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14. ABSTRACT Patient survival for highly aggressive advanced-stage neuroblastoma remains poor despite a multidisciplinary approach involving aggressive surgery, chemotherapy and adjuvant radiotherapy (RT). The large RT treatment volume, and concerns about the proximity of radiosensitive normal structures, restricts the tumoricidal dose of radiotherapy that can be delivered which limits the effectiveness of adjuvant RT. To address this, we delivered radiotherapy using an entirely novel treatment schedule designed to minimize normal tissue damage. The concept was to deliver 10 pulses of low-dose RT (PRT, 10 x 0.2 Gy) using a 3 minute inter-pulse interval to introduce the RT-induced damage at a level that spares tumor vasculature in order to prevent the development of treatment-induced hypoxia, since this increases tumor resistance to radiation and chemotherapy. Moreover, damage produced at this dose level evades ATM dose-dependent DNA damage detection and repair mechanisms. In vitro clonogenic survival experiments demonstrated that a single dose of PRT was not inferior to a continuously delivered standard 2 Gy dose (SRT). PRT was delivered at 0.25 Gy/min and SRT at 0.69 Gy/min using a Faxitron 160 kVp animal X-irradiator (0.5 mm Cu and Al filters; HVL: 0.77 (mm CU)). Female CB-17/SCID mice were xenotransplanted subcutaneously in the flank at 6-8 weeks of age with either SK-N-SH, SK-N-BE or MC-IXC neuroblastoma cells and the subsequent tumors irradiated with a total dose of 20 Gy given over 10 consecutive days (2 Gy/day) as either PRT or SRT. Tumor response was evaluated by physical measurements three times a week and by imaging using F18-FDG PET/CT 1 day prior to and 2 days post radiation treatment. For SK-N-BE tumors, significant differences in CT volume between PRT and SRT were evident at 5 days (p=0.008) and 21 days (p=0.014) post treatment, and animals reached endpoint criteria at 56 days after PRT compared with 43 days for SRT (p=0.012). Similar differences in response were seen between PRT and SRT for MC-IXC tumors. Studies with SK-N-SH are on-going. Quantitative immunohistochemistry identified differences in vascular density (CD34) and hypoxic markers (HIF1/CAXI) after PRT or SRT. These data indicate that PRT produces greater tumor control than SRT in this model.					
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INTRODUCTION

Neuroblastoma is the most common extra-cranial solid tumor of early childhood characterized by variable behavior, ranging from spontaneous regression to highly aggressive disease. Advanced-stage neuroblastoma is refractory to multi-modality therapy. Resistance is related to tumor microvessel density, therapy-induced damage to tumor vasculature and related effects on angiogenic factors [1-6]. However, paradoxically, vascular damage produces localized areas of hypoxia within the tumor volume, which can transiently protect surviving tumor cells since hypoxia reduces the effectiveness of radiotherapy and restricts the effective delivery of chemotherapeutic agents. The aim of this proposal was to test the **hypothesis that these deleterious vascular effects can be overcome by using a novel radiation delivery approach**. Conventional radiation therapy (RT) schemes involve daily treatments of 1.8–2 Gy/day to exploit reoxygenation, repair, redistribution and repopulation that occur in tumors and normal tissues between the individual daily RT dose fractions. This research proposal investigated a novel delivery scheme of pulsed radiotherapy; in which the same total daily dose of radiation is given as 10 individual pulses with a 3-minute inter-pulse time interval between each individual pulse, rather than as one continuous treatment used in conventional delivery. The rationale for giving pulsed treatments is that radiation-induced DNA lesions induced by pulsed radiotherapy occurs at a rate below the detection threshold of cellular repair processes in tumor cells, and thus DNA damage accumulates, causing cell death by apoptosis at the next mitosis. The hypothesis was investigated initially in subcutaneous tumors and then an orthotopic tumor model. Conventional standard radiation or pulsed radiation was given, and tumor response was monitored by non-invasive microPET/CT imaging. Tumor histology was used to investigate the underlying biological mechanisms.

BODY

The experimental plan has been successful and experiments have been conducted in a timely manner.

SOW FOR SPECIFIC AIM #1:

Specific Aim #1 – Establish and treat subcutaneous model on neuroblastoma (Months 1-7)

Overview: The aim is to develop a subcutaneous neuroblastoma model and treat with pulsed radiotherapy and standard RT to define radiation response and determine the best cell line for the orthotopic model.

Subtask1: Establish tumor implantation for three neuroblastoma cell lines (Months 1-2)

- a. Purchase and acclimatize female CB-17/SCID mice.
- b. Purchase and establish growth of cell lines in vitro.
- c. Establish in vivo tumors and measure tumor growth rate using calipers

Subtask2: Compare radiation schedules for subcutaneous tumors (Months 2-9)

- a. Implant female CB-17/SCID mice with subcutaneous tumors from in vitro cells.
- b. Treat in vivo tumors with conventional fractionated radiotherapy and pulse schedules.
- c. Assess tumor growth rate using calipers.
- d. MicroPET scan animals to assess RT treatment efficacy.
- e. Harvest tumors and process for immunohistochemistry to assess vascular damage.
- f. Analyze data set to determine effectiveness of two RT schedules against vascular injury.

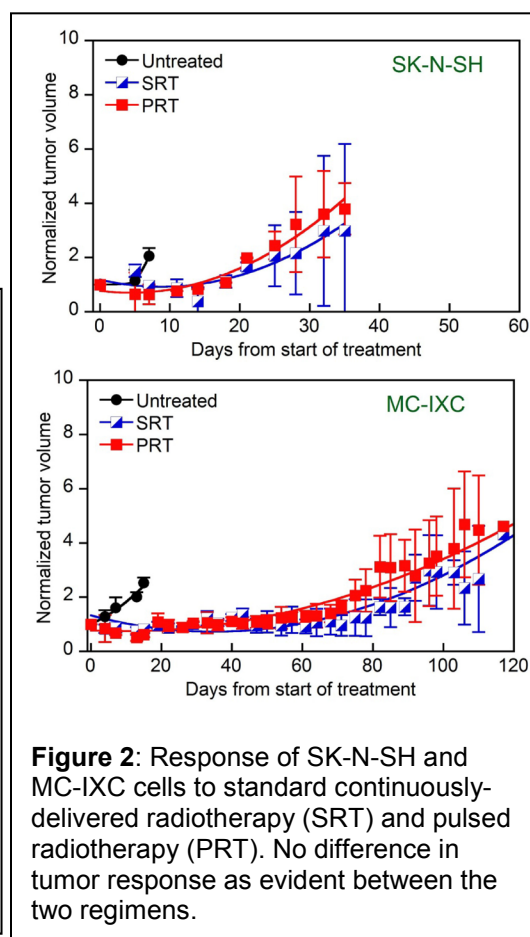
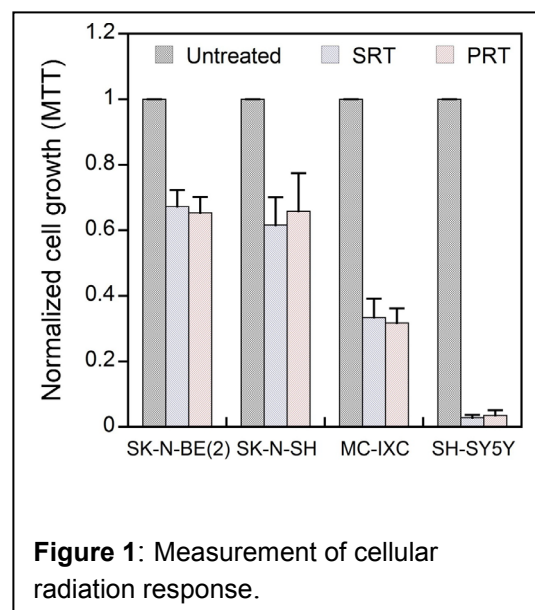
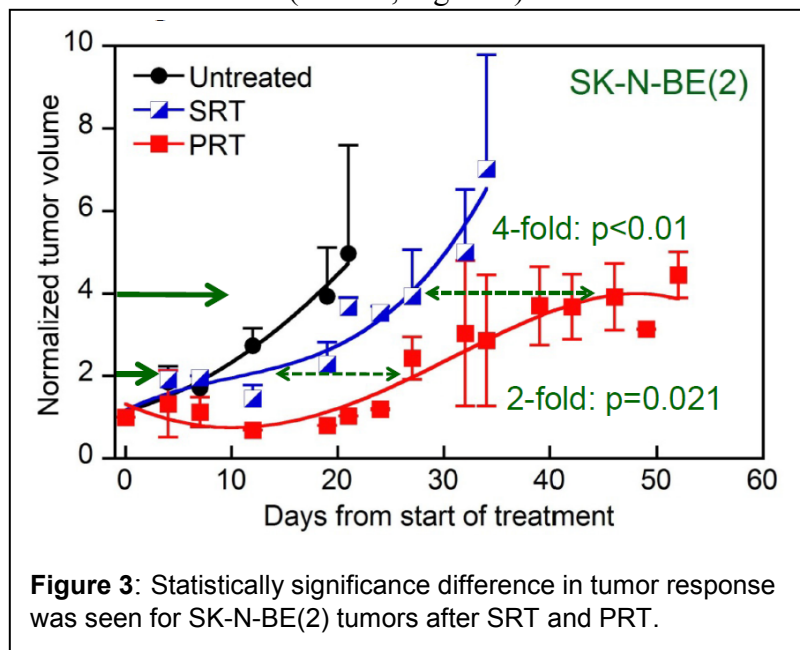
OUTCOMES OF SPECIFIC AIM #1:

Subtask 1 results: Four neuroblastoma cell lines, SK-N-BE(2), SK-N-SH, MC-IXC, and SH-SY5Y were purchased from ATCC. SK-N-BE(2), MC-IXC, and SH-SY5Y were grown in DMEM/F12 with 10% FBS and Pen/Strep. SK-N-SH cells were grown in DMEM with 10% FBS, Pen/Strep, L-glutamine, non-essential amino acids, and sodium pyruvate. All cell lines were grown as monolayer cultures at 37°C in a 5% CO₂ incubator.

SK-N-BE(2), SK-N-SH, MC-IXC exhibited similar responses *in vitro* to 2 Gy radiation given by standard continuously-delivered radiotherapy (SRT) or pulsed radiotherapy (PRT) (Figure 1). Comparing the cell lines, SK-N-BE(2) and SK-N-SH were almost identical in radiation sensitivity, while MC-IXC were more radiosensitive. The SH-SY5Y cells were very radiosensitive and discarded from future experiments.

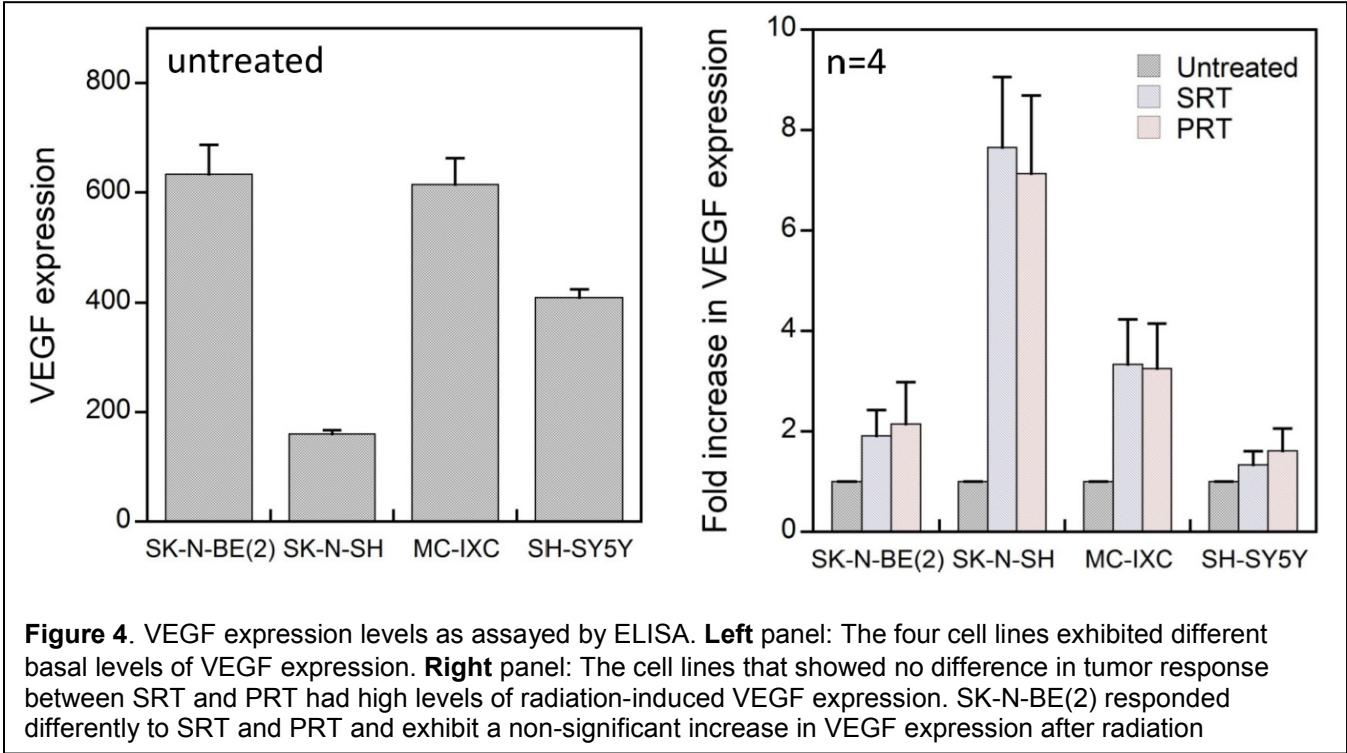
Female CB-17/SCID mice were purchased from Charles River Laboratories and implanted in the right rear flank with either 2x10⁶ SK-N-BE(2), SK-N-SH, or MC-IXC cells in 100uL Matrigel. Each cell line was implanted serially, that is once the experiment with SK-N-BE(2) was complete, SK-N-SH cells were implanted and once these were terminated the MC-IXC cells tumors were established. This methodological approach extended the time needed to generate the data but was undertaken due to the limitations of PET/CT scanning. We are limited to 5 animals per day, with a total of 10 animals per week as each group received a pre-radiation and post radiation scan.

The subcutaneous tumor response data for SK-N-SH and MC-IXC are shown in Figure 2, no difference in tumor response was seen between SRT and PRT. However, a **statistically significant** difference in tumor response was evident between SRT and PRT for SK-N-BE(2) subcutaneous tumors ($P < 0.01$, Figure 3).



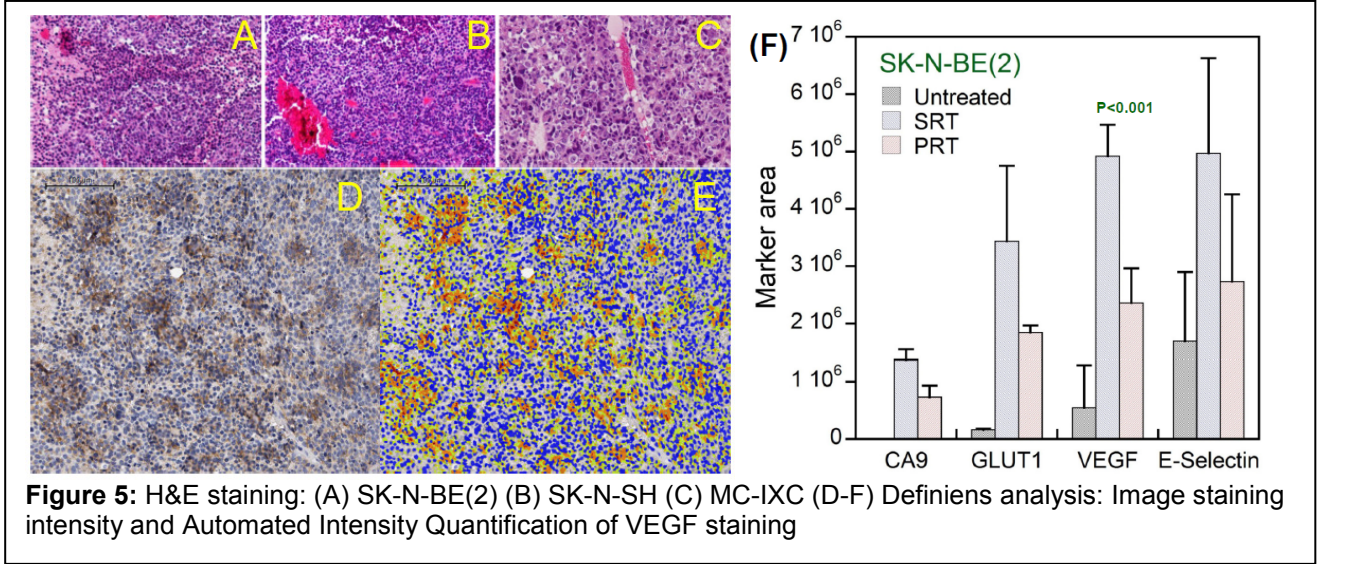
The difference in tumor response between these three cell types (Figure 2 and 3) does not reflect their radiation sensitivity, since SK-N-BE(2) and SK-N-SH cells have a similar radiation sensitivity (Figure 1). To explain this result, the level of baseline and radiation-induced vascular endothelial growth factor

(VEGF) expression was measured in cell culture (Figure 4). These data demonstrated an association between lower levels of radiation-induced VEGF expression and response to PRT; linking vascular biology to PRT effects.



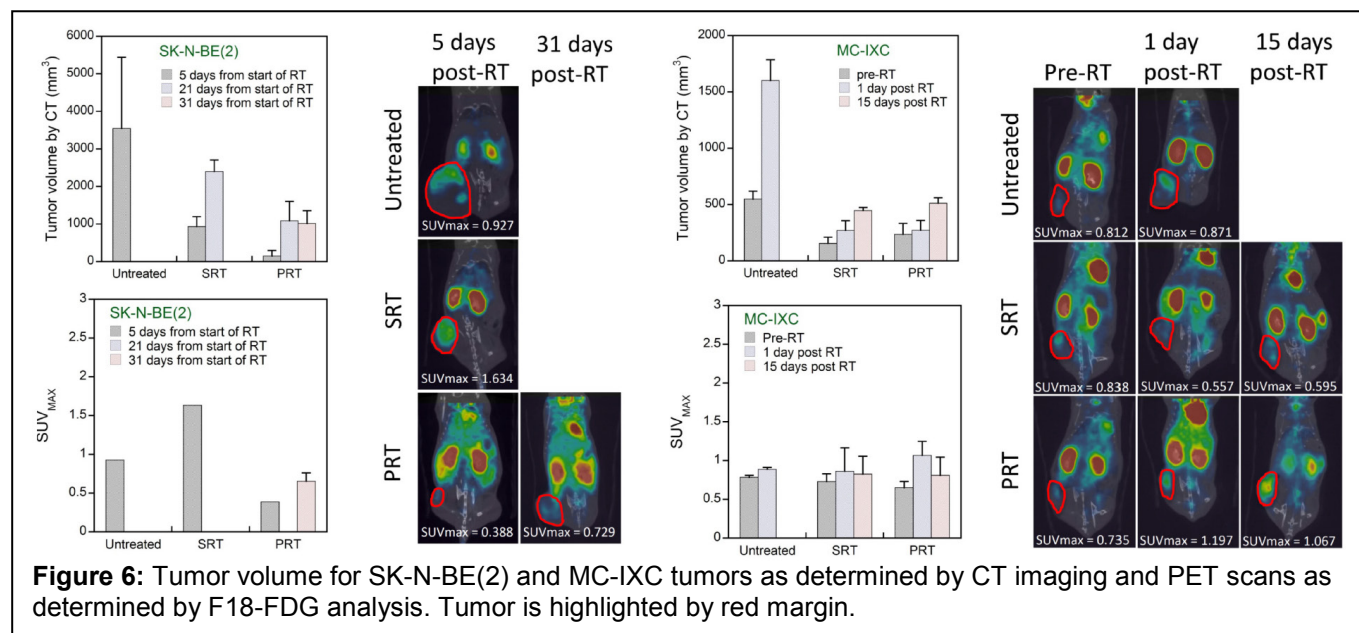
The data presented in Figure 4 demonstrate SK-N-BE(2) cells have low levels of radiation-induced VEGF expression, unlike SK-N-SH and MC-IXC cells that show more significant production of VEGF after radiation, given either as PRT or SRT, and that the extent of VEGF corresponds to tumor response after PRT. However, the fold increase in VEGF expression was not related to baseline levels of VEGF, since no relation was evident between baseline VEGF (Figure 4) and radiation response (Figures 2 & 3).

The difference in VEGF expression seen in the cell culture studies was also evident in tumor histology. Tissue samples were stained with several biological markers of tissue damage and tumor vascularity (Figure 5). The differential response to SRT or PRT was associated with the level of tumor vascularity. Panel A-C show H&E staining for the three tumor types and demonstrate that SK-N-BE(2) tumors are the less vascular. Antibody-specific staining of tissue sections demonstrated a difference in staining



intensity for markers of hypoxia (CA9 and GLUT1), VEGF and E-selectin for SK-N-BE(2) tumor irradiated with PRT and SRT. After PRT, tumors exhibited lower levels of hypoxia (CA9, GLUT1) and consistent with this less VEGF staining. These data support the hypothesis that PRT produces less tumor vascular damage and this maintains tumor oxygenation and therefore maintains tumor radiosensitivity.

PET imaging of SK-N-BE(2) and MC-IXC subcutaneous tumors did not identify differences between PRT and SRT but more complex analysis are on-going. Images were taken pre-radiation exposure and at two times after radiotherapy. These studies were undertaken to test each tumor model for PET avidity.



Aim 1 - Subtask 1 and subtask 2 are complete.

SOW FOR SPECIFIC AIM #2:

Overview: The aim is to develop an orthotopic neuroblastoma model and treat with pulsed radiotherapy and standard RT to define radiation response and determine treatment efficacy.

Subtask1: Establish surgical procedure for orthotopic tumor implantation (Months 7-12)

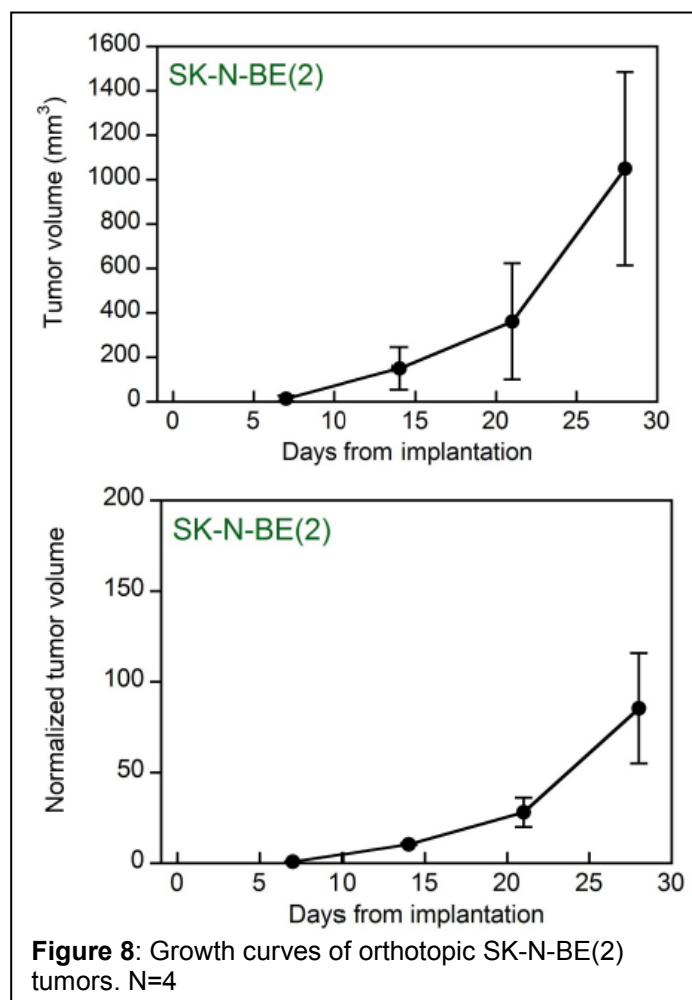
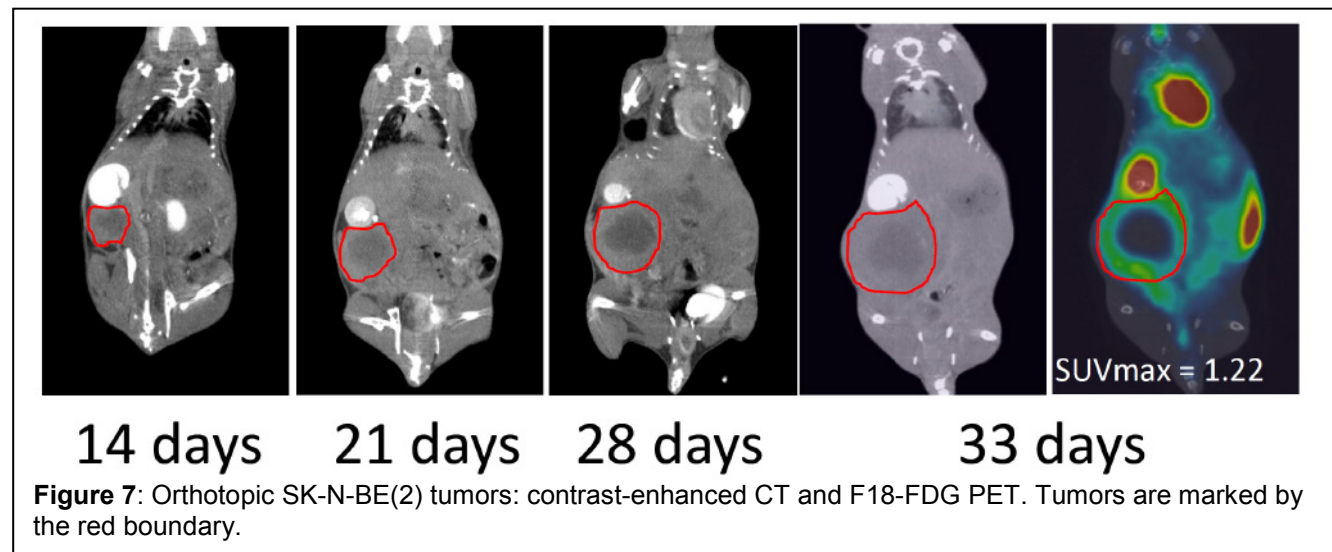
- Purchase and acclimatize female CB-17/SCID mice.
- Growth cell lines in vitro.
- Establish surgical technique for orthotopic tumor implant.
- Measure growth rate using microPET/CT.
- Excise tumor and normal tissues for histology.
- Compare tumor cells, normal cells and vessel density in histopathology sections.

Subtask2: Compare radiation schedules for orthotopic tumors (Months 9-18)

- Implant female CB-17/SCID mice with orthotopic tumors from in vitro cells.
- Treat animals with conventional fractionated radiotherapy and pulse schedules using image-guided irradiator and treated with chemotherapy.
- Assess tumor growth rate using microPET/CT.
- Harvest tumors and surrounding normal tissues and process for immunohistochemistry to assess vascular damage.
- Analyze data sets to determine effectiveness of two RT schedules against vascular injury.

OUTCOMES OF SPECIFIC AIM #2:

Given the success of the SK-N-BE(2) cells as a subcutaneous model, this cell line was used to establish an orthotopic neuroblastoma model (Figure 7). Orthotopically, these tumors grew more quickly than the subcutaneous implants (Figure 8), tumors are evident orthotopically with 14 days and 21 days are required before a similar sized palpable tumor is evident in the subcutaneous model.



These orthotopic SK-N-BE(2) neuroblastoma tumors have been excised and are being processed for histology.

Aim 2 - Subtask 1 is partially complete (a-e), subtask f is on-going; tumors have been harvested and histology is being analyzed.

Aim 2 - Subtask 2 is incomplete and experiments are in progress. The techniques have been established and the experiments are on-going.

KEY RESEARCH ACCOMPLISHMENTS

Demonstrated differences in subcutaneous tumor response after PRT or SRT were not related to in vitro radiation response for three neuroblastoma cell lines.

Established subcutaneous and orthotopic tumor models for neuroblastoma (Figure 3-5; 7)

Demonstrated that the degree of radiation-induced VEGF expression corresponded with tumor response to PRT or SRT (Figures 2-4, Figure 5).

Demonstrated that tumor immunohistochemistry after PRT or SRT suggested differential effects on tumor vasculature and hypoxic response (CA9, GLUT1 and VEGF: Figure 5).

Demonstrated the response of subcutaneous tumors to PRT treatment may be important in tumor metastasis since a role for E-selectin was established (Figure 5).

REPORTABLE OUTCOMES

PRT may provide a new treatment for MYCN amplified neuroblastoma tumors, since SK-N-BE(2) tumors are radiosensitivity to pulsed treatment regimes.

CONCLUSION

Pulsed RT may be a benefit for a subset of neuroblastoma tumors that are MYCN amplified, and show low levels of radiation-induced VEGF expression.

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APPENDICES

Work was presented at the 59th Radiation Research Society Annual Meeting in New Orleans. 9/15/2013 to 9/19/2013 as a research poster. Control/Tracking Number: 13-A-382-RRS

POSTER SESSION PS4-34: Wednesday 18th September 2013

Overcoming mechanisms of tumor radiation resistance: The use of low-dose pulsed radiotherapy.

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Jonathan Kane, BS; and George D. Wilson, PhD,
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Patient survival for highly aggressive advanced-stage neuroblastoma remains poor despite a multidisciplinary approach involving aggressive surgery, chemotherapy and adjuvant radiotherapy (RT). The large RT treatment volume, and concerns about the proximity of radiosensitive normal structures, restricts the tumoricidal dose of radiotherapy that can be delivered which limits the effectiveness of adjuvant RT. To address this, we delivered radiotherapy using an entirely novel treatment schedule designed to minimize normal tissue damage. The concept was to deliver 10 pulses of low-dose RT (PRT, 10 x 0.2 Gy) using a 3 minute inter-pulse interval to introduce the RT-induced damage at a level that spares tumor vasculature in order to prevent the development of treatment-induced hypoxia, since this increases tumor resistance to radiation and chemotherapy. Moreover, damage produced at this dose level evades ATM dose-dependent DNA damage detection and repair mechanisms. In vitro clonogenic survival experiments demonstrated that a single dose of PRT was not inferior to a continuously delivered standard 2 Gy dose (SRT). PRT was delivered at 0.25 Gy/min and SRT at 0.69 Gy/min using a Faxitron 160 kVp animal X-irradiator (0.5 mm Cu and Al filters; HVL: 0.77 (mm CU)). Female CB-17/SCID mice were xenotransplanted subcutaneously in the flank at 6-8 weeks of age with either SK-N-SH, SK-N-BE or MCIXC neuroblastoma cells and the subsequent tumors irradiated with a total dose of 20 Gy given over 10 consecutive days (2 Gy/day) as either PRT or SRT. Tumor response was evaluated by physical measurements three times a week and by imaging using F18-FDG PET/CT 1 day prior to and 2 days post radiation treatment. For SK-N-BE tumors, significant differences in CT volume between PRT and SRT were evident at 5 days ($p=0.008$) and 21 days ($p=0.014$) post treatment, and animals reached endpoint criteria at 56 days after PRT compared with 43 days for SRT ($p=0.012$). Similar differences in response were seen between PRT and SRT for MC-IXC tumors. Studies with SK-N-SH are on-going. Quantitative immunohistochemistry identified differences in vascular density (CD34) and hypoxic markers (HIF1/CAXI) after PRT or SRT. These data indicate that PRT produces greater tumor control than SRT in this model. The work was funded by DOD PRMRP Award W81XWH-12-1-0355.

Overcoming mechanisms of tumor radiation resistance: The use of low-dose pulsed radiotherapy

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Introduction

Neuroblastoma is the most common extra-cranial solid tumor of early childhood. Variable response is seen from spontaneous regression to highly aggressive disease which is resistant to multimodality treatment including conventionally delivered radiotherapy [1]. MYCN is amplified in 25% of tumors and is a genetic marker for treatment failure [2]. Tumor microvessel density [3, 4], angiogenic growth factor expression (e.g. VEGF) and down-regulation of angiogenic factors have been associated with advanced stage disease, along with apoptotic resistance owing to MYCN amplification [5, 6]. Therefore, novel approaches targeting tumor angiogenesis represent promising strategies for overcoming advanced disease. Our previous pre-clinical studies with glioblastoma multiforme (GBM) have demonstrated pulsed radiotherapy (PRT) offers a larger therapeutic benefit than continuously-delivered standard radiotherapy (SRT) [7-9]. In GBM, PRT spared tumor vasculature which maintained tumor oxygenation via a VEGF-based mechanism. Therefore, we hypothesized that PRT may be an effective treatment of neuroblastoma.

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Materials and methods

Neuroblastoma cells: SK-N-BE(2), SK-N-SH, MC-IXC, and SH-SY5Y cells were purchased from ATCC. SK-N-BE(2), MC-IXC and SH-SY5Y were grown in DMEM/F12 with 10% FBS and Pen/Strep. SK-N-SH cells were grown in DMEM with 10% FBS, Pen/Strep, L-glutamine, non-essential amino acids and sodium pyruvate. All cell lines were grown as monolayer cultures at 37°C in a 5% CO₂ incubator. SK-N-BE(2) cells are MYCN amplified, wild type ALK, with moderate levels of dopamine β hydroxylase activity. Conversely, SK-N-SH cells are not MYCN amplified have an ALK mutation (F1174L) and high levels of dopamine β hydroxylase activity. Doubling times: SK-N-BE(2)-35hr; SK-N-SH-75hr; SH-SY5Y-75hr; MC-IXC-30hr.

Animals: The protocol was approved by the William Beaumont Hospital IACUC. Female CB17-SCID mice (Charles River, MA) were housed in conventional micro-isolator cages and given standard rodent chow and water ad libitum. 2x10⁵ cells in 100 µl matrigel were implanted on the right rear flank or orthotopically.

Radiation schedule: Anesthetized animals were irradiated daily with 2-Gy, either by SRT (continuously delivered) or PRT (10 pulses of 0.2 Gy, separated by 3-minute intervals) to a total dose of 20 Gy over 10 consecutive days. RT was delivered using a Faxitron 160 kVp animal X-radiator (0.5 mm Cu and Al filters; HVL: 0.77 (mm Cu)) with a dose rate of 0.69 (Gy/min) (SRT) or 0.25 (Gy/min) (PRT).

Tumor imaging and endpoint: Tumor volumes were determined by microPET/CT imaging and calipers. A FLEX Triumph™ combined small-animal PET/CT system (Gamma Medica-Ideas) was used for contrast-enhanced CT (80 kV/250 µA, 170 µl Omnipaque 350, GE Healthcare) and [18F]-FDG-PET (22.2 MBq ±10% in 200 µl in 30 secs). Tumor volume by caliper measurement was calculated using the standard formula (πab²/6; a is the largest and b is the smallest diameter).

Cell Proliferation/MTT: 2,000-5,000 cells were plated into individual wells depending on cell type for MTT assays. Plates were irradiated with 2 Gy SRT, 10x0.2 Gy PRT or sham treated for five consecutive days.

Statistical analysis: Student t-test assuming unequal variances, with p values less than 0.05 considered biologically significant.

Histology: By Definiens Tissue Studio Software (DTSS). Regions of Interest (ROI) were determined from the entire histology image. A DTSS solution was created by initializing cellular analysis, detecting marker areas, and classifying stain intensities as low, medium or high, and then applied to all ROI. The results are marker area and percent area stained, all divided amongst the low, medium, and high intensity.

Results

Figure 1: Radiation response as determined by MTT. No differences in response were seen between SRT and PRT. γH2AX staining (DNA dsbs) are shown for SK-N-BE(2) and SK-N-SH.

Figure 2: *In vitro* VEGF expression by ELISA. SK-N-BE(2) had 3-fold higher levels of VEGF than SK-N-SH cells (p<0.001). SK-N-BE(2) and MC-IXC were comparable.

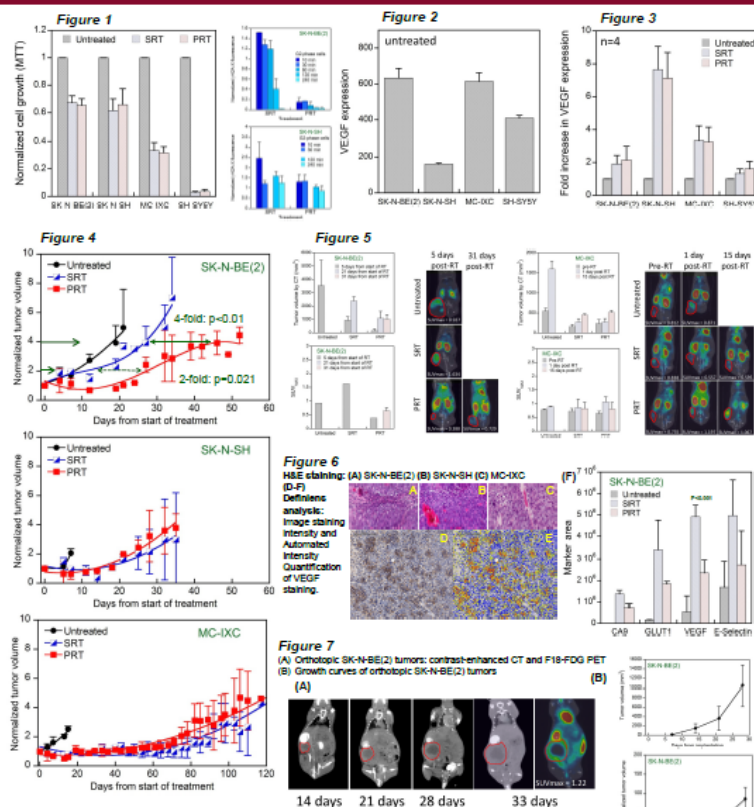
Figure 3: Fold increase in VEGF expression after 5 consecutive days of SRT or PRT. Significant increases were seen for SK-N-SH (p<0.001) and MC-IXC (p<0.05) cells.

Figure 4: Tumor regrowth delay as assayed by caliper measurements. The extent of growth delay corresponded with *in vitro* radiation response. SK-N-BE(2) tumors responded differently to PRT and SRT. For all three tumor types, the difference in tumor response to PRT and SRT corresponded with radiation-induced *in vitro* expression of VEGF.

Figure 5: Tumor response assayed by CT and PET. Responses after PRT or SRT were comparable.

Figure 6: Histological comparison of SK-N-BE(2) tumors. Differences in staining were evident between SRT and PRT treated tumors for E-Selectin, VEGF, CA9 and GLUT1.

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Conclusions

1. Differences in tumor response after PRT or SRT were not related to *in vitro* radiation response.
2. The degree of radiation-induced VEGF expression corresponded with tumor response to PRT or SRT (Figures 2-4, Figure 6).
3. Tumor immunohistochemistry after PRT or SRT suggested differential effects on tumor vasculature and hypoxic response (CA9, GLUT1 and VEGF: Figure 6). PRT response may be important in tumor metastasis (E-selectin; Figure 6).
4. These subcutaneous neuroblastoma studies are preliminary, and additional data are needed to confirm the therapeutic benefit of PRT. However, PRT may provide a new treatment for MYCN amplified tumors. An orthotopic tumor model is being used to address this question (Figure 7).