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High Throughput Genotyping in Osteosarcoma Identifies Multiple Mutations in PIK3CA and other Oncogenes

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Abstract

Background—Identification of new genes that are mutated in osteosarcomas is critical to developing a better understanding of the molecular pathogenesis of this disease and discovering new targets for therapeutic development.

Methods—We identified somatic non-synonymous coding mutations in oncogenes associated with human cancers and hotspot mutations from tumor suppressor genes that were either well-described in literature or seen multiple times in human cancer sequencing efforts. We then systematically characterized 961 mutations in 89 genes across 98 osteosarcoma tumor samples and cell lines. All identified mutations were replicated on an independent platform using homogeneous mass extend MALDI-TOF (Sequenom hME Genotyping).

Results—We identified 14 mutations in at least one osteosarcoma tumor sample or cell line. Some of the genetic changes identified were in tumor suppressor genes previously known to be altered in osteosarcoma: p53 (R273H, R273C, and Y163C) and RB1 (E137*). Notably, we identified multiple mutations in PIK3CA (H1047R, E545K, and H701P) which have never previously been observed in osteosarcoma. Additionally, we observed mutations in KRAS (G12S), CUBN (I3189V, seen in two separate tumor samples), CDH1 (A617T, seen in two separate tumor samples), CTNNB1 (N287S), and FSCB (S775L).

Conclusion—We performed the largest mutational profiling of osteosarcoma to date and identified for the first time several mutations involving the PI3 kinase pathway – adding osteosarcoma on to the growing list of malignancies with PI3 kinase mutations. Additionally, we initiated a mutational map detailing DNA sequence changes across a variety of osteosarcoma subtypes and offered new candidates for therapeutic targeting.

Keywords

Genotyping; Osteosarcoma; OncoMap; PIK3CA; Mutation

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Introduction

Osteosarcomas are aggressive primary bone malignancies that have a peak incidence in adolescence – accounting for 60% of primary bone cancers diagnosed in patients < 20 years old[1-5]. Patients with localized osteosarcoma develop metastatic disease in greater than 80% of cases when not treated with chemotherapy, and these patients usually die from their cancer if found to progress during or after treatment with standard chemotherapy regimens[6-8]. For patients with progressive or recurrent disease despite treatment using standard agents including cisplatinum, doxorubicin, ifosfamide, and methotrexate, therapeutic strategies other than cytotoxic drugs are needed[9-12].

Understanding the genetic mutations that drive cancer pathogenesis have recently led to identification of new treatments for several cancers such as EGFR-mutated lung cancers[13, 14], cKit mutated gastrointestinal stromal tumors[15, 16], and ALK-translocated tumors[17, 18]. As a result, recent research efforts on osteosarcoma have focused on identifying new treatment targets and prognostic markers[12, 19-21]. Of the molecular targets currently under evaluation for osteosarcoma, IGF1R[22-25], EGFR[26-29], STAT3[30, 31], PLK1[32-34], and mTOR[35-37], among others, are being intensely evaluated. To date, however, none of these targets have yet been proven to be of therapeutic benefit to patients with advanced osteosarcoma[22].

A whole genome sequencing approach in lung, breast, and colon cancer samples has identified numerous genetic alterations[38, 39], but many of these mutations are incidental and unlikely to play an important role in tumor pathogenesis or as therapeutic targets. Currently, a whole genome sequencing approach for osteosarcomas would be prohibitively expensive and results would be difficult to interpret. Therefore, taking advantage of insights gained in treatment of other tumor types, we sought to focus our analysis only on mutations that have a higher *a priori* chance at playing an important role in osteosarcoma pathogenesis (see Methods for Selection of Cancer Gene Mutations), and we genotyped for mutations known to occur in oncogenes or tumor suppressor genes that have been previously associated in literature with cancer pathogenesis.

Methods

Osteosarcoma tumor samples

Fresh frozen tumor specimens were obtained from the clinical archives of Dr. Francis Hornicek (Department of Orthopaedic Surgery, Massachusetts General Hospital) and the Massachusetts General Hospital Tissue Repository. Institutional review board (IRB) approval was obtained to study all samples from the Partners Human Research Office (2007P-002464).

Cell Culture

The human osteosarcoma cell line KHOS was kindly provided by Dr. Efstathios Gonos (Institute of Biological Research & Biotechnology, Athens, Greece), and U-2OS and SaOS were purchased from the ATCC (Rockville, MD). Cells were cultured in RPMI 1640 (Invitrogen,) supplemented with 10% FBS, 100-units/ml penicillin and 100μg/ml streptomycin (Invitrogen). Cells were incubated at 37°C in 5% CO_2-95% air atmosphere and passaged when near confluent monolayers were achieved using trypsin-EDTA solution. Cells were free of mycoplasma contamination as tested by MycoAlert(R) Mycoplasma Detection Kit from Cambrex (Rockland, ME).

Extraction of Genomic DNA

Extraction of DNA from osteosarcoma tumor tissues and cell lines were performed using QIAamp® DNA Micro kit (Qiagen). The extraction was carried out according to the manufacturer's instructions. Briefly, osteosarcoma tumor tissue sample or cell pellet from cultured cell lines of ~8 mg in weight was transferred to a 1.5 ml microcentrifuge tube and 180 ul of buffer ATL was added immediately. After equilibrating to room temperature (25C), 20 ul of proteinase K was added and mixed by vortexing for 15 seconds. The sample tube was incubated at 56°C overnight until the sample was completely lysed. In the next day, 200 ul buffer AL was added and mixed by vortexing for 15 seconds. Subsequently, 200 μl of ethanol (96-100%) was added. The mixture obtained was loaded on a QIAamp MiniElute spin column provided by the kit and washed with AW1 followed by AW2 buffers. DNA was eluted with 60 μl of buffer AE and preserved at −20 °C until use.

Selection of Cancer Gene Mutations and OncoMap Assay Design

Selection of cancer gene mutations for assay design and mass spectrometric genotyping were performed as previously described in Thomas et al. with modifications indicated in MacConaill et al.[40, 41].

In brief, we queried the Sanger Institute COSMIC database, PubMed, and The Cancer Genome Atlas (TCGA) databases for known somatic oncogene and tumor suppressor gene mutations. Non-synonymous coding mutations that have been previously reported to occur as somatic mutations in human cancers were selected and rank ordered based on (1) the frequency of mutation in cancers, (2) frequency across cancer subtypes, and (3) the feasibility of developing an inhibitor of the target gene. Most genes that were described in only one instance were excluded unless that gene was determined to be very important in tumorigenesis and/or the gene was currently in drug development pipelines of pharmaceutical companies. “Hotspot” mutations from selected well-known tumor suppressor genes were included based on the number of documented occurrences, with higher weight given to genes commonly deleted or genetically inactivated across cancer types.

For each mutation, the discriminating nucleotides for both wild-type and mutant alleles were determined, enabling insertions or deletions to be represented by single base changes. Subsequently, 250 bases of neighboring DNA were added to each side of the resulting mutation assay to enable primer design. These primers for PCR amplification and the extension probe were designed using the Sequenom MassARRAY Assay Design 3.0 software, applying default single base extension (SBE) settings and default parameters but with the following modifications: maximum multiplex level input adjusted to 24; maximum pass iteration base adjusted to 100. For complex mutations, genotyping assays were designed manually. The resulting 501 base pair DNA sequences were queried in the dbSNP database to avoid incorporation of SNPs during assay design. Resulting primer were then run through BLAT and modified where necessary to avoid pseudogene amplification. The resulting list of primer pairs and extension probes (OncoMap version 2.0) consists of 961 assays interrogating 89 oncogenes and tumor suppressor genes for mutations (single-base substitutions, insertions and deletions). All PCR primers and extension probes were synthesized unmodified using standard purification (Integrated DNA Technologies, Coralville, IA).

Mass Spectrometric Genotyping

Mass spectrometric genotyping was performed as previously described[40-42]. In brief, primers and probes were pooled, and all assays were validated on the CEPH panel of human HapMap DNAs (Coriell Institute) as well as a panel of human cell lines with known

mutational status. Genomic DNA from all tumor samples was quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, California) and subjected to whole-genome amplification (WGA), with the following modifications: 100ng of genomic DNA was used as input for WGA and a post-WGA cleanup step was implemented using a Nucleofast Purification Kit (Macherey-Nagel).

The Qiagen Repli-g kit was used for phi29-mediated WGA of fresh frozen and cell line DNA. After quantification and dilution of genome-amplified DNA, mass spectrometric genotyping using iPLEX chemistries was performed as previously published [41].

After iPLEX genotyping (32 iPLEX pools with an average pool plex size of 14.4 assays), samples harboring candidate mutations were further filtered by manual review. Samples harboring candidate mutations were selected for experimental confirmation using multi-base extension homogenous Mass-Extend (hME) chemistry with plexing of ≤6 assays per pool. Conditions for hME validation were consistent with the methods described by MacConaill et al. 2009. Primers and probes used for hME validation were designed using the Sequenom MassARRAY Assay Design 3.0 software, applying default multi-base extension (MBE) parameters but with the following modifications: maximum multiplex level input equal to 6; maximum pass iteration base adjusted to 200.

Western Blotting for PI3 kinase pathway proteins

The human AKT, pAKT(Thr308), and p4EBP1(Thr37/46) antibodies were purchased from Cell Signaling (Dedham, MA). The mouse monoclonal antibody to human actin was purchased from Sigma-Aldrich. Western blot analysis was performed as described previously[43]. Briefly, the cells were lysed in 1× radio-immunoprecipitation assay (RIPA) lysis buffer (Upstate Biotechnology) and protein concentration was determined by the DC Protein Assay (Bio-Rad). Total protein (25 μg) was resolved on NuPage 4% to 12% Bis-Tris gels (Invitrogen) and immunoblotted with specific antibodies. Primary antibodies were incubated in TBS (pH 7.4) with 0.1% Tween 20 with gentle agitation overnight at 4°C. Horseradish peroxidase–conjugated secondary antibodies (Bio-Rad) were incubated in TBS (pH 7.4) with 5% nonfat milk (Bio-Rad) and 0.1% Tween 20 at a 1:2,000 dilution for 1 h at room temperature with gentle agitation. Positive immunoreactions were detected by using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology).

Results

Characteristics of Clinical Tumor Samples

A total of 98 DNA samples were derived from cell lines or patients who had undergone operative resection of their osteosarcoma (Table 1). In summary, 68 specimens were obtained from fresh frozen tissue, 26 were derived from FFPE blocks, and 4 were derived from cell lines that were created from primary tumor samples. 83 samples had detailed pathologic subclassification information available, and the majority of samples were either of osteoblastic (21) or conventional (25) subtypes. The average known age of patients at time of surgery was forty years old (as this study only collected from patients age 20 or older). Because the available clinical dataset was incomplete, no available clinical characteristic or outcome correlated with the mutational status of the tumor.

OncoMap Results

98 osteosarcoma samples were tested for mutations across the 89 genes tested in the Oncomap version 2.0 panel (see Table 2 for list of genes tested). 89/98 samples passed our quality control check, and 96% of assays tested yielded results (see example of readout in Figure 1). 40/98 samples were identified to have at least one mutation. 14 mutations

occurring in 8 of the genes tested were identified; these were all validated using an alternate chemistry (hME genotyping) on unamplified DNA.

Among the 68 freshly frozen samples tested, 9 mutations were identified and validated. Among the 22 FFPE samples tested, 4 mutations were identified and validated. Among the 4 cell lines tested, 1 mutation was identified and validated. There was no significant difference in discovered mutation rates between freshly frozen and FFPE samples.

The 14 mutations that were identified involved 8 different genes. Some of these genes were previously associated with osteosarcoma pathogenesis: p53 (R273H, R273C, and Y163C) and RB1 (E137*). However, we also identified 3 mutations in PIK3CA (H1047R, E545K, and H701P) which have never previously been observed in osteosarcomas. Other mutations were identified in KRAS (G12S), CUBN (I3189V, seen in two separate tumor samples), CDH1 (A617T, seen in two separate tumor samples), CTNNB1 (N287S), and in FSCB (fibrous sheath CABYR binding protein) (S775L).

Although the study was designed with hopes of correlating mutation with clinical data, only 5 of the samples with identified mutations were annotated with detailed clinical information, a sample size too small to make statistically significant inferences. One patient with grade 2-3 osteoblastic osteosarcoma of the right femur had a mutation in FSCB (S775L) as well as in CDH1 (A617T). Another patient with grade 2 osteoblastic osteosarcoma of the femur had a mutation in PIK3CA (H701P). A patient with osteosarcoma with chondroid features, grade 2 of 3, had a P53 mutation (R273C). A patient with a low grade osteosarcoma of the right scapula had a mutation in CUBN (I3189V).

PI3 Kinase Pathway is activated in PIK3CA-mutated osteosarcoma tumor samples

PIK3CA is the gene coding for p110a, which is one of four catalytic units for class I PI3 kinase[44, 45]. Because the samples that revealed mutations in PIK3CA were from preserved tumor samples rather than cell lines, we were unable to perform functional assays for PI3 kinase activity. Therefore, we used western blot analysis to determine the relative levels of expression for members of the PI3 kinase pathway (Figure 2) when compared to three osteosarcoma tumor samples for which PI3 kinase mutations were not observed. PI3 kinase is known to activate both the AKT and mTOR pathway, so we looked for phospho-AKT as evidence of AKT activity and phospho-4EBP1 as evidence of mTOR activity. We found that all six samples expressed AKT. Two of the three samples with PIK3CA mutations revealed detectable phospho-AKT and all three samples revealed detectable phospho-4EBP1. Unexpectedly, one of the samples without an identified PIK3CA mutation also appeared to have high levels of phospho-AKT and phospho-4EBP1, suggesting an alternate mechanism for hyperactivation of PI3 kinase. Future studies including complete gene sequencing of PI3 kinase pathway members and gene copy number analysis may reveal the mechanism for PI3 kinase activation in this sample.

Discussion

Osteosarcomas have been well-described to have numerous chromosomal aberrations and are characterized by complex karyotypes[27, 46, 47]. Although high-level amplifications and homozygous deletions have been well described in this tumor type, with one study showing 28.6% and 3.8% of the osteosarcoma genome consisting of amplifications and homozygous deletions [46], a comprehensive genome-wide survey for high-yield mutations have not yet been performed across a large collection of osteosarcomas. Because whole-genome tumor sequencing of large collections of osteosarcomas are costly and prohibitively laborious, we aimed our screen to test only for those mutations that have been previously described in other tumors and implicated in tumor pathogenesis. Of course, results of our study are not

equivalent to what can be found by complete sequencing efforts that are currently being undertaken by large consortiums.

It is important to note that in the majority of our samples, we did not identify any mutations. This observation has two critical implications. First, a comprehensive gene sequencing study would likely identify more mutations. For example, although our screen was able to detect a few samples with mutations in p53 and RB, osteosarcomas have been well described to have frequent mutations in both p53[48] and RB[49]. Undoubtedly, complete gene sequencing of p53 and RB in the one hundred osteosarcoma samples tested in this study will yield many more samples with p53/RB mutations. Second, our mutation panel was quite thorough in its examinations of the more clinically relevant mutations found in lung and colon cancer, including mutations in the EGFR, KRAS, BRAF, and PDGFR. Therefore, the lack of identification of any mutations suggest that, for those genes, point mutations are unlikely to be involved in the pathogenesis of osteosarcoma.

We wanted to identify new mutations that could potentially serve as therapeutic targets for the treatment of osteosarcoma. Mutations newly identified in osteosarcoma need not be new to oncology. For example, mutations in cKIT were well-described to predict for the efficacy of the cKIT inhibitor, imatinib, in treating patients with gastrointestinal stromal tumors before a rare cKIT mutation in a melanoma led to a trial of treatment with imatinib and a major response[50]. Likewise, we are hopeful that other drug-mutation relationships that may be established in other cancer subtypes will point to effective drug targets for osteosarcoma.

We were interested in the finding that the validated mutation discovery rate in FFPE samples was similar to that in freshly frozen samples. Until recently, a major limitation to high-throughput multiplexed genotyping assays was the limitation in access to freshly frozen tissue. However, our study as well as other similar studies suggests that FFPE samples are sufficient for mutation discovery.

Although PIK3CA mutations have been described in myxoid/round cell liposarcomas[51], such mutations have never been previously described in osteosarcomas. PIK3CA is the gene coding for p110a, the catalytic subunit of class I PI3 kinase. These lipid kinases catalyze the conversion of phosphatidylinositol-3,4-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate. These lipid products in turn recruit AKT to the plasma membrane, where it is phosphorylated and itself catalyzes the phosphorylation and activation of other proteins, such as mTOR and 4EBP, that regulate glucose metabolism, cell proliferation, and survival[45]. In this study, we are the first to observe human osteosarcoma tumor samples harboring mutations in PIK3CA.

When these samples with PIK3CA mutations were analyzed for phosphorylation (and therefore activation) of proteins that signal downstream of PI3 kinase, we confirmed that all three samples expressed phosphorylated 4EBP-1 and that 2 of 3 samples expressed phosphorylated AKT. However, we were surprised to find one sample without an identified PIK3CA mutation also demonstrated high levels of phosphorylated 4EBP-1 and AKT. This observation can be due to either the hyperactivation of other pathways that also activates 4EBP-1 or AKT – such as the RAS/RAF/MAPK, the IGF-1R/IRS-1, and the mTOR pathways, or the loss of PTEN inhibition of AKT activation, or the existence of another PI3 kinase activating mutation that was not tested in this study - highlighting the limitation of this study as a genotyping study of particular mutations and underscoring the ultimate need for whole gene sequencing to comprehensively evaluate the mutation rate of PIK3CA in this cohort.

Further studies need to be done to establish PI3 kinase as a useful signal transduction pathway to target in osteosarcomas. There are at least nine PI3 kinase inhibitors in clinical

development[44] and, at the time of submission of this manuscript, there are eleven clinical trials using PI3 kinase inhibitors in cancer cohorts posted on ClinicalTrials.gov. The fact that activating mutations in PIK3CA have now been observed in osteosarcomas makes this disease group an interesting cohort to focus on for further pharmaceutical development of PI3 kinase inhibitors.

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References

1. Ferguson WS, Goorin AM. Current treatment of osteosarcoma. Cancer Invest. 2001; 19(3):292–315. [PubMed: 11338887]
2. Geller DS, Gorlick R. Osteosarcoma: a review of diagnosis, management, and treatment strategies. Clin Adv Hematol Oncol. Oct; 2010 8(10):705–718. [PubMed: 21317869]
3. Gorlick R. Osteosarcoma: clinical practice and the expanding role of biology. J Musculoskelet Neuronal Interact. Dec; 2002 2(6):549–551. [PubMed: 15758391]
4. Meyers PA, Gorlick R. Osteosarcoma. Pediatr Clin North Am. Aug; 1997 44(4):973–989. [PubMed: 9286295]
5. Mankin, HJ. Pathophysiology of orthopaedic diseases. Rosemont, IL: American Academy of Orthopaedic Surgeons; 2006.
6. Chi SN, Conklin LS, Qin J, et al. The patterns of relapse in osteosarcoma: the Memorial Sloan-Kettering experience. Pediatr Blood Cancer. Jan; 2004 42(1):46–51. [PubMed: 14752794]
7. Davis AM, Bell RS, Goodwin PJ. Prognostic factors in osteosarcoma: a critical review. J Clin Oncol. Feb; 1994 12(2):423–431. [PubMed: 8113851]
8. Goorin AM, Frei E 3rd, Abelson HT. Adjuvant chemotherapy for osteosarcoma: a decade of experience. Surg Clin North Am. Dec; 1981 61(6):1379–1389. [PubMed: 6976007]
9. Chou AJ, Gorlick R. Chemotherapy resistance in osteosarcoma: current challenges and future directions. Expert Rev Anticancer Ther. Jul; 2006 6(7):1075–1085. [PubMed: 16831079]
10. Guo W, Healey JH, Meyers PA, et al. Mechanisms of methotrexate resistance in osteosarcoma. Clin Cancer Res. Mar; 1999 5(3):621–627. [PubMed: 10100715]
11. Mankin HJ, Hornicek FJ, Rosenberg AE, Harmon DC, Gebhardt MC. Survival data for 648 patients with osteosarcoma treated at one institution. Clin Orthop Relat Res. Dec.2004 (429):286–291. [PubMed: 15577500]
12. O'Day K, Gorlick R. Novel therapeutic agents for osteosarcoma. Expert Rev Anticancer Ther. Apr; 2009 9(4):511–523. [PubMed: 19374604]
13. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med. May 20; 2004 350(21):2129–2139. [PubMed: 15118073]
14. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science. Jun 4; 2004 304(5676):1497–1500. [PubMed: 15118125]
15. Demetri GD, von Mehren M, Blanke CD, et al. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. N Engl J Med. Aug 15; 2002 347(7):472–480. [PubMed: 12181401]
16. Joensuu H, Roberts PJ, Sarlomo-Rikala M, et al. Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor. N Engl J Med. Apr 5; 2001 344(14):1052–1056. [PubMed: 11287975]
17. Butrynski JE, D'Adamo DR, Hornick JL, et al. Crizotinib in ALK-rearranged inflammatory myofibroblastic tumor. N Engl J Med. Oct 28; 2010 363(18):1727–1733. [PubMed: 20979472]

18. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J Med. Oct 28; 2010 363(18):1693–1703. [PubMed: 20979469]
19. Chou AJ, Geller DS, Gorlick R. Therapy for osteosarcoma: where do we go from here? Paediatr Drugs. 2008; 10(5):315–327. [PubMed: 18754698]
20. Gorlick R. Current concepts on the molecular biology of osteosarcoma. Cancer Treat Res. 2009; 152:467–478. [PubMed: 20213409]
21. Marina N, Gebhardt M, Teot L, Gorlick R. Biology and therapeutic advances for pediatric osteosarcoma. Oncologist. 2004; 9(4):422–441. [PubMed: 15266096]
22. Chugh R. Experimental therapies and clinical trials in bone sarcoma. J Natl Compr Canc Netw. Jun; 2010 8(6):715–725. [PubMed: 20581301]
23. Duan Z, Choy E, Harmon D, et al. Insulin-like growth factor-I receptor tyrosine kinase inhibitor cyclolignan picropodophyllin inhibits proliferation and induces apoptosis in multidrug resistant osteosarcoma cell lines. Mol Cancer Ther. Aug; 2009 8(8):2122–2130. [PubMed: 19638450]
24. Kolb EA, Kamara D, Zhang W, et al. R1507, a fully human monoclonal antibody targeting IGF-1R, is effective alone and in combination with rapamycin in inhibiting growth of osteosarcoma xenografts. Pediatr Blood Cancer. Jul 15; 2010 55(1):67–75. [PubMed: 20486173]
25. Ulaner GA, Vu TH, Li T, et al. Loss of imprinting of IGF2 and H19 in osteosarcoma is accompanied by reciprocal methylation changes of a CTCF-binding site. Hum Mol Genet. Mar 1; 2003 12(5):535–549. [PubMed: 12588801]
26. Do SI, Jung WW, Kim HS, Park YK. The expression of epidermal growth factor receptor and its downstream signaling molecules in osteosarcoma. Int J Oncol. Mar; 2009 34(3):797–803. [PubMed: 19212684]
27. Freeman SS, Allen SW, Ganti R, et al. Copy number gains in EGFR and copy number losses in PTEN are common events in osteosarcoma tumors. Cancer. Sep 15; 2008 113(6):1453–1461. [PubMed: 18704985]
28. Kersting C, Gebert C, Agelopoulos K, et al. Epidermal growth factor receptor expression in high-grade osteosarcomas is associated with a good clinical outcome. Clin Cancer Res. May 15; 2007 13(10):2998–3005. [PubMed: 17505002]
29. Wen YH, Koeppen H, Garcia R, et al. Epidermal growth factor receptor in osteosarcoma: expression and mutational analysis. Hum Pathol. Aug; 2007 38(8):1184–1191. [PubMed: 17509661]
30. Ryu K, Choy E, Yang C, et al. Activation of signal transducer and activator of transcription 3 (Stat3) pathway in osteosarcoma cells and overexpression of phosphorylated-Stat3 correlates with poor prognosis. J Orthop Res. Jul; 2010 28(7):971–978. [PubMed: 20063378]
31. Ryu K, Susa M, Choy E, et al. Oleanane triterpenoid CDDO-Me induces apoptosis in multidrug resistant osteosarcoma cells through inhibition of Stat3 pathway. BMC Cancer. 2010; 10:187. [PubMed: 20459702]
32. Duan Z, Ji D, Weinstein EJ, et al. Lentiviral shRNA screen of human kinases identifies PLK1 as a potential therapeutic target for osteosarcoma. Cancer Lett. Jul 28; 2010 293(2):220–229. [PubMed: 20144850]
33. Yamaguchi U, Honda K, Satow R, et al. Functional genome screen for therapeutic targets of osteosarcoma. Cancer Sci. Dec; 2009 100(12):2268–2274. [PubMed: 19725836]
34. Seong YS, Kamijo K, Lee JS, et al. A spindle checkpoint arrest and a cytokinesis failure by the dominant-negative polo-box domain of Plk1 in U-2 OS cells. J Biol Chem. Aug 30; 2002 277(35): 32282–32293. [PubMed: 12034729]
35. Gazitt Y, Kolaparthi V, Moncada K, Thomas C, Freeman J. Targeted therapy of human osteosarcoma with 17AAG or rapamycin: characterization of induced apoptosis and inhibition of mTOR and Akt/MAPK/Wnt pathways. Int J Oncol. Feb; 2009 34(2):551–561. [PubMed: 19148492]
36. Paoloni MC, Mazcko C, Fox E, et al. Rapamycin pharmacokinetic and pharmacodynamic relationships in osteosarcoma: a comparative oncology study in dogs. PLoS One. 2010; 5(6):e11013. [PubMed: 20543980]

37. Zhou Q, Deng Z, Zhu Y, Long H, Zhang S, Zhao J. mTOR/p70S6K signal transduction pathway contributes to osteosarcoma progression and patients' prognosis. Med Oncol. Dec; 2010 27(4): 1239–1245. [PubMed: 19936974]
38. Weir BA, Woo MS, Getz G, et al. Characterizing the cancer genome in lung adenocarcinoma. Nature. Dec 6; 2007 450(7171):893–898. [PubMed: 17982442]
39. Sjoblom T, Jones S, Wood LD, et al. The consensus coding sequences of human breast and colorectal cancers. Science. Oct 13; 2006 314(5797):268–274. [PubMed: 16959974]
40. Thomas RK, Baker AC, Debiasi RM, et al. High-throughput oncogene mutation profiling in human cancer. Nat Genet. Mar; 2007 39(3):347–351. [PubMed: 17293865]
41. MacConaill LE, Campbell CD, Kehoe SM, et al. Profiling critical cancer gene mutations in clinical tumor samples. PLoS One. 2009; 4(11):e7887. [PubMed: 19924296]
42. Macconaill LE, Garraway LA. Clinical implications of the cancer genome. J Clin Oncol. Dec 10; 2010 28(35):5219–5228. [PubMed: 20975063]
43. Duan Z, Weinstein EJ, Ji D, et al. Lentiviral short hairpin RNA screen of genes associated with multidrug resistance identifies PRP-4 as a new regulator of chemoresistance in human ovarian cancer. Mol Cancer Ther. Aug; 2008 7(8):2377–2385. [PubMed: 18687998]
44. Cleary JM, Shapiro GI. Development of phosphoinositide-3 kinase pathway inhibitors for advanced cancer. Curr Oncol Rep. Mar; 2010 12(2):87–94. [PubMed: 20425592]
45. Paez J, Sellers WR. PI3K/PTEN/AKT pathway. A critical mediator of oncogenic signaling. Cancer Treat Res. 2003; 115:145–167. [PubMed: 12613196]
46. Man TK, Lu XY, Jaeweon K, et al. Genome-wide array comparative genomic hybridization analysis reveals distinct amplifications in osteosarcoma. BMC Cancer. Aug 7.2004 4:45. [PubMed: 15298715]
47. Lau CC, Harris CP, Lu XY, et al. Frequent amplification and rearrangement of chromosomal bands 6p12-p21 and 17p11.2 in osteosarcoma. Genes Chromosomes Cancer. Jan; 2004 39(1):11–21. [PubMed: 14603437]
48. Ito M, Barys L, O'Reilly T, et al. Comprehensive mapping of p53 pathway alterations reveals an apparent role for both SNP309 and MDM2 amplification in sarcomagenesis. Clin Cancer Res. Feb 1; 2011 17(3):416–426. [PubMed: 21159888]
49. Feugeas O, Guriec N, Babin-Boilletot A, et al. Loss of heterozygosity of the RB gene is a poor prognostic factor in patients with osteosarcoma. J Clin Oncol. Feb; 1996 14(2):467–472. [PubMed: 8636759]
50. Hodi FS, Friedlander P, Corless CL, et al. Major response to imatinib mesylate in KIT-mutated melanoma. J Clin Oncol. Apr 20; 2008 26(12):2046–2051. [PubMed: 18421059]
51. Barretina J, Taylor BS, Banerji S, et al. Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy. Nat Genet. Aug; 2010 42(8):715–721. [PubMed: 20601955]

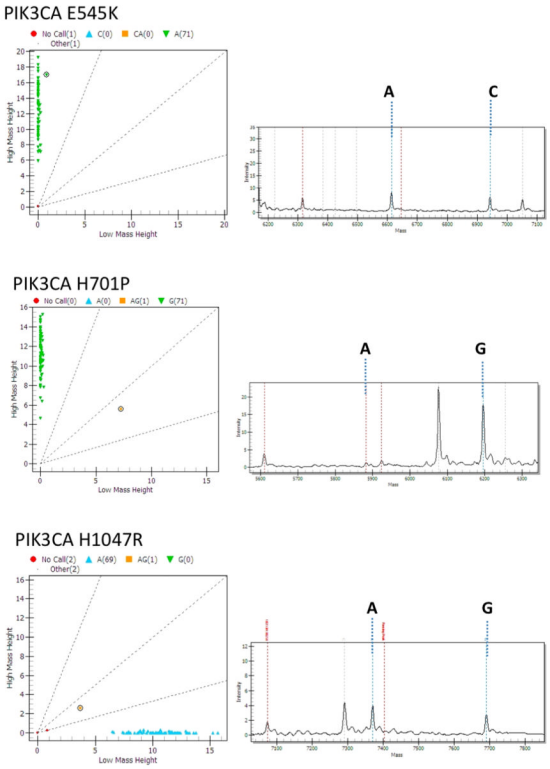

Fig 1.

Mass spectrometric cluster plots (left side) and spectral plots (right side) for the PIK3CA mutations identified in this study. The mutation interrogated is indicated above each plot. The sample with the mutation is indicated by a circle in the left panel. The corresponding spectral plot is indicated on the right. The mass of the alleles specific for the indicated assay are shown by dashed lines.

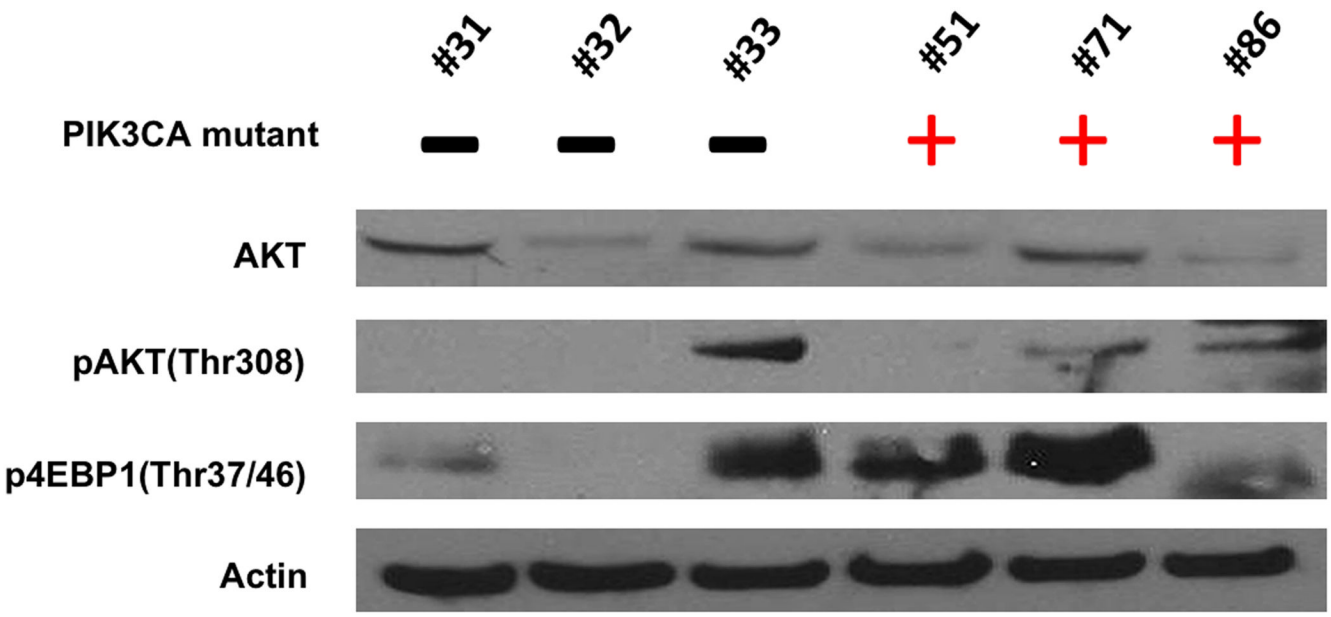

Fig 2.
Western Blot Analysis of AKT/mTOR Pathway Activation in Osteosarcoma Tissues, 3 without PIK3CA mutations and 3 with PIK3CA mutations.

Table 1

Patient and Tumor Characteristics

Subject #	Gender	age	date of tumor collection	histologic subtype	grade (1-3 of 3)	Location	Mutation
1	f	21	1993	OSTEOBLASTIC	3	right femur and acetabulum	
2	f	21	1993	OSTEOBLASTIC	3	right femur	
3	f	24	1996	OSTEOBLASTIC	3	left tibia	
4	f	24	1996	OSTEOBLASTIC	2 - 3	left tibia	
5	f	25	1997	CONVENTIONAL OSTEOSARCOMA	3	left tibia	
6	f	25	1994	OSTEOBLASTIC	3	left distal femur	
7	f	30	NA	NA	NA	NA	
8	f	31	1996	OSTEOBLASTIC	2	femur	PIK3CA H701P
9	f	32	2000	CHONDROSARCOMA	2	left acetabulum	
10	f	32	1995	MIXED OSTEOBLASTIC AND CHONDROBLASTIC	3	left pelvis	
11	f	32	1993	OSTEOBLASTIC	3	left tibia	
12	f	32	1993	OSTEOBLASTIC	3	left tibia	
13	f	33	NA	NA	NA	NA	
14	f	33	2001	TELANGIECTATIC	3	right distal femur	
15	f	34	1996	CONVENTIONAL OSTEOSARCOMA	3	left tibia	
16	f	34	1996	CONVENTIONAL OSTEOSARCOMA	3	left tibia	
17	f	34	1999	CONVENTIONAL OSTEOSARCOMA	2 - 3	left femur	
18	f	36	1996	MALIGNANT FIBROUS HISTIOCYTOMA	3	left knee	
19	f	42	1993	CHONDROBLASTIC	2	left iliac wing	

Subject #	Gender	age	date of tumor collection	histologic subtype	grade (1-3 of 3)	Location	Mutation
20	f	44	NA	NA	NA	NA	
21	f	47	1993	OSTEOBLASTIC	2	left distal femur	
22	f	47	1998	OSTEOBLASTIC	2 - 3	right femur	CDH1 A617T FSCB S775L
23	f	47	1998	OSTEOBLASTIC	2 - 3	right femur	
24	f	47	2000	OSTEOBLASTOMA	NA	left distal tibia	
25	f	48	1993	OSTEOBLASTIC	2 - 3	left femur	
26	f	51	1999	MIXED OSTEOBLASTIC AND FIBROBLASTIC	2 - 3	L 5 vertebrae	
27	f	54	2003	CONVENTIONAL OSTEOSARCOMA	2	left popliteal region	
28	f	55	1994	EPITHELIOID	3	right proximal humerus	
29	f	56	1994	EPITHELIOID	3	right humerus	
30	f	66	1975	CHONDROBLASTIC	NA	right tibia	
31	f	66	1975	CONVENTIONAL OSTEOSARCOMA	3	right proximal tibia	
32	f	73	1987	CONVENTIONAL OSTEOSARCOMA	2	left lower leg	
33	f	77	2001	MIXED OSTEOBLASTIC AND FIBROBLASTIC	1	right prlvis	
34	f	92	2005	CONVENTIONAL OSTEOSARCOMA	2 - 3	right femur	
35	m	20	2001	CONVENTIONAL OSTEOSARCOMA	3	right thigh	
36	m	20	2004	OSTEOBLASTIC	2-3	left distal femur	
37	m	23	1999	CHONDROBLASTIC	2	left femur	
38	m	24	2003	CHONDROBLASTIC	2	left tibia	
39	m	24	2003	CHONDROBLASTIC	2	left proximal tibia	
40	m	25	2000	CONVENTIONAL OSTEOSARCOMA	2	right femur	

Subject #	Gender	age	date of tumor collection	histologic subtype	grade (1-3 of 3)	Location	Mutation
41	m	25	1996	OSTEOBLASTIC	3	left humerus	
42	m	27	1994	MIXED OSTEOBLASTIC AND CHONDROBLASTIC	3	right proximal humerus	
43	m	27	1996	OSTEOBLASTIC	3	right proximal humerus	
44	m	27	1996	OSTEOBLASTIC	3	right humerus	
45	m	29	1995	CONVENTIONAL OSTEOSARCOMA	2 - 3	right tibia	
46	m	29	2004	OSTEOBLASTIC	2 - 3	left chest wall	
47	m	29	2004	OSTEOBLASTIC	2 - 3	left chest wall	
48	m	30	1993	CONVENTIONAL OSTEOSARCOMA	3	left proximal humerus	
49	m	30	1992	TELANGIECTATIC	2	left knee	
50	m	30	1992	TELANGIECTATIC	2	left knee	
51	m	31	1996	CONVENTIONAL OSTEOSARCOMA	2	right femur	
52	m	31	1991	OSTEOBLASTIC	3	proximal tibia	
53	m	32	1995	JUXTACORTICAL	1	right femur	
54	m	37		NA	NA	NA	
55	m	40	1990	CONVENTIONAL OSTEOSARCOMA	2	left proximal femur	
56	m	40	1987	OSTEOSARCOMA WITH CHONDROID FEATURES	2	left distal femur	TP53 R273H
57	m	40	1987	OSTEOSARCOMA WITH CHONDROID FEATURES	2	left femur	
58	m	40	1987	OSTEOSARCOMA WITH CHONDROID FEATURES	1	left femur	
59	m	41	2004	CONVENTIONAL OSTEOSARCOMA	3	left leg	
60	m	41	1997	SMALL CELL	2	right gluteal	
61	m	41	1997	SMALL CELL	2	right gluteal	

Subject #	Gender	age	date of tumor collection	histologic subtype	grade (1-3 of 3)	Location	Mutation
62	m	42	1994	CONVENTIONAL OSTEOSARCOMA	3	left illiac wing	
63	m	42	1994	CONVENTIONAL OSTEOSARCOMA	3	pelvis	
64	m	43	1994	SMALL CELL	2	sacrum	
65	m	44	2000	CONVENTIONAL OSTEOSARCOMA	1	right scapula	CUBN I3189V
66	m	44	2000	CONVENTIONAL OSTEOSARCOMA	1	right scapula	
67	m	45	2002	CHONDROBLASTIC	2	left tibia	
68	m	45	1997	CONVENTIONAL OSTEOSARCOMA	1	left femur	
69	m	45	NA	OSTEOCHONDROMA	NA	right distal femur	
70	m	46	1993	CONVENTIONAL OSTEOSARCOMA	2	right humerus	
71	m	47	1994	CONVENTIONAL OSTEOSARCOMA	2 - 3	left superior pubic ramus	
72	m	48	1991	CONVENTIONAL OSTEOSARCOMA	2 - 3	left femur	
73	m	49	NA	CONVENTIONAL OSTEOSARCOMA	NA	right leg	
74	m	51	1993	CHONDROBLASTIC	2 - 3	right tibia	
75	m	52	1993	CHONDROBLASTIC	2 - 3	proximal right tibia	
76	m	57	1994	OSTEOBLASTIC	2	left ischium	
77	m	60	1993	OSTEOBLASTIC	2 - 3	right distal femur	
78	m	61	1993	JUXTACORTICAL	2	left mid-humerus	
79	m	73	2005	CONVENTIONAL OSTEOSARCOMA	2	right femur	
80	m	73	1996	GIANT CELL OSTEOSARCOMA	NA	right femur	
81	m	79	2005	CHONDROBLASTIC	2 - 3	paravertebral mass	
82	m	79	2005	CHONDROBLASTIC	2 - 3	paravetebrae,T10	

Subject #	Gender	age	date of tumor collection	histologic subtype	grade (1-3 of 3)	Location	Mutation
83	m	80	2001	SPINDLE CELL SARCOMA	2	right femur	

Table 2

List of Genes Tested

ABL1	EGFR	KRAS	PTEN
ABL1	EPHA1	LRP1B	PTPN11
ABL2	EPHA3	MADH4	RAF1
ADAMTSL3	EPHB1	MAP2K4	RB1
AKT1	EPHB6	MET	RET
AKT2	ERBB2	MINK1	ROBO1
AKT3	ERBB2	MLL3	ROBO2
ALK	ERBB4	MOS	ROS1
AML1/RUNX1	FBXW7	MPL	SMAD2
APC	FES	MSH2	SMAD4
AR	FGFR1	MSH6	SMARCB1
AXL	FGFR2	MST1R	SMO
BMX	FGFR3	MYH1	SPTAN1
BRAF	FGFR3	NF1	SRC
BRCA1	FLJ13479	NOTCH1	STK11
BRCA2	FLNB	NPM	SUFU
BUB1B	FLT3	NPM1	TBX22
C-MYC	FMS	NRAS	TEC
C14orf155	GATA1	NTRK1	TFDP1
CDH1	GNAS	NTRK2	TIAM1
CDK4	GUCY1A2	NTRK3	TIF1
CDKN2A	HRAS	PDGFRA	TP53
CEBPA	IGF1R	PDGFRB	TRIM33
CTNNB1	JAK2	PDPK1	TSC1
CUBN	JAK3	PIK3CA	VHL
DBL	KDR	PKHD1	
DBN1	KIT	PRKCB1	