Loss of Heterozygosity at 2q37 in Sporadic Wilms' Tumor: Putative Role for miR-562

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Abstract Purpose: Wilms' tumor is a childhood cancer of the kidney with an incidence of ~1 in 10,000. Cooccurrence of Wilms' tumor with 2q37 deletion syndrome, an uncommon constitutional chromosome abnormality, has been reported previously in three children. Given these are independently rare clinical entities, we hypothesized that 2q37 harbors a tumor suppressor gene important in Wilms' tumor pathogenesis.

Experimental Design: To test this, we performed loss of heterozygosity analysis in a panel of 226 sporadic Wilms' tumor samples and mutation analysis of candidate genes.

Results: Loss of heterozygosity was present in at least 4% of cases. Two tumors harbored homozygous deletions at 2q37.1, supporting the presence of a tumor suppressor gene that follows a classic two-hit model. However, no other evidence of second mutations was found, suggesting that heterozygous deletion alone may be sufficient to promote tumorigenesis in concert with other genomic abnormalities. We show that miR-562, a microRNA within the candidate region, is expressed only in kidney and colon and regulates EYA1, a critical gene for renal development. miR-562 expression is reduced in Wilms' tumor and may contribute to tumorigenesis by deregulating EYA1. Two other candidate regions were localized at 2q37.3 and 2qter, but available data from patients with constitutional deletions suggest that these probably do not confer a high risk for Wilms' tumor.

Conclusions: Our data support the presence of a tumor suppressor gene at 2q37.1 and suggest that, in individuals with constitutional 2q37 deletions, any increased risk for developing Wilms' tumor likely correlates with deletions encompassing 2q37.1. (Clin Cancer Res 2009;15(19):5985–92)
implicated in the pathogenesis of Wilms' tumor. CTNNB1, which codes for β-catenin, is mutated in ∼15% of Wilms' tumors but rarely occurs without concomitant mutation of WT1 (7, 9), whereas somatic mutations of WTX are present in 11% to 29% of Wilms' tumors and occur with and without WT1 mutation (7, 9, 10). Interestingly, germ-line mutations of WTX were recently shown to underlie osteopetrosis striata congenita with cranial sclerosis, a X-linked sclerosing bone dysplasia, but these patients had no predisposition to Wilms' tumor or other malignancies, suggesting temporal or spatial constraints on the action of WTX during tumorogenesis (11).

Due to an improved combination of surgery, chemotherapy, and radiotherapy, there has been a dramatic improvement in Wilms' tumor survival over the past 40 years, with the cure rate now approaching 90% (12). Despite this, the molecular pathogenesis of Wilms' tumor and factors determining the subset that relapse remain largely unknown. Identification of other genes involved in the etiology of sporadic Wilms' tumor therefore remains an important priority. Studies of loss of heterozygosity (LOH), loss of imprinting, and constitutional chromosomal defects have implicated several recurrent changes in Wilms' tumor at chromosomes 11p15, 1p, 1q, 7p, 9q, 14q, 16q, and 22 (13–16).

2q37 deletion syndrome is a chromosomal disorder characterized by developmental delay, dysmorphic facies, skeletal abnormalities, and an increased risk of congenital heart defects (17–20). Although most cases have no associated malignancies, three children with constitutional 2q37 monosomy and Wilms' tumor have been reported (21–24). Two of these were de novo deletions and the third case resulted from unbalanced segregation of a reciprocal translocation from an unaffected parent with a balanced karyotype. All three cases showed additional urogenital anomalies: hypospadias and a small penis in a male patient (21); gonadal dysgenesis, bifid uterus, and dysplasia of the contralateral kidney in one female (24); and a horseshoe kidney and bilateral ovarian dysgenesis in the female translocation case (23). Features of urogenital anomalies and horseshoe kidney have also been noted in cases of constitutional 2q37 deletions without Wilms' tumor (19, 20, 25, 26), suggesting the presence of a gene at chromosome 2q37 that, like WT1, is important in both normal development and as a tumor suppressor gene.

We therefore hypothesized that chromosome 2q37 harbors a tumor suppressor gene, the deletion of which predisposes to Wilms' tumor, and that this gene, or a closely linked gene, is important in renal/urogenital development. To test this, we conducted LOH and candidate gene analyses in a large panel of sporadic Wilms' tumors.

Materials and Methods

LOH analysis in sporadic Wilms' tumors. Wilms' tumor samples and paired normal tissue or blood (where available) were accrued from centers in Europe and North America with appropriate ethics board approval and written informed consent. The initial panel used for LOH screening comprised 226 randomly selected tumors. Seventy-three of these were subjected to genome-wide screening and aggregate results for 2q have been reported previously (14). The remainder were specifically analyzed for LOH at 2q37 using a high-density microsatellite panel as described previously (19). Allele ratios were calculated as described, with ratios <0.45 classified as LOH and ratios of 0.45 to 0.66 as possible mosaic LOH or trisomy (14). Additional samples with known copy number loss were subsequently accrued to enrich the pool for candidate gene analysis (27). Copy number at 2q37 was determined by multiplex ligation–dependent probe amplification (28) using custom-designed oligonucleotide probes. Where sufficient DNA was available, precise breakpoints were defined by genome-wide single nucleotide polymorphism analysis using Illumina Hap300 arrays and Beadstudio software (Illumina). Mutation analysis and promoter methylation assays were done using standard methods as detailed in Supplementary Data.

miR-562 expression analysis. Total RNA from Wilms' tumor and normal adjacent kidney tissue was extracted using Trizol (Invitrogen). RNA from other human organs was purchased commercially (Agilent Technologies). Cell lines used included the fetal kidney-derived HEK-293 and 293T lines, the breast cancer–derived MCF-7 cell line, and WT-49 cells derived from a Wilms' tumor. RNA from these lines was extracted using the miRNeasy mini kit (Qiagen). RNase protection assay of miR-562 was done using 2 μg RNA with the mirVana miRNA detection kit (Ambion) according to the manufacturer's protocol. Probes for microRNA detection were end-labeled with γ-32P by using the mirVana probe and marker kit (Ambion).

For quantitative real-time PCR, two commercially available quantitative PCR assays for mature miR-562 failed completely even on control RNA samples that showed high expression in our RNase protection assay. We therefore opted to amplify the primary miR-562 transcript, designing primers within the precursor stem loop. Validation on the RNA panel used for RNase protection assays gave concordant results (data not shown). Total RNA was DNase I treated (Invitrogen) and reverse transcribed using SuperScript III (Invitrogen) and oligo(dT) primer. Samples were amplified on an Eppendorf Realpik Realplex MasterCycler (Eppendorf) with QuantiTect SYBR Green PCR master mix (Qiagen). The relative abundance of miR-562 was determined by using a standard curve generated from 5-fold serial dilutions of fetal kidney cDNA and normalized to GAPDH mRNA. To analyze changes in microRNA expression, ratios of the geometric means between control (fetal kidney) and experimental (Wilms' tumor and adjacent normal kidney) samples were calculated. Significance was determined by testing the difference of two means. One advantage of analyzing the primary miR-562 transcript is that the same aliquots of cDNA could also be used for analysis
Table 1. Wilms’ tumors showing LOH at 2q37

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Copy no.</th>
<th>Proximal breakpoint*</th>
<th>Distal breakpoint*</th>
<th>Size (Mb)</th>
<th>Genes affected</th>
<th>WT1 mutation</th>
<th>Additional abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>NWTS-99</td>
<td>1</td>
<td>232.24</td>
<td>234.37</td>
<td>2.2</td>
<td>PTMA-UTG1A cluster</td>
<td>No</td>
<td>LOH 11pter-p15.5</td>
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<tr>
<td></td>
<td>0</td>
<td>232.48</td>
<td>233.88</td>
<td>1.4</td>
<td>NPPC-ATG16L1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>239.39</td>
<td>240.55</td>
<td>1.2</td>
<td>TWIST2, HDAC4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>06-0116</td>
<td>2</td>
<td>219.54</td>
<td>Telomere</td>
<td>25</td>
<td>TNS1-telomere</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>232.58</td>
<td>233.60</td>
<td>1.1</td>
<td>DIS3L2-NEU2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA-31T</td>
<td>2</td>
<td>134.98</td>
<td>232.85</td>
<td>98</td>
<td>TMEM163-DIS3L2</td>
<td>No</td>
<td>dup 3pter-p14.3; del 11q12.1-qter</td>
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<td>MDA-28T</td>
<td>1</td>
<td>195.32</td>
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<td>Telomere</td>
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<tr>
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<td>1</td>
<td>230.39</td>
<td>233.74</td>
<td>3.4</td>
<td>TRIP12-INPP5D</td>
<td>Germ-line</td>
<td>del 7p; dup 7q; del 11pter-p11</td>
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<td>Rassekh et al. (16)</td>
<td>(case A7)</td>
<td>1</td>
<td>230.83</td>
<td>2.5</td>
<td>SP140-GIGYF2</td>
<td></td>
<td>Robinow syndrome</td>
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<tr>
<td>08-0144</td>
<td>1</td>
<td>231.98</td>
<td>239.79</td>
<td>7.8</td>
<td>ARMC9-HDAC4</td>
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<tr>
<td>RMH562</td>
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<td>Not defined</td>
<td>Telomere</td>
<td>&gt;13</td>
<td>Includes DIS3L2</td>
<td>No</td>
<td></td>
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<tr>
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<td>Not defined</td>
<td>Telomere</td>
<td>&gt;13</td>
<td>Includes DIS3L2</td>
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<td></td>
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<tr>
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<td>2</td>
<td>Not defined</td>
<td>Telomere</td>
<td>&gt;13</td>
<td>Includes DIS3L2</td>
<td>No</td>
<td></td>
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<tr>
<td>NWTS-11T</td>
<td>2</td>
<td>Not defined</td>
<td>Telomere</td>
<td>&gt;13</td>
<td>Includes DIS3L2</td>
<td>No</td>
<td></td>
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<tr>
<td>Germ-line 2q37 deletions</td>
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<td></td>
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<tr>
<td>Conrad et al. (21)</td>
<td>1</td>
<td>Not defined</td>
<td>Telomere</td>
<td>t(10;11)(q7;p13)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Viot-Szoboszlai et al. (24)</td>
<td>1</td>
<td>Not defined</td>
<td>Telomere</td>
<td>No</td>
<td>INGS-5 telomere</td>
<td>Somatic</td>
<td>der15, t(2;15) (q37.3; q24.1); +8</td>
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<td>MDA-74T (also refs. 22, 23)</td>
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<td>242.29</td>
<td>Telomere</td>
<td>0.6</td>
<td></td>
<td></td>
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</tbody>
</table>

*Approximate breakpoints are shown in Mb on chromosome 2, hg18 assembly. Tumors with homozygous deletions are shown first followed by new and published cases in order of proximal breakpoint.

Results

LOH and homozygous deletions at 2q37. In the initial panel of 226 Wilms’ tumors, 9 (4%) showed LOH with clonal loss of one allele, indicating that this was an early event in tumorogenesis. Six of these were copy neutral LOH and three harbored deletions. A further ~6% showed ratios in the range 0.45 and 0.66 and may represent later mosaic LOH events or trisomy. A mosaic deletion at 2q36.3-q37.1 has recently been documented (16). We subsequently ascertained an additional three tumors with known deletions, making a total of 12 samples for detailed analysis (Table 1). Two had a WT1 mutation, one was somatic, and the other was heterozygous in blood DNA and reduced to homozygosity in the tumor. In most of these tumors, the region of LOH is extensive, encompassing the terminal ≥13 Mb of chromosome 2q. Crucially, however, two tumors (NWTS-99 and 06-0116) were identified with homozygous deletion at 2q37.1 (Figs. 1 and 2). Additional cases showing interstitial LOH in this region define a minimal 360-kb interval bounded by the polymorphisms rs2679184 and rs13386477 (region A, Fig. 1). The only known gene within this region is DIS3L2, a homologue of the yeast mitotic control gene DIS3. Within intron 9 of DIS3L2 is miR-562, a previously uncharacterized microRNA. Case NWTS-99 also showed a heterozygous deletion of ~1 Mb at 2q37.3 (region B), encompassing histone deacetylase 4 (HDAC4) and TWIST2 (Figs. 1 and 2). The t(2;15) translocation case (MDA-74T) was the only tumor available from a patient with a known constitutional 2q37 rearrangement. Only the terminal ~500 kb of 2q37.3 was deleted in this patient, including the INGS5 gene (region C, Fig. 1). Thus, a total of three distinct candidate regions were identified within 2q37.

of miR-562 target genes (see below) and direct comparison could be made using a common housekeeping gene.

Bioinformatic analysis of miR-562 targets. miR-562 targets were identified using the Targetscan,9 miRBase,10 miRGator,11 and miRNA map12 databases. The targets were then analyzed using the KEGG gProfiler13 and Metacore14 pathway tools. Three target genes, EYA1, MET, and PSEN1, were chosen for further study based on their previously established expression in Wilms’ tumor or their roles in renal and urologic system development and function (Supplementary Table S1).

Luciferase assays. The putative miR-562 binding sites in the 3'-untranslated region from genes of interest were cloned into the pMIR-REPORT miRNA expression reporter vector system (Ambion). 293T cells (1 × 10^5 per well on a 12-well dish) were transfected with 200 ng of the reporter vector along with 200 ng β-gal-REPORT (Ambion) or an empty vector and pre-microRNA miR-562 precursor molecule (Ambion) and/or anti-microRNA miR-562 inhibitor (Ambion). The ratio of firefly luciferase to β-galactosidase activity was measured after 48 h using the Dual-Light combined reporter gene assay system (Applied Biosystems) following the manufacturer’s protocol. Changes in luciferase activity were determined by taking the ratios of the geometric means for reporters cotransfected with pMIR-REPORT and β-gal-REPORT. Variability of mean ratios for each reporter was determined by calculating the limits of a 95% confidence interval. Significance was determined by using Student’s t test.
Mutation analysis of candidate genes. The presence of homozygous deletions in two tumors strongly suggested that any tumor suppressor gene at 2q37.1 follows the classic two-hit hypothesis. We therefore analyzed the subset of Wilms' tumor samples that showed LOH in region A for evidence of a second mutation, screening candidate genes within the homozygously deleted region: DIS3L2, GIGYF2 (GRB10-interacting GYF protein 2), NPPC (natriuretic peptide precursor C), and miR-562. No additional genetic changes were identified. Bisulfite sequencing of the CpG island at the DIS3L2 promoter was also done, but there was no evidence for abnormal methylation in these tumors (data not shown). Similarly, there was no evidence of a second mutation in TWIST2 or HDAC4 among the tumors with LOH in region B.

As no second mutations were identified in the Wilms' tumors exhibiting LOH, it is possible that hemizygous deletion or mutation might be sufficient to contribute to tumorigenesis. We therefore screened a panel of 96 Wilms' tumor samples with no LOH at 2q37, looking for heterozygous mutations of genes within regions A and B: DIS3L2, miR-562, HDAC4, and TWIST2. A 18-bp deletion of miR-562 was identified in one tumor (Fig. 3A), the follow-up of which is detailed below. No pathogenic mutations were identified in DIS3L2, HDAC4, or TWIST2. We also screened for microdeletions in region A across the same panel using seven polymorphisms, ~60 kb apart (rs2679184, rs12988522, rs4973500, rs3100586, rs3116179, rs923333, and rs2633254). No additional microdeletions were detected.

Mutation and expression analysis of miR-562. To further characterize the 18-bp deletion of miR-562, we extended sequence analysis to a total of 176 Wilms' tumor samples and 210 controls. The heterozygote frequency was 3 of 176 Wilms' tumors (0.017) and 5 of 210 controls (0.024), suggesting that it is an uncommon polymorphism. miR-562 expression has

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Fig. 1. 2q37 deletions identified in Wilms' tumors. A, ideogram of chromosome 2 enlarged to show regions of LOH at 2q37. Pale gray columns, copy neutral LOH; dark gray columns, heterozygous deletion; black columns, homozygous deletion (06-0116 and NWTS-99T); thin black lines, minimum candidate regions A, B, and C. For simplicity, six additional tumors that show LOH across the entire region have been omitted. B, enlargement of region A showing the position of DIS3L2 and miR-562.
not been characterized previously, except for a single report of expression in colorectal cancer cells (29). We therefore characterized the expression pattern of miR-562 in a range of human tissues using RT-qPCR. Human fetal kidney and colon tissue, as well as Wt-49, 293T, and HEK-293 cells, all showed miR-562 expression (Fig. 3B). In contrast, no expression was detectable in human fetal heart and liver samples, adult heart, brain, ovary, testis, and lung tissue or MCF-7 cells.

This very tissue-restricted expression pattern made miR-562 an attractive candidate gene for a role in kidney development and Wilms’ tumor pathogenesis. Expression of miR-562 in Wilms’ tumors ranged from 0.11 to 1.15 times that observed in fetal kidney (Fig. 3C). The difference in mean miR-562 expression levels between Wilms’ tumor and normal adjacent kidney samples was significant (0.35 ± 0.075; P = 0.046). Closer inspection of the data suggests that there are two distinct groupings: three tumors show normal expression, whereas the remaining nine, which include two tumors with copy neutral LOH and one with the heterozygous miR-562 deletion polymorphism, show at least a 2-fold decrease in expression. This suggests that miR-562 expression is significantly downregulated in a subset of Wilms’ tumor cases.

Identification of miR-562 target genes. To elucidate the role of miR-562, we performed functional analysis to determine its target genes. Bioinformatic analyses of putative miR-562 targets revealed 37 genes with established roles in kidney or urogenital development and/or disease (Supplementary Table S1). Luciferase reporter assays were undertaken for the top three candidates: EYA1, MET, and PSEN1 to determine whether they are genuinely regulated by miR-562. Because microRNAs negatively regulate their target genes, a positive interaction in this assay results in a decrease in luciferase activity. MET and PSEN1 luciferase levels were not significantly altered in this system (data not shown), but EYA1 expression was significantly decreased (P ≤ 0.005), indicating that it is a genuine target of miR-562 (Fig. 4). Consistent with this, EYA1 was highly expressed in Wilms’ tumors but not in normal adjacent kidney tissue (Fig. 5), a result that is consistent with previous microarray expression analysis (30).

Discussion

The discovery of cytogenetic abnormalities in syndromes predisposing to Wilms’ tumor, such as 11p deletions in WAGR and 11p trisomies and translocations in Beckwith-Wiedemann syndrome, has been proven critical for the identification of Wilms’ tumor genes (31, 32). This combination of LOH data and rare constitutional chromosome abnormalities, used to pinpoint WT1, has also identified up to three loci on chromosome 7p that have a possible role in the etiology of Wilms’ tumor (13, 33–35). 2q37 deletion syndrome is a rare constitutional chromosome abnormality, and although most cases have no associated malignancies, reports of Wilms’ tumor in three cases (21–24) suggested the presence of a tumor suppressor gene.

Our analysis of a large panel of sporadic Wilms’ tumors identified clonal LOH at 2q37 in 4% of cases and allelic imbalance in another 6%. Remarkably, two of these tumors harbor homozygous deletions, strongly supporting our hypothesis of a tumor suppressor gene present in this region and suggesting that it follows a classic Knudson two-hit model (36). Furthermore, genome-wide single nucleotide polymorphism array analysis of these two tumors showed few additional cytogenetic abnormalities (Table 1). In contrast, the tumors that showed only a heterozygous loss at 2q37 harbored multiple additional chromosomal abnormalities, such as 11p LOH and isochromosome-7q (Table 1). We propose that homozygous deletion of one or more key genes at 2q37.1 is sufficient to initiate Wilms’ tumor development, whereas, in the absence of a second mutation, heterozygous loss can contribute to the pathogenesis in concert with other abnormalities elsewhere in the genome.

The significance of two additional regions of localized LOH (Fig. 1) is less clear-cut. Region B, encompassing HDAC4 and TWIST2, is defined by a 1-Mb deletion in sample NWTS-99. This same sample also harbors one of the homozygous deletions in region A, yet the intervening DNA shows normal copy number and no LOH. These deletions were all confirmed to be de novo in the tumor. One explanation is that they represent a more complex rearrangement, such as an inversion/deletion event that masquerades as contiguous deletions at the DNA microarray level. Dividing tumor cells are not available for the metaphase analysis needed to investigate this further, but it is possible that the region B deletion is a bystander and does not contribute to the pathogenesis of Wilms’ tumor. Similarly, the significance of the ~500-kb terminal deletion in the translocation case (region C) is somewhat unclear. It encompasses five genes, including ING5, a putative tumor suppressor gene that may modulate p53 function (37). The translocation is unbalanced and duplicates a 28-Mb region of distal 15q, a rearrangement that has also been implicated in the pathogenesis of Wilms’ tumor (15, 38). Thus, although this is one of the three germ-line rearrangements that inspired the study, the partial trisomy 15q likely also contributed...
to the pathogenesis of Wilms' tumor in this patient and the 2q37 deletion alone may not have been causal. In support of this, among the constitutional 2q37 deletions that have been well characterized at the DNA level, almost all harbor terminal deletions that extend proximally to HDAC4/TWIST2 and are therefore deleted for both regions B and C, yet these patients do not have Wilms' tumor (19, 39–42). In contrast, most of the proximal breakpoints localize distal of region A; in our panel of 30 deletion patients without malignancy, only one has a deletion of region A, with a breakpoint between NPPC and DIS3L2.15 Additionally, both of the constitutional deletion cases with Wilms' tumor had breakpoints in 2q37.1 (21–24). Combined with our identification of homozygous somatic deletions in two tumors, these data strongly suggest that region A in 2q37.1 is the primary Wilms' tumor susceptibility locus on 2q.

DIS3L2 is the only gene in the minimum 360-kb interval at 2q37.1. Because members of the yeast DIS3 family are essential in mitotic control and spindle formation (43, 44), we hypothesized that deletion of DIS3L2 may contribute to the pathogenesis of Wilms' tumor by disrupting normal cell division and predisposing to aneuploidy. However, no second mutations

\[ \text{Fig. 3. Characterization of miR-562.} \]

A, sequence analysis identified a heterozygous 18-bp deletion of miR-562 in 1.7% of Wilms' tumors and 2.4% of controls. The miR-562 hairpin is shown with the mature microRNA in bold text. Large arrows, terminal ends of miR-562 in the reference sequence; black box, 18-bp deletion. RNA folding analysis using the program Mfold (http://mfold.bioinfo.rpi.edu/) indicates that the 18-bp deletion will abolish hairpin formation. B, RNase protection assay detected miR-562 expression in human colon, fetal kidney, and several kidney-derived cell lines but not in other major organs. The miR-562 probe is 21 nucleotides in length. C, primary miR-562 expression in Wilms' tumor samples was determined by quantitative real-time PCR and normalized to GAPDH. NK, normal adjacent kidney from Wilms' tumor patients; 08-03XX samples are Wilms' tumors; *, a Wilms' tumor heterozygous for the miR-562 deletion polymorphism; #, Wilms' tumor samples with 2q copy neutral LOH. Bars, SE. Nine of the 12 tumor samples show at least a 2-fold decrease in expression compared with normal kidney.

15 M.A. Aldred, unpublished data.
or promoter methylation were identified in tumors with LOH of this region and no mutations or microdeletions were identified in our wider tumor panel. We therefore focused on miR-562, a microRNA that lies within intron 9 of DIS3L2. microRNAs are a group of noncoding ~22-nucleotide RNA molecules that post-transcriptionally regulate the expression of target mRNAs (45). These small RNAs are evolutionarily conserved and regulate processes as fundamental as cellular proliferation, differentiation, and apoptosis. It is increasingly recognized that dysregulation of microRNAs plays an important role in cancer (46, 47).

miR-562 showed a tissue-restricted expression pattern, strongest in fetal kidney. Real-time PCR analysis showed a significant reduction in Wilms’ tumors compared with normal kidney and suggested that tumors may stratify into two groups based on miR-562 expression level. Clinical data do not suggest a correlation with therapy response, histology, or survival between these two groups, but complete data were only available for 6 of the 12 cases where expression analysis was done, so no firm conclusions can be drawn. A polymorphic deletion of miR-562 was identified, which probably does not represent a major predisposing factor in the etiology of Wilms’ tumor but could potentially increase susceptibility to Wilms’ tumor in the presence of additional mutations. Decreased miR-562 expression was also observed in 8 of 11 tumors with no deletion. This suggests that miR-562 is frequently downregulated at the transcriptional level perhaps due to mutations of its promoter. In general, microRNAs residing in introns are coexpressed with their “parent” gene, presumably directed by that gene’s promoter (48), whereas miR-562 expression is apparently independent of the DIS3L2 promoter (data not shown). miR-562 is believed to be derived from a transposable element (49) and its expression may therefore be regulated by its own transposon-derived transcription machinery or by another locus. Defining the promoter for miR-562 will be important in further examining its regulation and its role in normal kidney development and Wilms’ tumor.

**Fig. 4.** miR-562 regulates EYA1 expression in vitro. A luciferase reporter construct containing the putative EYA1 3′-untranslated region binding site for miR-562 was transfected into 293T cells. Relative luciferase activity was measured after 48 h. Luciferase activity in the presence of three different concentrations of miR-562 was significantly reduced compared with empty vector (pMIIR-REPORT), indicating transcriptional downregulation of EYA1 by miR-562. This was reversed by addition of anti-miR-562 competitor, confirming specificity of the response. Bars, SD from three independent experiments.

**Fig. 5.** EYA1 is overexpressed in Wilms’ tumor. Expression of EYA1 was determined by quantitative real-time PCR and normalized to GAPDH. NK, normal adjacent kidney from Wilms’ tumor patients; 08-3XK samples are Wilms’ tumors; *, a Wilms’ tumor heterozygous for the miR-562 deletion polymorphism; †, Wilms’ tumor samples with 2q copy neutral LOH. Bars, SE. EYA1 is significantly overexpressed in all tumors compared with normal kidney. However, there was no clear inverse correlation with miR-562 expression, suggesting that multiple factors contribute to the regulation of EYA1.

EYA1, a gene essential for cell survival and proliferation in early metanephric development (50, 51), was validated as a target of miR-562. We confirmed that EYA1 was significantly overexpressed in Wilms’ tumors (30), suggesting that haploinsufficiency of miR-562 is likely one factor that contributes to increased EYA1 expression in Wilms’ tumors. Given that we did not see a strong inverse correlation between these two transcripts, it is clear that other genetic events also influence EYA1 expression. Notably, the gene is located on chromosome 8, the gain of which is observed in up to 30% of Wilms’ tumors (27). Downregulation of miR-562 in conjunction with gain of chromosome 8 would therefore be predicted to result in synergistic overexpression of EYA1.

In summary, we have shown LOH at 2q37 in at least 4% of sporadic Wilms’ tumors. Identification of two tumors with homozygous deletions strongly suggests the presence of a Wilms’ tumor suppressor gene at 2q37.1. Expression of miR-562, a microRNA within this region, is significantly reduced in Wilms’ tumors even in the absence of LOH or other detectable abnormality of the microRNA sequence. We showed that EYA1, which is overexpressed in Wilms’ tumors, is a target of miR-562, suggesting that haploinsufficiency of miR-562 contributes to the etiology of Wilms’ tumor by promoting deregulation of EYA1. Further study of the role of miR-562 in normal renal development and Wilms’ tumor is hampered by the fact it is primate-specific and no orthologue is present in model organisms such as mouse or zebrafish. Clinically though, our data may be helpful in clarifying the risk of Wilms’ tumor in children diagnosed with a constitutional 2q37 deletion. Our results from sporadic Wilms’ tumors are broadly concordant with published data on constitutional breakpoints: deletion patients who developed Wilms’ tumor had breakpoints in 2q37.1, whereas the majority of patients with no malignancy have smaller deletions encompassing only 2q37.2-q37.3. Some caution is still required, because we identified two regions of uncertain significance at 2q37.3 in sporadic Wilms’ tumors, but
overall our data suggest that any increased risk for developing Wilms' tumor likely correlates with deletions encompassing 2q37.1.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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