a) Model observed in the familial cases 1, 2, and 3, where a germline mutation in *DICER1* causes NMD and the acquired mutation affects either the RNase IIIa or RNase IIIb domain; (b) Model observed in somatic case N where specific missense mutations in RNase IIIb is required for WT formation.

Figure S1. Hereditary WT case 1

Figure S2. Hereditary WT case 2. (a) Pedigree; (b) germline mutation; (c) somatic mutation; (d) cDNA products seen in blood and WT from familial case 2 as well as sequences from gel purified cDNA products; (e) somatic mutation as seen in exon 25 depleted clones; (f) Cloning results.

Figure S3. Hereditary WT case 3. (a) Pedigree; (b) germline mutation; (c) somatic mutations.

Figure S4. Cloning results for somatic WT case N.

Figure S5. Additional ESS sites introduced by single base substitutions c.5429A>G and c.5438A>G. Output files from <http://genes.mit.edu/fas-ess/>[45]

Figure S6. Silent mutations seen in somatic Wilms tumours. ^{III}DNA extracted from FFPE samples.

Figure 1. Hereditary cases of WT

*<http://genetics.bwh.harvard.edu/pph2/index.shtml>

**http://sift.jcvi.org/www/SIFT_BLink_submit.html

Case	Somatic mutation and predicted protein change	Protein domain affected	Mutant sequence	Wild type sequence	Notes
C	c.2614G>A=rs149242330; p.A872T	before PAZ			Germline mutation
M	c.5330T>A;p.L1777H	RNAseIIIb			Germline mutation
N	c.5438A>G;p.E1788fsX41	RNAseIIIb			c.5438A>G and c.5452G>A are <i>in trans</i> and both were somatically acquired
	c.5452G>A;p.A1818T	RNAseIIIb
X	c.3328A>G; p.I1110V	Between PAZ and RNAseIIIa			No constitutional DNA available

Figure 2. Missense/deletion mutations in *DICER1* in sporadic WT.

Figure 3. Cloning strategy to demonstrate c.5429A>G and c.5438A>G act as exonic splicing silencer sites.

Figure 4. Western blot of FLAG-tagged DICER constructs.

Figure 5. Models for *DICER1*-related WT formation.

a **b** **c** **d**

Cloning results

c.1306insT only	c.5138A>C only	c.1306insT + c.5138A>C	No mutation
8	21	0	0

Figure S1. Hereditary case 1.

Figure S2. Hereditary case 2.

a **b** **c**

Figure S3. Hereditary case 3.

Cloning results

Just c.5438A>G	Just c.5452G>A	c.5438A>G + c.5452G>A	No mutation
24	13	0	2

Figure S4. Cloning results for somatic case N.

GGGGTA
 GGGGGT
 GGGGGG
 TGGGGG
 ATGGGG
 c.5429A>G ATATTGAAGTTCCAAAGGCCATGGGGGGTATTTTGTAGTC
 |.....|.....|.....|.....|
 2560 2570 2580 2590

GGGGAT
 GGGGGA
 TGGGGG TTTGGG
 ATGGGG TTTTGG
 c.5438A>G ATATTGAAGTTCCAAAGGCCATGGGGGATATTTTGGGTC
 |.....|.....|.....|.....|
 2560 2570 2580 2590

GGGGAT
 GGGGGA
 TGGGGG
 ATGGGG
 Wild type ATATTGAAGTTCCAAAGGCCATGGGGGATATTTTGTAGTC
 |.....|.....|.....|.....|
 2560 2570 2580 2590

Figure S5. Additional ESS sites introduced by missense mutations c.5429A>G and c.5438A>G.

Case	Somatic mutation and predicted protein change	Protein domain affected	Mutant sequence	Wild type sequence	Notes
A, B	c.1935G>A=rs61751177; p.P645P	DUF 283			Tumour possesses a somatic WT1 mutation
D, E	c.2997T>G=rs12018992; p.L999L	PAZ			
F	c.2997T>G=rs12018992; p.L999L	PAZ			c.2997T>G appears to be homozygous
G	c.3213A>G=rs148959399; p.R1071R	Between PAZ and RNase IIIa			germline
H	c.3033G>A=rs8019857; p.A1011A	Between PAZ and RNase IIIa			
	c.4515T>C=rs141308332; p.S1505S	Between RNase IIIa and RNase IIIb			c.4514T>C appears to be homozygous
	c.4680G>A=rs61729797; p.A1560A	Between RNase IIIa and RNase IIIb			
I ⁱⁱⁱ	c.3972G>A= rs45562437; p.K1324K	RNase IIIa			
W ⁱⁱⁱ	c.3972G>A=rs45562437; p.K1324K	RNase IIIa			germline
J	c.4680G>A=rs61729797; p.A1560A	Between RNase IIIa and RNase IIIb

Figure S6. Silent mutations seen in somatic Wilms tumours.ⁱⁱⁱDNA extracted from FFPE samples.

Table S1. Additional PCR primers used to screen *DICER1*.

Exon	Forward primer	Reverse primer
3	5'-TTTTTGTAATTTATGGAGGACGA-3'	5'- TGCCAGAAGAGATTAAATGAGTACA-3'
7	5'-CCCCACTGCTAACATTCTGGC-3'	5'- GAGCCGCATTAAGCATATTTTC-3'
10	5'- GGATTTAGAATGCCCAAGAGC-3'	5'- CCTCTTTTGAGATTATTGCCTGT-3'
22	5'- TTA CTGAATTCAGTGAAAAGACAGTG-3'	5'- TGAATGGGTAGAAAACTGTAGAGC-3'

Table S2. Primers used to screen *DICER1* RNase IIIa and RNase IIIb for FFPE samples.

Domain	Forward primer	Reverse primer
RNase IIIb	5'-GGGGATCAGTTGCTATGTGG-3'	5'-CGGGTCTTCATAAAGGTGCT-3'
	5'-ACTTCGGATCCCCTCAGATT-3'	5'-CGATGCAAAGATGGTGTGT-3'
	5'-CGCTTAGAATTCCTGGGAGA-3'	5'-TGACATGGAAGAGCTCAGGA-3'
	5'-CGCTGGCTGTAAAGTACGAC-3'	5'-TGCCGTCAGAACTCTGAAAC-3'
	5'-GGCCTTTTTGCTTACAAGTCAC-3'	5'-ATTAGTGGCCGCATCATGG-3'
	5'-TCACCTTATATCGGGGTTTGA-3'	5'-CACGATGAGATCAACACAAAGTC-3'
RNase IIIa	5'-GCAGAGCCCTTCTATTGGGTA-3'	5'-GCATCAGGGTAAGTGCAAAAT-3'
	5'-CTTGAAATGCTTGCGGACTC-3'	5'-CAAACCACTTTCAGGCACACT-3'
	5'-GGTAGAAAACTGTAGAGCCCTTA-3'	5'-GAGGAAGCCAATTCACAGG-3'

Table S3. Intronic variants seen in sporadic WTs

Variation	% carrying variation
rs114886684	0.51
c.438+18A>G	0.51
c.439-134T>C	0.51
c.573+96C>A	2.55
c.573+114T>A	6.63
c.573+113_115delTGinsAA	4.08
rs117349874	0.51
c.1377-93_94insA	0.51
rs192490028 ^I	1.02
rs2275182	17.35
rs11624081	7.14
rs74899136	1.53
rs188856063	0.51
rs57681406	1.02
rs112284114	0.51
c.2040+58_59delT	0.51
c.2040+60C>T	0.51
c.2040+60_61insT	3.57
c.2040+107C>A	0.51
rs2297730	21.94
c.2116+61_62insA	3.57
rs17091823	1.02
c.2256+95C>T	0.51
rs117996122	2.04
c.2804+66C>A	0.51
rs113189950	0.51
c.2805-217_219delAAT	1.02
c.2805-70C>T	0.51
c.2805-87_89delCAT	0.51
rs142857015	0.51
c.2988-93T>C	0.51
c.3093+149_153delGTTTT	0.51
c.3093+221A>G	0.51
c.3094-8_11delTTAC	0.51
c.4051-39A>T	0.51
c.4051-24G>T	0.51
c.4206+5_6insTT	3.06
rs1778057	3.06
c.4206+21G>T	0.51
c.5096-49_50delG	0.51
c.5096-41G>T	0.51
c.5364+51_52delCT	0.51
c.5527+7T>A	0.51
rs13078	59.69
c.5769+67G>A	0.51

^Ithis variant has previously been reported to be a splice mutation but detailed studies performed showed that it has no effect on splicing (data not shown)

Table S4. Characteristics of reported WT's with germline *DICER1* mutations

Case	Age at diagnosis	Histology	Germline mutation and predicted protein change	Somatic mutation, predicted protein change and domain involved	Other diagnoses in WT patient	Other diagnoses in family	Reference
1	2Y	Triphasic nephroblastoma with a prominent blastemal component; scattered areas dominated by immature tubules with primitive to more mature epithelium. No evidence of anaplasia. No evidence of nephrogenic rests. No involvement of one regional lymph node. Adjacent uninvolved kidney normal.	c.1306dupT; p.S436FfsX41	c.5138A>C; p.D1713A RNaseIIIb	-	PPB in brother	A
2	5Y	Triphasic nephroblastoma. No evidence of anaplasia. 2 of 7 regional lymph nodes involved. No rhabdomyoblastic differentiation. No evidence of nephrogenic rests.	c.2117-1G>A; p.G706AfsX8	c.5429A>G; r.5365_5527delCTTAGGAGATCTGAGG AGGATGAAGAGAAAGAAGAGGATAT TGAAGTTCCAAAGCCATGGGGGAT ATTTTGTGAGTCGCTTGCTGGTGCCAT TTACATGATAGTGGGATGTCACTGG AGACAGTCTGGCAGGTGTACTATCCC ATGATGCGGCCACTAATAG; p.E1788fsX41 RNaseIIIb	MNG	MNG + cERMS in mother; MNG in 3 maternal aunts	A
3	4Y	Triphasic nephroblastoma with a predominant primitive blastemal component (comprising 2/3 of the tumor), with primitive tubular structures and focal spindle cells indicating the presence of epithelial and stromal components respectively. Diffuse anaplasia, mostly involving blastema but focally mesenchymal tumor cells, with markedly enlarged hyperchromatic nuclei and multipolar mitotic figures. No evidence of nephroblastomatosis. Markedly necrotic WT tumor with variation in differentiation of tumor tissue with areas of differentiation into cartilage with whorls of tumor suggesting smooth muscle tumor; areas also suggesting osteoid and rhabdomyosarcoma. No involvement of several lymph nodes. (operation carried out in 1973)	c.912_919dupAGACTGTC; p.R307QfsX8	c.4031C>T; p.S1344L RNaseIIIa	-	CN + lung cyst in sister	A
4	2Y	WT, not otherwise described, with invasion of the renal vein and three lymph node metastases (operation carried out in 1964)	c.3079_3080delCT; p.L1027AfsX3	Tumour not available	MNG at age 17	CN in son	B
5	8Y	"Atypical histology"	c.2988-2_2988-1delAGinsCT	Not reported	MNG + R ovarian SLCT + L ovarian SLCT	None reported	C
6	3Y	WT, not otherwise described, with invasion of the renal vein and three lymph node metastases (operation carried out in 1964)	c.2379T>G; p.Y793X	Tumour not available	R ovarian cyst (endometriosis) Removed at age 23. 35mm peripelvic cyst in remaining L kidney	numerous extended family members affected; pituitary blastoma; PPB + CN; MNG; ovarian tumour (possibly SLCT) + "thyroid problems" + "calcified kidney"; SLCT	D

A= Foulkes et al., 2011; B = TY Tan, personal communication; C= Slade et al., 2011; D = Wildi-Runge et al., 2011 (WT not described in this report)

SLCT – Sertoli-Leydig cell tumour; MNG – multinodular goitre, cERMS – cervical embryonal rhabdomyosarcoma; CN – cystic nephroma, PPB – pleuropulmonary blastoma