Clinical Cancer Research

Clinical Genomic Sequencing of Pediatric and Adult Osteosarcoma Reveals Distinct Molecular Subsets with Potentially Targetable Alterations

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Abstract

Purpose: Although multimodal chemotherapy has improved outcomes for patients with osteosarcoma, the prognosis for patients who present with metastatic and/or recurrent disease remains poor. In this study, we sought to define how often clinical genomic sequencing of osteosarcoma samples could identify potentially actionable alterations.

Experimental Design: We analyzed genomic data from 71 osteosarcoma samples from 66 pediatric and adult patients sequenced using MSK-IMPACT, a hybridization capture-based large panel next-generation sequencing assay. Potentially actionable genetic events were categorized according to the OncoKB precision oncology knowledge base, of which levels 1 to 3 were considered clinically actionable.

Results: We found at least one potentially actionable alteration in 14 of 66 patients (21%), including amplification of *CDK4* ($n = 9$, 14%: level 2B) and/or *MDM2* ($n = 9$, 14%: level 3B), and somatic truncating mutations/deletions in *BRCA2* ($n = 3$, 5%: level 2B) and *PTCH1* ($n = 1$, level 3B). In addition, we observed mutually exclusive patterns of alterations suggesting distinct biological subsets defined by gains at 4q12 and 6p12-21. Specifically, potentially targetable gene amplifications at 4q12 involving *KIT*, *KDR*, and *PDGFRA* were identified in 13 of 66 patients (20%), which showed strong PDGFRA expression by IHC. In another largely nonoverlapping subset of 14 patients (24%) with gains at 6p12-21, *VEGFA* amplification was identified.

Conclusions: We found potentially clinically actionable alterations in approximately 21% of patients with osteosarcoma. In addition, at least 40% of patients have tumors harboring *PDGFRA* or *VEGFA* amplification, representing candidate subsets for clinical evaluation of additional therapeutic options. We propose a new genomically based algorithm for directing patients with osteosarcoma to clinical trial options.

Introduction

Osteosarcoma, the most common primary malignant bone tumor, accounts for approximately 1% of all cancer cases in the United States (1, 2). The incidence of osteosarcoma shows a bimodal distribution with one peak in childhood/adolescence and the other in adults over 50 years of age (1). The current standard therapies, which include combination chemotherapy and surgical resection, were originally developed in the 1980s and have significantly improved the 5-year disease-free survival of patients with osteosarcoma to approximately 70% (3, 4). Furthermore, the response to preoperative combination chemotherapy is highly prognostic in patients with localized disease (5). However, 20% to 30% of patients remain refractory to conventional treatment, and the survival rate for patients presenting with localized disease has remained essentially unchanged for over 20 years (4, 6). Patients with unresectable primary tumors or metastases have poor clinical outcomes (7, 8). Older studies have reported on kinases or their ligands including VEGF, IGF1, PDGF, HER2, and MET as potential therapeutic targets in osteosarcoma based on their overexpression by IHC analysis (9).

Next-generation sequencing (NGS) technology has made the comprehensive analysis of cancer-related genes more clinically accessible, opening new avenues in treatment modalities for a variety of tumor types (10, 11). The implementation of precision medicine for the treatment of rare tumors such as osteosarcoma

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Translational Relevance

The prognosis for patients who present with metastatic and/or recurrent osteosarcoma remains poor, but the potential of routine comprehensive genomic profiling to define additional therapeutic options in this subset of patients remains unclear. Here, we sought to define how often clinical genomic sequencing of osteosarcoma samples could identify potentially actionable alterations, based on large panel next-generation sequencing data obtained from 67 patients with osteosarcoma. This identified currently clinically actionable alterations in approximately 21% of patients. In another 40% of patients, we found a mutually exclusive pattern of *PDGFRA* or *VEGFA* amplification, representing candidate subsets for future clinical evaluation of additional therapeutic options. These data inform a proposal for genomically based algorithm that could be used to direct up to 50% of patients with osteosarcoma to targeted therapy options.

has been difficult due to a lack of targetable driver mutations or fusions involving well-established drug targets such as kinases (12). In the present study, we analyzed clinical sequencing data in osteosarcoma using the MSK-IMPACT (Integrated Mutation Profiling of Actionable Cancer Targets) panel assay (11) to identify the proportion of patients with potential somatic actionable alterations as defined by the OncoKB precision oncology knowledge base (13).

Materials and Methods

Patients and samples

This project was approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center (MSKCC) and was conducted in accordance with the U.S. Common Rule. A total of 92 formalin-fixed paraffin-embedded osteosarcoma samples from patients treated at MSKCC between 2004 and 2016 were submitted for clinical sequencing using the MSK-IMPACT panel (11). In all cases, the diagnosis of osteosarcoma was confirmed by sarcoma pathologists. The MSK-IMPACT assay generated data for 81 of the 92 osteosarcoma samples (Supplementary Table S1), with the remaining 11 samples (12%) being insufficient or inadequate for NGS. This percentage is in keeping with our general experience with MSK-IMPACT testing, where approximately 9% of samples overall are found to have insufficient tumor or insufficient DNA extracted to proceed with MSK-IMPACT NGS (11). The remaining 80 cases consisted of 71 samples of classic high-grade osteosarcoma (including six samples of postradiation osteosarcoma) that were used for the analyses of genomic and clinicopathologic correlates, and a separate group of nine cases of special osteosarcoma subtypes (extraskeletal osteosarcoma, $n = 7$; dedifferentiated osteosarcoma, $n = 2$) that were excluded from further analysis in this study (Supplementary Table S1).

Sample collection and sequencing

Among the 71 high-grade osteosarcoma samples (from 66 patients), 54 samples (from 49 patients) underwent clinical sequencing in a prospective manner, whereas 17 samples (from 17 patients) were selected and sequenced retrospectively. To confirm and select the tumor and corresponding normal tissue for the retrospective group, slides from all the tissue blocks were reviewed by a sarcoma pathologist (M. Hameed). In the prospective group, matched blood was used as the germline sample after obtaining patient consent. Tumor and germline DNA were sequenced using MSK-IMPACT, an FDA-cleared, hybridization capture-based NGS assay capable of detecting all somatic protein-coding mutations, copy-number alterations (CNA), and select promoter mutations and structural rearrangements in a panel consisting of 341 cancer-related genes (version 1) later expanded to 410 (version 2) and then 468 genes (version 3; ref. 11). Of the genes discussed in this study, only *VEGFA* was not present in all three versions (versions 2 and 3 only). The sequence read alignment processing, nonsynonymous mutations, and rearrangements were determined as previously described (11).

Copy-number aberrations were identified using an in-house–developed algorithm by comparing sequence coverage of targeted regions in a tumor sample relative to a standard diploid normal sample (11), as extensively validated for *ERBB2* (*HER2*) amplification (14). Specifically, coverage values were normalized for the overall coverage of the sample, square root transformed, and adjusted for the guanine/cytosine content of each target region using Loess normalization (14). The following criteria were used to determine significance of whole-gene gain or loss events: fold change >2.0 (gain) or <−2.0 (loss), $P < 0.05$ (FDR-corrected for multiple testing).

Somatic structural rearrangements including putative gene fusions were identified by Delly (v0.6.1; ref. 15) based on supporting read pairs and split reads (16). Candidate rearrangements were flagged for manual review if the tumor harbored $\geq$3 discordant reads with a mapping quality of $\geq$5 and the matched normal sample harbored $\leq$3 discordant reads (sites of known recurrent rearrangements) or if the tumor harbored $\geq$5 discordant reads with mapping quality of $\geq$20 and the matched normal sample harbored $\leq$1 discordant read (novel rearrangement sites). All candidate somatic structural rearrangements were annotated using in-house tools and manually reviewed using the Integrative Genomics Viewer (17).

The somatic genomic alterations in the sequenced osteosarcoma samples were then analyzed using cBioPortal for Cancer Genomics tools (18, 19). Germline alterations in cancer susceptibility genes were not evaluated in this study as consent issues did not allow germline variant calling across this entire set of patients with osteosarcoma. A systematic analysis of germline cancer susceptibility across pediatric solid cancers (including osteosarcoma) in the MSK-IMPACT dataset is in progress and will be published separately.

Identification of potentially actionable alterations by OncoKB

Potentially actionable genetic events were categorized into one of four levels using MSK-Precision Oncology Knowledge base (OncoKB; www.OncoKB.org; ref. 13). The level of evidence on a specific molecular alteration is based on FDA labeling, National Comprehensive Cancer Network (NCCN) guidelines, disease-focused expert group recommendations, and scientific literature (13). Tumors with two or more level 1–4 oncogenic drivers were grouped with the highest level actionable driver alteration per the following OncoKB criteria. Individual mutational events are annotated by the level of evidence that supports the use of a certain drug in an indication that harbors that mutation. The levels of evidence are tiered as follows:

OncoKB level 1. FDA-recognized biomarkers that are predictive of response to an FDA-approved drug in a specific indication.

OncoKB level 2A. Standard care biomarkers that are predictive of response to an FDA-approved drug in a specific indication.

OncoKB level 2B. FDA-approved biomarkers predictive of response to an FDA-approved drug detected in an off-label indication.

OncoKB level 3A. FDA- or non–FDA-recognized biomarkers that are predictive of response to novel targeted agents that have shown promising results in clinical trials in a specific indication.

OncoKB level 3B. FDA- or non–FDA-recognized biomarkers that are predictive of response to novel targeted agents that have shown promising results in clinical trials for another indication.

OncoKB level 4. Non–FDA-recognized biomarkers that are predictive of response to novel targeted agents on the basis of compelling biologic data.

Results

Clinicopathologic characteristics

The clinical characteristics of the 67 patients with high-grade osteosarcoma are summarized in Table 1, whereas clinical, pathologic, and predominant molecular characteristics of all osteosarcoma cases with DNA sequencing belonging to multiple cohorts are shown in Supplementary Tables S1 and S7. The cutoff age of disease presentation for pediatric osteosarcoma was defined as up to 18 years. The median age at diagnosis was 14 for the pediatric group ($n = 33$; age range, 8–18) and 32 for the adult group ($n = 34$; age range, 19–80). Thirty-eight (56.7%) of the patients were male, and 29 (43.3%) were female. The primary sites included extremities ($n = 53$, 79.1%), trunk ($n = 9$, 13.4%), and other ($n = 5$, 7.5%). The histologic subtypes for high-grade osteosarcoma and all sequenced cohorts are shown in Supplementary Table S1. Thirty-five samples were collected from the primary site, five from local recurrences, and 32 from metastatic lesions. Upon NGS, one sample (No. 40) failed QC metrics for tumor content (flat copy-number profile + no nonsynonymous somatic variants + no silent somatic variants) and therefore the subsequent MSK-IMPACT data analyses were performed on the remaining 71 osteosarcoma samples from 66 patients.

Table 1. Clinicopathologic characteristics of 72 osteosarcoma samples (67 patients)

Features	Number of cases (%)	Total
Age (in years)		67
Range	8–80	
Median	19	
Gender		67
Male	38 (56.7%)	
Female	29 (43.3%)	
Primary site		67
Extremity	53 (79.1%)	
Trunk	9 (13.4%)	
Other	5 (7.5%)	
Type		72
High-grade osteosarcoma	66 (91.7%)	
Postradiation osteosarcoma	6 (8.3%)	
Histologic subtype		72
Osteoblastic	32 (44.5%)	
High-grade NOS	13 (18.2%)	
Telangiectatic	8 (11.2%)	
Chondroblastic	7 (9.7%)	
Fibroblastic	6 (8.3%)	
Pleomorphic	2 (2.7%)	
Giant cell rich	2 (2.7%)	
Spindle	2 (2.7%)	
Sample type		72
Primary	35 (48.7%)	
Local recurrence	5 (6.9%)	
Metastasis	32 (44.4%)	

Somatic mutations

Somatic alterations detected by MSK-IMPACT in the 71 high-grade osteosarcoma samples from 66 patients are shown in Fig. 1A and listed in Supplementary Tables S2 and S3. Among the common mutations, *TP53* mutations were identified in 22 samples (31%; Fig. 1A; Supplementary Table S2). As MSK-IMPACT is not designed to pick up *TP53* intron 1 rearrangements, recently reported in osteosarcoma (20), the prevalence of *TP53* mutations may even be higher. We also identified alterations in *ATRX* (nine mutations in seven samples, 10%), *RB1* (seven mutations in seven samples, 10%), and *SETD2* (five mutations in five samples, 7%; Supplementary Table S2). Approximately 13% of samples (9/71) did not show alterations in any of the genes in Fig. 1A but did show other somatic mutations and/or CNAs. Tumor adequacy was not deemed to be an issue in these cases because they showed similar tumor mutational burdens (TMB) as the cases with the more common alterations (range, 0.9–16.7 mutations/Mb). The mutations seen in these nine cases are listed in Supplementary Table S8.

CNAs

With respect to CNAs (Fig. 1A; Supplementary Table S3), amplifications at 6p12-21 harboring *VEGFA* ($n = 17/64$ samples; 27%), often also including *CCND3*, were the most frequent CNAs. Deletions at 9p21 involving *CDKN2A* ($n = 16$; 22%) and *CDKN2B* ($n = 16$; 22%) were the second most frequent CNAs (Table 2). Amplifications at 12q14 harboring *MDM2* ($n = 11$; 15%) and *CDK4* ($n = 9$; 13%) were frequent (Figs. 1 and 2; Table 2; Supplementary Table S4). As expected, *MDM2* and *CDK4* amplifications were mutually exclusive with *TP53* and *CDKN2A* alterations, respectively (Supplementary Fig. S1; Supplementary Tables S5 and S6), consistent with previous data in osteosarcoma (21, 22). Furthermore, *CDK4* and *CDKN2A* alterations were mutually exclusive with *RB1* alterations, such that, in aggregate, this pathway was altered in about half of osteosarcoma samples. Likewise, the *TP53*/*MDM2* pathway is altered in at least half of cases.

Notably, we also identified a subset of tumors with 4q11-12 amplification, including *KIT* ($n = 11$; 15%), *KDR* ($n = 11$; 15%), and *PDGFRA* ($n = 13$; 18%). Consistent with their chromosomal proximity, amplifications of *PDGFRA* and *KDR* frequently cooccurred with *KIT* amplification ($P < 0.001$; Fig. 1A and B; Table 2; Supplementary Table S4). Tumors with 4q11-12 amplification were mutually exclusive from those with 6p12-21 amplification with the exception of a single 4q12-amplified case that also showed borderline 6p12 gain (Fig. 1A). In addition, cases with 4q12 gene amplification were mutually exclusive not only with 6p12-21 amplification, but also with 12q14 gene amplification

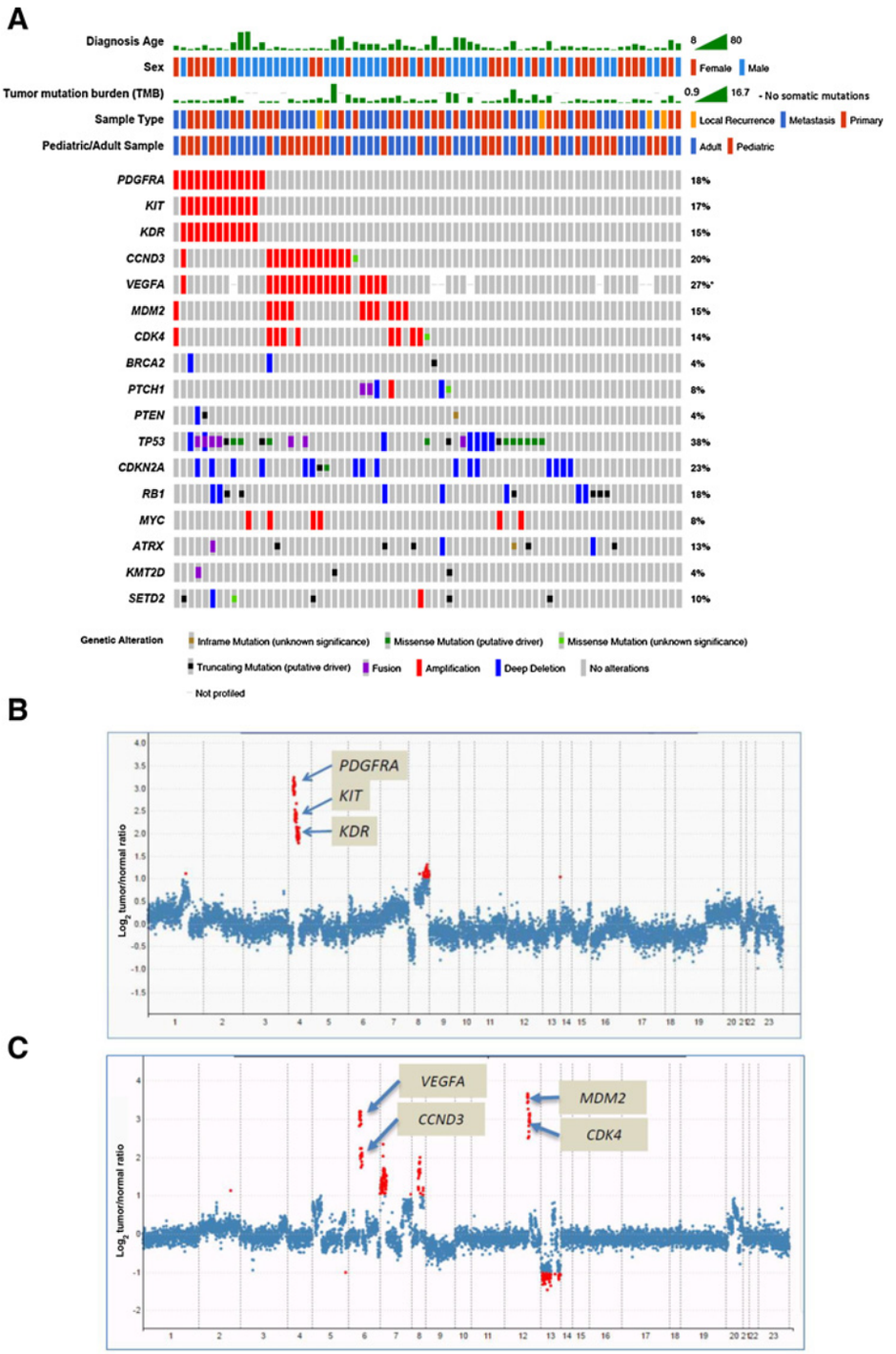

Figure 1.

A, Oncoprint of commonly occurring and potential targetable somatic alterations and TMB in 71 osteosarcoma samples. As *VEGFA* was not present on the first version of MSK-IMPACT, some samples are missing data for *VEGFA*. TMB estimation was not possible in samples that showed no somatic mutations in the MSK-IMPACT panel. **B,** Copy-number plot of an osteosarcoma case (sample 4) showing 4q12 gene amplification. **C,** Copy-number plot of an osteosarcoma case (sample 7) showing 6p12-21 and 12q14 gene amplification.

involving *MDM2* (Supplementary Tables S5 and S6). Perhaps not unexpectedly, given that cases with 4q12 gain were mutually exclusive with *MDM2* amplification, they appeared enriched for *TP53* alterations. In addition, four cases with 11q13 gene amplification involving *CCND1* and the *FGF* cluster were nonoverlapping with *CCND3* gains at 6p12 and *PDGFRA/KIT/KDR* gains at 4q12 (Supplementary Table S6). Other less common regions of recurrent amplification are shown in Fig. 1A and Supplementary Table S3.

Potentially actionable alterations annotated by OncoKB

Among the 66 patients with MSK-IMPACT data, 14 (21%) had at least one potentially actionable alteration (level 2 or 3) as defined by the OncoKB classification (www.OncoKB.org;

Table 2. Frequent CNAs in 72 osteosarcomas

Gene	Cytoband	CNA	Number of CNAs	Freq
JUN	1p32-p31	AMP	4	5.6%
MCL1	1q21	AMP	6	8.3%
TMEM127	2q11.2	AMP	4	5.6%
KDR[a]	4q11-q12	AMP	11	15.3%
PDGFRA[a]	4q12	AMP	13	18.1%
KIT[a]	4q12	AMP	11	15.3%
FAT1	4q35	DEL	6	8.3%
TERT	5p15.33	AMP	4	5.6%
VEGFA[a]	6p12	AMP	17	23.6%
CCND3[a]	6p21	AMP	13	18.1%
PIM1	6p21.2	AMP	6	8.3%
CARD11	7p22	AMP	4	5.6%
RAD21[a]	8q24	AMP	5	6.9%
MYC[a]	8q24.21	AMP	6	8.3%
CDKN2A[a]	9p21	DEL	16	22.2%
CDKN2B[a]	9p21	DEL	16	22.2%
CCND1[a]	11q13	AMP	4	5.6%
FGF3[a]	11q13	AMP	4	5.6%
FGF19[a]	11q13.1	AMP	4	5.6%
FGF4[a]	11q13.3	AMP	4	5.6%
GLI1	12q13.2-q13.3	AMP	4	5.6%
CDK4[a]	12q14	AMP	9	12.5%
MDM2[a]	12q14.3-q15	AMP	11	15.3%
RB1	13q14.2	DEL	7	9.7%
NCOR1[a]	17p11.2	AMP	8	11.1%
FLCN[a]	17p11.2	AMP	7	9.7%
MAP2K4[a]	17p12	AMP	4	5.6%
TP53	17p13.1	DEL	7	9.7%
ALOX12B[a]	17p13.1	AMP	4	5.6%
AURKB[a]	17p13.1	AMP	4	5.6%
CCNE1	19q12	AMP	6	8.3%
DNMT1[a]	19p13.2	AMP	4	5.6%
KEAP1[a]	19p13.2	AMP	4	5.6%
INSR[a]	19p13.3-p13.2	AMP	4	5.6%

Abbreviations: AMP, amplification; DEL, deletion.
[a]Significant cooccurrent CNAs at that genomic region (cytoband).

ref. 13; Table 3). Overall, 32 of 66 cases (48%) were annotated as levels 2 to 4 by OncoKB. None of the alterations were level 1, reflecting the lack of biomarker-driven FDA approvals in this disease.

OncoKB level 2. Nine patients (14%) with *CDK4* amplification were classified as level 2B potentially actionable somatic alterations by OncoKB. *CDK4*, an intracellular kinase, is altered by amplification in a diverse range of cancers, including liposarcoma, and *CDK4* inhibitors, including abemaciclib (NCT02846987) and palbociclib (23, 24) are treatment options for patients with well-differentiated and dedifferentiated liposarcomas in the NCCN compendium. A somatic *BRCA2*-truncating mutation and two cases with *BRCA2* deletions were annotated as a level 2B alteration. *BRCA2* is a tumor-suppressor gene involved in DNA damage repair by homologous recombination (25, 26). PARP inhibitors olaparib (25) and rucaparib (26) are currently approved by the FDA for use in the treatment of *BRCA2*-mutant ovarian cancer. Interestingly, a recent analysis identified a genomic signature of homologous recombination deficiency in approximately 27% of osteosarcoma samples (27).

OncoKB level 3. *MDM2* amplifications, detected in nine patients (14%), are classified as a level 3B alteration. *MDM2*, an ubiquitin ligase that negative regulates p53, is amplified in a diverse range of cancers, including well-differentiated and dedifferentiated liposarcomas (28, 29). There are promising clinical data supporting the use of *MDM2*-inhibitors such as RG7112 (28) and DS-3032b (29) in patients with *MDM2*-amplified liposarcoma. A *GULP1-PTCH1* fusion, likely inactivating, was detected in one case and was classified as a level 3B potentially actionable alteration by OncoKB. *PTCH1*, a tumor-suppressor gene and inhibitor of the hedgehog pathway, is recurrently mutated in basal cell carcinoma (30, 31). Currently, there are promising clinical data to support the use of hedgehog pathway inhibitors such as sonidegib (30) and vismodegib (31) in patients with basal cell carcinoma harboring truncating *PTCH1* mutations.

OncoKB level 4. *PTEN* deletion and truncating mutation were identified in two of 66 patients (3%). *PTEN*, a tumor-suppressor gene and phosphatase, is one of the most frequently altered genes in cancer. Although there are no FDA-approved or NCCN-compendium listed treatments specifically for patients with *PTEN*-deleted bone cancer, functional studies and clinical trials using ARQ 751, AZD5363+olaparib, AZD8186, GSK2636771, and palbociclib + gedatolisib are in progress for various malignancies (32–41). *CDKN2A* alterations were identified in 18 cases (27%), and an *NF1* deletion was identified in a single case.

4q12 amplification and overexpression of PDGFRA and KDR

A previously underappreciated prevalence of 4q12 amplification, including *KIT*, *KDR*, and *PDGFRA*, was noted in this series, being identified in 13 of 66 patients (20%; Figs. 1A and B and 2; Tables 2 and 4). Of the 13 patients with 4q12 amplifications, IHC was performed for PDGFRA [Clone: 1C10; Novus (NBP2-46357); 1:600 (1.7 μg/mL)] on nine patients with available material: tumors from eight of nine patients showed strong cytoplasmic expression (2+ to 3+ intensity; Fig. 2), whereas one showed weak expression (1+). IHC was also performed for KDR [VEGF Receptor 2; Clone: 55B11; Cell Signaling Technology (2479); 1:250 (0.1 μg/mL)] on five patients with available material and two of these showed focal cytoplasmic expression (Supplementary Fig. S1). IHC for KIT [Clone: YR145; Cellmarque (117R); 1:300 (0.1 μg/mL)] was negative in this subset of cases.

These findings may provide a rationale for closer evaluation of multikinase inhibitors targeting these kinases. For example, pazopanib and regorafenib both target *VEGFR*, *PDGFR*, and *KIT* (42–44). Interestingly, both agents have been recently shown to produce objective responses in a subset of patients with osteosarcoma. Furthermore, olaratumab, an mAb to PDGFRA (45), could be evaluated in patients in this 4q12-amplified subset of osteosarcoma.

6p12 amplification involving *VEGFA*

VEGFA at 6p12 was amplified in 14 of 59 patients (24%), pointing to angiogenesis pathways as potential targets in this subset of patients with osteosarcoma (Fig. 1A and C). Several antiangiogenic agents have shown *in vitro* and *in vivo* antitumor activity in osteosarcoma in association with amplification of *VEGF* (46–51). Clinical studies have reported activity of antiangiogenic therapies such as antibodies and small-molecule inhibitors which target the *VEGF–VEGFR* axis in some patients with osteosarcoma (52–54), a subset that we now speculate may represent *VEGFA*/6p12-amplified cases. Sorafenib has also been shown to produce long-lasting partial responses in a small subset of osteosarcoma (55), and intriguingly, it has also been shown to be effective in *VEGFA*-amplified hepatocellular carcinoma (56).

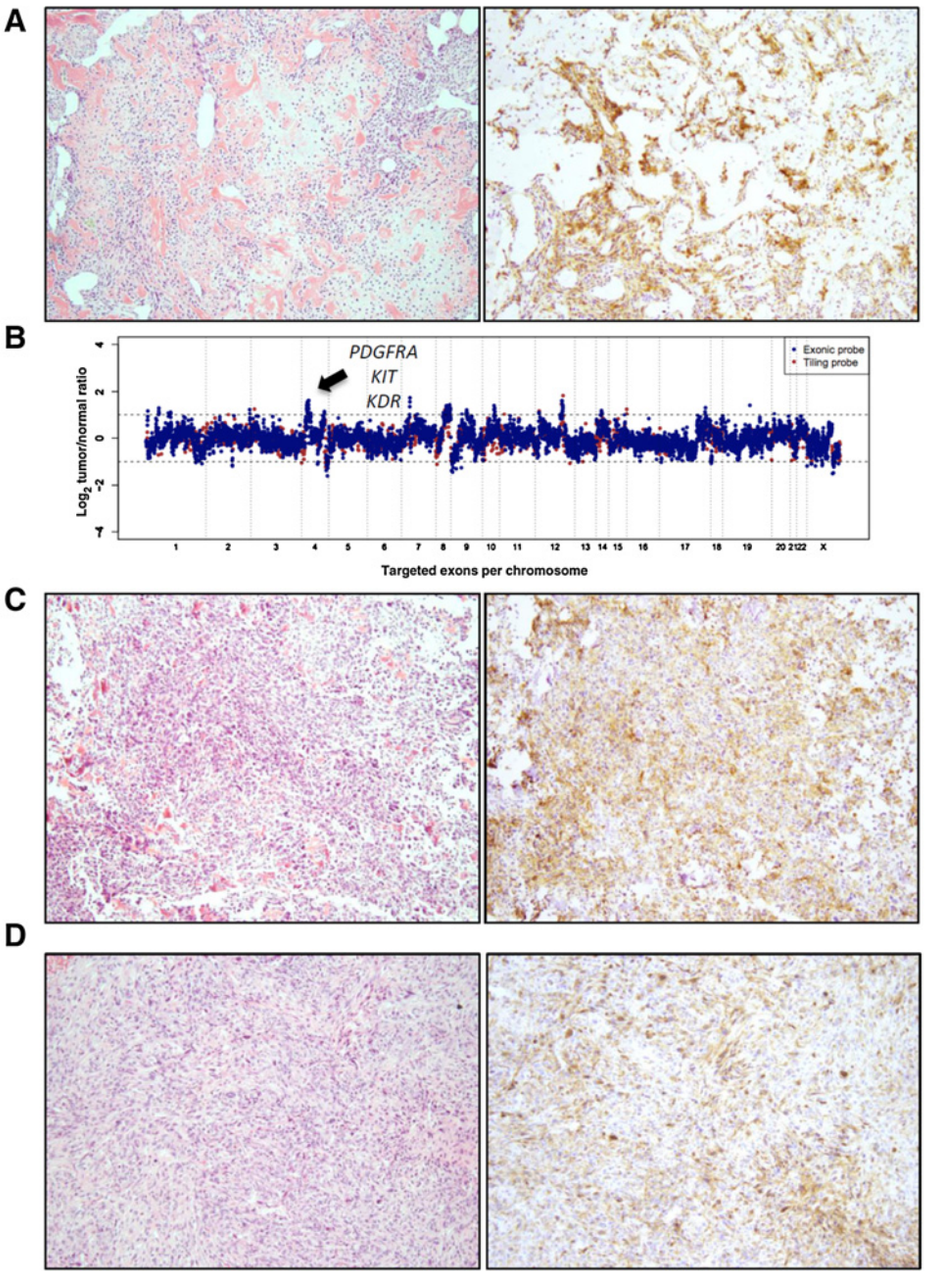

Figure 2.
PDGFRA IHC staining in cases identified with 4q12 amplification. **A,** Hematoxylin and eosin (H&E) and PDGFRA IHC in a case of telangiectatic osteosarcoma (sample 57) showing strong PDGFRA expression. **B,** Copy-number plot of **A** showing 4q12 amplification. **C,** H&E and PDGFRA IHC in a case of osteoblastic osteosarcoma (sample 17) showing strong PDGFRA expression. **D,** H&E and PDGFRA IHC in a case of pleomorphic osteosarcoma (sample 55) showing strong PDGFRA expression.

Table 3. Potentially actionable alterations identified by OncoKB in 67 osteosarcoma cases

Gene name	Mut/CNA	Annotated cases	OncoKB levels	% of cases
CDK4	Amplification	9 cases	Level 2B	13.4%
BRCA2	Deletion/truncating mutation	3 cases	Level 2B	4.5%
MDM2	Amplification	9 cases	Level 3B	13.4%
PTCH1	Fusion	1 case	Level 3B	1.5%
CDKN2A	Deletion/mutation	18 cases	Level 4	26.9%
PTEN	Deletion/truncating mutation	2 cases	Level 4	3.0%
NF1	Deletion	1 case	Level 4	1.5%

Comparison of alterations between pediatric and adult osteosarcoma

No significant differences were found between pediatric and adult osteosarcoma groups in the frequency of potentially actionable alterations, commonly altered genes, or distinct molecular subsets. Furthermore, we did not identify any molecular alterations that were unique to pediatric or adult osteosarcoma cases. However, we did find differences in overall TMB (see below).

Clinical outcome correlates of genomic alterations

The samples obtained from primary site included samples from pretreatment biopsies (24 samples) as well as posttreatment

Table 4. Frequent genomic CNAs based on sample type in 72 osteosarcoma samples

Locus	Number of samples	Pretreatment biopsy samples	Posttreatment resection samples	Posttreatment metastatic/recurrent samples
Total	72 samples	24 samples	11 samples	37 samples
6p12-21 gain	17	2	1	14/34
	23.60%	8.30%	9.10%	41.2%[a]
9p21 loss	16	4	6	6
	22.20%	16.70%	54.50%	16.20%
4q12 gain	13	5	2	6
	18.10%	20.90%	18.20%	16.20%
12q14 gain	14	4	0	10
	19.40%	16.70%	0%	27%
***RB1* alterations**	14	4	3	7
	19.40%	16.70%	27.30%	18.90%
***TP53* alterations**	27	8	5	14
	37.50%	33.30%	45.50%	37.90%

[a]Statistically significant difference between posttreatment metastatic/recurrent samples and primary samples (pretreatment biopsies and posttreatment resections), $P < 0.01$ (χ^2 test). Denominators are as indicated in the totals for each column unless otherwise indicated.

resections (11 samples; Table 4). The frequency of the most common CNAs was then calculated for each of the specimen types. Amplification of 6p12-21 including *VEGFA* was identified in 14 of 34 metastatic/recurrent samples (41.2%) as compared with three of 31 primary samples (9.7%; Fig. 1A; Table 4). This difference was found to be statistically significant ($P < 0.01$, χ^2 test). Overall, the 37 metastatic/recurrent samples in the cohort were enriched for amplification of 12q14 including *MDM2* (10 samples, 27%), but the differences did not reach statistical significance (Fig. 1A; Table 4). When cases were divided into two prognostic groups based on the development of recurrence and/or metastasis within 5 years of diagnosis, cases with 6p12-21 gain showed a trend toward faster disease progression (recurrence and/or metastasis within 5 years) when compared with the rest of the cohort (32.1% vs. 12.8%, $P = 0.05$, χ^2 test). No differences were observed in overall or disease-free survival between groups with different genomic alterations (data not shown).

Intermetastatic heterogeneity

Four cases had two or more samples tested (highlighted samples in Supplementary Table S7). All cases with multiple samples were posttreatment metastatic specimens that lacked matched primary tumor data. In three of four cases, the alterations found were concordant across samples, with some alterations identified at subthreshold levels that did not meet criteria for clinical reporting (Supplementary Table S7). In one patient, where both samples were posttreatment lung metastases resected one and 1.5 years after initial presentation, only one of the two samples showed an *MDM2* amplification (samples 34 and 35, Supplementary Table S7).

TMB

The range of TMB scores, based on the ratio of nonsynonymous somatic mutations to sequencing territory (adjusted for MSK-IMPACT version), spanned 0.9 to 16.7 mutations/Mb (Fig. 1A). The average TMB for patients with an age of diagnosis up to 18 years was lower (1.9 mutations/Mb) than patients aged 19 years or older at disease presentation (2.9 mutations/Mb; *t* test, $P < 0.05$).

Discussion

Knowledge of a tumor's genetic profile has proved to be useful in diagnosis, prognosis, and targeted therapy selection for a variety of common and rare cancers including sarcomas (11, 57–61). High-grade osteosarcomas are genetically unstable tumors with generally complex, chaotic karyotypes (62). Their genomic instability is highlighted by high levels of somatic structural variations and many CNAs (63–67). Whole-genome sequencing studies have shown recurrent *TP53*, *RB1*, and *ATRX* somatic mutations (64, 68–70). *TP53*, *RB1*, *CDKN2A/B*, *CDKN2AP14ARF*, and *CDKN2AP16INK4A* have been previously shown to be frequently affected by deletions and/or LOH, whereas *MDM2* and *VEGFA* have been the most frequent amplified genes previously reported (64, 68–74).

In the present study, the findings of recurrent gene amplifications of *CDK4*, *MDM2*, *KIT*, *PDGFRA*, *KDR*, and *VEGFA* raise the possibility of an umbrella protocol using targeted therapeutics in distinct subsets of patients with osteosarcoma (Fig. 3). Approximately 20% of tumors in this study harbored a chromosome 4q12 amplification, encompassing the genes encoding the targetable receptor tyrosine kinases *PDGFRA*, *KDR*, and *KIT*. KIT has been previously proposed as a target in osteosarcoma (75). IHC analysis of this cohort confirmed strong expression of PDGFRA, moderate expression of KDR, and only weak expression of KIT, suggesting a rationale for combined PDGFRA/KDR inhibition. Recent reports have described patients with osteosarcoma with clinical responses to single-agent multikinase inhibitors with activity against PDGFRA and KDR (42, 76, 77). Although correlative genomic data for these responders were not reported, these findings are compelling for a formal trial of combined PDGFRA/KDR inhibition in 4q12-amplified osteosarcoma. If possible, it would be informative to correlate responses in trials of regorafenib (77, 78) and pazopanib (NCT01759303) for patients with recurrent osteosarcoma with the genomic amplification profiles of the tumor specimens. In a recent study by Holme and colleagues, 18 osteosarcoma cell lines were tested for chemosensitivity to 79 small-molecule inhibitors, and MG-63, an osteosarcoma cell line with *PDGFRA* amplification, showed sensitivity to imatinib and sunitinib (79).

Approximately 24% of patients in our cohort harbored a 6p12 amplification, involving *VEGFA* and *CCND3*. Moreover, our study identified this group of tumors as almost entirely mutually exclusive from tumors harboring 4q12 gene amplifications. Similar to *PDGFRA* and *KDR* in 4q12-amplified tumors, *VEGFA* is a candidate driver that is potentially targetable through kinase inhibition. In IHC studies, the expression of VEGF has been detected in 63% to 74% of osteosarcoma samples and has been associated with

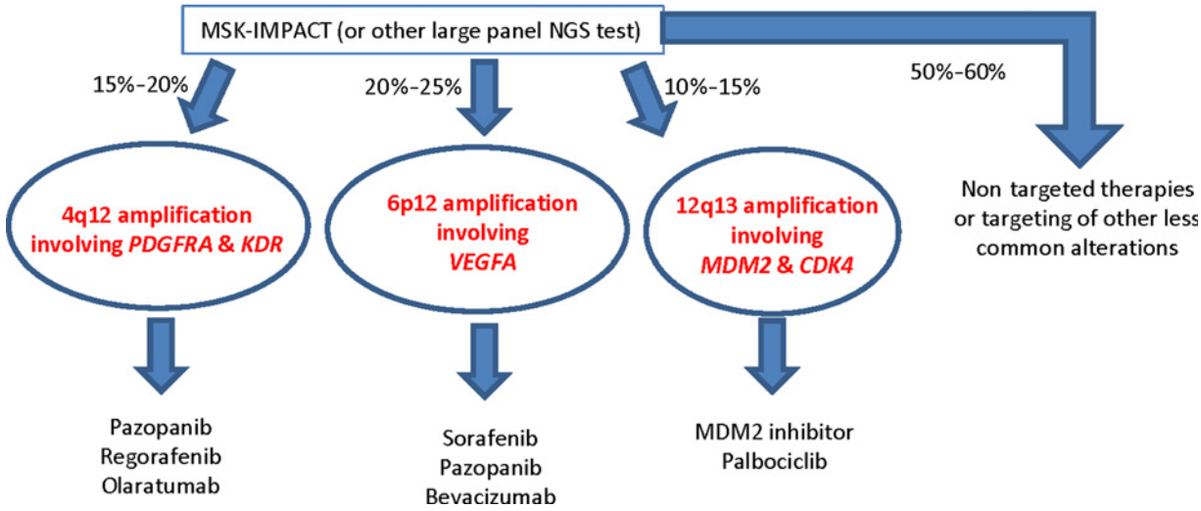

Figure 3.

Recurrent gene amplifications and their potential for an umbrella protocol of targeted therapeutics in distinct subsets of patients with osteosarcoma. Percentages are approximate ranges. Examples of drugs are for illustrative purposes only.

pulmonary metastasis, decreased disease-free survival, and overall survival (46, 80). Our study shows a significantly higher proportion of metastatic/recurrent samples harboring *VEGFA* (14/34 samples, 41.2%) as compared with samples procured from primary sites (3/31 samples, 9.7%; $P < 0.01$). Furthermore, VEGF signaling inhibition has been reported to suppress cell growth and enhance apoptosis in osteosarcoma cell lines (81, 82). In another study, 32 of 50 osteosarcoma showed *VEGFA* amplification (46) which was associated with decreased tumor-free survival and increased microvascular density (46, 83). Several antiangiogenic agents have been shown to have antitumor activity against osteosarcoma *in vitro* and *in vivo* (44–47, 49). In particular, pazopanib, which targets VEGF, has shown activity in preclinical mouse models with high expression of VEGF (84). As mentioned above, recent reports of clinical responses to pazopanib in small patient cohorts have been published (42). Sorafenib, another multikinase inhibitor with activity against VEGF, demonstrated significant clinical activity in a very small subset of patients with recurrent osteosarcoma (55). In hepatocellular carcinoma, tumors with VEGFA amplifications are distinctly sensitive to sorafenib (56). In a recent study by Sayles and colleagues, whole-genome sequencing performed on tumor specimens from 23 patients with osteosarcoma showed *VEGFA* amplification in 23% (85). In the same study, patient-derived tumor xenografts with *VEGFA* amplification showed significant decrease in tumor volume on treatment with sorafenib (85). Together, these findings suggest that osteosarcoma with 6p12 amplifications may be good candidates for VEGF inhibition (42, 76).

Among other potentially targetable alterations, we identified *MDM2* amplification in 9 of 66 (14%) patients, including 6 cases (9%) with coamplification of *CDK4* and *MDM2*. Earlier studies using a variety of methods have reported *MDM2* amplification in 6.6% to 14.3% of osteosarcoma (21, 86, 87), and recently whole-genome sequencing studies identified *MDM2* amplification in 3.1% to 5.1% of osteosarcoma (70). In clinical trials, *MDM2* inhibitors have shown significant antitumor activity in patients with liposarcoma (23, 24). Some *MDM2* inhibitors also display significant activity in *MDM2*-amplified osteosarcoma cell lines (e.g., SJSA) in comparison with non–*MDM2*-amplified cell lines (88, 89). *CDK4* overexpression has been reported in about 10% of osteosarcoma (22, 87, 90). However, to the best of our knowledge, there have been no studies examining the association between *CDK4* amplification and the activity of *CDK4* inhibitors in osteosarcoma. In well-differentiated and dedifferentiated liposarcomas, several clinical trials have shown that treatment with a *CDK4* inhibitor was associated with favorable progression-free survival in patients with *CDK4* amplification (23, 24). Based on these findings, targeting of *MDM2* and *CDK4* appears to be a potential therapeutic option for the 12q13-amplified subset of patients with osteosarcoma.

Mutually exclusive genetic alterations often point to important alternative oncogenic pathways. There were several notable relationships of this type in our dataset. The 17 samples with *VEFGA*/*CCND3* amplification at 6p12-21 were mutually exclusive with the 13 samples with amplification of *PDGFRA*, *KIT*, and *KDR*, at 4q12, with one exception (Log OR, −1.87; Supplementary Table S5). In the single case with gains at both loci, the 4q12 amplification was higher, whereas the 6p12 gain was borderline (results not shown). Amplification of 12q14 (*MDM2* and *CDK4*) was found in 20% (14/71) of the samples and was mutually exclusive with 4q12 amplification (Log OR←10; Supplementary Table S5). These mutually exclusive and targetable oncogenic pathways may represent distinct biological subsets of osteosarcoma with important therapeutic implications. It should be noted that the major copy-number gains highlighted in Fig. 3 could also be detected by methods other than the one used in the present study, such as FISH or array-based copy-number profiling, which might be more widely available. In summary, we were able to identify potentially actionable (OncoKb levels 1–3) somatic alterations in approximately 21% of patients with osteosarcoma 66/14)). In addition, distinct osteosarcoma subsets defined by amplification of *PDGFRA* and *KDR* at 4q12 or *VEGFA* at 6p12-21 may offer new therapeutic opportunities.

Disclosure of Potential Conflicts of Interest

G. Jour is a consultant/advisory board member for Bristol-Myers Squibb. E. Slotkin reports receiving other commercial research support from Eli Lilly. P. Myers has immediate family members who have received speakers bureau honoraria from Genentech; holds ownership interest (including patents) in Amgen; and is a consultant/advisory board member for Eli Lilly, Astellas, Takeda, and Boehringer. M. Ladanyi is a consultant/advisory board member for Bayer. No potential conflicts of interest were disclosed by the other authors.

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