Recurrent Somatic Structural Variations Contribute to Tumorigenesis in Pediatric Osteosarcoma

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http://dx.doi.org/10.1016/j.celrep.2014.03.003
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SUMMARY

Pediatric osteosarcoma is characterized by multiple somatic chromosomal lesions, including structural variations (SVs) and copy number alterations (CNAs). To define the landscape of somatic mutations in pediatric osteosarcoma, we performed whole-genome sequencing of DNA from 20 osteosarcoma tumor samples and matched normal tissue in a discovery cohort, as well as 14 samples in a validation cohort. Single-nucleotide variations (SNVs) exhibited a pattern of localized hypermutation called kataegis in 50% of the tumors. We identified p53 pathway lesions in all tumors in the discovery cohort, nine of which were translocations in the first intron of the TP53 gene. Beyond TP53, the RB1, ATRX, and DLG2 genes showed recurrent somatic alterations in 29%–53% of the tumors. These data highlight the power of whole-genome sequencing for identifying recurrent somatic alterations in cancer genomes that may be missed using other methods.

INTRODUCTION

Osteosarcoma is the most common malignant bone tumor in children and adolescents, with approximately 400 new cases each year in the United States (Ottaviani and Jaffe, 2009). Although most cases are sporadic, the risk of osteosarcoma is increased in patients with various genetic diseases, including hereditary retinoblastoma, Li Fraumeni syndrome, and germline mutations of RecQL4 (Hicks et al., 2007; Kleinerman et al., 2005; McIntyre et al., 1994). Current multimodal therapies that incorporate surgical excision and combination chemotherapy (i.e., doxorubicin, methotrexate, and cisplatin) cure approximately 70% of patients (Meyers et al., 2005). However, clinical outcomes and therapeutic strategies have remained virtually unchanged over the past 20 years (Smith et al., 2010).

In this study, we characterized the genomic landscape of osteosarcoma by performing whole-genome sequencing (WGS) on 34 osteosarcoma tumor and matched nontumor tissue samples from 32 patients. Our results demonstrate that pediatric osteosarcomas have one of the highest rates of SVs of any pediatric cancer sequenced to date (Downing et al., 2012), but relatively few recurrent single-nucleotide variations (SNVs). However, when SVs and SNVs were combined, inactivating mutations were identified in several cancer pathways. Taken together, our results provide insights into the molecular pathology of pediatric osteosarcoma and demonstrate that comprehensive WGS is required to elucidate the complete genetic landscape of osteosarcoma.

RESULTS

WGS of Primary and Metastatic Osteosarcomas

Using a paired-end sequencing approach, we generated 10,265 Gb of sequence data for DNA in 20 osteosarcomas and matched...
Figure 1. WGS of Osteosarcoma

(A) Representative CIRCOS plots of validated mutations and chromosomal lesions in diagnostic and metastatic osteosarcoma tumors from different patients. LOH (orange), gain (red), and loss (blue) are shown. Intrachromosomal (green lines) and interchromosomal (purple lines) translocations are indicated. Sequence mutations in RefSeq genes included silent SNVs (green), nonsense and missense SNVs (brown), splice-site mutations (dark blue), and insertion/deletion mutations (red). An additional track was added to the innermost ring of the plot showing the density of SNVs to highlight regions adjacent to SVs characteristic of kataegis.

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normal DNA from 19 osteosarcoma patients in a discovery cohort, and 14 tumor specimens and matched normal DNA from 13 patients in a validation cohort (Table S1); 9,671 Gb (94%) were successfully mapped to the reference genome (Table S2). In the discovery cohort, the samples included 17 pre-treatment diagnostic samples (16 primary and one metastatic), one recurrent metastatic sample (SJOS001), and two tumor specimens (SJOS010_D and SJOS010_M) from the same patient with metachronous osteosarcoma (Table S1).

The average genome coverage was 44× and the average exon coverage was 39×; 99% of SNPs detected across all 39 genomes showed concordance with their corresponding SNP array genotype calls (Table S2). Validation was carried out using custom liquid capture for all SNVs, SVs, and insertions or deletions (indels) identified in the original sequence data. Combining the discovery and validation cohorts, we identified 50,426 validated somatic sequence mutations and 10,806 SVs (Table S3). These included 856 nonsilent tier 1 mutations in genes, 4,651 tier 2 mutations in evolutionarily conserved regions of the genome, and 43,782 tier 3 mutations in nonrepetitive regions of the genome that are not part of tier 1 or tier 2 (Table S3). The average number of sequence mutations was 1,483.1 per case (range 610–5,178), with 25.2 mutations per case (range 5–103) resulting in amino acid changes (Table S3). The estimated mean mutation rate was 1.15 × 10⁻⁹ per base (range 4.90 × 10⁻⁷–3.99 × 10⁻⁵). Among the validated SVs, 377 were predicted to produce an in-frame fusion protein (Table S3). Good-quality RNA sequencing (RNA-seq) data were available for five tumors with 64 predicted fusion SVs. Among them, 15 SVs (23%) were expressed (Table S3).

Primary and metastatic osteosarcomas had high rates of validated SVs (Figures 1A and S1). The number of SVs and CNVs, background mutation rate, and number of nonsilent tier 1 mutations were significantly higher in osteosarcoma compared with medulloblastoma and T-ALL (Robinson et al., 2012; Zhang et al., 2012; Figure 1B). However, only the number of SVs was significantly higher in osteosarcoma compared with another pediatric solid tumor with high rates of somatic alterations (embryonal rhabdomyosarcoma) (Chen et al., 2013; Figure 1B). The global patterns revealed by the WGS analysis of osteosarcoma suggest that the majority of SVs and CNVs were generated by sequential accumulation of SVs (Figures 1C and 1D), but chromothripsis (Stephens et al., 2011) was detected at specific genomic regions in four samples (chr14 in SJOS002_D, chr17q in SJOS003_D, chr6q in SJOS005_D, and chr13 in SJOS010_M; Supplemental Experimental Procedures). We used a modified version of GISTIC analysis to identify regions of the osteosarcoma genome with recurrent copy number alterations in the discovery cohort. The TP53, RB1, MYC, and PTEN pathways, as well as ATRX, LSAMP-AS3, CCNE1, and a genomic region on chromosome 16 containing COP3, PMP22, MAPK7, NCOR1, and UBB, were recurrently mutated (Figure S1C). Among SNVs with sufficient coverage in both SJOS010 samples (20×), we validated 673 SNVs in both samples, 1,686 in diagnostic-only samples, and 1,408 in metastasis-only samples, indicating that these two tumors shared a limited amount of common mutations and were divergent early in the progression.

Applying the GRIN method (Pounds et al., 2013) on functional mutations (including SNVs and indels) and SVs, we identified TP53 (false discovery rate [FDR] = 3.6E-51, mutated in 28/34 samples) RB1 (FDR = 1.1E-5, mutated in 10/34 samples), ATRX (FDR = 2.4E-4, mutated in 10/34 samples), and DLG2 (FDR = 0.044, mutated in 18/34 samples) as significantly mutated genes. All genes except DLG2 were mutated by point mutations (nine for TP53, three for RB1, and five for ATRX) and SVs in multiple tumors (18 for TP53, seven for RB1, and five for ATRX). DLG2 was exclusively mutated by SVs.

**Osteosarcoma Tumor Purity and Tumor Heterogeneity**

Using the purity-adjusted mutant allele fraction (MAF) derived from deep sequencing of all SNVs by capture enrichment and Illumina sequencing, we analyzed intratumor heterogeneity. Eleven tumors (SJOS001_M, SJOS004, SJOS005, SJOS008, SJOS012, SJOS013, SJOS015, SJOS001103_D1, SJOS001105_D1, and SJOS001123_D1, and SJOS001125_D1) were excluded from quantitative heterogeneity analysis due to an insufficient number of SNVs in copy-neutral regions. Statistical modeling demonstrated that 61% (14/23) of osteosarcomas in this group had evidence of multiple clones, including metastatic samples SJOS010_M, SJOS001107_M1, and SJOS001107_M2 (Figure S2).

**Kataegis in Osteosarcoma**

To determine whether there was any relationship between the SVs and location, distribution, or type of SNV in the osteosarcoma genomes, we plotted the validated SVs and SNVs for each sample and analyzed the intermutation distance (Figure S2). Hypermutable regions with the five hallmarks of kataegis (Nik-Zainal et al., 2012) were identified in 17 of the osteosarcoma tumors (Figure 2A). These five hallmarks of kataegis are (1) enriched C->T and C->G substitutions at CpG or CpX trinucleotides (Figures 2B and 2C), (2) the same class of nucleotide mutation occurring for contiguous stretches before switching to a different class (Figure 2D), (3) mutations within short stretches of the genome occurring on the same parental chromosome (Figure S2), (4) clustering of heavily mutated short stretches of the genome at multiple scales (Figure 2E), and (5) association of the hypermutated region with SV breakpoints (Figure 2E). The regions of the genome with kataegis were not recurrent in our cohort and were not associated with recurrently mutated genes.
Figure 2. Kataegis in Osteosarcoma

(A) Rainfall plot showing the Log_{10} of the intermutation distance versus genomic position for a representative osteosarcoma sample (SJOS005) with evidence of kataegis. The chromosomes are demarcated by gray shading and the number of SVs in each chromosome is shown in brown at the bottom. The validated SNVs are plotted and color-coded by the type of mutation.

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in osteosarcoma (Figure S2). Tier 1 SNVs in kataegis regions were not significantly associated with the expression status (p = 0.16 by Fisher’s exact test).

**Chronology of Kataegis, SVs, and Aneuploidy in SJOS005**

SJOS005 had the highest proportion (11%) of kataegis SNVs in our cohort. The large number of kataegis SNVs (n = 212) coupled with the accurate measurement of the MAFs of all SNVs derived from deep sequencing allowed us to analyze the chronology of kataegis in relation to other mutational events in this tumor. First, we examined MAFs of SNVs in kataegis microclusters containing five or more consecutive kataegis SNVs within 10 kb. The MAF variance was relatively small (6.7% of overall variance) within a microcluster, although there was a wide range of MAFs across microclusters (range 0.142–0.839, median 0.364; Figure S2). This pattern, along with the observation that SNVs in a microcluster occurred on the same parental chromosome, supports the hypothesis that SNVs in a kataegis microcluster originated from a single event. MAF analysis of SVs flanking “kataegis” clusters (range 0.132–0.866, median 0.396) also showed a significant positive correlation (p = 4.56E-5) with those of “kataegis” SNVs, and there was no significant difference between them (p = 0.143 by Wilcoxon signed rank test), indicating that neighboring SVs likely arise simultaneously with kataegis SNVs (Figure S2).

**SVs in TP53**

The p53 pathway was mutated in all 20 tumor samples from the 19 patients in our discovery cohort. The majority (95%, 19/20) had either sequence mutations or SVs in the TP53 gene, and one (SJOS018) had an MDM2 amplification (see Figures 3A–3C; Table S4). Surprisingly, 55% of the tumors (11/20) had SVs in the TP53 gene, and the majority of those were translocations with breakpoints that were confined to the first intron of the gene (90%, 19/21 SV breakpoints; Figures 3A–3C; Table S4). Indeed, some tumors had rearrangements in both alleles of TP53, resulting from two or more independent translocations (Table S4). One patient’s tumor (SJOS006) had a germline SNV (R337H), one (SJOS012) had a somatic splice-site mutation, and two (SJOS004 and SJOS010) had somatic missense SNVs (Figures 3A–3C; Table S4). The remaining four patients had tumors that harbored indels in the TP53 gene. Loss of heterozygosity (LOH) at the TP53 locus

**Figure 3. Validated Mutations in TP53**

(A) Structure of the TP53 gene showing the trans-activation, proline, DNA binding, and oligomerization domains with splice-site, frameshift, and missense mutations in tumors of the 19 patients in the discovery cohort.

(B) Structure of the genomic locus of the TP53 gene showing the exon boundaries color-coded in accordance with the protein domains shown in (A).

(C) A magnified view of intron 1 of TP53 showing the deletions (blue arrowheads), intrachromosomal translocations (red arrowheads), and interchromosomal translocations (black arrowheads).

See also Figure S3 and Table S4.
was evident in 40% (8/20) of the osteosarcoma tumors. In total, 15 tumors had biallelic inactivation of TP53, four had monoallelic inactivation of TP53, and one had MDM2 amplification (Figure 1C; Table S4).

To further validate the translocations in the TP53 gene identified by WGS, we developed a break-apart fluorescence in situ hybridization (FISH) assay with separate probes spanning the 5' and 3' regions of the gene (Figure 4A). We also developed a FISH assay with a probe spanning the entire TP53 gene (Figure 4A) to assess ploidy and determine whether the gene was deleted. To complement the FISH analysis, we performed p53 immunostaining to verify that the tumors with missense mutations had accumulated high levels of nuclear p53 protein. We successfully performed FISH in 18 of 20 tumors and p53 immunostaining on all 20 tumors (Table S4). Overall, there was perfect concordance between the WGS data and the FISH data (Figures 4B–4M; Table S4).

In an additional cohort of patient tumor samples, we found that 50% (16/32) had TP53 rearrangements, 22% (7/32) had missense mutations, 16% (5/32) had nonsense mutations, 6% (2/32) had a TP53 deletion, and 3% (1/32) had an MDM2 amplification (Table S5). Three patients with tumor showed no evidence of a p53 pathway mutation.

We did not find any significant difference in CNV (p = 0.20 by Wilcoxon rank sum test), SV (p = 0.85), SNV (p = 0.43), nonsilent tier 1 mutations (p = 0.66), or background mutation rate (p = 0.43) in the osteosarcoma samples with mutant p53 versus those with inactivating (nonsense, deletion and truncation) mutations in TP53. Survival analysis, including event-free survival and overall survival, did not show a significant difference in outcome for the patients whose tumors carried TP53-missense mutations (ten patients) versus those with TP53-truncating mutations (34 patients), with log rank test p values of 0.88 and 0.64, respectively.

RB1, ATRX, and DLG2 Are Recurrently Mutated in Osteosarcoma

ATRX is part of a multiprotein complex that regulates chromatin remodeling, nucleosome assembly, and telomere maintenance. It was recently shown that ATRX mutations in neuroblastoma are associated with age at diagnosis (Cheung et al., 2012). Most neuroblastomas with ATRX mutations show evidence of alternative lengthening of telomeres (ALT), as measured by WGS, telomere FISH, and telomere quantitative PCR (qPCR) (Cheung et al., 2012). In our osteosarcoma discovery cohort, we identified five tumors (SJOS001, SJOS002, SJOS007, SJOS001112-M2, and SJOS001117-D1) with point mutations in ATRX, and five
Figure 5. ATRX Mutations Correlate with ALT in Osteosarcoma

(A) Diagram of the five SNVs, four deletions, and one interchromosomal SV found in the ATRX genes of the osteosarcoma cohort. Three of the samples with ATRX SVs (SJOS006, SJOS018, and SJOS011) had matching RNA-seq data. SJ006 has a short deletion at exon 23 and the RNA-seq data confirmed a readthrough.

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with focal deletions or SVs affecting the coding region of the gene (Figure 5A; Table S6). There was no significant gender bias in ATRX mutations (p = 0.25 by Fisher’s exact test) even though it is located on the X chromosome. By immunohistochemistry, 31% (6/19) of the tumors in the discovery cohort were ATRX negative (Figure 5B; Table S6). The sample with a missense mutation (SJOS007-R1803C) and one with an SV (SJOS018) were heterogeneous for ATRX protein expression. Analysis of telomere sequence reads from the WGS data and qPCR of telomeres showed that the majority of osteosarcomas had longer telomeres (Figures 5C and 5D), and ALT was found in 85% (12/14) of the samples using telomere FISH (Table S6).

Beyond TP53 and ATRX, there were significant recurrent mutations in RB1 (10/34, FDR q = 1.1E-5) and DLG2 (18/34, FDR q = 0.044). DLG2 encodes a multi-PDZ domain protein that is involved in epithelial polarity during cell division and has been implicated in cancer cell invasion. In Drosophila, DLG is a tumor suppressor, but a clear tumor-suppressor function has not yet been confirmed for DLG2 in human cancer.

**SVs in Cancer Genes**

SVs contributed 91% (9,605/10,523) of all functional genetic lesions in our osteosarcoma cohort. In total, 122 cancer genes had at least one SV breakpoint (Table S7) and all but one tumor (SJOS01118_D1) had at least one breakpoint (range 1–40) in a cancer gene. SV breakpoint enrichment in the cancer genes was highly significant even when we excluded TP53 from the list (p = 2.5E-6). Twelve of the 34 tumors (35%) achieved significant enrichment of SV breakpoints in cancer genes individually. In addition, some tumors have “fold-back intrachromosomal translocations” (Campbell et al., 2010) to inactivate tumor-suppressor genes (Figure S3). These results further support the hypothesis that genomic instability leads to lesions in various cancer genes.

**Discussion**

WGS of osteosarcoma demonstrated that the rate of SNVs was similar to that in other pediatric solid tumors, and only a few recurrent SNVs were detected. Approximately half of the osteosarcomas in our discovery cohort had a pattern of hypermutation associated with SVs, called kataegis (Nik-Zainal et al., 2012). The regions of the genome with kataegis were not recurrent, and none of the most recurrently mutated genes were found in regions of kataegis. Chromosomal lesions, rather than SNVs, were the major mechanism of recurrent mutations, and many of the most significant chromosomal lesions were found in known cancer genes, including TP53, RB1, and ATRX.

**Genomic Stability and Osteosarcoma Initiation and Progression**

The most frequent mutation in osteosarcoma is in TP53. By our estimates, both alleles are mutated in as many as 80% of tumors, and at least one allele was mutated in >90% of tumors. These data suggest that p53 mutations are a major oncogenic driver in osteosarcoma. Although this finding is not novel, what is surprising is the mechanism of inactivation. Most TP53 mutations are SVs in intron 1, which suggests that either the TP53 locus is particularly susceptible to SVs or SVs occur at a high rate in the osteosarcoma tumor-initiating cell. Aside from osteosarcomas and prostate cancers (Baca et al., 2013; Berger et al., 2011), there is no evidence of TP53 SVs in any other cancer, so the locus is probably not uniquely susceptible to chromosomal rearrangements. These data raise an intriguing possibility: genomic instability characterized by high rates of CNVs and SVs may precede TP53 inactivation, and may be the underlying mechanism that initiates and promotes osteosarcoma.

**Kataegis in Osteosarcoma**

In a recent WGS study, Nik-Zainal et al. (2012) described a distinct hypermutation phenomenon in breast cancer that they termed kataegis. Here, we found SNV clusters with the same five characteristics of kataegis in 50% of the osteosarcomas analyzed by WGS. Interestingly, genomic regions encoding TP53 and ATRX, the two most frequently mutated genes in osteosarcoma, did not exhibit this pattern of local hypermutation. Furthermore, there was no association between kataegis and TP53 mutation type (i.e., SNV, indel, or SV).

**TP53-Mutant or -Null Osteosarcomas**

Previous studies have estimated that 20%–70% of osteosarcomas carry mutations in the p53 pathway (Lonardo et al., 1997; Wunder et al., 2005), but our data suggest that the proportion is much higher. For example, Wunder et al. (2005) sequenced exons 4–10 of the TP53 gene in 196 osteosarcoma samples and found that 19.4% (38/196) had TP53 SNVs. The investigators concluded that the remaining 80.6% (158/196) had wild-type TP53 (Wunder et al., 2005). They went on to show that event-free survival was indistinguishable between the two groups (wild-type and mutant TP53) (Wunder et al., 2005). SVs in the first intron of TP53 were not analyzed in that study, even though such lesions had previously been reported in osteosarcoma (Miller et al., 1990). Our data suggest that the majority of the tumors identified as TP53 wild-type in the study event that would result in a T1885 frameshift. For SJOS011, the RNA-seq and WGS data supported a junction connecting exon 1 to exon 28, creating a nonsense mutation (M6R*). For SJOS018, the RNA-seq and WGS data supported a deletion connecting exon 1 to exon 13, thereby creating an in-frame fusion protein (M6R11406). The WGS for SJOS016 predicts a deletion that connects exon 1 to exon 16, producing a frameshift (M6fs).
by Wunder et al. (2005) actually had inactivating SVs in TP53. Therefore, it may be useful to revisit the association of TP53 pathway inactivation with osteosarcoma outcome in a large cohort of patient samples.

EXPERIMENTAL PROCEDURES

Full details regarding sample acquisition, molecular and biochemical procedures, informatics, and WGS are provided in the Supplemental Information. All tumors in this study were obtained from St. Jude Children’s Research Hospital (SJCRH) patients. The SJCRH IRB approved experiments involving human subjects and informed consent was obtained from all subjects.

ACCESSION NUMBERS

The European Bioinformatics Institute accession number for the sequencing data reported in this paper is EGAS0000100263.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.003.

ACKNOWLEDGMENTS

This work was supported, in part, by Cancer Center Support (CA21765) from the NCI, grants to M.A.D from the NIH (EY014867, EYO18599, and CA168875), and the American Lebanese Syrian Associated Charities (ALSAC). M.A.D. is an HHMI Investigator. The whole-genome sequencing was supported as part of the St. Jude Children’s Research Hospital -Washington University Pediatric Cancer Genome Project.

Received: May 10, 2013
Revised: November 22, 2013
Accepted: March 3, 2014
Published: April 3, 2014

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