Complementary genomic approaches highlight the PI3K/mTOR pathway as a common vulnerability in osteosarcoma

Osteosarcoma is the most common primary bone tumor, yet there have been no substantial advances in treatment or survival in three decades. We examined 59 tumor/normal pairs by whole-exome, sequencing. Only the TP53 gene was mutated at significant frequency across all samples. The mean nonsilent somatic mutation rate was 1.2 mutations per megabase, and there was a median of 230 somatic rearrangements per tumor. Complex chains of rearrangements and localized hypermutation were detected in almost all cases. Given the intertumor heterogeneity, the extent of genomic instability, and the difficulty in acquiring a large sample size in a rare tumor, we used several methods to identify genomic events contributing to osteosarcoma survival. Pathway analysis, a heuristic analytic algorithm, a comparative oncology approach, and an shRNA screen converged on the phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) pathway as a central vulnerability for therapeutic exploitation in osteosarcoma.

Osteosarcoma cell lines are responsive to pharmacologic and genetic inhibition of the PI3K/mTOR pathway both in vitro and in vivo. We present, to our knowledge, the first comprehensive next-generation sequencing of osteosarcoma in combination with a functional genomic screen in a genetically defined mouse model of osteosarcoma. Our data provide a strong rationale for targeting the phosphatidylinositol 3-kinase/mammalian target of rapamycin pathway in osteosarcoma and a foundation for rational clinical trial design. These findings present an immediate clinical opportunity because multiple inhibitors of this pathway are currently in clinical trials.

Significance

We present, to our knowledge, the first comprehensive next-generation sequencing of osteosarcoma in combination with a functional genomic screen in a genetically defined mouse model of osteosarcoma. Our data provide a strong rationale for targeting the phosphatidylinositol 3-kinase/mammalian target of rapamycin pathway in osteosarcoma. We present, to our knowledge, the first comprehensive next-generation sequencing of osteosarcoma in combination with a functional genomic screen in a genetically defined mouse model of osteosarcoma. Our data provide a strong rationale for targeting the phosphatidylinositol 3-kinase/mammalian target of rapamycin pathway in osteosarcoma and a foundation for rational clinical trial design. These findings present an immediate clinical opportunity because multiple inhibitors of this pathway are currently in clinical trials.
investigating somatic alterations in specific genes were performed on small numbers of tumors with low-throughput techniques. More recently, next-generation sequencing of 34 osteosarcomas identified recurrent alterations in ATRX and Dlk2, in addition to TP53 and RB1 (13). This next-generation sequencing study used in depth analysis of whole-genome sequence data and demonstrated that OS tumors have multiple rearrangements across the genome, kataegis, and a high degree of intratumor heterogeneity. Chromothripsis has been described in selected cases (14, 15). To date, none of these discoveries have led to the development of molecularly targeted therapies or improved survival for OS patients.

Because treatment for OS has not changed appreciably in the past 30 years, new approaches to treating the disease are imperative. We sought to identify tractable therapeutic targets in OS by performing next-generation sequencing and a comprehensive analysis of the genomic alterations in OS. We anticipated challenges in identifying a gene or genes altered in a large proportion of cases because of the difficulty collecting a large sample size in such a rare tumor, genomic complexity and heterogeneity. We hypothesized that we could identify genes or pathways required for OS survival by combining sequencing data with four methods for identifying essential genes, including: (i) pathway analysis, based on the mutational profile; (ii) a heuristic algorithm used to identify alterations with the potential to be clinically actionable; (iii) a comparative oncology approach using whole-exome sequencing from a mouse model of OS; and (iv) a genome-wide functional shRNA screen in murine OS to identify essential genes. Taken together, the data implicate the phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) pathway as a key target for the treatment of OS.

Results

Clinical and Sequence Data Characteristics. We examined OS tumor/normal (blood) sample pairs by whole-genome sequencing (WGS; 13 cases), whole-exome sequencing (WES; 59 cases), and RNA-sequencing (RNASeq; 35 cases) (Fig. 1). Clinical features of the patient population including outcome were typical for OS, with the exception of a higher proportion (47%) with metastases at diagnosis (Fig. S1A). All cases except two were sporadic, and no patients had clinically apparent germline syndromes associated with OS.

WES of ~23 Mbp of protein-coding sequence yielded a median of 103x tumor (range 60x–1880x) and normal (range 56x–206x) depth of coverage. A median of 88.2% (range 84–91%) of exonic bases were powered for discovering mutations. WGS yielded a median of 36x tumor (range 30x–61x) and 35x normal (range 27x–38x) depth of coverage. In WGS, a median of 89% of genomic bases (range 87–90%) were powered for discovering mutations. Bases were considered powered for discovering mutations if they were covered with at least 14 reads in the tumor sample and at least 8 reads in the normal sample. The thresholds were selected to achieve 80% power for mutations at an allelic fraction of 0.3 (16).

Deleterious Germline Variants in TP53 Are Common in Osteosarcoma. We examined germline variants of the 59 normal samples by WES. Twenty rare nonsilent variants were present in nine candidate genes after excluding missense variants classified as “benign” by PolyPhen-2 (Table S1) (17). Variants present in more than two normal samples were detected in only two genes: TP53 (n = 7) and SQSTM1 (n = 4). Germline mutations in TP53 were present at a higher frequency (12%) than previously reported (3%) (4). All seven TP53 variants were previously reported. We also examined somatic loss of heterozygosity of the germline variant alleles and found that only TP53 germline variants were subject to a “second hit” by loss of heterozygosity of the wild-type allele (Table S1). Four rare nonsilent nucleotide variants in SQSTM1 were identified. Three of these were identical: p.K238E, a SNP (rs11548633) in the TRAF6 binding domain, and p.A426V, a mutation previously linked to Paget’s disease of bone (18).

Osteosarcoma Marked by High Mutation Rate and Kataegis. In the coding regions of the OS genomes (59 WES samples), we detected a median of 37 somatic, nonsilent mutations per case (range 7–237), corresponding to a median somatic mutation rate of 1.2 mutations per megabase. To better understand the mechanisms leading to mutations in OS, we examined the distribution and categories of somatic point mutations across the genome. We observed a prevalence of localized hypermutation, in particular involving closely spaced C > T and C > G mutations in TpCpX trinucleotide contexts in several samples. This phenomenon, termed kataegis, was recently described in breast cancer and, subsequently, OS (13, 19). Using “rainfall” plots of C > T and C > G mutations derived from WGS, we identified kataegis in 11 of 13 (85%) OS samples. As previously described, kataegis events overlapped with genomic rearrangements in OS (Fig. 2). Kataegis has been linked, in other diseases, to APOBEC family proteins, which play a role in innate antiviral defense (19). To test the hypothesis of viral involvement, we examined WGS, WES, and RNASeq data for the presence of viral nucleic acid sequences by PathSeq but did not detect any enrichment (20).

Osteosarcomas Exhibit Genomic Complexity and Heterogeneity. Copy number analysis using the WES data from 59 samples with GISTIC2 revealed 15 significant arm-level events, 15 significant focal amplifications, and 22 significant focal deletions (Fig. S1B and Datasets S1 and S2) (21). The observed copy number alteration rates in this study are consistent with previous reports. The most significant focal deletions included the genes RB1, TP53, and CDKN2A/B (9, 10, 12). The most significant focal amplifications were in the COP3, CCNE1, CDK4, and MYC genes, and 6p12.3 amplifications (8, 22). In the 13 WGS samples, a median of 230 (range 3–1,059) rearrangements per OS genome were observed, which is much higher than that seen in 275 TCGA (The Cancer Genome Atlas) tumor/normal WGS samples from other tumor types (median 48 (range 0–699), P < 3.5e-5, one-sided Mann–Whitney test) (23). No recurrent fusion events were found; however, rearrangements with breakpoints near TP53 occurred with highest frequency among 2,586 genes (Dataset S3). Additionally, RNASeq data revealed that TP53 was a recurrent fusion partner (3 of 35 samples) (Dataset S3). Integrative analysis of genomic breakpoints and copy number data were performed using the ChainFinder algorithm (24). Complex rearrangements indicative of chromoplexy were abundant in OS, and could be identified in samples both with high and low degrees of overall genomic rearrangement (25). We detected complex chains of rearrangements in 11 OS genomes (Fig. 2 and Dataset S4). The percentage of chromosomal breakpoint points involved in a chain ranged from 27–70% (median 44%) across osteosarcomas. Chains of rearrangements from all tumors involved multiple chromosomes (median 4, range 3–6) and all but one involved multiple “deletion bridges” (median 6, range 0–29), which indicate large segments of DNA deletions at fusion junctions. Consistent with observations in other cancer types, regions of kataegis were highly correlated with copy number breakpoints and rearrangement sites.

OS samples are heterogeneous and highly complex. Among the 59 WES samples we analyzed, estimates of sample purity ranged from 19 to 100% (median 55%) and average cancer cell ploidy ranged from 1.42 to 4.31 (median 2.09). This finding is consistent with the massive complexity revealed by rearrangement analysis and points to frequent occurrence of genome doublings in OS: indeed, 12 of 55 (22.7%) samples for which ploidy estimations were obtained had ploidy above 3 (Dataset S5).
Frequent Alterations in TP53 and RB1 and in TP53 and RB1 Interacting Genes. Analysis of sequencing data using algorithms optimized for identifying recurrent events across samples identified a limited number of events, previously known to occur in OS. The MutSigCV (mutation significance) algorithm identified only one gene mutated at significant frequency across the 59 WES samples (at q-value threshold of 0.1): TP53 (Fig. 1 and Dataset S6) (26). TP53 was mutated in 22% of samples. 85% of the TP53 mutations were missense or frameshift mutations in the DNA binding domain (Fig. S1C). Of the genes listed in the COSMIC database, the only other genes with nonsilent mutations occurring in more than one case were RB1 and PTEN (27, accessed May, 2014).

We examined each sample for the presence of known mechanisms of TP53 and RB1 inactivation and for alterations in known TP53 and RB1 interacting genes (Fig. 1). The results are summarized in Table 1. Fifty-six percent of cases had alterations resulting in both TP53 and RB1 inactivation. Inactivation of TP53 was observed in 75% of cases and surprisingly, 34% of cases have multiple mechanisms of TP53 inactivation. The types and relative frequency of genomic events in TP53 and RB1 are similar to prior reports (10, 11). Amplification of MDM2 and COPS3, proteins proposed to negatively regulate TP53 protein stability, were observed in 5% and 39% of cases, respectively (8, 9).

Alterations in PI3K/mTOR Pathway Genes Are Frequent in Osteosarcoma. To overcome difficulties in identifying significant common alterations in a tumor with high intra- and intertumor heterogeneity and relatively small sample size, we combined multiple analytical and genomic approaches to deepen the analysis.
First, we combined gene set enrichment analysis (GSEA) with mutational analysis and identified 32 pathways as being significantly affected by mutations across the 59 WES samples using the GSEA MSigDB Canonical Pathway collection ($q < 0.1$) (Dataset S7) (28, 29). Twenty-two pathways, including the 21 most significant pathways, contained TP53 and are likely significant because of the frequency of mutations in this gene (TP53 was the only significantly mutated gene across samples) (Fig. 3A). Of the remaining 10 significant pathways, 4 implicate the PI3K/mTOR signaling pathway: PTEN, IGF1/mTOR, RAS, and AKT (Fig. 3B). Four more pathways were statistically significant and contained members of the PI3K/mTOR signaling pathway but not TP53: IL2RB, RACCYCD, IL-7, and HCMV.

Pathway analysis indicated that the PI3K/mTOR pathway may be altered in OS, as we evaluated each genome individually with the objective of identifying mutations and copy number alterations in known cancer pathways, particularly those potentially involved with targeted therapies. To identify the most clinically relevant alterations in each sample, analysis of sequencing data and ranking of gene alterations by biologic and clinical relevance was performed using the Precision Heuristics for Interpreting the Alteration Landscape (PHIAL) algorithm (Dataset S8) (30). Samples contained a range of one to five potentially actionable alterations. This method identified 24% of patients as having alterations in the PI3K/mTOR pathway (Fig. 1 and Table 2). There were five PTEN deletions, a PTEN nonsense mutation, and a PTEN frameshift mutation. There were two TSC2 tuberin domain mutations, both previously reported to occur in individuals affected by tuberous sclerosis. There was an NF1 frameshift mutation. A PIK3CA mutation at amino acid position 545, a mutation hotspot, was identified (27, accessed April 9, 2013). Mutations were identified in PI3K/mTOR pathway components PDPK1, AKT1, and EIF4B and are predicted to be damaging by PolyPhen-2 (31). For PDPK1 and AKT1, mutations resulted in amino acid changes in key protein domains. The PIK3RI and one of the EIF4B mutations were not predicted to be damaging. In addition to PI3K/mTOR pathway alterations, PHIAL identified the presence of additional alterations, suggesting the potential for response to targeted agents (Table 2). The genes affected include MYC, growth factor receptor tyrosine kinases, Wnt pathway members, cell cycle regulatory molecules, and coordinators of DNA repair. In total, 20 of 59 patients (34%) had alterations with potential clinical implications. This method also identified alterations in epigenetic regulators SUZ12, ATRX, and ARID1A (Fig. 1).

Table 1. Frequent alterations in TP53 and RB1 and in TP53 and RB1 interacting genes in 59 osteosarcoma cases

<table>
<thead>
<tr>
<th>Alteration</th>
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<th>%</th>
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<tr>
<td>TP53 alterations</td>
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<tr>
<td>TP53 mutation</td>
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<td>39</td>
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<td>COP53 amplification</td>
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<tr>
<td>MDM2 amplification</td>
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</tr>
<tr>
<td>One mechanism TP53 inactivation</td>
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<td>41</td>
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<td>34</td>
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<tr>
<td>Total TP53 inactivation</td>
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<td>RB1 alterations</td>
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<td>3</td>
</tr>
<tr>
<td>RB1 deletion</td>
<td>36</td>
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<tr>
<td>Only RB1 inactivation</td>
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<td>7</td>
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<td>Both TP53 and RB1 inactivation</td>
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<td>56</td>
</tr>
<tr>
<td>Neither RB1 nor TP53 inactivation</td>
<td>11</td>
<td>19</td>
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Next, in a comparative oncology approach, we sequenced nine tumor/normal pairs (WES) from a mouse model of OS created via conditional deletion of Tp53 and Rb1 in the preosteoblast (Dataset S9) (32). Two PI3K/mTOR pathway genes were affected by somatic mutations in both mouse and human tumors: PTEN and PIK3RI. The Pten mutation in the murine tumor was a nonsense mutation p.Y16*. The Pik3r1 missense mutation, p.S629N, lies within the C-terminal Src homology 2 domain, a region demonstrated to be affected by mutations in endometrial cancer (33).

**Pik3ca and Mtor Are Essential for Osteosarcoma Proliferation and Survival.** Finally, we performed a functional genomics screen to try to understand the relationship between the complicated genotype in OS and the functional dependencies in an unbiased fashion. To identify genes essential for the proliferation and survival of OS, we performed a genome-wide, pooled shRNA screen in a primary cell line derived from murine OS (mOS482) (Fig. 4A) (32). Cells were infected with a pool of 40,000 lentivirally delivered shRNAs, targeting ~8,400 genes (Dataset S10).
relative abundance of each shRNA at the endpoint (18 doublings) relative to the initial reference pool was determined by massively parallel sequencing of the lentiviral DNA. Individual hairpins were rank ordered and RNAi gene enrichment (RIGER) analysis was used to collapse the normalized shRNA ranked list to gene rankings by two statistical methods: a weighted second best (\( P \leq 0.05 \)) method and the Kolmogorov–Smirnov (\( P \leq 0.05 \)) nonparametric rank statistic (Dataset S11 and S12). At the intersection were 172 genes. The 172 genes were enriched for cell cycle genes but also included \( {\text{Pik3ca}}, \) the \( \alpha \)-catalytic subunit of PI3K, and \( {\text{Mtor}} \) (Fig. 4B and Dataset S13). shRNAs against these genes were inhibitory to mOS cell growth (mOS482 and mOS202) while having minimal effect on a noncancerous bone cell (Kusa4b10) (Fig. 4C) (34). To determine whether gene candidates identified in the in vitro screen were essential for OS proliferation and survival in vivo, we screened for shRNAs that become depleted upon OS formation in mice. A pool of shRNAs targeting \( {\text{Pik3ca}}, {\text{Mtor}}, \) and controls (four shRNAs per gene) was introduced into mOS482 cells that were then transplanted into recipient mice (Fig. S2A). Six weeks later, orthotopic tumors were isolated and massively parallel DNA sequencing was used to determine the abundance of each shRNA from tumors and preinjection cells. shRNAs against \( {\text{Pik3ca}} \) and \( {\text{Mtor}} \) were significantly depleted compared with control shRNAs (Fig. 4D). To confirm isoform specificity of PI3K’s role in OS cell growth, three distinct mouse OS cell lines (mOS202, mOS482, and mOS493) were infected with shRNAs targeting \( {\text{Pik3ca}} \) or \( {\text{Pik3cb}} \) (Fig. S2B). Compared with both control and \( {\text{Pik3cb}} \) knockdown, \( {\text{Pik3ca}} \) knockdown caused growth arrest in mOS cells (Fig. 4E). Taken together, the in vitro and in vivo shRNA screen data indicate that OS cell line proliferation and tumor formation are dependent on the \( {\text{Pik3ca}} \) and \( {\text{Mtor}} \) genes.

**PI3K/mTOR Inhibitors Suppress Osteosarcoma Proliferation.** The occurrence of alterations in the PI3K/mTOR pathway in 24% of human OS samples and the identification of \( {\text{Pik3ca}} \) and \( {\text{Mtor}} \) in the murine OS genomic shRNA screen suggest that the PI3K/mTOR pathway is a potential unifying vulnerability to be exploited for targeted therapy in OS. Consequently, the PI3K/mTOR dual inhibitors GSK2126458 and BEZ235 and the PIK3CA-selective inhibitor PIK75 were tested against four human and three murine OS cell lines and MCF7 cells, a breast cancer cell line with a PIK3CA p.E545K mutation previously demonstrated to undergo

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### Table 2. PI3K/mTOR pathway alterations and other potentially actionable alterations identified in 20 osteosarcoma samples using the PHIAL algorithm

| Gene Alterations | \( n \) | Sample ID | Molecularly targeted agent
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<tr>
<td><strong>PI3K/mTOR pathway alterations</strong></td>
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<tr>
<td>( {\text{TSC2}} )</td>
<td>p.R611W, p.H593R</td>
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<td>BZ04, BZ18</td>
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<td>( {\text{PTEN}} )</td>
<td>p.E288*, p.P244fs</td>
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<td>BZ04, BZ09</td>
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<td>5</td>
<td>DF01, BZ03, SJ12, BZ06, SJ03</td>
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<td>( {\text{PIK3CA}} )</td>
<td>p.E545D</td>
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<td>DF01</td>
</tr>
<tr>
<td>( {\text{AKT1}} )</td>
<td>p.T443M</td>
<td>1</td>
<td>BZ29</td>
</tr>
<tr>
<td>( {\text{PIK3R1}} )</td>
<td>p.P84L</td>
<td>1</td>
<td>SJ13</td>
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<tr>
<td>( {\text{PDPK1}} )</td>
<td>p.G530E</td>
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<tr>
<td>( {\text{EIF4B}} )</td>
<td>p.R304K, p.R331K</td>
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<td>SJ02</td>
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<td><strong>Growth factor receptor tyrosine kinases</strong></td>
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<td>( {\text{KDR}} )</td>
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<td><strong>Other</strong></td>
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<tr>
<td>( {\text{CDKN2A/B}} )</td>
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*Only samples with preserved RB1 counted, because CDK inhibitors do not function in the absence of RB1 protein.
apoptosis in response to treatment with the dual inhibitors (35). All three inhibitors inhibited cell proliferation in all cell lines tested (Figs. 5A and 5B and Fig. S2C), but a PIK3CB-selective compound, TGX-221, had little effect on cell proliferation at concentrations up to 10 μM (Fig. S2D). In the murine and human OS cell lines, the IC50s of GSK2126458 and PIK75 ranged from 7 to 169 nM, similar to the IC50 observed in the MCF7 cells, demonstrating the sensitivity of OS cell lines to inhibition of PIK3CA or PI3K/mTOR. PIK75 and GSK2126458 readily induced apoptosis with treatment as shown by caspase 3/7 activation (Fig. S2E) and poly(ADP-ribose) polymerase (PARP) cleavage (Fig. 5C, assessed only for PIK75). Growth inhibition and apoptosis were seen at drug levels at which phosphorylated AKT (Ser473) and, in the case of GSK2126458, phosphorylated S6 (Ser235/236) levels were reduced (Fig. 5C and Fig. S2F). The IC50 of BEZ235 was 8–51 nM in human cell lines and 178 nM to 1.2 μM in murine cell lines. BEZ235 appeared to cause cytostatic cell growth and did not induce caspase 3/7 activation or PARP cleavage in OS cell lines (Fig. 5C and Fig. S2F). These data are consistent with a recent study that reports the anti-proliferative and antitumor effects of BEZ235 on OS cell lines (36).

**Discussion**

Although none of the patients included in this study had documented Li Fraumeni syndrome, rare nonsilent mutations were present in TP53 in 12% cases. In previous studies, germline mutations in TP53 were present in fewer than 3% of patients with OS without a history suggestive of Li Fraumeni syndrome (4). The higher rate of germline mutations detected in this study may be due to or attributable to the nature of the patient population, with a large number of patients from Brazil, where there is a 0.3% population incidence of the p.R337H mutation (37). In our study, 39 of 59 patients were from Brazil and 6 patients had germline TP53 mutations (15% of Brazilian patients), and 3 of these patients harbored the p.R337H mutation (8% of Brazilian patients). Another possible explanation is the improved ability to detect TP53 mutations using modern techniques compared with previously used techniques. If these results are confirmed in other study populations, patients with sporadic OS should be referred to a genetic counselor for discussion of testing for Li Fraumeni syndrome, which would constitute a change in clinical practice.

We found four rare nonsilent nucleotide variants in SQSTM1, three of which were identical: p.K238E is a SNP (rs11548633) in the TRAF6 binding domain with a minor allele frequency of 0.003 (National Heart, Lung, and Blood Institute Exome Variant Server). Because of the small study size, strong statistical conclusions regarding the association between the SQSTM1 p.K238E SNP and OS are not possible. The relationship between SQSTM1 SNPs and OS...
should be evaluated using methodologies more suited to identifying germline associations, such as genome-wide association studies.

In the context of pediatric cancers, OS is unique with regards to the somatic mutation rate and the somatic rearrangement rate (26). Pediatric cancers, such as retinoblastoma and rhabdoid tumor, both aggressive malignancies occurring in very young children, have extremely low somatic mutation frequencies. Even those pediatric cancers with higher somatic mutation frequencies, such as neuroblastoma and medulloblastoma, have $\sim 0.4$–0.5 mutations per megabase (26). In contrast to these tumor types, the median somatic nonsilent mutation frequency in OS was much higher at 1.2 mutations per megabase. This frequency approaches the median somatic mutation frequency of 1.5 mutations per megabase found in a study of 3,083 tumors of 27 different types, including both childhood and adult cancers (26). This finding suggests an alternative mechanism of transformation in OS tumors compared with other common childhood tumors.

We observed kataegis in 11 of 13 (85%) WGS samples, which is higher than the prevalence of kataegis reported in another cohort of OS samples (50%) (13). Both cohorts are small and so the true prevalence of kataegis remains to be determined once a larger cohort of samples has been analyzed. Similarly, sequencing depth and purity decrease the sensitivity for detecting alterations, so it is possible that we are underestimating the frequency of mutations and structural alterations, and thus kataegis, in OS.

In their study of breast cancers, Nik-Zainal et al. (19) suggested that APOBEC1 and APOBEC3s cytidine deaminases may be responsible for the kataegis phenomenon. Because our results and others point to kataegis as a mutational mechanism in OS, we suggest further investigation of the role of APOBEC enzymes in this tumor type.

Complex chains of rearrangements were present in 92% of the OS tumors studied and we observed a median of 230 rearrangements per OS genome, which is higher than previously suggested (15). Although direct comparison of the number and complexity of the rearrangements seen in OS to other pediatric cancers is not possible, OS appear to have a much greater frequency and complexity of structural variations (38). The frequent inactivation of TP53 may play a role in structural instability in OS, as has been reported in medulloblastoma (39). However, whether genomic instability is simply a byproduct of TP53 inactivation or is, in and of itself, a mechanism of osteosarcomagenesis remains to be answered. In summary, OS is one of the only cancers identified to date that occurs in younger patients in a site not exposed to carcinogens that has a relatively high somatic mutation rate and a very high rate and degree of complexity of structural alterations. This makes OS a model tumor in which to identify mechanisms driving processes, such as kataegis and chromoplexy, and the role of these processes in oncogenesis.

This study confirms the importance and prevalence of TP53 and RB1 inactivation by genomic events, which either affect the TP53 and RB1 genes directly or which alter TP53/RB1-interacting genes. Seventy-five percent of samples contained at least one somatic event in TP53, which is higher than the 40% previously reported in studies using candidate gene methods (5, 9). We report a slightly lower proportion (75%) of somatic events in TP53 than the 90% rate reported by Chen et al. in their comprehensive next generation sequencing study (13). This result is likely a result of lack of detection of structural variations in TP53 in our study, because only 22% of tumors were subjected to WGS. MDM2 amplification was seen in only 5% of tumors, less often than the 25% frequency previously reported (5, 8, 9, 40–43). Whereas previous studies have reported lack of overlapping mechanisms of TP53 inactivation,
specifically lack of *MDM2* or *COPS3* amplification in osteosarcomas with *TP53* mutations, 34% of our cases have multiple mechanisms of *TP53* inactivation. The analysis of mutations (germline and somatic), copy number changes, and rearrangements of multiple genes in one study also allowed us to determine that 56% of the tumors have both *TP53* and *RB1* inactivated.

Pathway analysis of mutated genes, clinical interpretation of individual genomes (PHIAL), a comparative oncology approach, and a genomic screen all point to a dependence on PI3K/mTOR pathway activation in OS. To our knowledge, this is the first study to report genomic alterations in PI3K/mTOR pathway members in a significant proportion of OS tumors (24%). *PTEN* deletion has been evaluated in a single study in which 4 of 27 osteosarcomas had biallelic deletion of *PTEN*, a similar rate of occurrence as seen in this study (44). A *PTEN* mutation has been detected in a single canine OS cell line but mutations in *PTEN* have not been previously described in human OS samples (45). One recent study using a sequenom assay identified mutations in *PIK3CA* in 3 of 89 tumors (46). However, the other PI3K/mTOR pathway members (*PDPK1, AKTI, and EIF4B*) containing somatic mutations in this study have not previously been recognized to harbor alterations in OS. Additionally, the PI3K/mTOR pathway is activated by many receptor tyrosine kinases (RTKs), and the pathway has been recognized as a critical effector of alterations that activate RTKs in cancer (47). PHIAL analysis implicated six alterations in RTKs in eight patients (*PDGFRα, PDGFRβ, IAK1, ALK, KDR, and FGFR4*). These patients may benefit from selective RTK inhibitors, but it also reasons that these tumors may be sensitive to inhibition with PI3K/mTOR inhibitors.

In addition, our in vitro and in vivo shRNA screen data strongly suggest that OS cell line proliferation and tumor formation are dependent on the *Pik3ca* and *Mtor* genes signifying that kinase activity of the PI3K/mTOR pathway is required for OS survival. These data are consistent with an earlier report that used kinome profiling to show that AKT (an intermediary kinase in the PI3K/mTOR pathway) is active in OS and its inhibition blocks cell proliferation (48). Interestingly, genetic ablation of either *Pik3ca* or *Mtor* is sufficient to halt cell proliferation and slow tumor formation. This finding suggests that agents that can inhibit either *Pik3ca* or *Mtor* individually should have a similar effect, but experimental evidence from other tumor types demonstrate that tumors that harbor mutations in PI3K/mTOR pathway members may acquire resistance to single-target inhibitors (49). Thus, a combination of PI3K and mTOR inhibitors or a dual inhibitor may help overcome feedback loops and pathway cross-talk that can mediate resistance.

The universal sensitivity of both human and murine OS cells demonstrates that mutation of PI3K/mTOR is not necessary to confer sensitivity to PI3K/mTOR inhibitors in OS. Using data from the Cancer Cell Line Encyclopedia, we determined that HOS cells, which harbor an *NF1* deletion, are the only human OS cell line used in these studies with an alteration in a PI3K/mTOR pathway member (50). In the HOS cell line the IC₅₀ of BEZ235 was 40 nM, the second highest IC₅₀ of the cell lines studied, and the IC₅₀ of PIK75 was 82 nM, the highest IC₅₀ of the cell lines studied. The presence of an *NF1* deletion does not seem to confer increased sensitivity to PI3K/mTOR inhibitors in this panel of human OS cell lines. However, because all of the human OS cell lines display a high degree of sensitivity to PI3K/mTOR pathway inhibitors, it would be difficult to detect increased sensitivity.

Genomic alterations in PI3K/mTOR pathway members is likely only one way in which the PI3K/mTOR pathway becomes active in OS. p70S6K expression, a measure of pathway activity, is present in 70–80% of OS and the level of expression has been correlated with overall and disease-free survival (51). Activation of PI3K-AKT signaling is downstream of several biological processes involved in OS metastasis, such as c-Met and Ezrin expression (52, 53). Micro-RNA-221, expressed at high levels in OS targets PTEN, increases cell survival, decreases apoptosis, and induces cisplatin resistance (54). These alternative mechanisms of PI3K/mTOR pathway activation in OS may explain the universal sensitivity to PI3K/mTOR inhibitors observed in human osteosarcoma cell lines. In agreement with our data, the dual PI3K/mTOR inhibitor BEZ235 and the α-selective PI3K inhibitor BYL719 have been shown to induce cell cycle arrest and decrease tumor formation in vivo (36, 55). In addition, in preclinical models, mTOR inhibition potentiates the effect of bispophonates and sorafenib, drugs being studied in clinical trials in OS (56–58). Other studies have focused on one gene or small molecule but this work demonstrates the convergence of multiple different approaches in human and mouse on the PI3K/mTOR pathway as a whole. In using an unbiased screen and comprehensive genomic assessment, we strengthen the existing evidence regarding a role for the PI3K/mTOR pathway in OS.

Prior clinical studies of mTOR inhibition in sarcomas and our identification of a range of alterations in the PI3K/mTOR pathway serves as a basis for rational clinical trial design. In a phase II trial of ridaforolimus, a second-generation mTOR inhibitor, two patients with OS had confirmed partial responses and one patient with OS had an unconfirmed partial response. The number of OS patients enrolled in this phase II trial is not reported (54 bone tumor patients were enrolled) and so a response rate cannot be determined (59). Given the promising results seen with mTOR inhibition in tuberous sclerosis-associated subependymal giant-cell astrocytomas and renal angiomylipoma, one could hypothesize that the two patients with partial responses had tumors harboring TSC2 mutations (60, 61). In a follow-up phase III study, ridaforolimus was administered as maintenance therapy to patients with advanced sarcomas. Only 69 of the 711 patients enrolled in this phase III trial had bone tumors and consequently, the subgroup analysis of bone tumors does not achieve statistical significance (62). To ensure adequate enrollment of OS patients, future clinical trials of PI3K/mTOR inhibition should be limited to OS patients. Given the frequency and range of PI3K/mTOR pathway alterations identified in this study, patient’s tumors should be genotyped and activity of the PI3K/mTOR pathway assessed by additional methods so that clinical responses can be correlated with genotype and pathway activity.

### Materials and Methods

#### Sample Selection and Clinical Data

Samples were contributed by hospitals in Brazil, Spain, Mexico, and the United States. All patients had histopathology confirmed to be OS by a pathologist at the local institution. All patients had consented to protocols approved by the Institutional Review Board at the appropriate institution. All but five tumor specimens sequenced were obtained prechemotherapy from the primary tumor at the time of diagnosis. Three tumor specimens were obtained postchemotherapy from a metastatic tumor (*S102, S113, DF01*). Survival curves were compared using a log-rank (Mantel-Cox) test. Patients who died from treatment toxicity were censored at the time of death.

WGS, WES, RNA Sequencing, and Analysis. Sequencing data reported in this paper is available via National Center for Biotechnology Information dbGap: phu000959.v1.p1 Osteosarcoma Genomics. Detailed methods for WGS, WES, and RNAseq and analysis are provided in SI Materials and Methods. Human genomics data were used as follows: WES data were used for the detection of variants (germline SNV/Indel, somatic SNV/Indel), copy number changes, purity/ploidy, and clonality analysis. WGS data were used for the validation of somatic variants and copy number variation detected in WES and detection of structural variants. RNAseq data were used for the validation of somatic variants detected in WES, analysis of gene expression levels, and detection of gene fusions.

Evaluation for Known Mechanisms of *TP53* and *RB1* Inactivation and for Alterations in Known *TP53* and *RB1* Interacting Genes. A sample was classified as having TP53, RB1, or CDKN2A/B, deletion if GISTIC2 score for
the gene was −1 or −2 (focal) and COPS3 or MDM2 amplification if GISTIC2 score for the gene was positive, or 2 or 1 (Dataset S14). Both WGS and RNASeq data were examined for evidence of TPS3 or RB1 rearrangements.

Murine Osteosarcoma. Murine OS spontaneously arising in a mouse model based on conditional deletion of Tp53 and Rb1 restricted to the preosteoblast were obtained by killing the animal once a visible tumor causing symptoms was present (32). Tumors were dissected free of normal tissue and frozen at −80 °C. Murine OS cell lines were derived by mincing fresh tumors and culturing in xMEM.

shRNA Constructs and Lentiviruses. Lentiviral shRNAs were obtained from The RNAi Consortium (TRC) collection of the Broad Institute (www.broad-institute.org/rnai/public). shRNAs were expressed from the lentiviral expression plasmid P.LKO.1. Virus was produced as previously described (63, 64). The TRC numbers for the shRNAs used in the in vitro and in vivo pooled shRNA screens are listed in Datasets S10 and S15, respectively. For individual targets of focal somatic copy-number alteration in human cancers. Genes Chromosomes and Cancer 12(4):R41.

PI3K/mTOR Inhibitor Studies. All inhibitors used in this study were purchased from Selleck Chemicals. Human OS cell lines were obtained from ATCC. Human OS cell lines were cultured with drug or DMSO control for 24 h and viability was assessed with the CellTiter-Glo luminescent cell viability assay (Promega). Murine OS cell lines were cultured with drug or control for 72 h and viability was assessed with the cell proliferation reagent WST-1 (Roche). Caspase 3/7 activation after 16 h of treatment with inhibitors was determined with the Caspase-Glo 3/7 luminescence assay (Promega). For Western blot analysis, human OS cell lines were treated with inhibitors for 4 h; murine OS cell lines were treated for 24 h. Western blotting was performed as previously described (65). All primary antibodies were purchased from Cell Signaling. HRP-conjugated secondary antibodies were from Jackson Laboratories.

ACKNOWLEDGMENTS. We thank Ben Hubbell-Engler for technical support. This work was conducted as part of the Sloan initiative for Genomic Medicine at The Children’s Hospital of Philadelphia, a project of The Children’s Hospital of Philadelphia and the Charles S. Loeb and Potentiated by loss of Rb, mimics the human disease. Cancer Lett 1876.


Supporting Information

Perry et al. 10.1073/pnas.1419260111

SI Materials and Methods

Whole Genome, Whole Exome, RNA Sequencing, and Analysis.

Library construction. Libraries are constructed using the protocol described previously, with several modifications: first, initial genomic DNA input into shearing has been reduced from 3 μg to 100 ng in 50 μL of solution (1). Second, for adapter ligation, Illumina paired-end adapters have been replaced with palindromic forked adapters with unique eight-base index sequences embedded within the adapter. These index sequences enable pooling of libraries before sequencing. Third, custom sample preparation kits from Kapa Biosciences are now used for all enzymatic steps of the library construction process.

In-solution hybrid selection (for whole-exome libraries). In-solution hybrid selection was performed as described by Fisher et al. (1).

Size selection (for whole-genome shotgun libraries). For a subset of samples, size selection was performed using gel electrophoresis, with a target insert size of either 340 bp or 370 bp ± 10%. Multiple gel cuts were taken for libraries that required high sequencing coverage. For another subset of samples, size selection was performed using Sage’s Pippin Prep.

Preparation of libraries for cluster amplification and sequencing. Following sample preparation, libraries were quantified using PicoGreen. Based on PicoGreen quantification, libraries were normalized to equal concentration and pooled by equal volume. Library pools were then quantified using a Sybr Green-based quantitative PCR (qPCR) assay, with PCR primers complementary to the ends of the adapters (kit purchased from Kapa Biosciences). After qPCR quantification, library pools were normalized to 2 nM, denatured using 0.2 N NaOH, and diluted to 20 pM, the working concentration for downstream cluster amplification and sequencing.

Cluster amplification and sequencing. Cluster amplification and sequencing of denatured templates was performed according to the manufacturer’s protocol (Illumina) using v3 cluster amplification kits, v3 flowcells, v3 Sequencing-by-Synthesis kits. Multiplexing Sequencing Primer kits, and the latest version of Illumina’s RTA software.

Exome analysis. Pair-ended reads were aligned to the hg19/GRCh37 build of the reference human genome using BWA 0.5.9. WES data were generated for 59 pairs using in-solution hybrid capture followed by Illumina sequencing. Reads were aligned to build hg19/GRCh37 of the human reference genome sequence BWA. PCR-duplicated reads were flagged using Picard (2). Alignments near putative indel sites were refined using GATK, using both the tumor and the normal samples. The degree of contamination by other samples was estimated using ContEst (3). Somatic point mutations were detected using MuTect (4). Somatic short insertions and deletions were identified using indelocator (www.broadinstitute.org/cancer/indelocator). Artifactual mutations caused by the oxidative DNA damage during library preparation were removed using D-ToxoG (www.broadinstitute.org/cancer/dt toxog). Somatic mutations were annotated using Oncotator (www.broadinstitute.org/oncotator). Total copy number ratios were computed as the ratio of tumor fraction read depth to the average fractional read depth in the normal samples in the region, followed by Circular Binary Segmentation (5, 6). Copy number profiles were analyzed using GISTIC2 (7). Absolute copy number, purity/ploidy, and clonality analysis was done using ABSOLUTE (8).

RNA sequencing and analysis. RNA Reads were aligned to the hg19/GRCh37 build of the reference human genome using an improved algorithm described previously, followed by PCR dupli-
resequencing was performed by PCR using a microfluidic device (Fluidigm), following the manufacturer’s instructions. PCR primers were designed with 200-bp flanking tails around mutations of interest. All amplicons for a given sample were given the same barcode. Constructed libraries were loaded onto an Illumina MiSeq and sequenced using paired-end 150-bp reads, followed by the standard alignment pipeline. The resulting BAM files were used for validation in the sense of the method described above.

**Identification of samples with kataegis.** C > T and C > G mutations were plotted according to intermutation distance (WES), along with copy number calculated by SegSeq (WGS) and genomic rearrangements analyzed with dRanger (WGS) and ChainFinder (WES) (9, 16–18). Regions of characteristic co-occurrence of local hypermutation and genomic rearrangement were identified as “kataegis” (19).


**Analysis of copy number, genomic breakpoints, and rearrangements.** Somatic copy number alterations were assessed in WGS using SegSeq (17). Integrative analysis of genomic breakpoints and copy number in WES samples was performed using the ChainFinder algorithm (16). Somatic rearrangements were identified in WGS samples with dRanger (18). Rearrangements in 13 OS WGS samples were compared with those found in a pan-cancer dataset consisting of 275 tumor/normal WGS pairs from The Cancer Genome Atlas (TCGA). The distribution of tumor types across samples was as follows: 49 THCA (papillary thyroid carcinoma), 40 LUAD (lung adenocarcinoma), 31 LUSC (lung squamous cell carcinoma), 31 GBM (glioblastoma multiforme), 25 SKCM (skin cutaneous melanoma), 24 STAD (stomach adenocarcinoma), 20 PRAD (prostate adenocarcinoma), 18 BLCA (bladder urothelial carcinoma), 17 HNSC (head and neck squamous cell carcinoma), 16 LGG (brain lower grade glioma) and 7 KIRC (kidney renal clear cell carcinoma). All TCGA datasets are available through The Cancer Genome Atlas Data Portal (tcga-data.nci.nih.gov/tcga) and GChub (gchub.ucsc.edu).

**Identification of significantly altered pathways.** Gene sets from the Molecular Signatures Database (MSigDB) Canonical Pathway set (GSEA) were treated analogously as single genes for the purpose of calculation of the footprint and the background mutation rate (i.e., gene territory and composition was combined in each gene set). MutSig2.0 was then used to identify significantly mutated gene sets.

**Heuristic algorithm for analysis of clinically relevant somatic mutations (PHIAL).** All exome-derived alterations (somatic point mutations, short insertions and deletions, and copy number alterations) were analyzed using a heuristic algorithm that interprets the clinical and biological significance of each alteration in the exome (21). Clinical significance was defined by whether a specific alteration may predict sensitivity or resistance to a treatment, or has prognostic or diagnostic ramifications. All alterations scored as being potentially clinically actionable were manually reviewed.

**shRNA Screening.**

**In vitro shRNA screening.** Primary mouse OS cells were seeded into 12-well dishes at a density of 1 × 10^5 cells per well, with a total of 3 × 10^7 cells infected per replicate (four replicates total). Cells were infected with a pool of lentivirally delivered shRNAs, composed of 40,021 shRNAs targeting ~8,400 mouse genes with a multiplicity of infection of 0.3–0.5. Cells were incubated overnight with virus and 5 μg/mL puromycin. The next day, cells from each replicate were pooled and cultured in 0.5 μg/mL puromycin for 18 population doublings. During propagation, 1 × 10^5 cells were passaged every 3–4 d to maintain initial representation, and remaining cells at each passage were stored in PBS at −80 °C. Genomic DNA was extracted from the final cell pellets, and 60 μg of gDNA was used as template for PCR amplification in eight parallel bar-coded reactions for each experimental replicate. PCR reactions were prepared for massively parallel sequencing (Illumina), as previously described (22, 23). All samples were sequenced to obtain at least 8e6 raw reads. The number of reads per individual shRNA was normalized between samples using the following calculation: Log2{[(raw read count for hairpin/ sum of raw reads for entire sample) × 1e6]+1}.

shRNAs were rank ordered by their log-twofold change value, which was calculated as the average normalized log2 of the fold-change in the abundance of each shRNA in the average of endpoint samples compared with the initial pDNA reference pool. Next, the shRNA gene enrichment (RIGER) algorithm in the GENE-E program (www.broadinstitute.org/cancer/software/GENE-E) was used to collapse the normalized shRNA ranked list to gene rankings by two comp methods: (i) the weighted second best score (ranked top shRNA 25% weight + second best shRNA 75% weight) and (ii) a KS statistic, which is a Kolmogorov–Smirnov nonparametric rank statistic representing the positional distribution of a set of shRNAs within an ordered list of shRNAs (22, 24). Lists were generated from the top 500 genes from each ranking method. The lists were trimmed by P value (P ≤ 0.05) and common essential genes, including ribosomal proteins proteosomasal proteins, and splicing factors (22). A union of the remaining 348 genes (weighted second best) (Dataset S11) and 313 genes (KS) (Dataset S12) was taken (Dataset S13).

**In vivo shRNA screening.** Plasmids encoding shRNAs targeting Pic3ca, Mior, and control genes listed in Dataset S15 were used to generate lentivirus-containing supernatants, as previously described (22). Equivalent amounts of supernatants were pooled and primary mouse OS cells were infected as described for the genomic screen. Cells were selected for 2 d with 0.5 μg/mL puromycin. Next, 1 × 10^6 cells were injected in 100 μL PBS subcutaneously into the flanks of NCRNU-M mice (Taconic). Tumors were harvested 5 wk after implantation. Genomic DNA was isolated from tumors and all available genomic DNA from each tumor was prepared for massively parallel sequencing as described above. The log-twofold change values reported are the average log base 2 of the fold-change in the abundance of each shRNA in the tumors compared with the preinjection cells, n = 5 tumors. All experiments involving mice were carried out with approval from the Boston Children’s Hospital Animal Use and Care Committee.

**Primers for amplifying shRNAs encoded in genomic DNA.** Barcoded forward primer (N indicates location of sample-specific barcode sequence): AATGATACCGCGACACACGAGAAGATATTTCGATTCCTTGTGCTTTATATATCTTGTGGAAAGANNGACGAAA-AC. Common reverse primer: CAAGCAGAAGACGCTATCGAGCTTCTTCGA TCTTGTGGATGAACTACTGCTATGTCTCAGAGTC. Illumina sequencing primer: AGATTTCGTATT CTTGGCCITTATATCTTGTGGAA.


Fig. S1.  (A) Overall survival (Left) and overall survival by disease status at time of diagnosis (Right) for 59 osteosarcoma patients in the study population. (B) Genomic positions of significant focal amplifications and deletions. Amplifications are depicted on the left (red) and deletions are depicted on the right (blue). Chromosomes are listed on the y axis. The x axis represents the normalized amplification signal (Top x axis) and the significance by \( q \) value (Bottom x axis). The green line represents the significance cutoff at \( q \) value = 0.25. (C) Schematic of the TP53 protein showing the distribution and type of somatic TP53 mutations (numbers indicate amino acid position). TP53 mutations were detected in 22% of samples by MutSigCV. Twelve of 13 TP53 mutations were in the DNA binding domain with 9 missense, 2 frameshift, and 1 splice site mutation. An additional missense mutation was found outside the DNA binding domain.
**Fig. S2.** (A) Outline of experimental design of targeted shRNA screen in osteosarcoma xenograft development. (B) Knockdown of PIK3CA in mOS cells. mOS cells were lentivirally transduced with shRNAs against luciferase (L), Pik3ca (α) or Pik3cb (β). Cell lysates were analyzed by Western blot 4 d postinfection and selection with puromycin. (C) mOS cells were exposed to BEZ235, PIK75, and GSK2126458 at the indicated concentrations for 72 h. Survival was measured by WST-1 assay using DMSO as the control. (D) mOS cells were exposed to 5 and 10 μM TGX-221, a Pik3cb-selective inhibitor, for 72 h and proliferation was measured as a percentage of control-treated (DMSO) cells. (E) Caspase 3/7 activation was measured in mOS cells after 16 h of exposure to indicated concentrations of PIK75, BEZ235, and GSK2126458. (F) mOS cells were treated with BEZ235, GSK2126458, or PIK75 at the indicated drug concentrations. Cell extracts were analyzed by Western blotting with antibodies against phosphorylated AKT (Ser473), AKT, phosphorylated S6 (Ser235/236), S6, and GAPDH (loading control). Error bars are SEM, n = 3.
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Sixteen candidate genes were analyzed and 20 rare nonsilent variants were present after excluding missense variants classified as “benign” by PolyPhen2. For each germline mutated gene, the rationale, the specific mutation, and sample ID are listed. Genes with germline mutations were analyzed for somatic loss of heterozygosity (LOH) in tumor samples. Unk, unknown because of inability to determine LOH with data available.

## Other Supporting Information Files

- Dataset S1 (XLSX)
- Dataset S2 (XLSX)
- Dataset S3 (XLSX)
- Dataset S4 (XLSX)
- Dataset S5 (XLSX)
- Dataset S6 (XLSX)
- Dataset S7 (XLSX)
- Dataset S8 (XLSX)
- Dataset S9 (XLS)
- Dataset S10 (XLS)
- Dataset S11 (XLSX)
- Dataset S12 (XLSX)
- Dataset S13 (XLS)
- Dataset S14 (XLSX)
- Dataset S15 (XLS)