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Research Paper

Intra-Tumor Genetic Heterogeneity in Wilms Tumor: Clonal Evolution and Clinical Implications

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ABSTRACT

The evolution of pediatric solid tumors is poorly understood. There is conflicting evidence of intra-tumor genetic homogeneity vs. heterogeneity (ITGH) in a small number of studies in pediatric solid tumors. A number of copy number aberrations (CNA) are proposed as prognostic biomarkers to stratify patients, for example 1q+ in Wilms tumor (WT); current clinical trials use only one sample per tumor to profile this genetic biomarker. We multisampled 20 WT cases and assessed genome-wide allele-specific CNA and loss of heterozygosity, and inferred tumor evolution, using Illumina CytoSNP12v2.1 arrays, a custom analysis pipeline, and the MEDICC algorithm. We found remarkable diversity of ITGH and evolutionary trajectories in WT. 1q+ is heterogeneous in the majority of tumors with this change, with variable evolutionary timing. We estimate that at least three samples per tumor are needed to detect >95% of cases with 1q+. In contrast, somatic 11p15 LOH is uniformly an early event in WT development. We find evidence of two separate tumor origins in unilateral disease with divergent histology, and in bilateral WT. We also show subclonal changes related to differential response to chemotherapy. Rational trial design to include biomarkers in risk stratification requires tumor multisampling and reliable delineation of ITGH and tumor evolution.

1. Introduction

Intra-tumor genetic heterogeneity (ITGH) has been documented in several adult tumors. Such tumors typically evolve over long periods before diagnosis, with most demonstrating branched evolutionary trajectories (Nowell, 1976; Greaves and Maley, 2012; Gerlinger et al., 2012, 2014; de Bruin et al., 2014). However, the prevalence and relevance of ITGH are poorly understood in pediatric solid tumors: since they carry lower burdens of mutational changes and have evolved for shorter periods of time before diagnosis, they may be expected to show less complex evolutionary histories (Vogelstein et al., 2013).

Although there are relatively few sequence mutations in pediatric malignancies, DNA copy number aberrations (CNA) and rearrangements are often characteristic features of these tumors. Some common CNA have recognized prognostic significance in pediatric tumors. For example, in neuroblastoma, *MYCN* amplification or subchromosomal genomic gains and losses are used to stratify therapy (Schleiermacher et al., 2011). In Wilms tumor (WT), gain of 1q (1q+) is increasingly being proposed as a common prognostic biomarker to select patients for more intensive treatment (Natrajan et al., 2006; Gratias et al., 2013; Segers et al., 2013). However, these studies have relied on a single tumor sample from each case.

Indeed, there has been limited investigation of ITGH in pediatric solid tumors. A recent multisampling study reported genetic homogeneity in multi-sampled embryonal brain tumors (Remke et al., 2015). However, a study of four pediatric small round cell tumors, with two samples from each, reported heterogeneous CNA in three out of four tumors (Mengelbier et al., 2015).

In WT, a large study showed that combined loss of heterozygosity (LOH) of chromosomes 1p and 16q, while rare, was not only associated

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with poorer outcome, but also showed concordance in the vast majority of the 10% of tumors from which two separate samples were assessed (Grundy et al., 2005). In contrast, heterogeneous *WTX* deletion has been reported in two multi-sampled WTs (Wegert et al., 2009), and heterogeneous activation of *MYCN* and inactivation of *TP53* have been reported in a case of bilateral WT (Popov et al., 2013; Williams et al., 2015).

Such variable heterogeneity complicates clinical decision making because of a poor understanding of the evolution of pediatric tumors. It also means that most previous studies showing prognostic significance for specific CNA did not take into account potentially significant ITGH. Therefore, here we assess the extent and significance of ITGH in a prospective study of unselected multi-sampled Wilms' tumors.

2. Methods

2.1. Samples

We obtained multiple samples from WT nephrectomy/nephron-sparing surgery specimens at Great Ormond Street Hospital between May 2011 and June 2013. All patients were enrolled on the SIOP WT 2001 trial (Pritchard-Jones et al., 2015), the current IMPORT study or their parents had consented for additional tissue to be used in research as part of the UK Children's Cancer and Leukaemia Group tissue bank. The research reported here was approved by a national research ethics committee. Patients received preoperative chemotherapy as per the SIOP WT 2001 trial protocol or according to national clinical guidelines based on this trial. Tumors were classified as previously described (Vujanić et al., 2002). A histological section from each tissue sample was reviewed to determine viable tumor content, and only samples with more than 50% viable tumor (the remainder consisting of necrotic tumor/post-chemotherapy change) were used. DNA was extracted using standard techniques from each tumor sample, and from adjacent non-tumorous kidney where it was available in 19 cases, and from peripheral blood lymphocytes in 3 cases.

2.2. Imaging

In two cases, different regions within the same tumor were identified prospectively as distinct nodules in the same overall tumor mass on T1- and T2-weighted MR imaging, and matched on comparison of pre- and post-chemotherapy images. Assessed diffusion coefficient (ADC) was calculated by one observer (ØEO) as previously described (McDonald et al., 2010).

2.3. Molecular Analyses

Illumina® HumanCytoSNP-12 v2·1 microarrays (~300,000 SNP probes) were hybridized with 250 ng DNA per sample according to the manufacturer's instructions. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) for 11p15 was carried out as previously described (Scott et al., 2008a), using the Salsa MS-MLPA BWS/RSS ME030-C3 probemix (MRC-Holland), and data visualized in Coffalyser.NET (MRC-Holland).

2.4. Detection of Allele-Specific Copy Number Aberrations and Inference of Tumor Evolution

Log R ratio ($\log_2$[observed intensity/reference intensity], LRR) and B-allele frequency (BAF) were calculated using the Illumina® GenomeStudio software for each array using default settings. LRR genomic waves (Diskin et al., 2008) were detected in normal tissue samples and corrected from all arrays. The LRRs from each array were segmented and copy number states were called using the 'CGHcall' R package (van de Wiel et al., 2007) in Bioconductor (Gentleman et al., 2004). For each case, the boundaries between adjacent regions were compared, smoothed and summarized between samples using the 'CGHregions' R package (van de Wiel and van Wieringen, 2007) in Bioconductor (Gentleman et al., 2004). A region was removed if it contained fewer than 100 probes or its probe density was an outlier. The mean tumor-specific mirrored BAF (mBAF) was calculated for each aberrant region and copy number aberrations were rejected if they did not show expected allelic imbalance. Aberrant regions detectable only in the BAF (i.e. copy number neutral LOH) were incorporated into our analysis using a custom pipeline. Allele-specific copy number was interpreted by the phylogenetic algorithm MEDICC (Schwarz et al., 2014) to infer clonal evolution of samples in each case. Normal tissue samples were used to root phylogenetic trees. Annotated code of our entire analysis pipeline is available as a GitHub repository at: https://github.com/luslab/multiregion-cnv-phylogenetics.

2.5. Role of the Funding Source

The study sponsors did not participate in study design, in the collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication.

3. Results

3.1. Intra-Tumor Genetic Heterogeneity in Wilms Tumor Demonstrates Unexpected Diversity

We studied 70 distinct tumor samples from 24 tumors in 20 patients (mean 3.5 samples/case, range 2–6 samples), with matched DNA from non-tumorous kidney and/or peripheral blood leukocytes in 19 cases. Five patients (Cases 9, 10, 16, 17, 20) had bilateral WT, and we obtained samples from both tumors in four of them; in Case 9, the contralateral tumor had been removed prior to the start of our study. Patient characteristics and samples are summarized in Table 1. We applied a custom-built pipeline to determine reproducibly genome-wide allele-specific CNA and LOH events using high-resolution SNP arrays hybridized with genomic DNA from each sample, and automatically compare these events across samples in a tumor. Fig. 1 shows a graphical representation of all CNA and copy number neutral LOH (CNNLOH) events across the 70 tumor samples. We detected most known recurrent WT CNA/LOH, including those associated with poor outcome (Natrajan et al., 2006; Gratias et al., 2013; Segers et al., 2013; Grundy et al., 2005). Surprisingly, 1q+ was heterogeneous in four of seven (57%) multi-sampled tumors with this change (see below). In general, we found remarkable diversity in the extent of intra-tumor CNA and CNNLOH heterogeneity, ranging from cases with unique CNA/CNNLOH events in all or most samples (such as Cases 1 and 15) to tumors exhibiting no CNA/CNNLOH heterogeneity across samples. The latter either lacked somatic CNAs/CNNLOH (Case 12) or showed a consistent pattern of somatic CNAs/CNNLOH across all samples (Cases 2, 3, 6), and the single dominant clone in each tumor showed few (0–4) CNAs/CNNLOH events. The four patients with these homogeneous (and unilateral) tumors were not statistically significantly younger than the other twelve patients with heterogeneous unilateral tumors (Welch two-sample one-tailed t-test, $t = -1.52$, $p = 0.08$), suggesting that heterogeneity does not arise purely as a consequence of later age at diagnosis.

3.2. Diverse Evolutionary Pathways in Wilms Tumor: From Homogeneity to Branched Evolution

In order to infer the evolutionary history of tumors, we generated phylogenetic trees depicting the relationships between samples. Our pipeline requires a minimum of four samples, and this condition was satisfied in seventeen cases. In thirteen cases, all tumor samples were from the same kidney (unilateral). We obtained a flat phylogenetic