Cancer Chemotherapy and Pharmacology (2019) 83:809–815
https://doi.org/10.1007/s00280-019-03782-w

ORIGINAL ARTICLE

Regorafenib regressed a doxorubicin-resistant Ewing's sarcoma in a patient-derived orthotopic xenograft (PDOX) nude mouse model

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Received: 23 December 2018 / Accepted: 17 January 2019 / Published online: 13 February 2019

Abstract

Purpose Ewing's sarcoma (ES) is a rare and recalcitrant disease which is in need of a development of a novel effective therapy. The aim of this study was to investigate the efficacy of regorafenib on an ES tumor in a patient-derived orthotopic xenograft (PDOX) model.

Methods The ES PDOX models were established orthotopically in the right chest wall of nude mice to match the site of the tumor in the donor patient. The ES PDOX models were randomized into three groups (G) when the tumor volume reached 75 mm^3: G1: untreated control; G2: doxorubicin (DOX) (i.p., 3 mg/kg, weekly, 2 weeks); G3: regorafenib (REG) (p.o., 30 mg/kg, daily, 2 weeks). Tumor volume and body weight were measured twice a week. All mice were sacrificed on day 15.

Results DOX was ineffective compared to the control group ($P = 0.229$). REG regressed the tumor size ($P < 0.001$ and $P < 0.001$, relative to control and DOX, respectively).

Conclusions Our findings suggest that REG has clinical potential for ES patients whose tumors respond to REG in a PDOX model.

Keywords Ewing's sarcoma · Patient-derived orthotopic xenograft · PDOX · Nude mouse · Regorafenib

Introduction

Ewing's sarcoma (ES) is an aggressive, poorly differentiated tumor derived from bone and/or soft tissue. ES is the second most common primary bone tumor, which occurs mainly in children and adolescents [1]. The overall survival (OS) of ES patients with localized disease has improved up to approximately 70% with the development of multidisciplinary treatment [2]. However, the OS of ES patients with metastases and/or recurrence is still very low [3]. Thus, more effective therapy is needed to improve the outcome of ES patients.

Regorafenib (REG) is a multi-tyrosine-kinase inhibitor that binds to the following receptors: platelet-derived growth factor receptor (PDGFRB), vascular endothelial growth factor receptors (VEGFR), angiopoietin-1 receptor, fibroblast growth factor receptor 1 (FGFR1) and mutant kinases such as c-KIT [4]. Initially, REG was approved for patients with advanced colorectal cancer based on previous clinical trials which showed an OS benefit [5–7]. Subsequently, REG was approved for patients with gastrointestinal stromal tumors

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after progression and as standard treatment for hepatocellular carcinoma as a second-line therapy [8–11]. A randomized double-blind, placebo-controlled, Phase II of REG showed improved progression-free survival (PFS) in non-adipocytic soft-tissue sarcomas [7]. REG improved the quality-adjusted survival for patients with DOX-pretreated soft-tissue sarcoma (STS) [12]. REG showed clinical antitumor efficacy in metastatic osteosarcoma [13], REG was effective in metastatic biliary tract adenocarcinoma [14], glioblastoma [15], lung squamous-cell carcinoma [16], multiple myeloma [17], and adenoid-cystic carcinomas [18].

Previously, we have developed many types of PDOX nude mouse models using the surgical orthotopic implantation (SOI) technique [19]. The PDOX models can be metastatic and mimic the original patient's tumor, contrary to the classical subcutaneous model. We previously developed the chest-wall ES PDOX model with a rare genetic alteration which is FUS-ERG fusion and cyclin-dependent kinase 2A/B deletion [20]. We identified clinically effective therapy for bone marrow and organ metastases using the ES PDOX model [21, 22]. Bone marrow and organ metastases responded the regimens identified in the PDOX models, and the OS of the patient was prolonged. The PDOX models can also be utilized as an alternative to clinical trials which can save time and money, although further large-scale studies are warranted to confirm the concordance of clinical and PDOX drug response.

The aim of this study was to investigate the efficacy of REG on a chest wall ES using the ES PDOX model.

Materials and methods

Animals

We performed this study using 4–6 weeks old athymic nu/nu female nude mice (AntiCancer, Inc., San Diego, CA). All animals were maintained under the high efficiency particulate arrestance (HEPA)-filtered racks under standard conditions of 12-h light/dark cycles. All experiments were performed with an AntiCancer Institutional Animal Care and Use Committee (IACUC)-protocol specifically approved for this study and in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1. All animal procedures were conducted as we described before [20].

Establishment of the ES PDOX model

This ES tumor originally occurred in the right chest wall of a patient. Tumor specimens were previously brought to AntiCancer, Inc. from the Department of Surgery, University of California, Los Angeles (UCLA) when the patient received curative-intent surgery. The patient signed an informal consent form. This study was approved by the Institutional Review Board of UCLA. Tumor specimens were initially implanted into nude mice subcutaneously. The subcutaneous tumors, grown in nude mice, were harvested and cut into 2 mm-diameter fragments. The fragments were implanted into the right chest wall of nude mice by SOI technique, as described previously [21].

Treatment protocol for the ES PDOX models

The treatment protocol is illustrated in Fig. 1. The ES PDOX models were randomly divided into 3 groups (G) when the tumor volume reached 80 mm^3; G1: untreated control; G2: DOX (i.p., 3 mg/kg, weekly, 2 weeks); G3: REG (REG: p.o., 30 mg/kg, daily, 2 weeks). All regimens were determined by considering previous reports [20, 23]. Tumor size and body weight were measured twice a week. Tumor volume was calculated by the following formula: tumor volume (mm^3) = length (mm) × width (mm) × width (mm) × 1/2. All mice were sacrificed on day 15.

Histological findings

Tumors were fixed in 10% formalin and embedded in paraffin. Sections were cut at 5 µm and deparaffinized in xylene and rehydrated in an ethanol series. Hematoxylin and eosin (H&E)-staining was performed by our standard protocol [24]. Histopathology was observed using a BHS system microscope (Olympus, Japan). Pathological response to treatment was evaluated in accordance with a previous report [25].

Statistical analysis

All data were analyzed statistically by free statistical software EZR (Saitama Medical Center, Jichi Medical University) [26]. This free software is a graphical-user interface for R (The R Foundation for Statistical Computing, version 3. 4. 1.). EZR is a modified version of R commander (version 2. 4-0) which includes statistical functions for biostatistics. All statistical analyses were performed as we previously reported [27]. Probability values less than 0.05 were considered as statistical significance.

Results

Quantitative treatment efficacy

The treatment response transition of the tumor volume ratio is shown in Fig. 2a. DOX did not have significant

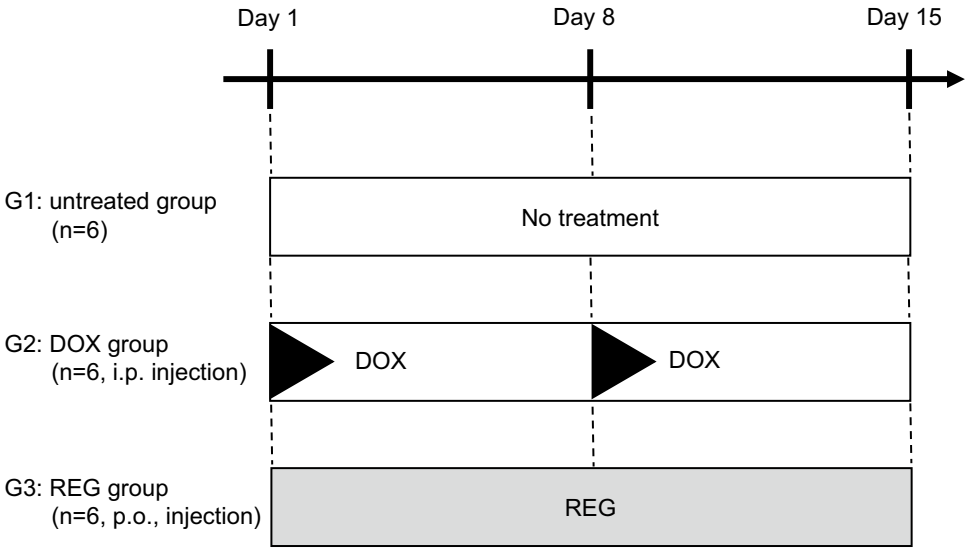

Fig. 1 Treatment protocol. G1: untreated group; G2: treated with DOX (i.p., 3 mg/kg, weekly, 2 weeks); G3: treated with REG (p.o., 30 mg/kg, daily, 2 weeks). Each group consisted of six mice. Tumor volume and body weight were measured twice a week. All mice were sacrificed on day 15

efficacy compared to the control group ($P = 0.229$), matching the previous results in the patients [21, 22]. REG regressed tumor growth relative to the control and the DOX group ($P < 0.001$ and $P < 0.001$, respectively). The final tumor volume ratios (day 15/day 0) were as follows: untreated control (G1) (2.55 ± 0.42); DOX (G2) (2.20 ± 0.34); REG (G3) (0.60 ± 0.12). Representative

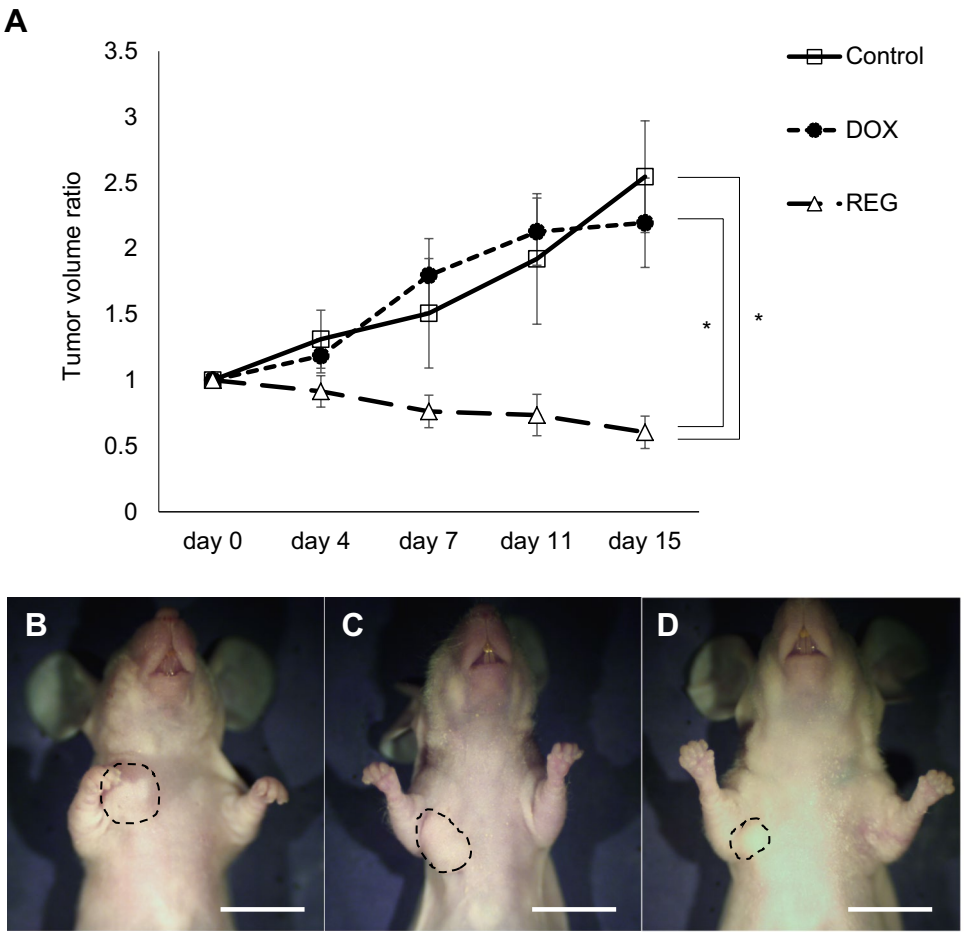

Fig. 2 Treatment response of the tumor volume ratio. Line graphs illustrate the tumor volume ratio (tumor volume at each day/day 0). REG significantly regressed the tumor compared to the control group and the DOX group ($P < 0.001$ and $P < 0.001$, respectively, $N = 6$). *$P < 0.001$. Error bars: ± 1 SD. B–D. Representative images of the ES PDOX mice from each group on day 15. The area circled by black broken line is the tumor treated by each drug. Scale bar is 10 mm

images of the ES PDOX models from each group on day 15 are shown in Fig. 2b–d.

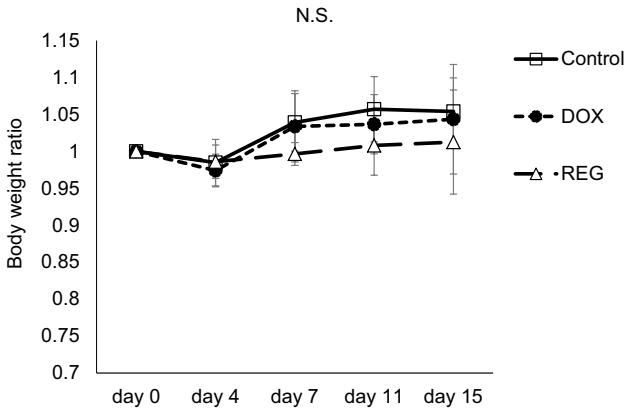

Fig. 3 Body weight ratio. Line graphs show the body weight ratio in each group on each day/day 0. Significant body weight loss was not observed in any group on any day ($N=6$). Error bars: ±1 SD

Effect of treatment on body weight

The body weight ratio is shown in Fig. 3. No significant differences were observed among groups on any day. Significant body weight loss was not detected through the treatment.

Effect of treatment on tumor histology

Figure 4a–c shows representative pathological findings. The tumor in the untreated control group is composed of uniform small round cells and cleared out cytoplasm mimicking the original patient's tumor, as we previously reported (Fig. 4a) [20]. DOX showed a slight reduction of cellularity (Fig. 4b). REG showed significant reduction of cellularity and extensive necrosis (Fig. 4c). Relative cell density in the tumor was extensively reduced in REG treatment compared to the control and DOX treatment groups (Fig. 5a). The extent of necrosis is quantified in Fig. 5b. There was no significant difference between the control group and the DOX group. REG caused significant broad necrosis compared to control group and the DOX group ($P<0.001$ and $P<0.001$, respectively). The

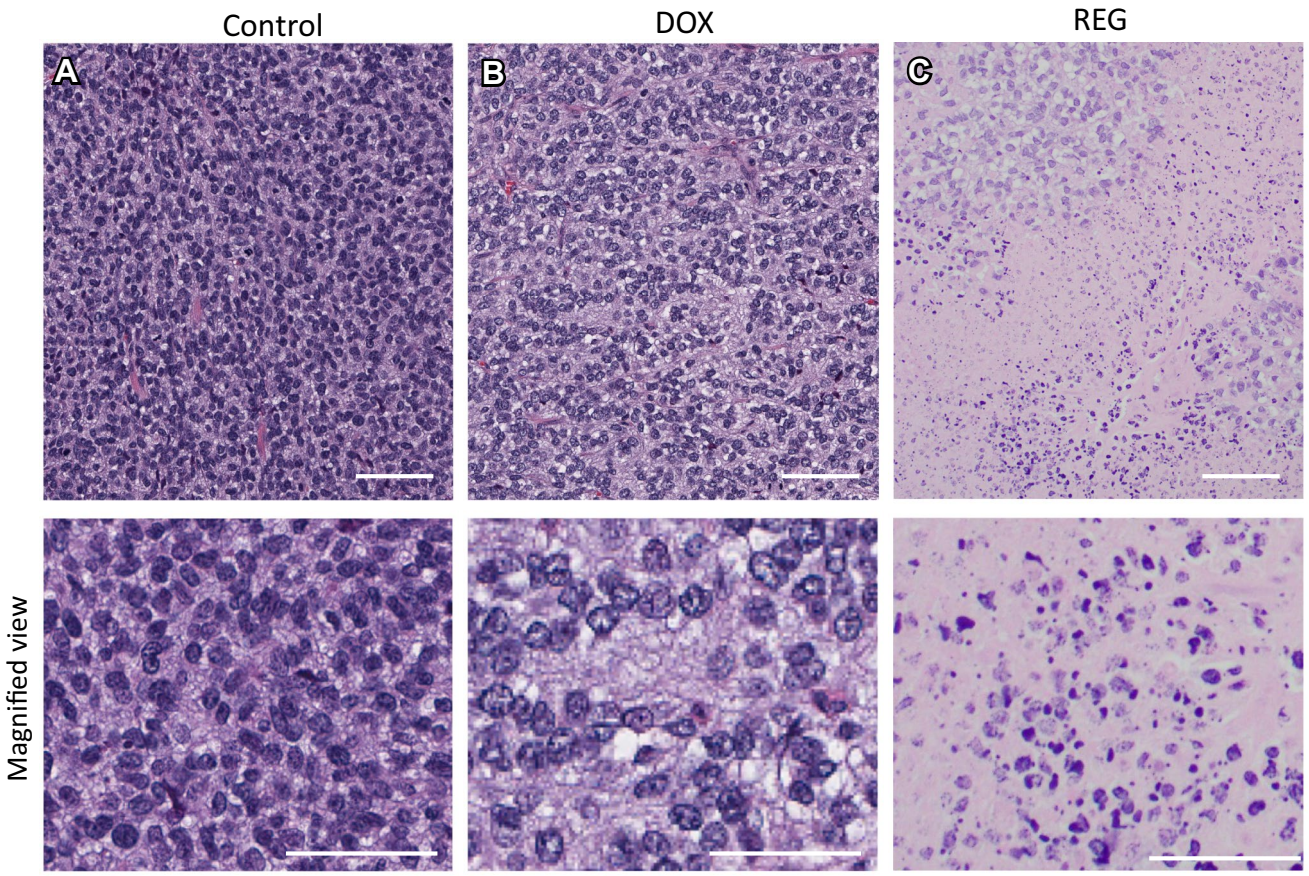

Fig. 4 Pathological findings. **a–c** H & E staining of the ES PDOX tumor on day 15 ($N=6$). Scale bar: 100 µm

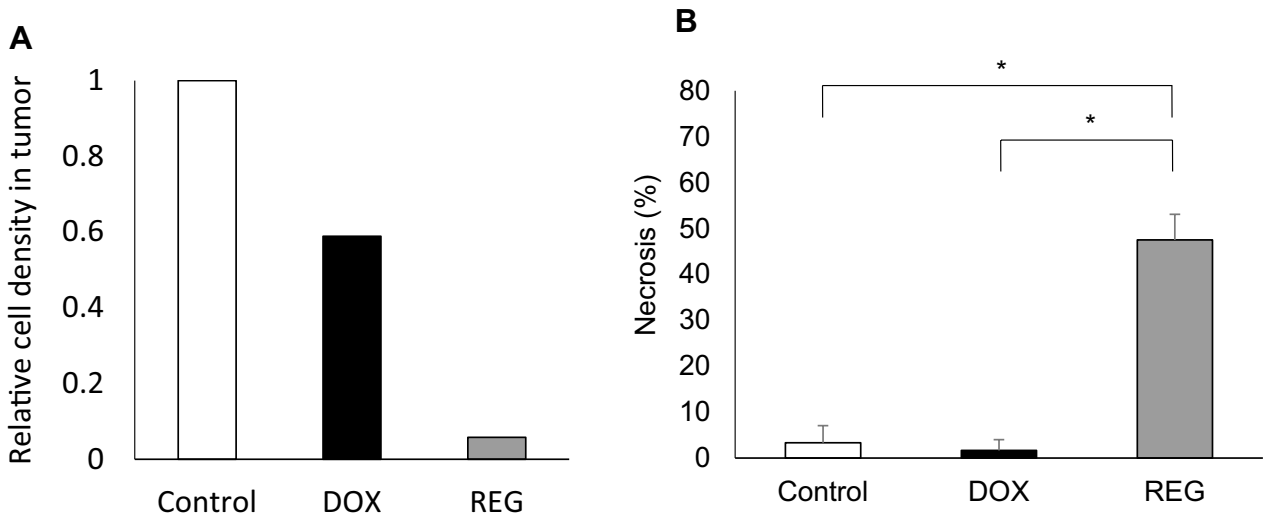

Fig. 5 Effect of treatment on tumor cellularity and necrosis. **a** Relative cell density in tumor. Extensive reduction of tumor cellularity occurred with REG treatment compared to the control and DOX treatment groups. **b** There was no significant difference between the control group and the DOX group. REG caused significant broad necrosis compared to the control group and the DOX group ($P<0.001$ and $P<0.001$, respectively, $N=6$). *$P<0.001$. Error bars: ± 1 SD

percent of the necrotic area was as follows: untreated control (G1) (3.33 ± 3.73); DOX (G2) (1.67 ± 2.36); REG (G3) (47.5 ± 5.60).

Discussion

We have developed PDOX mouse tumor models for discovery of transformative therapy for recalcitrant cancer including ES [19–22, 28–30]. ES is generally defined by a genetic translocation related to the EWSR1 gene [31]. There are several subtypes with a fusion between EWSR1 gene and the regulatory ETS genes. However, EWSR1-FLI1 fusion accounts for approximately 90% of ES patients, which occurs due to a translocation involving chromosomes 11 and 22 [32]. ES patients with a EWSR1-FLI1 fusion have a heterogeneous response to therapy [33, 34].

The patient donor in the present study had a rare FUS-ERG fusion combined with a CDKN2A deletion, for which there is no standard effective therapy. In our previous studies, we observed that this patient's PDOX model was sensitive to palbociclib and linsitinib [20]. The patient responded extensively when treatment was based on the PDOX results. Subsequently, we found the patient's PDOX model was sensitive to irinotecan combined with temozolomide and eribulin [21, 22] and the patient again responded to treatment based on these PDOX results. The patient donor of the ES PDOX model was resistant to DOX, consistent with present and previous PDOX studies of this tumor.

Currently REG is being tested for ES patients in a Phase II clinical trial SARC024 (NCT02048371) which already achieved a primary endpoint of increased PFS [35]. Therefore, REG is expected to be a promising drug for ES patients. However, the efficacy of REG varies in each patient due to heterogeneity of this type of tumor and need to be tested first directly in PDOX models of each patient. The present and previous PDOX studies described above indicate the potential of the PDOX model to identify both ineffective and effective drugs for each patient.

In conclusion, REG regressed the chest wall ES tumor in a PDOX model. Our findings suggest that REG have a potential to be novel effective therapy for ES patients who test positively in a PDOX model.

Acknowledgements This paper is dedicated to the memory of A. R. Moossa, M.D., and Sun Lee, M.D.

Funding This work was supported in part by a Yokohama City University research grant "KAMOME Project" which had no role in the research or writing the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. K.M., T.K., K.K., T.H., H.O., Z.Z., S.W., S.R., T.M., Y.H., and RMH are or were unsalaried associates of AntiCancer, Inc. AntiCancer, Inc. uses PDOX models for contract research.

Ethical approval All experiments were performed with an AntiCancer Institutional Animal Care and Use Committee (IACUC)-protocol specifically approved for this study and in accordance with the principals and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1.

Informed consent The patient signed an informed consent form. This study was approved by the Institutional Review Board of UCLA.

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