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Multiple mechanisms of MYCN dysregulation in Wilms tumour

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ABSTRACT

Genomic gain of the proto-oncogene transcription factor gene MYCN is associated with poor prognosis in several childhood cancers. Here we present a comprehensive copy number analysis of MYCN in Wilms tumour (WT), demonstrating that gain of this gene is associated with anaplasia and with poorer relapse-free and overall survival, independent of histology. Using whole exome and gene-specific sequencing, together with methylation and expression profiling, we show that MYCN is targeted by other mechanisms, including a recurrent somatic mutation, P44L, and specific DNA hypomethylation events associated with MYCN overexpression in tumours with high risk histologies. We describe parallel evolution of genomic copy number gain and point mutation of MYCN in the contralateral tumours of a remarkable bilateral case in which independent contralateral mutations of TP53 also evolve over time. We report a second bilateral case in which MYCN gain is a germline aberration. Our results suggest a significant role for MYCN dysregulation in the molecular biology of Wilms tumour. We conclude that MYCN gain is prognostically significant, and suggest that the novel P44L somatic variant is likely to be an activating mutation.

INTRODUCTION

Wilms tumour (WT, nephroblastoma) is the commonest paediatric renal malignancy. A significant minority of WT cases have mutations in known genes, including WT1 [1-6], CTNNB1 [7, 8], AMER1 (WTX) [9], and TP53 [10]. Less frequent mutations targeting genes such as FBXW7 [11] and GPC3 [12] have also been reported, and epigenetic lesions affecting the IGF2/H19 locus are common [13]. A recent whole exome study has identified mutations in microRNA processing genes including DROSHA, DGCR8 and DICER1 [14]. Numerous recurrent copy number aberrations and loss of heterozygosity (LOH) events have been described, some of which affect known genes (e.g. 11p LOH, 17p loss, focal deletion of AMER1 and FBXW7), while the functional significance of others (e.g. 1q gain, 1p loss, 16q loss) is unclear. Only a few of these aberrations have
known associations with histology or outcome. TP53 mutations are largely confined to (high risk) anaplastic tumours [10], while WT1 mutations (which are frequently coincident with CTNNB1 mutations) are associated with stromal predominant histology [15]. We have previously noted an association between 1q gain and relapse [16, 17], but the only genomic biomarker with a fully validated association with poor outcome that is currently used in treatment planning is simultaneous loss of heterozygosity of 1p and 16q [18].

One recurrent focal copy number gain on 2p24.3, encompassing the MYCN locus, has been observed in several previous WT studies [11, 19-23]. The MYCN gene encodes a proto-oncogenic MYC family transcription factor, MYCN. MYCN is known to be amplified in 16-25% of neuroblastomas, an aberration associated with a poorer prognosis, and it undergoes prognostically relevant copy number gain, and more rarely amplification, in other paediatric tumours including medulloblastoma [24, 25] and rhabdomyosarcoma [26]. In an earlier study [11], we used SNP arrays to analyse tumours from patients who had received pre-operative chemotherapy under International Society of Paediatric Oncology (SIOP) protocols, and noted an apparent association between MYCN gain and the high risk diffuse anaplastic subtype. In a larger study [22], in which genomic real-time PCR was used to measure MYCN copy number in a more heterogenous group of tumours from patients treated under various protocols (including immediate nephrectomy), we confirmed that MYCN gain is a common event, but found no particular subtype association. In the current report, we describe the largest analysis of MYCN status in WT to date, using multiplex ligation dependent probe amplification (MLPA) to assess its copy number in a series of samples drawn entirely from the SIOP WT 2001 clinical study and trial.

Recent developments in neuroblastoma genomics suggest that gain or amplification is not the only mechanism that affects MYCN function in paediatric tumours. Maris and co-workers, using whole genome sequencing, have shown that a specific somatic variant, P44L, can be detected in 2% of all neuroblastomas analysed [27], while isolated cases of the same variant have also been described in glioma, medulloblastoma, endometrial carcinoma, and neoplastic cysts of the pancreas [28-31]. This variant is postulated to be an activating ‘gain of function’ mutation that, like increased MYCN dosage due to copy number gain, could cause oncogenic upregulation of downstream MYCN-dependent pathways.

Here we report the discovery of MYCN P44L mutations through whole exome analysis of Wilms tumour, leading us to hypothesise that multiple routes to MYCN-oncogenesis may exist in this paediatric tumour. We present targeted MYCN sequencing analysis of a large WT series to determine the frequency of these mutations, and investigate potential correlations between MYCN expression levels, copy number events, and gene-specific hypomethylation.

**RESULTS**

**Point mutations in the MYCN coding sequence**

In a whole exome analysis of a series of 51 WTs, enriched in cases with high risk histology or poor outcome, heterozygous somatic point mutations in the MYCN coding sequence were detected in three tumours. In all three cases, the same variant, a c.131C>T transition substituting proline with leucine at codon 44 (p.P44L), was detected (Table 1). 45 of the 51 cases gave informative exome data at this position, with sufficient depth of coverage for variant calling. Thus, 6.7% of the cases that could be analysed in the exome series carried this mutation. To determine the proportion of WTs harbouring this or other MYCN mutations in a larger, unselected cohort (Supplementary Table 1), we sequenced the complete MYCN coding region in 168 additional tumour samples. Where a mutation was detected, we sequenced matched normal kidney or blood germline DNA (if available) from the same case. Five additional tumours carrying P44L were identified (3.0%, Table 1) and, as in the exome series, all mutations were heterozygous in the tumour and absent from the germline. The overall frequency of P44L in the extended series of 213 cases was therefore 3.8%. The subset carrying the mutation had no particular distinguishing features. Multiple histological subtypes (as defined by SIOP) and clinical stages were represented (Table 1). Only one of the patients – the complex bilateral case described in Table 2 and below - has so far suffered a recurrence (minimum follow-up 25 months, median follow-up 54.5 months), and none have died.

Four further non-synonymous heterozygous variants elsewhere in the MYCN coding sequence, each affecting a single case, were also found (Table 1). The first of these, c.853C>T (p.R285W), was present in the germline. R285 is a highly conserved amino acid residue in protein domain pfam01056 (‘Myc amino-terminal region’ [32]). This mutation was predicted as damaging by both SIFT [33] and PolyPhen2 [34]. The second variant, c.227G>A (p.S76N), was found in a case for which no blood or unambiguous normal kidney tissue was available, so it was not possible to determine if it was a germline variant. S76 is a poorly conserved residue, and the S76N variant was not predicted as damaging by either SIFT or PolyPhen. A third variant, c.473C>T (p.A158V), was located in another poorly conserved region and predicted as tolerated by SIFT, but possibly damaging by PolyPhen, and was confirmed as present in the germline. The fourth variant, c.1093C>G (p.P365A), was also predicted to be a benign change by both algorithms, and is a known rare variant.
previously observed in the 1000 Genome Project data [35]. Data from unambiguous germline material were not available. No congenital abnormalities or family history of cancer were reported in three of these cases; no data were available for the A158V case. Two further variants (c.189C>A, c.207G>A), one of which was a rare known SNP, were also identified in three samples (Table 1), but they did not alter the amino acid sequence or overlap with splice sites and were not analysed further.

MLPA copy number analysis of all the cases with

**Table 1: MYCN single nucleotide variants in Wilms tumour.**

<table>
<thead>
<tr>
<th>CH ID</th>
<th>Exome</th>
<th>Genomic (Chr2)</th>
<th>CDS</th>
<th>Protein</th>
<th>Germline</th>
<th>ESP5400</th>
<th>1000g</th>
<th>dbSNP 132</th>
<th>SIFT</th>
<th>PolyPhen2</th>
<th>Histology</th>
<th>Stage</th>
<th>MYCN CN</th>
</tr>
</thead>
<tbody>
<tr>
<td>2560</td>
<td>Y</td>
<td>g.16082317C&gt;T</td>
<td>c.131C&gt;T</td>
<td>p.P44L</td>
<td>Wild type</td>
<td>D</td>
<td>D</td>
<td>Focal Anaplastic</td>
<td>3</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3863</td>
<td>Y</td>
<td>g.16082217C&gt;T</td>
<td>c.131C&gt;T</td>
<td>p.P44L</td>
<td>Wild type</td>
<td>D</td>
<td>D</td>
<td>Regressive</td>
<td>1</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4788</td>
<td>Y</td>
<td>g.16082317C&gt;T</td>
<td>c.131C&gt;T</td>
<td>p.P44L</td>
<td>Wild type</td>
<td>D</td>
<td>D</td>
<td>Regressive</td>
<td>3</td>
<td>Gain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4956</td>
<td>Y</td>
<td>g.16082317C&gt;T</td>
<td>c.131C&gt;T</td>
<td>p.P44L</td>
<td>Wild type</td>
<td>D</td>
<td>D</td>
<td>Regressive</td>
<td>2</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6613</td>
<td>Y</td>
<td>g.16082317C&gt;T</td>
<td>c.131C&gt;T</td>
<td>p.P44L</td>
<td>Wild type</td>
<td>D</td>
<td>D</td>
<td>Mixed</td>
<td>1</td>
<td>Gain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8783</td>
<td>Y</td>
<td>g.16082317C&gt;T</td>
<td>c.131C&gt;T</td>
<td>p.P44L</td>
<td>Wild type</td>
<td>D</td>
<td>D</td>
<td>Blastemal</td>
<td>2</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8810</td>
<td>Y</td>
<td>g.16082317C&gt;T</td>
<td>c.131C&gt;T</td>
<td>p.P44L</td>
<td>Wild type</td>
<td>D</td>
<td>D</td>
<td>Regressive</td>
<td>3</td>
<td>Gain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9710</td>
<td>Y</td>
<td>g.16082317C&gt;T</td>
<td>c.131C&gt;T</td>
<td>p.P44L</td>
<td>Wild type</td>
<td>D</td>
<td>D</td>
<td>Mixed</td>
<td>1</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3136</td>
<td>Y</td>
<td>g.16082413G&gt;A</td>
<td>c.227G&gt;A</td>
<td>p.S76N</td>
<td>N/A</td>
<td>T</td>
<td>B</td>
<td>Regressive</td>
<td>3</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7512</td>
<td>Y</td>
<td>g.16082659C&gt;T</td>
<td>c.473C&gt;T</td>
<td>p.A158V</td>
<td>Variant</td>
<td>T</td>
<td>P</td>
<td>Stromal</td>
<td>2</td>
<td>Gain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2451</td>
<td>Y</td>
<td>g.16085917C&gt;G</td>
<td>c.1093C&gt;G</td>
<td>p.P365A</td>
<td>N/A</td>
<td>T</td>
<td>B</td>
<td>Regressive</td>
<td>3</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8914</td>
<td>Y</td>
<td>g.16082375C&gt;A</td>
<td>c.189C&gt;A</td>
<td>p.P63P</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>Mixed</td>
<td>1</td>
<td>Gain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9140</td>
<td>Y</td>
<td>g.16082393G&gt;A</td>
<td>c.207G&gt;A</td>
<td>p.E69E</td>
<td>ND</td>
<td>0.0027</td>
<td>0.0009 rs41264199</td>
<td>-</td>
<td>-</td>
<td>Regressive</td>
<td>1</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>9224</td>
<td>Y</td>
<td>g.16082393G&gt;A</td>
<td>c.207G&gt;A</td>
<td>p.E69E</td>
<td>ND</td>
<td>0.0027</td>
<td>0.0009 rs41264199</td>
<td>-</td>
<td>-</td>
<td>Regressive</td>
<td>1</td>
<td>Normal</td>
<td></td>
</tr>
</tbody>
</table>

SIFT/PolyPhen2: D = damaging, T = tolerated, B = benign, P = possibly damaging. 1000g = variant frequency in 1000 Genome Project (Feb 2012). ESP5400 = variant frequency in Exome Sequencing Project (Dec 2011). N/A = material not available. ND = Not done.

**Figure 1: Focal gain of MYCN in the germline of a bilateral WT case.** (a) Illumina CytoSNP-12 raw copy number data (Log R Ratio) from blood sample ICH-10529 showing probes aligned to the 2p region. (b) Copy number segmentation by OncoSNP (linear scale). Arrows: position of region of focal gain. LOH = segmented loss of heterozygosity (none detected).
Table 2: MYCN and TP53 aberrations in a bilateral WT case.

<table>
<thead>
<tr>
<th>ICH ID</th>
<th>Side</th>
<th>Sample</th>
<th>Histology</th>
<th>TP53</th>
<th>MYCN seq data</th>
<th>MYCN CN</th>
<th>Other aberrations</th>
<th>Treatment Month</th>
</tr>
</thead>
<tbody>
<tr>
<td>5179</td>
<td>Blood</td>
<td>wild type</td>
<td>wild type</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3474</td>
<td>Left</td>
<td>Initial biopsy</td>
<td>Wilms tumour</td>
<td>wild type</td>
<td>ND</td>
<td>Gain</td>
<td>+1q, +6, +8, -10p, +12p, +13, -20p</td>
<td>1</td>
</tr>
<tr>
<td>6583</td>
<td>Left</td>
<td>Partial nephrectomy</td>
<td>Wilms tumour (stromal area)</td>
<td>wild type</td>
<td>wild type</td>
<td>NA</td>
<td>NA</td>
<td>6</td>
</tr>
<tr>
<td>6580</td>
<td>Left</td>
<td>Partial nephrectomy</td>
<td>Wilms tumour (anaplastic area)</td>
<td>c.517G&gt;T: p.173V&gt;L</td>
<td>wild type</td>
<td>NA</td>
<td>NA</td>
<td>6</td>
</tr>
<tr>
<td>3864</td>
<td>Left</td>
<td>Partial nephrectomy</td>
<td>Wilms tumour (diffuse anaplastic)</td>
<td>c.517G&gt;T: p.173V&gt;L</td>
<td>wild type</td>
<td>Gain</td>
<td>+2, +6, -10p, +13, -14q, -17p, +17q, -20p</td>
<td>6</td>
</tr>
<tr>
<td>3865</td>
<td>Left</td>
<td>Partial nephrectomy</td>
<td>Wilms tumour (diffuse anaplastic)</td>
<td>c.517G&gt;T: p.173V&gt;L</td>
<td>wild type</td>
<td>Gain</td>
<td>+2, LOH 6, +8, -10p, +10q, +13, +17p, +17q, -20p</td>
<td>6</td>
</tr>
<tr>
<td>6579</td>
<td>Left</td>
<td>Partial nephrectomy</td>
<td>Normal kidney</td>
<td>wild type</td>
<td>wild type</td>
<td>NA</td>
<td>NA</td>
<td>6</td>
</tr>
<tr>
<td>6578</td>
<td>Left</td>
<td>Partial nephrectomy</td>
<td>Nephrogenic rest</td>
<td>wild type</td>
<td>wild type</td>
<td>NA</td>
<td>NA</td>
<td>6</td>
</tr>
<tr>
<td>3475</td>
<td>Right</td>
<td>Initial biopsy</td>
<td>Wilms tumour</td>
<td>wild type</td>
<td>wild type</td>
<td>normal</td>
<td>Neutral profile</td>
<td>1</td>
</tr>
<tr>
<td>6581</td>
<td>Right</td>
<td>Partial nephrectomy</td>
<td>Wilms tumour (regressive)</td>
<td>c.131T:p.P44L</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>6</td>
</tr>
<tr>
<td>3863</td>
<td>Right</td>
<td>Partial nephrectomy</td>
<td>Wilms tumour (regressive)</td>
<td>c.131T:p.P44L</td>
<td>Normal</td>
<td>+1q, LOH 14</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>6582</td>
<td>Right</td>
<td>Partial nephrectomy</td>
<td>Normal kidney</td>
<td>wild type</td>
<td>wild type</td>
<td>NA</td>
<td>NA</td>
<td>6</td>
</tr>
<tr>
<td>9934</td>
<td>Right</td>
<td>Partial nephrectomy</td>
<td>Nephrogenic rest</td>
<td>n/a</td>
<td>wild type</td>
<td>NA</td>
<td>NA</td>
<td>6</td>
</tr>
<tr>
<td>8426</td>
<td>Right</td>
<td>Recurrence</td>
<td>Wilms tumour (diffuse anaplastic)</td>
<td>c.584T&gt;C: p.I195T</td>
<td>wild type</td>
<td>NA</td>
<td>NA</td>
<td>54</td>
</tr>
</tbody>
</table>

NA = Data not available

Figure 2: Copy number heterogeneity in a bilateral WT case. Affymetrix SNP 6.0 smoothed log2 ratios. (a) ICH-3474 (left, initial biopsy). (b) ICH-3864 (left, partial nephrectomy sample 1). (c) ICH-3865 (left, partial nephrectomy sample 2). (d) ICH-3475 (right, initial biopsy). (e) ICH-3863 (right, partial nephrectomy). Arrows: position of MYCN gain. x-axis: genomic position (divided by chromosome). y-axis: relative copy number (log2 scale). Known copy number variants detected by CNV-specific probes on the SNP 6.0 array were not excluded from this analysis, and are visible as multiple focal peaks in all profiles; the most prominent peak on 2p, labelled ‘*’ in panel (a), corresponds to the variable ANKRD36BP2 locus, and should not be confused with the MYCN peak.
MYCN sequence variants (as below) showed that three of the eight P44L tumours (ICH-4788, 6613 and 8819) also had evidence of MYCN gain, while single control probes on 2p (DYSF) and 2q (PAK3) detected normal copy number, indicating a focal or regional event rather than whole arm or chromosome gain. ICH-7512, the A158V case, had large scale or complete gain of chromosome 2, as did one of the samples with a silent mutation, ICH-8914.

Germline MYCN gain in a bilateral WT case

In a SNP array analysis of a bilateral case, a relatively focal single copy gain of MYCN was detected in both contralateral tumours. To determine if this was a germline aberration, or a somatic change (an early event in kidney development or a contralateral metastasis), copy number profiles were also obtained from the (right) normal kidney and the patient’s blood DNA. Both samples harboured the region of gain, confirming that this was a germline event. Segmentation of the copy number data with OncoSNP detected a <0.5 Mb region of single copy gain (Figure 1, Supplementary Figure 1), spanning 98 SNP probes from rs2287273 (chr2: 15658523) to rs648658 (chr2: 16145517), and flanked by rs2287272 (chr2: 15651846) and rs13008990 (chr2: 16150760), the nearest probes which detected normal copy number. The region of gain contained the complete coding sequences of MYCN and DDX1, and the 5’ region of NBAS, and was consistent with the focal gains typically observed in WT samples in our previous SNP array study [11].

Parallel Evolution of MYCN mutations and copy number aberrations in a bilateral WT case

One of the samples in which a P44L mutation was detected by exome sequencing was taken from the right side of a second bilateral case we have previously described [36]. This case is notable for its significant intra-tumour heterogeneity on the left side (Figure 2); all showed clear evidence of MYCN gain. Thus, in the contralateral kidneys of a single case with considerable spatial heterogeneity we observed separate MYCN aberrations (P44L on the right, copy number gain on the left) and distinct TP53 mutations (I195T on the right, V173L on the left).

MYCN gain is associated with anaplastic histology and poorer outcome

MYCN gain was measured in 293 WT cases (Supplementary Table 1) using the P380 Wilms Tumour probemix (gain threshold = 1.2, determined empirically with reference to Affymetrix and Illumina SNP array data). A single sample with copy number loss at the MYCN locus was excluded from further analysis. Associations between histological subtype and gain were assessed by two-tailed Fisher’s exact test. There was no significant association (p > 0.05) between histology and MYCN gain for the blastemal, epithelial, stromal, mixed, regressive or focal anaplastic subtypes. However, for the diffuse anaplastic subtype, where 7/23 (30.4%) samples had MYCN gain compared to 30/269 (11.2%) in the remaining series, the association was significant (p = 0.0159). Survival analyses factored by MYCN copy number status (gained / normal) were carried out using the Kaplan-Meier model (Figure 3); two samples with no outcome data were excluded, and two further samples from patients who died without a defined relapse event were included in the analyses of overall, but not relapse-free, survival. MYCN gain was associated with significantly poorer relapse-free (p = 0.005) and overall (p = 0.002) survival by the Log Rank (Mantel-Cox) test. Using the Cox Proportional Hazards model, we calculated the hazard ratios for MYCN gain as 2.443 (relapse) and 3.545 (death). However, since anaplastic histology is itself associated with poor outcome, survival analysis was also carried out with the anaplastic samples removed from the series (Figure 3). Again, the MYCN gain group had significantly poorer relapse-free (p = 0.006) and overall (p = 0.003) survival by the Log Rank test, with hazard ratios of 2.697 (relapse) and 4.610 (death). 11 of the 37 samples with MYCN gain (2p24.3) also had gain of the DYSF locus (control probe) on 2p13.3, indicating that MYCN gain is not always focal. We therefore determined whether gain of DYSF, as an example of a 2p locus that is distinct from MYCN, is associated with a particular subtype or outcome, using the same analyses we applied to MYCN (excluding cases with DYSF loss). There was no association between...
DYSF gain and any of the histological subtypes, and no significant difference between the relapse free or overall survival of cases with gained or normal DYSF at the p < 0.05 level.

**MYCN overexpression is associated with hypomethylation of specific loci**

In a parallel study in our laboratory, we have recently completed an integrated genomic analysis of WT in which 65 samples were profiled on Affymetrix 250K Nsp / SNP 6.0 arrays for copy number, Illumina HT-12 arrays for mRNA expression, and Illumina 450K arrays for DNA methylation (manuscript in preparation). We therefore examined these data to determine if there is evidence of significant differential regulation of MYCN expression in this tumour series. MYCN expression levels were assessed in each of six histological subtypes (Figure 4, Supplementary Table 2). Levels of expression appeared notably higher in the high risk diffuse anaplastic (DA) and blastemal type (BT) tumours than in the four intermediate risk (IR: epithelial, stromal, mixed and regressive type) tumours examined. Univariate class comparisons (two-sample t-test) between the IR tumours (taken together as a group) and the tumours in either of the high risk groups identified MYCN as a relatively overexpressed gene in both DA and BT WT. When DA tumours were compared with IR tumours at the p < 0.01 significance level, 1786/34339 (5%) of the probes on the array detected differential expression, including both probes for MYCN (ILMN_1653761: p = 0.0001, fold-change 2.10; ILMN_2219767: p = 0.0002, fold-change 3.34). Similarly, for BT vs IR, 2521/34339 (7%) of probes detected differential expression, including both MYCN probes (ILMN_1653761: p = 0.0019, fold-change 1.66; ILMN_2219767: p = 0.0013, fold-change 2.87). MYCN overexpression also appeared to be associated with poorer outcome. Applying the Cox proportional hazards model to the expression data (Wald statistic, p < 0.01) identified 803 probes that detected genes in which overexpression was associated with poorer relapse-free survival (hazard ratio ≥ 1.5), including both MYCN probes (ILMN_1653761: p = 0.0082, hazard ratio = 1.9; ILMN_2219767: p = 0.0097, hazard ratio = 1.5). To establish whether the expression patterns we observed were potentially driven by DNA methylation events or by copy number changes, we carried out Spearman rank correlation analyses across the sample series between the expression data (log2 intensity) and either the segmented log2 copy number data, or the logit-transformed methylation value (M value), using all probes with annotation that mapped them to MYCN. By

![Figure 3: Survival analysis in SIOP patients with or without MYCN gain.](image)

(a) Relapse-free survival (RFS) in cases of all histologies with MYCN gain (blue) or with normal MYCN copy number (green). (b) Overall survival (OAS) in patients of all histologies. (c) Relapse-free survival in cases with diffuse anaplasia excluded. (d) Overall survival in cases with diffuse anaplasia excluded.
this metric we detected only a weak positive correlation between MYCN expression and copy number that did not quite reach statistical significance (ILMN_1653761: rho = 0.23, p = 0.0672; ILMN_2219767: rho = 0.24, p = 0.0522). However, we detected a strong negative correlation (rho = -0.50 to -0.71, p < 0.0001) between MYCN expression detected by both probes and methylation level at five loci probed by the 450K array (Figure 5). Two of these loci (cg20431766 and cg07083806) are in the intron downstream of the first MYCN coding exon, and the more 5’ probe, cg20431766, is within the CpG island that spans the MYCN promoter. The other three loci (cg19623054, cg04609952, cg25074809) overlap with the second coding exon. Although the methylation array includes another 18 probes that are annotated to MYCN and either overlap with the gene or are located in the intergenic region immediately upstream of its start site, none of these detected differential methylation that significantly correlated with gene expression (Spearman rho < -0.5 or > 0.5; p < 0.05).

**DISCUSSION**

The results presented here suggest that MYCN function in Wilms tumour is potentiated by several mechanisms. Copy number gains that included the MYCN locus were detected in 37/292 (12.7%) of tumours overall, and in 7/23 (30.4%) of diffuse anaplastic WTs. Relative overexpression of MYCN is associated with hypomethylation of two differentially methylated regions within the MYCN gene. 8/213 (3.8%) of samples carried a recurrent point mutation, c.131C>T (p.P44L), that has been identified in neuroblastoma as an acquired somatic variant with presumed gain of function [27]. MYCN mutations had not previously been described in WT, but during the review of this manuscript another group [37] reported two further examples of the P44L mutation. We note also that our previous discovery of FBXW7 aberrations in a small proportion of WTs [11] may represent an additional mechanism of MYCN regulation, since FBXW7 is part of a ubiquitin ligase complex that degrades MYCN.

Our results suggest that MYCN dysregulation is significantly associated with adverse outcome. MYCN gain was associated with high risk diffuse anaplastic histology, and with poorer relapse-free and overall survival, even when diffuse anaplastic cases were excluded from the analysis. This is consistent with a previous study that found an association between MYCN expression and relapse [38]. MYCN was relatively overexpressed in diffuse anaplastic and blastemal type tumours, and there was an association between expression level and poorer outcome.
relapse-free survival. However, it is unclear if all potential mechanisms of dysregulation have the same functional effect on downstream pathways or tumour behaviour. The number of P44L mutations detected to date is too small to draw firm conclusions about associations with outcome or histology. A recurrent somatic variant at a single highly conserved residue is most consistent with an activating mutation, though it has not yet been subject to a full functional analysis in WT or in neuroblastoma. We did detect one additional somatic variant, R285W, that is predicted to be deleterious, but its exact functional impact is currently unknown. Three of the P44L mutations were also associated with MYCN copy number gain. This may indicate further selection by the tumour cell for multiple copies of a variant that confers a growth advantage.

The case with MYCN mutation in one kidney and copy number gain in the other is a striking example of parallel evolution in bilateral disease. As we reported previously [36], this case also has independent contralateral TP53 mutations, albeit with apparently dissimilar timing of the mutational events. In the left kidney, MYCN gain was detected in all specimens examined (Table 2), including the initial pre-chemotherapy biopsy, while the V173L TP53 mutation was absent at initial biopsy and in a precursor lesion (nephrogenic rest). A copy number profile was not available for the nephrogenic rest, so it was not possible to determine if MYCN gain was present in the earliest phase of tumourigenesis, but it is notable that the MYCN aberration, together with the large-scale copy number changes on 10p, 20p and 13 that were common to all profiled samples, appear to have preceded disruption of TP53 by point mutation and 17p loss. In the right kidney, both genes were wild type at initial biopsy, but the MYCN P44L mutation was detected at the time of the first (partial) resection, again before any TP53 aberrations were observed. A subsequent relapse, 4 years after the original tumour was excised, carried the I195T TP53 mutation, concomitant with the development of anaplasia, but the MYCN sequence was wild type. It therefore appears that similar but independent evolutionary solutions affecting two different pathways have been selected for at different stages of the development of contralateral tumours in the same patient, with MYCN disruption the earlier event on each side. We note, however, that interpretation of this case is complicated by spatial heterogeneity, which is obviously extensive, at least at the copy number level. It is, for example, not possible to determine from our current analysis whether the relapse in the right kidney, which has a novel TP53 mutation but lacks the MYCN mutation, arose from some minority clone not sampled or undetectable in the first (partial) nephrectomy, or if the relapse itself (of which there was only a single sample) had developed significant spatial heterogeneity. It is even possible that the ‘recurrence’ was a novel primary tumour.

MYCN gain, even when focal, typically also results in gain of the adjacent DDX1 gene. All the focal events at this locus analysed by SNP arrays in our previous study [11] had DDX1 gain, as did the germline gain detected here. Recently, a similar region of germline gain encompassing both genes was detected in a patient with bilateral nephroblastomatosis and a family history of WT [39]. A genome-wide association study has also identified potential WT susceptibility loci in this commonly gained region, mapping to SNPs that flank DDX1 [40]. We cannot therefore rule out a significant role for DDX1 in Wilms tumour. However, our finding that MYCN is also subject to putatively activating point mutations strongly suggests that MYCN is a primary target for dysregulation at this locus.

In addition to neuroblastoma and WT, MYCN gain or amplification is now emerging as a common aberration associated with adverse outcome in multiple paediatric malignancies, including rhabdomyosarcoma [26] and medulloblastoma [24, 25]. This raises the possibility that any novel therapies that target the MYCN pathway and prove useful in one of these tumours could be of significant value in the others. In WT, anaplastic tumours that are metastatic at presentation respond poorly to current treatment. While TP53 mutation remains the most common aberration in this group of tumours, our finding of an association between MYCN gain and anaplasia, as well as outcome, makes the MYCN pathway an attractive target for further research into new approaches to treatment.

MATERIALS AND METHODS

Clinical samples

Frozen Wilms tumour samples were obtained with informed consent and ethical approval according to national regulations from UK, German, Dutch, and Swedish centres participating in the SIOP WT 2001 clinical study and trial (2001-2011) (Supplementary Table 1). A frozen section was prepared directly or paraffin-embedded from the same specimen used in nucleic acid extraction, stained with haematoxylin and eosin, and assessed for tumour content by an experienced paediatric pathologist. Only samples from WT cases treated by the standard neoadjuvant chemotherapy treatment approach were included in this study.

Nucleic acid extraction

Frozen tumour material was pulverised at liquid nitrogen temperatures using a Sartorius Mikro-Dismembrator. Genomic DNA was prepared by standard detergent lysis and phenol-chloroform extraction methods. Total RNA was extracted using Trizol reagent (Life Technologies) according to the manufacturer’s instructions.
Whole exome sequencing

Target enrichment using the Agilent SureSelect V4+UTR system and sequencing on the Illumina HiSeq 2000 instrument were carried out by an external service provider. Data were analysed using a somatic variant detection pipeline on the UCL Computer Science HPC cluster. Sequence reads were aligned to human genome build hg19 using BWA [41]. Removal of PCR duplicates, local realignment around indels, and quality score recalibration were carried out using GATK [42] and Picard (http://picard.sourceforge.net/), according to the GATK ‘best practice’ recommendations. Somatic variants were detected with MuTect [43], annotated with ANNOVAR [44], and assessed in IGV [45, 46]. All MYCN variants are described with reference to the translational start codon of NCBI RefSeq cDNA NM_005378.4, corresponding to protein NP_005369.2.

PCR and Sanger sequencing

The complete coding sequence of MYCN was amplified from genomic DNA using primers: Ex2Af: 5’- GGTATTAAAACGAACGGGGC-3’; Ex2Ar: 5’-GGACTGGGCGGTGGAAC-3’; Ex2Bf: 5’- ATCCTCAGGACTGATGTG-3’; Ex2Br: 5’-CAGGCCAAGACATACGAGC-3’; Ex3Af: 5’- GCCGGAAGAGACAGATAAGC-3’; Ex3Ar: 5’- GATGTTGTGGTTTCTTGCAGC-3’; Ex3Bf: 5’- GCCCAGCACCACTATATG-3’; Ex3Br: 5’- TACTGCCCACCCAGAGCC-3’. PCR products were sequenced using the ABI BigDye cycle sequencing kit on an ABI Prism 3100 Genetic Analyzer. Variants were detected and assessed with GeneScreen [47] with reference to the hg19 reference sequence.

MLPA

MLPA was performed according to the manufacturer’s instructions using the P380 Wilms Tumour probemix, developed by MRC-Holland in association with our laboratory. This probemix includes 3 probes for MYCN, a DYSF control probe on 2p outside the common region of genomic gain previously observed on SNP arrays, a PAX3 control probe on 2q, further test probes to other loci of interest in the Wilms tumour genome, and 9 reference probes to loci on chromosomes 3, 5, 15, 19 and 21 found to have comparatively stable copy number in previous Wilms tumour microarray experiments. Since MYCN binding sites are distributed throughout the genome, we reasoned that the most biologically relevant measurement of MYCN status was its copy number relative to the genomic baseline copy number. All copy number measurements were therefore made with respect to the 9 reference probes; the DYSF and PAX3 probes served only to determine whether any MYCN probes served in the context of larger scale gain of 2p or of the whole chromosome.

Methylation arrays

DNA methylation profiling was carried out according to the manufacturer’s instructions using the Zymo EZ DNA Methylation kit kit and Illumina Infinium HumanMethylation450 BeadChip. Arrays were run and scanned at the UCL Genomics ICH Microarray facility. Data were normalised and analysed using the Bioconductor [48] packages minfi [49] and limma [50].

Expression arrays

Expression profiling was carried out on the Illumina HumanHT-12 v3 Expression BeadChip at the UCL Genomics ICH Microarray facility, according to the manufacturer’s instructions. Data were normalised and analysed using the lumi [51] Bioconductor package and BRB-ArrayTools [52].

SNP arrays

DNA samples were profiled on Affymetrix Human Mapping 250K Nsp or Genome-Wide Human SNP Array 6.0 arrays at UCL Genomics, UK or AROS Applied Biotechnology A/S, Denmark. Copy number and LOH analysis was carried out using the Affymetrix Genotyping Console, using HapMap reference samples, and visualised in CGH Explorer [53] or IGV [45, 46]. Samples from a single case with germline MYCN gain were profiled on the Illumina HumanCytoSNP-12 BeadChip, and analysed with Illumina GenomeStudio and OncoSNP [54]. Raw data are available from the authors on request.

Survival analysis

Survival analyses were carried out in IBM SPSS 21 using the Kaplan-Meier model, with the Log Rank test for equality of the survival distributions. Hazard ratios were determined using the Cox Proportional Hazards model.

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**CONFLICTS OF INTEREST**

The authors declare no conflict of interest.

**Foot note**

Jan de Kraker deceased (chief investigator of the SIOPWT2001 trial, who sadly passed away during the conduct of the study).

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