Aberrant AKT activation drives well-differentiated liposarcoma

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Well-differentiated liposarcoma (WDLPS), one of the most common human sarcomas, is poorly responsive to radiation and chemotherapy, and the lack of animal models suitable for experimental analysis has seriously impeded functional investigation of its pathobiology and development of effective targeted therapies. Here, we show that zebrafish expressing constitutively active Akt2 in mesenchymal progenitors develop WDLPS that closely resemble the human disease. Tumor incidence rates were 8% in p53 wild-type zebrafish, 6% in p53 heterozygotes, and 29% in p53-homozygous mutant zebrafish (P = 0.013), indicating that aberrant Akt activation collaborates with p53 mutation in WDLPS pathogenesis. Analysis of primary clinical specimens of WDLPS, and of the closely related dedifferentiated liposarcoma (DDLPS) subtype, revealed immunohistochemical evidence of AKT activation in 27% of cases. Western blot analysis of a panel of cell lines derived from patients with WDLPS or DDLPS revealed robust AKT phosphorylation in all cell lines examined, even when these cells were cultured in serum-free media. Moreover, BEZ235, a small molecule inhibitor of PI3K and mammalian target of rapamycin that effectively inhibits AKT activation in these cells, impaired viability at nanomolar concentrations. Our findings are unique in providing an animal model to decipher the molecular pathogenesis of WDLPS, and implicate AKT as a previously unexplored therapeutic target in this chemoresistant sarcoma.

Regions of chromosome 12q13-15 are often amplified in well-differentiated and dedifferentiated liposarcomas, typically involving MDM2, CDK4, and HMGAI2, along with several other genes (6, 10, 12, 13); JUN can also be amplified in WDLPS cases that have a dedifferentiated component (14). Further dissection of WDLPS molecular pathogenesis has been greatly impeded by the lack of animal models suitable for experimental analysis. Oncogenic signal transduction through the PI3K-AKT pathway, which is widely dysregulated in human cancer, is normally downregulated by the PTEN tumor suppressor (15). Individuals with germ-line PTEN-inactivating mutations frequently develop multiple lipomas (benign adipocytic neoplasms) (16), and AKT activation has been described in human liposarcomas (17), suggesting that the PI3K-AKT pathway is involved in adipocyte transformation. Here we show that expression of constitutively active Akt2 in zebrafish mesenchymal progenitors induces WDLPS, thus being unique in providing an animal model for future investigation of this disease. Moreover, we also show that AKT pathway inhibition impairs viability in human cell lines derived from patients with WDLPS and DDLPS, thus implicating AKT as a previously unexplored therapeutic target in these chemoresistant sarcomas.

**Results**

**Expression of Constitutively Active Akt2 Induces Well-Differentiated Liposarcoma.** To test the hypothesis that Akt is a WDLPS oncogene that collaborates with p53 inactivation during adipocyte transformation, we in-crossed zebrafish harboring heterozygous p53 mutations, which encode a transactivation-defective p53 protein (18), and all resultant embryos were microinjected at the one-cell stage with a rag2myr-mAkt2 expression construct (Fig. 1). This construct encodes a myristoylated, constitutively active mouse Akt2 transgene (19) driven by a zebrafish rag2 promoter that drives expression in mesenchymal progenitors. Thus, homozygous p53-deleted embryos injected at the one-cell stage with the Akt2 overexpression construct developed WDLPS-like tumors (Fig. 2F), whereas littermate controls did not. This result was confirmed in a repeat experiment (Fig. 2G), and was validated by the finding that ectopic Akt2 expression was present in WDLPS tumors (Fig. 3A). Further, these tumors were addicted to Akt activation, as treatment with the Akt inhibitor GDC-0941 (20) increased cell death in two WDLPS lines derived from these tumors (Fig. 3B).


**Conflict of interest statement:** C.P.R. is a consultant for Novartis and participates in clinical trials of Novartis. G.D.D. is a consultant for Novartis, Pfizer, Ariad, Johnson & Johnson, PharmaMar, Genentech, Infinity Pharmaceuticals, EMD-Serono, Glaxo Smith Kline, Amgen, Daiichi-Sankyo, ArQule, Enzon, Millenium/Takeda; is a member of the scientific advisory board of Flexikon, ZioPharm, Nereus, N-Of-One, and Koltan Pharmaceuticals; and participates in clinical trials of Novartis, Pfizer, Ariad, Johnson & Johnson, PharmaMar, and Infinity Pharmaceuticals. A.J.W. is a consultant for Novartis, Roche/Genentech, Sanofi, Pfizer, EMD-Serono, and participates in clinical trials supported by Novartis, Roche/Genentech, Pfizer, and Exelixis.

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A 58% of pure WDLPS (n = 22) and 22% of the pure DDLPS (n = 13) tumors analyzed (Fig. 3J). We also included human tumors containing both well-differentiated and dedifferentiated liposarcoma components in our analysis (n = 22), which revealed phospho-AKT positivity in 32% of the well-differentiated components and 45% in the dedifferentiated components of these cases (Fig. 3J). Given that phospho-AKT is a labile epitope in clinical speci-

Aberrant AKT Pathway Activation in Primary Human Well-Differentiated and Dedifferentiated Liposarcomas. To test whether AKT activation is also involved in human liposarcoma pathogenesis, we performed immunohistochemical analysis for phospho-AKT (Ser473) on clinical specimens from 58 patients with well-differentiated and dedifferentiated liposarcomas. These studies demonstrated AKT activation in a subset of the human WDLPS or DDLPS cases (Fig. 3 A–F), including 22% of the pure WDLPS (n = 23) and 46% of pure DDLPS (n = 13) tumors analyzed (Fig. 3J). Given that phospho-AKT is a labile epitope in clinical speci-
mens, we also performed immunohistochemistry for phospho-S6 (Ser235/236), a downstream target of the AKT pathway (21). Similar results (Fig. 3 G–I) were obtained, with 17% of pure WDLPS (n = 23), 41% of well-differentiated WDLPS/DDLPS components (n = 22), and 47% of DDLPS (pure DDLPS, n = 13; dedifferentiated WDLPS/DDLPS components, n = 21), demonstrating evidence of S6 activation (Fig. 3K). We found no immunohistochemical evidence of AKT or S6 phosphorylation in lipomas or in normal adipose tissue (Fig. 3 D and G, and Fig. S1).

To determine whether AKT activation is aberrant in human WDLPS and DDLPS, we took advantage of a panel of cell lines derived from patients with these sarcomas to evaluate phosphorylation of AKT and of its downstream target GSK3β after 4 h of growth in serum-free conditions. Strikingly, Western blot analysis revealed persistent phosphorylation of AKT and of its downstream target GSK3β in all eight cell lines examined, even under such serum-starved conditions. In contrast, serum starvation resulted in silencing of AKT and GSK3β phosphorylation in control SU-CCS-1 clear cell sarcoma cells (Fig. 4).

PI3K-AKT-Mammalian Target of Rapamycin Pathway Inhibition Impairs the Viability of Human Liposarcoma Cells. To determine whether human WDLPS and DDLPS cells are dependent on aberrant AKT pathway activation, we treated four cell lines (two WDLPS and two DDLPS) with BEZ235, a dual-specificity inhibitor of PI3K and both mammalian target of rapamycin (mTOR) complexes (22) that effectively silences AKT pathway activation in these cells (Fig. 5A). BEZ235 treatment for 72 h decreased the viability of all cell lines tested, with IC50 values ranging from 13 to 75 nM (Fig. 5B). Treatment with rapamycin, an mTORC1 inhibitor, had less of an effect on viability (Fig. 5C), suggesting that the PI3K-AKT pathway plays both mTORC1-dependent and -independent roles in the pathobiology of WDLPS and DDLPS. Analysis of cell-cycle profiles revealed that BEZ235 treatment induced G1 arrest at nanomolar concentrations (Fig. 5D), whereas apoptosis was induced only at a 1-μM concentration (Fig. 5E).
Moreover, treatment with the PI3K-AKT pathway inhibitor BEZ235 inhibited viability in all cell lines derived from patients with WDLPS and DDLPS that we tested. Taken together, our findings implicate a central role for oncogenic AKT signaling in the molecular pathogenesis of human WDLPS and DDLPS, and suggest the need for clinical trials of AKT pathway inhibitors in patients with unresectable disease, for whom there are currently no known effective therapies.

Our analyses of primary human tumors revealed an increased frequency and intensity of staining for phospho-AKT and phospho-S6 ribosomal protein in dedifferentiated liposarcomas compared with their well-differentiated counterparts. These findings suggest the intriguing possibility that AKT activation may define a subset of WDLPs, which is particularly prone to dedifferentiation, a process that may be caused in part by the acquisition of additional oncogenic abnormalities further potentiating signaling through the AKT pathway. However, we cannot rule out the possibility that this apparent difference may simply be related to the greater difficulty of detecting phosphorylated epitopes in WDLPs sections, in which most of the tumor mass consists of large fat vacuoles within malignant yet well-differentiated adipocytes, whereas the densely cellular DDLPS tumors have a much greater number of cellular elements in which AKT phosphorylation can be assessed per section. Further studies will be required to establish the mechanisms underlying this observation.

Recent work has revealed that 18% of myxoid/round-cell liposarcomas harbor activating mutations in PIK3CA, encoding the catalytic subunit of class IA PI3K, whereas these tumors lack the 12q amplifications characteristic of WDLPs/DDLPS, leading most investigators to believe that these liposarcoma subtypes are biologically distinct (1, 2). S6K1, a direct target of mTORC1 downstream of PI3K-AKT, has recently been shown to be required for the earliest stages of adipogenesis (23), providing one plausible mechanism to explain selection for AKT pathway activation in diverse liposarcoma subtypes. Nevertheless, the fact that expression of activated Akt2 in mesenchymal progenitors drives WDLPs in transgenic zebrafish, and that nearly one-third of clinical specimens from primary cases of human WDLPs and DDLPS showed immunohistochemical evidence of AKT pathway activation. Moreover, treatment with the PI3K-AKT pathway inhibitor BEZ235 inhibited viability in all cell lines derived from patients with WDLPs and DDLPS that we tested. Taken together, our findings implicate a central role for oncogenic AKT signaling in the molecular pathogenesis of human WDLPs and DDLPS, and suggest the need for clinical trials of AKT pathway inhibitors in patients with unresectable disease, for whom there are currently no known effective therapies.

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Current knowledge of the molecular pathogenesis of WDLPs has been driven by genetic analyses of human tumors, which have revealed that almost all cases harbor MDM2 amplification or TP53 mutations (7–10). Evidence also suggests that individuals with germ-line TP53 mutations are at increased risk of WDLPs development at a very young age (11). By demonstrating that activated Akt2 and p53 mutations collaborate in the zebrafish, we have now experimentally demonstrated the long-suspected role of p53 as a tumor suppressor in WDLPs. These tumors are also characterized by recurrent amplifications of distinct regions of chromosome 12q13-15 (6, 10, 12), but until now it has not been possible to identify which of the involved genes are WDLPs oncogenes driving the selection for these amplifications, and which are merely nonpathogenic “passengers.” Furthermore, although dedifferentiated liposarcoma is thought to arise because of further malignant transformation of WDLPs, it has previously been impossible to directly test the ability of candidate genetic lesions to drive this transformation in a physiological context. The zebrafish model we describe now provides a platform for experimental
studies to dissect molecular pathogenesis and discover novel therapeutic targets in this chemoresistant tumor.

Materials and Methods

Zebrafish Husbandry, Mutant Lines, and Imaging. Zebrafish husbandry was performed as previously described (24), in accord with protocols approved by the Dana-Farber Cancer Institute Animal Care and Use Committee. The p53ΔMTAK2-mutant zebrafish line was previously described (18). Zebrafish images were obtained using a Nikon SMZ1500 microscope, Nikon DS2MBWc camera, and NIS-Elements F Package Ver. 3.00 (Nikon Instruments Inc.).

Expression Constructs. The rag2:myr-mAkt2 construct was generated by placing a myristoylated murine Akt2 transgene (19), which is constitutively activated as a result of constitutive membrane localization, downstream of a zebrafish rag2 promoter fragment (25) in a modified pBlauscript vector, wherein the rag2:myr-mAkt2 construct is flanked by recognition sequences for I-SceI meganuclease.

Generation of Transgenic Zebrafish. Circular rag2:myr-mAkt2 plasmid DNA (30 pg) was microinjected along with I-SceI meganuclease (New England Biolabs) into one-cell stage zebrafish embryos from the A8 wild-type strain, as previously described (26).

Zebrafish Paraffin Embedding and Sectioning. Zebrafish were killed in tricaine anesthetic, fixed in 4% paraformaldehyde at 4 °C for 2 d, decalcified with 0.25 M EDTA (pH 8.0) for 2 d, dehydrated in alcohol, cleared in xylene, and embedded in paraffin. Tissue sections from paraffin-embedded tissue blocks were placed on charged slides, deparaffinized in xylene, rehydrated through graded alcohol solutions, and stained with H&E or analyzed by immunohistochemistry.

Patient Samples. Human well-differentiated liposarcoma, dedifferentiated liposarcoma, lipoma, and normal fat specimens were removed at surgery and collected from patients treated at Brigham and Women’s Hospital, who gave informed consent for use of anonymized surgical specimens for research purposes after all clinically relevant evaluations were performed, with approval of the Partners Health Care Institutional Review Board. The diagnosis of well-differentiated liposarcoma/atyypical lipomatous tumor, dedifferentiated liposarcoma, or lipoma was made by institutional pathologists and reviewed by E.L.S. and C.D.M.F. to ensure diagnostic accuracy based on criteria of the World Health Organization (2).

Immunohistochemistry. Immunohistochemistry on human samples was performed on a tissue microarray containing three 0.4-mm cores from each individual tumor, and on selected whole tumor sections. Zebrafish immunohistochemistry was performed on slides of whole zebrafish sections. Slides were deparaffinized and pretreated with 10 mM citrate (pH 6.0) in a steam pressure cooker (Decloaking Chamber, BioCare Medical) according to the manufacturer’s instructions, followed by washing in distilled water. Slides were pretreated with Peroxidase Block (Dako) for 5 min, followed by serum-free protein block (Dako) for 20 min. Primary rabbit antibody to Ser473 phospho-AKT (#4058; Cell Signaling Technology) or to Ser235/236 phospho-S6 (Cell Signaling Technology; #9231; 1:1,000), GSK3 (Santa Cruz; #sc-7936; 1:1,000), GSK3 (Santa Cruz; #sc-7291; 1:500), and vinculin (Sigma-Aldrich; #V9495; 1:500). The hybridization signals were detected by chemiluminescence imaging system (ECL, Amersham Biosciences) and captured using a LAS1000-plus chemiluminescence imaging system (Fujiﬁlm).

Cell Viability Assays. Liposarcoma cells were plated in 96-well plates at 2,000 cells per well in 100 μL of medium containing 15% FBS. After 24 h, cells were exposed to increasing concentrations of compounds. Each concentration was tested in triplicate. Cell viability was determined after 72 h using the Cell-Titer-Glo Luminescent Cell Viability Assay Kit (Promega) with a modification to the manufacturer’s protocol wherein the Cell-Titer-Glo reagent was diluted 1:3 with PBS. The relative luminescence units (RLU) were measured using the FLUOstar Optima plate reader (BMG Labtech GmbH) and relative cell number was calculated by normalization to the RLU of the control treated cells. The inhibitory concentrations 50 (IC50) were calculated using sigmoidal dose-response (variable slope) curve ﬁtting with Prism version 5.0 (GraphPad Software).

Cell Cycle and Apoptosis Analyses. Human liposarcoma cells were exposed to inhibitors or 0.1% DMSO for 24 h and harvested. For cell cycle analysis, cells were washed with ice-cold PBS, fixed in 70% ethanol at 4 °C overnight, and stained in PBS containing 10 μg/mL RNase A and 20 μg/mL propidium iodide (Sigma) in the dark. DNA content analysis was performed by flow cytometry (FACSscan; Becton Dickinson) with CellQuest and ModFIT LT software (Becton Dickinson).

For apoptosis analysis, cells were exposed to BEZ235 or 0.1% DMSO for 38 h and harvested. Annexin V and 7-aminomethine dye D (7-AAD) staining was performed using the PE Annexin V Apoptosis Detection Kit I (#558763; BD Pharmingen) according to the manufacturer’s instructions. Stained cells were quantitated as viable (Annexin V−/7-AAD−), early apoptotic (Annexin V+/7-AAD+), or late apoptotic (Annexin V+/7-AAD+) by flow cytometry (FACSscan; BD Biosciences) with CellQuest software (BD Biosciences).

Statistical Analyses. Differences in sarcoma incidence between zebrafish of different p53 genotypes were tested by the log-rank test. Differences in categorical data were assessed via Fisher’s exact test.

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