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Supplementary Discussion

Our 5-year experience with obtaining consent from patients for the production of O-PDX tumors has helped to establish an efficient pipeline for production and characterization of models. Fewer than 1% of patients who were approached to consent to the MAST protocol refused to participate. The biggest challenge of producing O-PDX tumors was procuring enough tissue. For biopsy specimens or small tumor samples, there is rarely enough tissue for injection. This is particularly challenging for retinoblastoma due to the difficulty in isolating viable tumor from the vitreous of the eye. Also, tumor viability can limit engraftment efficiency. For patients on active chemotherapeutic treatment regimens, such as those with neuroblastoma, a substantial proportion of the tumor is necrotic, which may contribute to the relatively lower engraftment efficiency. A larger cohort of patients will be required to determine if particular subsets of tumors defined by their genetic lesions are more likely to engraft than others. Indeed, we have some matched pairs of diagnostic/recurrent/autopsy tumors, and more will be developed from St. Jude patients' tumors over the next several years. To date, we have received and injected tumors from 15 patients at autopsy and 33% (5/15) have engrafted (E.S. and S.F. in preparation). This is a particularly important source of tissue for O-PDX production because at autopsy, multiple recurrent metastatic tumors are often present and represent the most aggressive form of the disease.

We favor orthotopic implantation over flank implantation for 5 reasons. First, the microenvironment may be more favorable to tumor engraftment if it recapitulates the organ site in the patient[1-4]. Second, the pharmacokinetics and associated normal cells from the microenvironment are tumor site specific[5,6]. For example, ocular astrocytes infiltrate the retinoblastoma tumors, and those cells are not present in the flank. Third, the imaging modalities used in patients (MRI, ultrasound, PET-CT) will be more relevant in orthotopic PDX tumor models. For example, X-ray and PET-CT were particularly valuable for Ewing sarcoma orthotopic tumors, but this would not have been feasible in the flank[7]. Fourth, orthotopic implantation is amenable to tumor cell dissociation. This is important because dissociation prior to injection may reduce tumor heterogeneity and lead to more reproducible engraftment and preservation of individual O-PDX subline clonal composition. Indeed, clonal analysis of 8 regions in a subset of our O-PDX models showed little evidence of regional heterogeneity. Fifth, the cells engraft more efficiently from cryopreservation of single-cell suspensions into orthotopic sites than from that of tissue pieces in the flank in our experience over the past 5 years (data not shown). Therefore, although flank implantation is a useful approach for producing PDXs, the orthotopic approach, when feasible, provides some advantages.

The O-PDX tumors retained many but not all of the cellular features of the patient tumors. In general, it was much easier to appreciate the unique cellular features of each tumor type in the O-PDX

tumors because there were fewer infiltrating normal cells and less necrosis. In the patient tumors, necrosis may be caused by ongoing treatment or regions of hypoxia and nutrient depletion. The O-PDX tumors had more proliferation and less cell death, while retaining the expression of proteins that are diagnostic for each tumor type. However, in patient tumors such as neuroblastomas, which are a mixture of postmitotic differentiated cells and undifferentiated proliferating cells, the differentiated cell population was lost in the O-PDX tumor. It is not known whether the clonal shift in those O-PDX tumors reflects this loss of differentiated tumor cells or a selection of subclones of proliferative cells. In addition to histopathologic analysis, TEM analysis demonstrated that these diverse pediatric solid tumors maintained the subcellular features that are a hallmark of their developmental origins. These data may also help identify tumor vulnerabilities, such as the swollen endoplasmic reticulum in osteosarcoma or the lipid biosynthesis in liposarcoma.

By combining RNA-seq with WGS and WES, we cross-validated individual coding SNVs and insertions/deletions, as well as information on larger genomic events (e.g., CNVs and SVs). Some genomic lesions that contribute to tumorigenesis would have been missed if we did not perform all 3 types of sequencing. For example, SVs that juxtapose an enhancer adjacent to the *TERT* promoter in neuroblastoma and SVs that inactivate the *TP53* gene in osteosarcoma would have been missed with WES and RNA-seq alone[8,9]. Most differences that we observed were apparent gains of mutations in the O-PDX tumors, but custom capture and Illumina deep sequencing demonstrated that few of those were new mutations. Most of the mutations detected in the O-PDXs were present in the patient tumor but were masked by low sequence coverage and low tumor purity. Thus, the O-PDX tumors were very useful for validating mutations with low allele frequency in the patient tumor. It is important to emphasize the value of custom capture and deep sequencing for validating the mutations in the patient tumor and the O-PDX models. These data are useful for not only validating SNVs but also performing the clonal analysis of the O-PDX relative to the patient tumor. It is not surprising that we observed clonal shifts in the O-PDX tumors. Osteosarcoma had the best clonal preservation in O-PDX models and neuroblastoma had the worst. Neuroblastomas also had the longest engraftment time and the lowest engraftment efficiency. The tumors that undergo clonal shifts in the O-PDX (neuroblastoma) and have lower engraftment efficiency and growth rate may be under greater selective pressure in mice than are those that preserve their clonal composition in the O-PDXs (osteosarcoma and rhabdomyosarcoma). In addition, only a minimal shift in clonal composition occurred with passage in immunocompromised mice. In some O-PDX tumors, later passages were a better match to the patient tumor because a minor clone that was unique to the O-PDX at initial engraftment was lost with subsequent passage. Moreover, there were several examples where the distinct O-PDX sublines encompassed different clonal populations in the patient tumor, thereby enabling researchers to study those populations and their contribution to tumor recurrence.

Several previous studies using patient-derived xenografts or organoids of adult cancers have demonstrated the value of testing drug sensitivity in culture and in vivo[10-13]. Our high-throughput drug screening showed that pediatric solid tumor cell lines were more sensitive to chemotherapy than were the primary cultures of the O-PDX tumors. In addition, the drugs that are used to treat patients at diagnosis and recurrence were active, which is consistent with clinical experience. Our data suggest that HDAC and proteasome inhibitors are broadly active against virtually all of our O-PDX tumors and cell lines. However, the combination of an HDAC inhibitor (panobinostat) and a proteasome inhibitor (bortezomib) were not active in vivo for rhabdomyosarcoma. The failure to achieve an antitumor response in vivo was most likely not due to poor tumor penetration, because the levels of each drug was at least 10-fold higher than the EC_{50} for each drug in culture. There are several possible explanations for the discrepancy between the ex vivo sensitivity and in vivo sensitivity to these drugs. Tumor cells may compensate for HDAC and proteasome inhibition in vivo in ways that are not observed in culture. Pathways may be activated in tumor cells in culture that make them particularly vulnerable to HDAC and proteasome inhibitors, but those pathways may be less vulnerable in vivo. Additional pharmacodynamics studies will be required to distinguish between these possibilities. Our data demonstrate that ex vivo activity does not always translate in vivo, and this is why it is important to perform preclinical phase I, II, and III studies before moving new drug combinations into clinical trials for pediatric solid tumors.

A drug that was more selective for rhabdomyosarcoma (AZD1775) was active in vivo when combined with standard-of-care therapy for recurrent rhabdomyosarcoma. Our data are consistent with previously published data using rhabdomyosarcoma cell lines[14]. The efficacy of AZD1775+IRN+VCR as measured by the proportion of complete responses (CR) was greater for ARMS than ERMS. However, it is important to emphasize that the ARMS O-PDX tumors were also more responsive to standard-of-care therapy. Therefore, the improvement in outcome relative to standard-of-care therapy was actually greater for the ERMS O-PDX tumors than ARMS O-PDXs. These data emphasize the importance of comparing new drug combinations to relevant standard treatment regimens used in the clinic and suggest that patients with the most aggressive recurrent RMS (ARMS and ERMS) may benefit from AZD1775+IRN+VCR. The combination of AZD1775 with IRN and VCR is particularly attractive for rhabdomyosarcoma; a combination phase I study of AZD1775 with IRN from the COG is now complete. The addition of VCR to the phase II–recommended doses of AZD1775 and IRN should be straightforward because the toxicities do not overlap and because VCR is routinely combined with IRN in patients with recurrent rhabdomyosarcoma. Previous studies have demonstrated that neuroblastoma cell lines are sensitive to drug combinations including AZD1775[Ref. 15]. The neuroblastoma O-PDX models described here will be useful for extending those observations in future preclinical studies. We tested 4 different high-risk rhabdomyosarcoma O-PDX tumors in our preclinical

phase III study; 3 of which were from patients with recurrent disease. Although all 4 showed improved outcome with the addition of AZD1775 to VCR and IRN, relative to VCR and IRN alone, there were differences in the sensitivity to VCR and IRN. SJRHB013757_X2 from a patients with ARMS at diagnosis and SJRHB013759_X1 from a patient with recurrent ARMS had very good response to IRN and VCR, so it was more difficult to document any improvement with the addition of AZD1775. It will be important to test this triple-drug combination in a broader panel of high-risk recurrent rhabdomyosarcoma O-PDX tumors in future studies.

All of our O-PDX models and associated data are freely shared through the Childhood Solid Tumor Network (CSTN; www.stjude.org/CSTN/), and we have already filled 314 requests from 122 investigators at 46 institutions across 11 countries. We have nearly 20,000 vials of cells cryopreserved for shipment to investigators interested in pediatric solid tumors. All samples, protocols, and associated data are distributed freely, with no obligation to collaborate. We update the O-PDX models available through the CSTN every 6 months, with approximately 40 new O-PDX tumors entered into the database every year. These models and their associated molecular, cellular, and drug sensitivity data will be valuable resources for the pediatric oncology research community, and as we collect more diagnostic/recurrent tumor pairs, we hope to identify therapies that will improve outcomes for children who are not cured by conventional upfront therapy.

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