Osteosarcoma Patient-derived Orthotopic Xenograft (PDOX) Models Used to Identify Novel and Effective Therapeutics: A Review

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Abstract. *Background/Aim:* Recurrent osteosarcoma is recalcitrant with poor response rates to first-line chemotherapy due to heterogeneity and metastatic potential. This disease requires novel drug discovery and precision treatment. *Materials and Methods:* The osteosarcoma patient-derived orthotopic xenograft (PDOX) mouse model mimics the clinical disease and has identified effective clinically-approved drugs and experimental agents, especially drug combinations, that hold much clinical promise. *Results:* Effective treatment for drug-resistant osteosarcoma includes regorafenib, as monotherapy, and temozolomide-irinotecan, trabectedin-irinotecan, sorafenib-everolimus, sorafenib-palbociclib, and olaratumab-doxorubicin-cisplatin, as combinations. *Conclusion:* The PDOX model can be used to improve the outcome of osteosarcoma patients, including individualized, precision therapy.

Doxorubicin (DOX), cisplatin (CDDP), ifosfamide (IFO), and methotrexate (MTX) are first-line therapy for osteosarcoma (1-4). However, osteosarcoma frequently develops resistance to chemotherapy after initial treatment,

resulting in tumor recurrence, often metastatic and thereby fatal to the patient (5, 6). Therefore, development of new precision treatment is necessary.

The patient-derived orthotopic xenograft (PDOX) model has been developed for sarcoma and mimics the malignant behavior of osteosarcoma due to the natural tumor micro-environment (TME) of the orthotopically-implanted tissue (7-12). Surgical orthotopic implantation (SOI) was used to establish the PDOX models (7-12).

The present report reviews the osteosarcoma PDOX models, and their potential to discover effective agents, especially combinations for drug-resistant disease.

Patient No. 1. Establishment of a Drug-Resistant Recurrent Osteosarcoma PDOX

The first osteosarcoma PDOX was established from a chondroblastic osteosarcoma of the left distal-femur from a 16-year-old patient who had pre-operative chemotherapy containing CDDP and a limb-salvaging tumor resection with an endoprosthesis (10). One year after the primary surgery, lung metastases were found and metastasectomy was performed. Fresh specimens of the lung metastasis were obtained and immediately taken to our laboratory on wet ice (Figure 1A). The tumor sample was divided into small fragments with surrounding normal tissue in cell-culture medium and subcutaneously implanted on the flank of nude mice (Figure 1B and C). For PDOX establishment, subcutaneous tumors were harvested and divided into 3-4 mm fragments (Figure 1D and E). The vastus-lateralis muscle of the nude mouse was incised and the biceps-femoris muscle was split to expose the distal femur. Subsequently, an incision

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Figure 1. Establishment of the osteosarcoma patient-derived orthotopic xenograft (PDOX) nude-mouse model. (A) Biopsy or surgical specimens were provided from the osteosarcoma patient. (B, C) The tumor specimens were fragmented and subcutaneously implanted in nude mice. (D, E) Before implantation, harvested tumors were fragmented in cell-culture medium. (F) The lateral side of the distal femur was opened by splitting the biceps-femoris muscle and osteotomized. (G) A tumor fragment was orthotopically implanted into the space created by osteotomy. The tumor was covered by the joint capsule, muscle and skin with a nylon suture. Modified after (10, 19).

was made to open the lateral patellofemoral ligament in order to preserve the knee joint. A scissors was used to cut the lateral femoral condyle of the femur (Figure 1F). The space created by the osteotomy was used to implant a single tumor fragment (Figure 1G). The joint, muscle, and skin were repaired using 6-0 nylon suture (Figure 1H). Histologically, the original patient tumor (Figure 2A) was a chondroblastic osteosarcoma, and the established PDOX tumor mimicked it (Figure 2B) with dense osteosarcoma cells displaying aggressive mitoses and an abundant chondroid matrix (13).

Screening of single drugs and their combinations in the osteosarcoma PDOX. Distant recurrence to the lung was seen in the patient after CDDP treatment. CDDP was also not effective in the established PDOX model, concordant with the clinical results (13). Temozolomide (TEM), used in glioma, and trabectedin (TRAB), used in advanced soft-tissue sarcoma (STS), inhibited tumor growth and caused histological necrosis in the CDDP-resistant osteosarcoma-PDOX model (10). In subsequent experiments, the combination of TEM and irinotecan (IRT), a topoisomerase inhibitor, regressed this osteosarcoma PDOX, thereby demonstrating a new combination therapy for drug-resistant

osteosarcoma (14). Eribulin (ERI), a synthetic tubulin inhibitor derived from a sea sponge, clinically used for breast cancer and advanced STS, arrested the growth of this osteosarcoma PDOX, while first-line osteosarcoma drug DOX, and sunitinib (SUN) or pazopanib (PAZ), both multi-targeted tyrosine-kinase receptor inhibitors, were not effective (15). We have also demonstrated that the combination of everolimus (EVL), an mTOR inhibitor, and PAZ were effective for this drug-resistant osteosarcoma PDOX, showing strong anti-angiogenesis efficacy as demonstrated by the Gelfoam angiogenesis assay (16).

Patient No. 2. PDOX Established From a 14-year-old Patient With Drug-resistant Osteosarcoma of the Pelvis

The next osteosarcoma PDOX model was established from a 14-year-old boy with pelvic osteosarcoma. A biopsy specimen was fragmented in our laboratory and implanted subcutaneously in nude mice (17). The patient was not treated before the biopsy. SOI into the femur of nude mice was performed following harvesting and fragmentation of the subcutaneously-grown tumors as described above to establish

Figure 2. Pathological similarity between the osteosarcoma patient-derived orthotopic xenograft (PDOX) model and the original patient tumor. (A) The original patient tumor from a 16-year-old patient (patient #1) with chondroblastic osteosarcoma. (B) The PDOX tumor established from the patient. Both tumors contained high-grade malignant osteosarcoma cells with neoplastic osteoid and chondroid matrix. The osteosarcoma PDOX model is faithful to the original patient osteosarcoma. Scale bars: 80 μ m. Modified after (13).

Figure 3. A representative osteosarcoma patient-derived orthotopic xenograft (PDOX) study identifying effective approved therapeutics. Identification of regorafenib by the osteosarcoma PDOX as a highly effective therapy compared to other multi tyrosine-kinase inhibitors. (A) The line graphs show the tumor volume at each time point after treatment initiation relative to the initial tumor volume for each group ($n=6$). $*p<0.05$; $***p<0.001$. Error bars: $\pm$ standard error of the mean. (B) Representative photographs of the control or drug-treated osteosarcoma PDOX mouse models. Arrows show the margin of the tumors. (C) Hematoxylin and eosin-stained sections of control and drug-treated tumors. Scale bars: 100 μ m. Modified after (27).

the PDOX model (Figure 1). Chemotherapy drugs were screened against this PDOX, including traditional chemotherapy and molecular-targeted drugs. CDDP and DOX were ineffective in this osteosarcoma PDOX model (17, 18). Monotherapy with targeted drugs including, palbociclib (PAL), a cyclin-dependent kinase 4/6 (CDK4/6) inhibitor, sorafenib (SFN), a multi tyrosine-kinase inhibitor, olaratumab (OLA), a monoclonal antibody for platelet-derived growth-factor receptor alpha (PDGFR α), and EVL were not effective in this osteosarcoma PDOX model (17-21). The combination of TRAB and IRT, whose clinical efficacy has been studied only in Ewing's sarcoma, ovarian cancer, and rhabdomyosarcoma, regressed the PDOX osteosarcoma, resulting in extensive necrosis. Ki-67-positive cells were also decreased by the

combination (17). A SFN and EVL combination, whose synergistic efficacy has been clinically demonstrated in hepatic, pancreatic, and renal carcinomas, significantly inhibited the growth of this PDOX osteosarcoma (18). A SFN and PAL combination, which had strong clinical synergy on hepatic and pancreatic carcinoma, regressed the osteosarcoma PDOX model, making it necrotic (20). An OLA and DOX combination, which was approved for advanced STS, was partially effective on this osteosarcoma PDOX model (19). An OLA-DOX-CDDP combination regressed the PDOX osteosarcoma, made it necrotic, and significantly reduced the number of proliferating cells (19). Pioglitazone (PIO), a thiazolidinedione derivative, is a potential activator of peroxisome proliferator-activated receptor gamma (PPAR γ)

(22). A decrease in the chemoresistance of several cancers by activation of PPAR γ has been reported (23, 24). PIO combined with CDDP significantly inhibited the tumor growth of the CDDP-resistant osteosarcoma PDOX (25). Moreover, we showed that PIO blocked DOX-induced P-glycoprotein (P-gp), a drug transporter which plays a critical role in drug resistance. PIO could overcome DOX resistance in the osteosarcoma PDOX (26).

Patient No. 3. PDOX Established From a 23-year-old Patient With Metastatic Osteosarcoma

This osteosarcoma PDOX model was established from a patient with femoral osteosarcoma who underwent surgery for tibia-bone metastasis after CDDP-based chemotherapy. A surgical sample of the metastatic lesion was obtained and the PDOX model was established as described above. We confirmed that this PDOX model was CDDP resistant, in accordance with the patient's tumor (27). To determine an effective second-line chemotherapy for this patient, the efficacy of major oral multi-targeted receptor tyrosine-kinase inhibitors, including PAZ, SUN, SFN, crizotinib (CZT), and regorafenib (RGF) were tested in this metastatic osteosarcoma-PDOX model. Only RGF significantly inhibited and regressed the tumor in this osteosarcoma PDOX (Figure 3) (27). To confirm the efficacy of RGF in the osteosarcoma treatment, these multi-targeted receptor tyrosine-kinase inhibitors were also tested on the pelvic osteosarcoma PDOX from patient #2 (27). SFN, SUN, and RGF significantly inhibited the osteosarcoma PDOX growth, and RGF regressed the tumor in the pelvic osteosarcoma PDOX, indicating RGF as a potential effective chemotherapy for osteosarcoma in the clinic (27).

Identification of Effective Experimental Drugs for Osteosarcoma With the PDOX Model

Tumor-targeting Salmonella typhimurium A1-R. We have previously developed tumor-targeting *Salmonella typhimurium* A1-R (*S. typhimurium* A1-R) and reported its efficacy on PDOX models of major cancers (28). Intra-arterial administration of *S. typhimurium* A1-R was more effective than intra-venously administrated bacteria on the CDDP-resistant osteosarcoma PDOX model (13).

Efficacy of methionine restriction by methioninase on osteosarcoma PDOX. We have previously developed recombinant methioninase (rMETase) to target methionine addiction, a general hall mark of cancer (29-31). The osteosarcoma PDOX from patient #2 was resistant to docetaxel (DOC), which arrests the cell cycle in the M phase. DOC and rMETase together were complementary because of the late S/G₂ phase arrest of cancer cells caused by rMETase, thereby, overcoming DOC resistance of the osteosarcoma PDOX (32).

CDDP-resistant lung-metastatic osteosarcoma from patient #1 was also implanted in the lung of nude mice to produce a lung-metastatic osteosarcoma PDOX model (33). This PDOX model also responded to rMETase. The combination of *S. typhimurium* A1-R, rMETase, and CDDP was able to eradicate the lung metastasis in the PDOX model due to the decoy of *S. typhimurium* A1-R to induce S-phase in the osteosarcoma cells where they were trapped by rMETase and killed by CDDP, which targets cells in S-phase (33).

Caffeine (CAF) increased the sensitivity of cancer cells arrested by oral rMETase (o-rMETase) by inducing mitotic catastrophe (34, 35). Oral administration of the CAF, CDDP and o-rMETase combination regressed the osteosarcoma PDOX from patient #2. This combination thus overcame the resistance to CDDP in this osteosarcoma PDOX (36).

Azacitidine (AZA), a DNA methyltransferase inhibitor, induces DNA hypomethylation in cancer cells (37). rMETase and AZA have a strong DNA-demethylating effect (29, 30, 38). We demonstrated that the combination of AZA and o-rMETase significantly inhibited the growth of the CDDP-resistant osteosarcoma PDOX model of patient #2, while also decreasing Ki-67-positive cells, suggesting the clinical potential of this combination (38).

Efficacy of a novel anioninc-phosphate-platinum complex on osteosarcoma PDOX. An anioninc-platinum complex, which contains 3Pt and bisphosphonates, can have high-bone and low-kidney accumulation, leading to increased anti-tumor efficacy on bone tumors and decreased nephrotoxicity compared to CDDP (39). We demonstrated that 3Pt inhibited tumor growth more effectively than CDDP in the pelvic-osteosarcoma PDOX model from patient #1 (39).

Conclusion

Recurrent osteosarcoma is a recalcitrant cancer that is often resistant to the first-line drugs DOX, CDDP, IFO, and MTX (1, 2). Recurrence is often seen in osteosarcoma after treatment with these drugs (5, 6). Targeted therapy and immunotherapy have limited success in osteosarcoma (40). Identifying treatment options for individual osteosarcoma patient is necessary.

We have reported 18 osteosarcoma PDOX studies evaluating approved and experimental drugs since 2017 (Table I). Effective therapies, including novel drug combinations for osteosarcoma, were identified in the osteosarcoma PDOX model. Most important, RGF, as monotherapy, and TEM-IRT, TRAB-IRT, SFN-EVL, SFN-PAL, and OLA-DOX-CDDP as combinations, regressed the osteosarcoma PDOX tumor, indicating clinical potential for drug-resistant osteosarcoma. Experimental therapy with rMETase, tumor-targeting *S. typhimurium* A1-R, or combinations of these agents and other drugs have shown

Table I. Summary of effective and ineffective drugs on the osteosarcoma patient-derived orthotopic xenograft models.

Case	Ineffective treatment (Ref)	Effective treatment which arrested the PDOX (Ref)	Highly-effective treatment which regressed the PDOX (Ref)	Effective experimental treatment (Ref)
Patient #1: Recurrent chondroblastic femoral osteosarcoma.	CDDP (10, 13, 14, 33, 39) DOX (15, 16) PAZ (15, 16) SUN (15) EVL (16)	TRAB (10) TEM (10, 15) GEM+DOC (14) ERI (15) EVL+PAZ (16)	TEM+IRT (14)	A1-R i.a. (13) rMETase+A1-R (33) rMETase+A1-R+CDDP (33) 3Pt (39)
Patient #2: Primary pelvic osteosarcoma.	CDDP (17, 19, 20, 25, 27, 36) DOX (18, 26, 38) TRAB (17) IRT (17) EVL (18) SFN (18, 20, 27) OLA+DOX (19) DOX+CDDP (19) OLA+CDDP (19) PAL (20) ERI (21) PIO (25, 26) SUN (27) CZT (27) PAZ (27) DOC (32) AZA (38)	CDDP+PIO (25) DOX+PIO (26)	TRAB+IRT (17) SFN+EVL (18) OLA+DOX+CDDP (19) SFN+PAL (20) RGF (27)	o-rMETase+DOC (32) CDDP+o-rMETase+CAF (36) o-rMETase+AZA (38)
Patient #3. Metastatic tibial osteosarcoma	CDDP (27) PAZ (27) SUN (27) SFN (27) CZT (27)		RGF (27)	

CDDP: Cisplatin; DOX: doxorubicin; PAZ: pazopanib; SUN: sunitinib; EVL: everolimus; TRAB: trabectedin; TEM: temozolomide; GEM: gemcitabine; ERI: eribulin; IRT: irinotecan, A1-R: *Salmonella typhimurium* A1-R; rMETase: recombinant methioninase; 3Pt: anioninc platinum complex; SFN: sorafenib; OLA: olaratumab; PAL: Palbociclib; PIO: pioglitazone; CZT: crizotinib, DOC: docetaxel; AZA: azacytidine; RGF: regorafenib; o-rMETase: oral-recombinant methioninase; CAF: caffeine; i.a.: intra-arterial administration.

surprising efficacy in the recalcitrant-osteosarcoma PDOX models. The PDOX model offers unique opportunities for osteosarcoma patients to provide improved, precision, and personalized treatment, and should be universally implemented.

Conflicts of Interest

TH, KI, NY, KH, HK, SM, and RMH are or were unsalaried associates of AntiCancer Inc. which uses PDOX models for contract research. There are no other competing financial interests regarding this study.

Authors' Contributions

Conception and design: TH and RMH. Acquisition, analysis, and interpretation of data: TH, KI, NY, KH, HK, SM, and MB. Writing, review, and revision of the article: TH, HT, and RMH.

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