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¹⁸F-FDG MicroPET Imaging Detects Early Transient Response to an IGF1R Inhibitor in Genetically-Engineered Rhabdomyosarcoma Models

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Abstract

Background—Alveolar rhabdomyosarcoma (ARMS) and embryonal rhabdomyosarcoma (ERMS) are among the most common and most treatment resistant soft tissue sarcomas of childhood. Here, we evaluated the potential of ¹⁸F-Fluorodeoxyglucose (FDG) as a marker of therapeutic response to picropodophyllin (PPP), an IGF1R inhibitor, in a conditional mouse model of ARMS and a conditional model of ERMS/undifferentiated pleomorphic sarcoma (UPS).

Procedure—Primary tumor cell cultures from *Myf6Cre*, *Pax3:Fkhr*, *p53* and *Pax7CreER*, *Ptch1*, *p53* conditional models of ARMS and ERMS/UPS were found to be highly sensitive to PPP (IC50 values 150 and 200 nM, respectively). Animals of each model were then treated with 80 mg/kg/day PPP by intraperitoneal injection for 12 days and imaged by ¹⁸F-FDG microPET.

Results—Tumor volumes on day 4 for PPP treated ARMS and ERMS mice were lower than untreated control mouse tumor volumes, although treated tumors were larger than day 0. However, tumor FDG uptake was significantly reduced on day 4 for PPP treated mice compared to pretreatment baseline or untreated control mice on day 4 (*p*<0.05). Nevertheless, by day 12 tumor volumes and FDG uptake for treated mice had increased significantly, indicating rapidly evolving resistance to therapy.

Conclusions—¹⁸F-FDG PET imaging is a potential imaging biomarker of molecular susceptibility to targeted agents early in treatment for this aggressive form of sarcoma, but may

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find best use serially for Phase I/II studies where chemotherapy and targeted agents are combined to cytoreduce tumors and abrogate Igf1r inhibitor resistance.

Keywords

rhabdomyosarcoma; picropodophyllin; FDG; microPET; imaging; efficacy

Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children and adolescents [1]. The prognosis for RMS is dependent on the site of tumor origin, extent of disease, and histological subtype. Alveolar (ARMS) and embryonal (ERMS) are the two major subtypes of RMS and have distinct genetic abnormalities and biological behavior [1–4]. While advances in surgery, radiation, and chemotherapy have improved outcome for patients with localized RMS, the prognosis for patients with metastatic RMS has remained unchanged for decades. Metastatic RMS of any subtype has been associated with poor prognosis with overall survival rates of less than 30% [1,3]. Resistance to cytotoxic chemotherapeutic agents and metastatic spread are the two main reasons for failure of therapy in patients with RMS. As a result, new molecularly-targeted therapies are actively being investigated as adjuvants to chemotherapy.

Growth factor receptors in rhabdomyosarcoma, particularly receptor tyrosine kinases (RTKs), are attractive targets for non-chemotherapeutic, small molecule agents [5]. These RTKs regulate cell proliferation, growth and survival through signaling pathways and are thought to be involved in the tumorigenesis of many cancers [6,7]. Some of the RTKs implicated in the development of RMS are IGF1R, PDGFR, c-Met and c-Kit [7–9]. Therefore these RTKs are actively being investigated as potential targets for therapy [7,10–12]. IGF1R has been extensively investigated as a therapeutic target in childhood sarcoma and studies have shown that inhibition of IGF1R using antibodies and small molecule inhibitors results in reduced tumorigenesis, invasion and metastasis *in vivo* [13,14]. Several ongoing clinical trials are evaluating the efficacy of IGF1R targeting in RMS. Furthermore, while monoclonal antibodies against IGF1R are becoming clinically well studied in cancer, small molecule inhibitors are also showing clinical promise [15]. Therefore, in our study we have focused on Picropodophyllin (PPP), a potent small molecule inhibitor of the IGF1R pathway [16–18].

In the new era of molecularly-targeted therapies, imaging-based biomarkers of response may be of increasing value for evaluating solid tumors such as rhabdomyosarcoma. ^{18}F -fluorodeoxyglucose (FDG) based positron emission tomography (PET) is used clinically for diagnosis, staging, treatment planning and monitoring tumor response to therapy of many forms of solid tumors [19]. Functional imaging with FDG-PET is more sensitive to treatment associated early changes in tumor compared to computed tomography (CT) or magnetic resonance imaging (MRI) since biochemical changes occur earlier than tumor shrinkage [20]. In one study, early response by FDG-PET was shown to correlate with improved long-term outcome in patients with soft-tissue sarcomas [21]. As a method to ‘personalize’ therapy, ^{18}F -FDG PET-based prediction of early response to therapy might allow for immediate modification of therapy in cases in which patients are receiving ineffective treatment, thereby reducing the probability of relapse and recurrence. Although ^{18}F -FDG PET has not been widely used for monitoring therapeutic response in RMS, studies have suggested that ^{18}F -FDG PET may be an early predictor of tumor response after RTK inhibitor therapy [22–24]. The Children’s Oncology Group is currently evaluating early response by ^{18}F -FDG PET as a surrogate (biomarker) of response in a pilot study of an IGF-IR antibody for newly diagnosed metastatic RMS (clinicaltrials.gov

identifier, NCT01055314). To complement these clinical studies, we have evaluated the potential of ^{18}F -FDG as a biomarker for therapeutic response to the small molecule IGF1R inhibitor, PPP, in our conditional mouse models of ARMS and spindle cell ERMS/undifferentiated spindle cell sarcoma (UPS) [25,26].

Materials and Methods

Mice

All animal procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Health Science Center at San Antonio (UTHSCSA) and the Oregon Health & Science University (OHSU). The *Myf6Cre*, *Pax3:Fkhr*, *p53* conditional mouse model of ARMS has been described previously [25,27,28]. The *Pax7CreER*, *Ptch1*, *p53* conditional model of spindle ERMS/UPS has also recently been described [26].

PPP synthesis

PPP was synthesized by Southwest Research Institute (SwRI, San Antonio, TX). The structure and chemical purity of PPP was established by SwRI using ^1H NMR, ^{13}C NMR and HPLC/MS analysis. PPP was dissolved in dimethyl sulfoxide (DMSO) and vegetable oil (10:1 v/v) and stored at 4°C.

In vitro assays

Mouse RMS primary culture cells (U33915, ERMS; U48484, ARMS) were plated at 3×10^3 cells per well in a 96-well plate. After 24h, PPP was added to the wells in a range of concentrations by serial dilution. After cells were incubated with PPP for 72 h, cytotoxic effects were assayed using Cell Titer-Glo Luminescent cell viability assay system (Promega, Madison, WI) and SpectraMax M5 luminometer (Molecular Devices, Sunnyvale, CA). For western blotting, the cells were grown overnight in serum-free medium followed by treatment with 50 ng/ml recombinant mouse Igf-1 (Sigma-Aldrich, St. Louis, MI) and picropodophyllin for 10 minutes. The cells were then lysed for protein extraction followed by western blotting.

Western blotting

Cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors. Lysates were then centrifuged at 13,000 rpm for 10 minutes. The supernatant was used for western blotting using anti-phospho-Igf1r antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Igf1r antibody (Cell signaling Technology, Beverly, MA), anti-insulin receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-phospho-insulin receptor antibody (Novus Biologicals, Littleton, CO). Chemiluminescence was detected by using SuperSignal West Pico chemiluminescent substrate and SuperSignal West Dura chemiluminescent substrate (Pierce Biotechnology, Rockford, IL).

In vivo treatment

ARMS (n=6) and ERMS (n=6) mice were treated with 80 mg/kg of PPP daily for at least 4 days by intraperitoneal (i.p.) injections. Mice with smaller tumors were treated and followed for 12 days [ARMS (n=3), ERMS (n=3)]. Control ARMS (n=3) and ERMS (n=2) mice were treated with vehicle alone. The length (l), width (w) and depth (d) of the palpable tumor were measured with vernier calipers. Tumor volume was calculated as $V = (\pi/6)lwd$, assuming tumors to be spheroid.

FDG-PET imaging protocol

Mice were fasted overnight and their body temperature maintained using heating pads for one hour prior to ^{18}F -FDG injection to reduce brown fat uptake. ^{18}F -FDG (200 μCi) was injected intravenously through the tail vein and images acquired 45 minutes post injection. Animals were anesthetized using 1.5% isoflurane (with O_2 balance) and placed in the gantry of the Siemens Focus 220 (Siemens, Munich, Germany) microPET scanner. Static PET images were acquired for 10 minutes and reconstructed using proprietary scanner software (microPET manager 2.4.1.1).

Image analysis

Images were analyzed using ASIPro VM microPET analysis software (Siemens Preclinical Solutions, Knoxville, TN). Volume of interest (VOI) was drawn around the tumor and SUV_{mean} of tumor was calculated before and after therapy. In this study we use the definition, $\text{SUV}_{\text{mean}} = [\text{Activity in tumor } (\mu\text{Ci}) / \text{Tumor weight (g)}] / [\text{Injected activity } (\mu\text{Ci}) / \text{Body weight (g)}]$.

Statistical analysis

All data are presented as mean $\pm$ sd. Pearson's correlation was used to determine correlation between tumor volume and SUV_{mean} . Exact Wilcoxon test was used to determine differences between FDG uptake in tumor and cardiac muscle. Student t-test was used to determine statistical differences in tumor volume and FDG uptake between control and treated groups. ANOVA was used to determine statistical significance of FDG uptake before and after therapy (4d and 12d) for ARMS and ERMS treated group. $P < 0.05$ was considered statistically significant.

Results

In vitro efficacy

The effect of PPP on cell growth of RMS primary cell cultures was examined. After exposing the cell cultures to PPP for 72 h, the relative number of viable cells was determined. The IC_{50} for ARMS and ERMS were 150 nM and 200 nM, respectively (Fig. 1a). To investigate the effect of PPP on activation of Igf1r, mouse ARMS and ERMS primary cell cultures (U48484 and U33915) were treated with PPP along with recombinant mouse Igf-1 for 10 minutes. Western blot analysis of the cell lysates after treatment showed a decrease in Igf1r phosphorylation upon treatment with PPP in ARMS and ERMS (Fig. 1b). These biochemical studies also show that PPP does not interfere with insulin receptor expression nor does PPP prevent phosphorylation of insulin receptor in ARMS cells (Fig. 1c). For ERMS cells, however, a small dose-dependent decrease in insulin receptor phosphorylation was observed with PPP (Fig. 1c) (see Discussion).

FDG uptake in ARMS and ERMS

Since ARMS and ERMS can arise from different cells of origin, we sought to determine whether such tumor subtypes also had distinctly different metabolic (glycolytic) phenotypes. ^{18}F -FDG uptake in ARMS and ERMS tumors is shown in Fig. 2a. The average tumor volumes for ARMS and ERMS at baseline were $0.35 \pm 0.41 \text{ cm}^3$ and $0.62 \pm 0.45 \text{ cm}^3$, respectively. SUV_{mean} for ARMS and ERMS were 1.89 ± 0.91 and 2.56 ± 1.41 , respectively. Accounting for differences in tumor size at diagnosis, FDG uptake was not significantly different between ARMS and ERMS tumors. There was no correlation between tumor volume and FDG uptake for ARMS and ERMS tumors. Fig. 2b and c show representative images of tumor size-matched ARMS and ERMS mice at baseline. To determine if ARMS and ERMS tumors had basal glucose uptake rates different from a

metabolically active muscle, FDG uptake in tumors were compared to that of cardiac muscle. Both ARMS and ERMS tumors had significantly less glucose uptake ($SUV_{mean} - 2.18 \pm 1.11$) than cardiac muscle (5.11 ± 3.83) ($p=0.01$).

^{18}F -FDG PET imaging is a biomarker of therapeutic efficacy

Tumor volumes for control and treated ARMS and ERMS mice measured on the fourth day of therapy are shown in Fig. 3a. Treated ARMS and ERMS mice showed 12% and 25.2% increases, respectively, in tumor volume at 4d post therapy compared to their baseline values. Although tumors of treated ARMS and ERMS mice were increased in size at this time point, the tumor volumes of vehicle-treated ARMS and ERMS control mice were yet higher (62.7% and 76.2%, respectively) ($p<0.05$). FDG-PET imaging studies were also performed at baseline and 4d post therapy for control and treated groups (Fig. 3b). Representative images of treated and control ARMS and ERMS mice are shown in Fig. 3c and d, respectively. A significant decrease in FDG uptake 4d post therapy is seen for tumors treated with PPP compared to their baseline or in comparison to FDG uptake of control mice at the same time point for both ARMS ($p<0.01$) and ERMS ($p<0.05$) groups. FDG uptake decreased by 42.6% and 33.3% for treated ARMS and ERMS mice, respectively, on day 4 post therapy compared to baseline. Image analysis showed no statistical difference in the cardiac SUV_{mean} before (5.11 ± 3.83) and after treatment (6.17 ± 4.33).

FDG uptake at 12d post therapy

Tumor volume and SUV_{mean} change of ARMS ($n=3$) and ERMS ($n=3$) mice treated and followed up to 12 days are shown in Fig. 4. Treated ARMS and ERMS mice showed 59.8% and 133.7% increases, respectively, in tumor volume measured by calipers at 12d compared to baseline values, respectively. SUV_{mean} calculated from the PET images on 12d for ARMS and ERMS mice were increased to the pre-treatment tumor FDG uptake values.

Discussion

Biomarkers such as imaging have the potential to be early, predictive measures of long term response to cancer therapy. If truly reliable, imaging biomarkers can allow therapy to be modified in real time before symptomatic relapses occur. Studies have shown that ^{18}F -FDG PET imaging has the potential to be useful for monitoring therapeutic response in RMS after radiation therapy and/or chemotherapy in children [24,29]. Emerging targeted therapy including RTK inhibitors impede tumor growth and proliferation by inhibiting key signaling pathways such as those involved in growth and metabolism [6,12]. Therefore, functional imaging with ^{18}F -FDG PET may be able to provide information about tumor glucose metabolism which could potentially serve as early response biomarker after RTK inhibition therapy [22,23]. Particularly for muscle cancers, whose tumors take advantage of growth factor signaling [5,30], ^{18}F -FDG PET has the potential to be a powerful imaging biomarker. ^{18}F -FDG PET/CT has been shown to be a valuable predictor of therapy outcome and may further improve survival by adaptive therapy [31]. In the context of this study, RMS has been shown to over express IGF1R, and therefore the IGF1R inhibitor picropodophyllin (PPP, AXL1717) is an attractive therapeutic option for RMS [7,11,17] and a potential paradigm for evaluating ^{18}F -FDG PET as a biomarker of response to RTK inhibitors.

Anti-tumor activity of PPP has been demonstrated in many tumors such as myeloma, melanoma, medulloblastoma, glioblastoma and lung carcinoma [32–35]. Related studies suggest PPP strongly inhibits IGF-1R activity without interfering with the insulin receptor and other related receptors [16,36]. In our studies, effectiveness of PPP as a therapeutic agent against RMS was initially tested using *in vitro* assays. Cell growth assay showed

effective growth inhibition of RMS primary cell cultures at IC₅₀ concentrations of 150–200 nM. Also, treatment with PPP caused a dose-dependent decrease in phospho-Igf1r levels in mouse rhabdomyosarcoma primary cell cultures. Whereas our biochemical data supports the conclusion for ARMS that the decrease in FDG uptake on PET imaging is more likely due to response to PPP therapy than interference with insulin receptor/uptake of glucose by the tumor, for ERMS the possibility remains that some of the decrease in FDG uptake may be attributable to PPP action on not only Igf1r, but also Insulin receptor. Given the lack of PPP effect on cardiac muscle FDG uptake, we speculate that the ERMS-specific decrease in Insulin receptor phosphorylation may be attributable to ERMS-specific Igf1r/Insulin receptor heterodimers or that the fetal IR-A isoform known to bind IGF ligand in reports of breast and colon cancer [37] is expressed in ERMS and susceptible to PPP.

In vivo therapeutic efficacy of PPP was determined by tumor volume measurements as well as by ¹⁸F-FDG uptake in tumors. Tumor volume measurements of ARMS and ERMS tumors treated with PPP showed a relative slowing of growth at 4d post therapy compared to vehicle treated control mice. On the other hand, ¹⁸F-FDG uptake in tumors calculated from the microPET images at 4d post therapy revealed a significant overall decrease in tumor uptake compared to their baseline values and also compared to tumor uptake of control mice at 4d. To confirm that the effect of PPP was exclusively on the tumor, we evaluated the glucose uptake in cardiac muscle before and after treatment and found no significant difference in glucose uptake. This result also indicates that PPP is not interfering with the ability of normal tissue to uptake the FDG tracer and hence the decrease in tumor FDG uptake would have to be due to tumor-specific early therapeutic response. These data support the premise that PET imaging can reveal early response of tumors to IGF1R inhibition therapy. Despite the eventual resistance to PPP, PPP was nevertheless effective at slowing tumor growth as evidenced by the lower relative tumor volumes at 12d in treated mice compared versus control mice. Control mouse tumors were 1.5 – 2 fold larger than pretreatment baseline by 4d; however, PPP treated ARMS or ERMS tumors were only 1.5 – 2 fold larger at 12d. Disappointingly, ¹⁸F-FDG uptake in tumors calculated from the microPET images at 12d post therapy revealed FDG uptake similar to baseline indicating a near complete reversal of treatment effect - an indicator of acquired resistance to treatment. PPP is an interesting compound that has shown significant initial success in stopping metastatic progression of some cases of non-small cell lung cancer in Europe [18], with clinical trials now open in the US (clinicaltrials.gov identifier NCT01062620). The progression of tumor after PPP treatment seen in our study is similar to the result observed in RMS xenograft model after treatment with another IGF1R inhibitor BMS-754807 [38]. Our results do not necessarily indicate that PPP would fail as an agent in rhabdomyosarcoma, only that combination therapy with other receptor tyrosine kinases or chemotherapy may be indicated [14].

While preclinical models have been used to validate FDG uptake as an early predictor of response of hematological xenografts to cytotoxic chemotherapy [39], other key early studies have shown the effectiveness of RTK inhibition *in vivo* and use of ¹⁸F-FDG to monitor therapeutic efficacy with sustained decrease in tumor growth and tumor FDG uptake [40–42]. These studies were conducted in xenograft models of human tumors where the tumor site and size for treatment is controlled. The differences in the results seen in our study to the previous study could be due to animal model used. Use of transgenic mouse model allows for authentic recapitulation of the tumor microenvironment and signaling pathway as seen in human RMS, and therefore the results observed in our study could be encountered in clinical situation. In our study tumor volume continuously increased despite early treatment decrease in FDG-PET uptake at day 4. Clinical studies in GIST and squamous non-small cell lung carcinoma (NSCLC) have noted tumor size to increase although tumors were becoming necrotic as seen by the decrease in the FDG uptake on ¹⁸F-

FDG PET imaging [32,43]. However, our result increases in tumor size was not attributable to necrosis, but instead the result of progression.

FDG PET appears to have a potential advantage in detecting metastatic sites of disease and in staging rhabdomyosarcoma patients [45]. A recent COG report has recommended that FDG-PET should be considered a part of the initial workup of a patient with extremity RMS, whenever possible [46]. On the other hand, molecular determination of early therapeutic response can be important as a personalized cancer therapy tool in allowing identification of tumors with intrinsic or developing drug resistance such that altering therapy can prevent overt relapse. While initial responses to PPP were seen nearly uniformly in all mice treated for our study, all mice also developed resistance to this IGF1R inhibitor – so rapidly that only more frequent FDG PET scanning could have identified relapse before the physical enlargement of tumors. If tumors can grow quickly enough – and evolve Igf1r inhibitor resistance rapidly enough – that molecular imaging has no advantage over anatomical tumor volume assessment (for this disease or this mouse model), then in certain preclinical or clinical instances FDG PET may be much more useful for Phase 0 (microdosing) studies for determining initial molecular target susceptibility than for Phase I or II studies of single agents where rapid cytoreduction is unlikely. Furthermore, FDG-PET imaging for clinical trials may be best used in evaluating combination therapies whereby early decrease in FDG-PET signal has a better chance of becoming a predictor of tumor size decreasing over time because in this context the drug combination's cytotoxicity may successfully abrogate tumor cell evolution and Igf1r inhibitor resistance.

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a

b

Fig. 1. *In vitro* efficacy

(a) Relative cell viability of mouse ARMS and ERMS primary cell cultures as a function of increasing PPP concentration (tumor cultures U48484 and U33915, respectively). IC₅₀ was approximately 150 nM and 200nM, respectively, for ARMS and ERMS models. (b) Western blot showing a dose-dependent reduction in phospho-Igf1r levels in primary tumor cell cultures (U33915 and U48484) upon treatment with PPP. (c) Western blot showing almost no reduction in phospho-insulin receptor levels in primary tumor cell cultures (U33915 and U48484) upon treatment with PPP.

Fig. 2. FDG uptake in ARMS and ERMS tumors

(a) Differences in the FDG uptake as measured by SUV_{mean} as a function of tumor volume for untreated ARMS ($R^2 = 0.045$) and ERMS tumors ($R^2 = 0.351$). Statistically significant differences in slopes for SUV_{mean} as tumor size increased were not found ($p=0.303$). (b) Coronal, sagittal and transverse FDG-PET images of a representative ARMS tumor (volume = 0.235 cc). (c) Coronal, sagittal and transverse FDG-PET images of a representative ERMS tumor (volume = 0.279 cc). [white arrow, tumor; b, bladder; h, heart]

Figure 3. FDG-PET as an imaging biomarker to evaluate early therapeutic response to PPP (a) Tumor growth as measured by vernier calipers and expressed as tumor size relative to tumor size at diagnosis is shown for control and treated ARMS and ERMS mice (* $p < 0.05$, compared to control at 4d post therapy) (b) FDG uptake in terms of SUV_{mean} before and after therapy for control and treated ARMS and ERMS mice (* $p < 0.05$, ** $p < 0.01$ compared to control at 4d post therapy, # $p < 0.05$, ## $p < 0.01$ compared to baseline). Tumor size and SUV_{mean} did not correlate strongly pre- and post-therapy ($R^2 = 0.062$ and 0.001 , respectively). (c, d) Representative images of control and treated ARMS mice (tumor volumes are as labeled on figures). (e, f) Representative images of control and treated ERMS mice, respectively (tumor volumes are also as labeled on figures).

Fig. 4. Longer term response to therapy reflected by FDG-PET scans

(a) Tumor growth trend measured by vernier calipers and expressed as tumor size (at 4d and 12d) relative to tumor size at diagnosis is shown for treated ARMS and ERMS mice (b) FDG uptake in terms of SUV_{mean} at baseline, 4d and 12d post therapy for treated ARMS and ERMS mice. SUV_{mean} values at 12d increased to pre-treatment values for both treated ARMS and ERMS mice. Note that not all mice in Figure 3a or 3b are represented in Figure 4a or 4b, as some mice tumors enlarged to the point of requiring termination before treatment day 12. (c, d) Representative images of treated ARMS and ERMS mice, respectively (tumor volumes are as labeled on figures).