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Loss of heterozygosity at 2q37 in sporadic Wilms tumor: a 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.

Co-occurrence of Wilms tumor with 2q37 deletion syndrome, an uncommon constitutional chromosome abnormality, has previously been reported 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 (LOH) analysis in a panel of 226 sporadic Wilms tumor samples and mutation analysis of candidate genes.

Results—LOH 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 classical 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

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Translational Relevance

2q37 deletion syndrome is an uncommon constitutional chromosome abnormality. An association with Wilms tumor has been reported in three cases, suggesting the presence of a tumor suppressor gene. In a panel of 226 sporadic Wilms tumors, we identified loss of heterozygosity in at least 4% of tumors. Homozygous deletions in two independent tumors strongly implicate a 360kb region containing the *DIS3L2* gene and microRNA *miR-562*. Previously it has been unclear whether children with 2q37 deletions are at increased risk for developing Wilms tumor; approximately a hundred cases have been described, only three of whom had this malignancy. However, the majority of cases without malignancy where the breakpoints have been molecularly defined do not encompass the critical 360kb region we identified, suggesting a genotype-phenotype correlation. Overall, our results therefore suggest that any increased susceptibility to Wilms tumor in children with a constitutional 2q37 deletion likely correlates with deletions encompassing 2q37.1.

in kidney and colon and regulates *EYAI*, a critical gene for renal development. *miR-562* expression is reduced in Wilms tumor and may contribute to tumorigenesis by deregulating *EYAI*. Two other candidate regions were localized at 2q37.3 and 2qter but available data from patients with constitutional deletions suggest 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.

Keywords

Wilms tumor; chromosome deletion; loss of heterozygosity; microRNA

Introduction

Wilms tumor (nephroblastoma) is the most common pediatric renal malignancy, affecting approximately 1 in 10,000 children (1). Most cases of Wilms tumor are sporadic and unilateral, but a minority (2%) have a family history (2). Wilms tumors appear to develop from nephrogenic rests, an abnormal structure in the kidney that is formed by a failure of the mesenchymal tissue to differentiate into nephrons (3). Additional genetic events are then required to transform these undifferentiated cells, thus causing uncontrolled growth, which can lead to the formation of Wilms tumor.

Wilms tumor is also a feature in a number of syndromes, notably WAGR (Wilms, aniridia, genitourinary abnormalities and mental retardation), Simpson-Golabi-Behme and Beckwith-Wiedemann (4). Analysis of chromosome deletions in patients with WAGR led to the identification of the Wilms tumor-suppressor gene, *WT1*, on chromosome 11p13 (5, 6). Homozygous mutations in *WT1* are found in approximately 18% of Wilms tumors (7), while point mutations of *WT1* in patients with Denys-Drash and Frasier syndromes underscore its importance in normal renal and urogenital development (8). Two other genes, *WTX* and *CTNNB1*, have also been implicated in the pathogenesis of Wilms tumor. *CTNNB1*, which codes for β -catenin, is mutated in approximately 15% of Wilms tumors, but rarely occurs without concomitant mutation of *WT1* (7, 9), whereas somatic mutations of *WTX* (Wilms Tumor on the X) are present in 11-29% of Wilms tumors and occur with and without *WT1* mutation (7, 9, 10). Interestingly, germline mutations of *WTX* were recently shown to underlie osteopathia striata congenita with cranial sclerosis, an 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 tumorigenesis (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 LOH, loss of imprinting and constitutional chromosomal defects have implicated a number of 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, 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 loss of heterozygosity 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 loss of heterozygosity (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 previously described (19). Allele ratios were calculated as described, with ratios less than 0.45 classified as LOH and ratios of 0.45-0.66 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 (MLPA) (28) using custom-designed oligonucleotide probes. Where sufficient DNA was available, precise breakpoints were defined by genome-wide single nucleotide polymorphism (SNP) analysis using Illumina Hap300 arrays and Beadstudio software (Illumina, San Diego, CA). Mutation analysis and promoter methylation assays were performed using standard methods, as detailed in the 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, Cedar Creek, TX). Cell lines used included the fetal kidney derived HEK-293 and 293T lines, the breast cancer derived MCF-7 cell line and WiT-49 cells, derived from a Wilms tumor. RNA from these lines was extracted using the miRNeasy mini kit (Qiagen). Ribonuclease protection assay of *miR-562* was performed using 2 µg of RNA with the mirVana miRNA detection kit (Ambion, Austin, TX) according to the manufacturer's protocol. Probes for miRNA 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-I 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-I 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 Realplex MasterCycler (Eppendorf, Westbury, NY) 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 miRNA 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 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 (www.targetscan.org), miRBase (<http://microrna.sanger.ac.uk>), miR Gator (genome.ewha.ac.kr/miRGator) and miRNA map (<http://mirnamap.mbc.nctu.edu.tw>) databases. The targets were then analyzed using the KEGG gProfiler (<http://biit.cs.ut.ee/gprofiler>) and Metacore (www.genego.com/metacore.php) pathway tools. Three target genes, *EYAI*, *MET* and *PSEN1*, were chosen for further study based on their previously established expression in Wilms tumor or their roles in renal and urological system development and function (Supplementary Table 1).

Luciferase assays

The putative *miR-562* binding sites in the 3' UTR from genes of interest were cloned into the pMIR-REPORT miRNA expression reporter vector system from Ambion. 293T cells (1×10^5 cells per well on a 12-well dish) were transfected with 200 ng of the reporter vector along with 200 ng of 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.

Results

Loss of heterozygosity 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 tumorigenesis. 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, 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 or more of chromosome 2q. Crucially, however, two tumors (NWTS-99 and 06-0116) were identified with homozygous deletion at 2q37.1 (Figures 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, Figure 1). The only known gene within this region is *DIS3L2*, a homolog 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 approximately 1 Mb at 2q37.3 (Region B), encompassing histone deacetylase 4 (*HDAC4*)

and *TWIST2* (Figures 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 *ING5* gene (Region C, Figure 1). Thus a total of three distinct candidate regions were identified within 2q37.

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 classical 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 performed, 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* amongst 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 19 bp deletion of *miR-562* was identified in one tumor (Figure 3A), 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 19 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 not previously been characterized, 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 RNase protection assays. Human fetal kidney and colon tissue, as well as WiT-49, 293T and HEK-293 cells, all demonstrated *miR-562* expression (Figure 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. *miR-562* expression was also undetectable in the mouse kidney, confirming genome sequence data that suggests *miR-562* is only present in primate genomes (data not shown).

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 (Figure 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 two-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 1). Luciferase reporter assays were undertaken for the top three candidates: *EYA1*, *MET* and *PSEN1* to determine whether they are genuinely regulated by *miR-562*. Since 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* (Figure 4). Consistent with this, *EYA1* was highly expressed in Wilms tumors but not in normal adjacent kidney tissue (Figure 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, have proved 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 classical Knudson two-hit model (36). Furthermore, genome-wide SNP 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 (Figure 1) is less clear-cut. Region B, encompassing *HDAC4* and *TWIST2* is defined by a 1Mb deletion in sample NWT5-99. 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 discontinuous 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 germline 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* (MAA, unpublished data). 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. Since 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 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 posttranscriptionally 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 demonstrated a significant reduction in Wilms tumors compared with normal kidney and suggested 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 six of the twelve cases where expression analysis was performed and 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 co-expressed 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.

EYAI, 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 *EYAI* was significantly overexpressed in Wilms tumors (30), suggesting that haploinsufficiency of *miR-562* is likely one factor that contributes to increased *EYAI* 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 *EYAI* expression. Notably, the gene is located on chromosome 8, 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 *EYAI*.

In summary, we have demonstrated loss of heterozygosity 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 demonstrated that *EYAI*, which is over-expressed 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 *EYAI*. 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 ortholog 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, since 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. 2q37 deletions identified in Wilms tumors

A. Ideogram of chromosome 2 enlarged to show regions of LOH at 2q37. Pale gray bars indicate copy neutral LOH; dark gray bars indicate heterozygous deletion; and black bars (06-0116 and NWTs-99T) indicate homozygous deletion. Minimum candidate regions A, B and C are indicated by thin black lines. 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*.

Figure 2. homozygous deletion at 2q37.1

Illumina SNP microarray data are shown as the difference in log intensity ratio between tumor NWTs-99T and matched normal tissue. A homozygous deletion is evident at 2q37.1, flanked by small regions of heterozygous deletion. An additional heterozygous deletion is present at 2q37.3, with normal copy number and heterozygosity in the intervening DNA.

Figure 3. characterization of *miR-562*

A. Sequence analysis identified a heterozygous 19 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 indicate the terminal ends of *miR-562* in the reference sequence,

while the black box indicates the 19 bp deletion. RNA folding analysis using the program Mfold (<http://mfold.bioinfo.rpi.edu/>) indicates that the 19 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 denotes normal adjacent kidney from Wilms tumor patients, 08-03XX samples are Wilms tumors, * is a Wilms tumor that is heterozygous for the *miR-562* deletion polymorphism and # denotes Wilms tumor samples with 2q copy neutral LOH. Error bars represent the standard error of the mean. Nine of the 12 tumor samples show at least a two-fold decrease in expression compared with normal kidney.

Figure 4. miR-562 regulates *EYA1* expression *in vitro*

A luciferase reporter construct containing the putative *EYA1* 3'UTR 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 (pMIR-REPORT), indicating transcriptional downregulation of *EYA1* by *miR-562*. This was reversed by addition of anti-miR-562 competitor, confirming specificity of the response. Error bars represent the standard deviation from three independent experiments.

Figure 5. *EYA1* is overexpressed in Wilms tumor

Expression of *EYA1* was determined by quantitative real-time PCR and normalized to *GAPDH*. NK is normal adjacent kidney from Wilms tumor patients, 08-03XX samples are Wilms tumors, * is a Wilms tumor heterozygous for the *mir-562* deletion polymorphism and # denotes Wilms tumor samples with 2q copy neutral LOH. Error bars represent the standard error of the mean. *EYA1* is significantly overexpressed in all tumors compared to normal kidney. However, there was no clear inverse correlation with *mir-562* expression, suggesting that multiple factors contribute to the regulation of *EYA1*.

Wilms tumors showing LOH at 2q37

Table 1

Tumor	Copy #	Proximal brkpt [*]	Distal brkpt [*]	Size (Mb)	Genes affected	WT1 mutation	Additional abnormalities
NWTS-99	1	232.24	234.37	2.2	<i>PTMA-UTG1A</i> cluster	No	LOH 11pter-p15.5
	0	232.48	233.88	1.4	<i>NPPC-ATG16L1</i>		
	1	239.39	240.55	1.2	<i>TWIST2, HDAC4</i>		
06-0116	2	218.54	telomere	25	<i>TNSI</i> -telomere		
	0	232.58	233.60	1.1	<i>DIS3L2-NEU2</i>		
MDA-31T	2	134.98	232.85	98	<i>TMEM163-DIS3L2</i>	No	dup 3pter-p14.3; del 11q12.1-qter
MDA-28T	1	195.32	telomere	48		No	
MDA-30T	2	221.72	telomere	22		No	
MDA-1T	1	230.39	233.74	3.4	<i>TRIP12-INPP5D</i>	Germline	del 7p; dup 7q; del 11pter-p11
Rassekh, et al. 2008 (16) (Case A7)	1	230.83	233.29	2.5	<i>SPI40-GIGYF2</i>		Robinow syndrome
08-0144	1	231.98	239.79	7.8	<i>ARMC9-HDAC4</i>		None
RMH562	2	Not defined	telomere	>13	Includes <i>DIS3L2</i>	No	
RMH781	2	Not defined	telomere	>13	Includes <i>DIS3L2</i>	No	
NWTS-7T	2	Not defined	telomere	>13	Includes <i>DIS3L2</i>	No	
NWTS-11T	2	Not defined	telomere	>13	Includes <i>DIS3L2</i>	No	
Germline 2q37 deletions							
Conrad, et al. 1995 (21)	1	Not defined	telomere				t(10;1)(q?p13)
Viot-Szoboszlai, et al. 1998 (24)	1	Not defined	telomere			No	
MDA-74T (also refs. 22, 23)	1	242.29	telomere	0.6	<i>ING5</i> -telomere	Somatic	der 15, t(2;15)(q37.3;q24.1);+8

^{*} 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.