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The Role of Temozolomide-Irinotecan in Doxorubicin-Resistant Cell Lines of Recurrent Ewing's Sarcoma, in A Patient Derived Orthotopic Xenograft (PDOX) with EWS-ERG Fusion and CDKN2A Loss: What Impact the Combination has in Real Life Practice?

ABSTRACT

Introduction: A patient-derived orthotopic xenograft (PDOX) nude-mouse model and irrespective of genetic profile can help guiding our treatment in progressive and resistant Ewing Sarcoma (ES) patients progressed after Doxorubicin containing regimens.

Patients and methods: In our study, the PDOX mice established model with ES were divided into two arms once tumor mass exceeded 60 mm3: (1) untreated control (2) irinotecan plus temozolomide (irinotecan: intra-peritoneal injection; temozolomide: orally, daily for 14 days). A similar study was carried out on patients from whom the graft was taken. We have collected data from 35 patients of ES: diagnosed, treated and progressed after the first line chemotherapy. Those patients received oral temozolomide 100mg/m² on days 1 through 5 plus IV irinotecan 10-20 mg/m²/day on days 1 through 5 and day 8 through 12 (repeated every 3-4 weeks).

Results: Irinotecan plus temozolomide was found to be an effective combination when compared to the untreated control (p = 0.022) in mice model where tumor shrinkage was observed on day 10. To assure these findings, we have conducted in part, a study employing both Temozolomide and irinotecan in patients resistant to Doxorubicin containing regimens and demonstrating EWS-ERG fusion and CDKN2A loss. However, the same protocol was used to the same patient where the graft was taken showing not complete concordance between the lab and real practice.

Conclusion: PDOX is a good model to test a library of drugs on. It shows to be a promising method in several studies and in ours as well. Studies demonstrated that the combination of temozolomide/irrinotecan is effective in ES patients in progression after Doxorubicin resistance.

KEYWORDS: Ewing Sarcoma; Xenograft; Progression; ERG; EWS; CDKN2A


INTRODUCTION

Ewing's sarcoma (ES) is the second most frequent bone tumor after osteosarcoma with a young age predisposition [1]. The origin of this tumor was unclear until recently, when electron microscopec and immunohistochemistry suggested that it is of neurogenic origin [2,3]. The overall survival was improved using a combination chemotherapy protocols and the multimodality treatment using both surgery and radiotherapy [4] however; response is still very poor in resistant cases [5]. Furthermore, new genes implicated in ES pathogenesis make treatment decision more and more challenging [6]. Most patients are treated with induction (Doxorubicin, Cyclophosphamide, and Vincristine) followed by IE (Ifosfamide, Etoposide) [7] however; most patients relapse, metastasize or become resistant. The most common type of fusion gene is the EWS-FLI1 fusion caused by the t(11;22) which is found in 85% of ES [8,9]. 10% of cases express a fusion of EWS-ERG as a result of t(21;22)(q24;q12) [10]. Another rare fusion is EWS-ETV1 which is the result of t(7;22)(q22;q12) [11].

Doxorubicin belongs to the class of anthracyclines and acts by topoisomerase II poisoning, creation of double-strand DNA breaks (DSBs), and impairment of DNA repair and supercoiling, leading to changes in epigenetic processes. Mechanisms involved in resistance to doxorubicin include drug efflux transporters, alterations in the ability of doxorubicin to form DSBs, and alterations in downstream apoptosis signaling triggered by DNA damage [12]. We have established a (PDOX) model of several cases of ES with both a EWS-ERG fusion [13] and CDKN2A loss according to every patient's underlying genetic aberration [14]. In 2004, a study conducted by Wagner LM, et al. firstly mentioned the maximum tolerated dose (MTD) of oral temozolomide combined with protracted irinotecan in solid tumors refractory to other chemo-lines [15]. In 2007, the same researcher found that TMZ-IRI combination is effective and well tolerated in patients with ES in relapse [16]. The Memorial Sloan-Kettering cancer center experience demonstrated a good tolerability of the combination as well as a good response in both recurrent and progressive ES [17].

MATERIALS AND METHODS

The study was conducted at Al Bairouni University Cancer Center, HÔpitalmilitaire Principal d'Instruction de Tunis and CH de Chicas in France. Data was collected from 35 patients of ES diagnosed, treated, and progressed after the first line chemotherapy. All patients were resistant to Doxorubicin and having EWS-ERG fusion, CDKN2A loss and EWS-ETV1. For this purpose, a new biopsy was taken from our patients and they were subjected to cell culture and constitution of the cell line of interest as illustrated in figure 1.
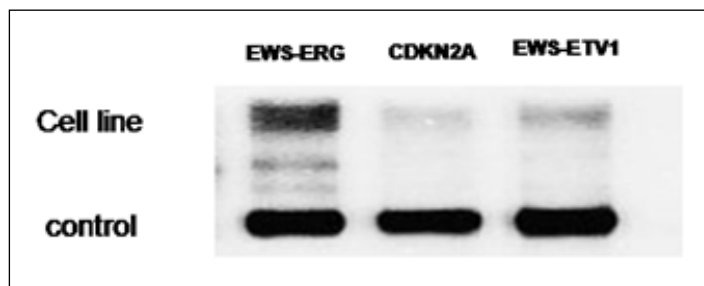
Figure 1: Southern blotting performed on cell culture from a normal control and a progressed case (superior raw) which shows low expression of both CDKN2A and EWS-ETV1.

Xenograft fresh tissue from progressed patients was taken and inserted under the abdominal skin of a nude mice and tumor growth was observed over 15 days as illustrated in figure 2.

Day 3   day 9   day 15
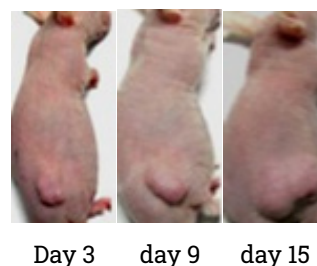
Figure 2: Xenograft insertion and observation over 15 days showing an increase in tumor volume to reach a plateau on day 15.

A nude mouse, were housed in a barrier facility on a highly filtered rack respecting the standard criteria used to grow up and maintain mice in these conditions [10]. A special approval was taken from our local committee supervising working on animal models. Anesthesia was used before every surgical procedure aiming at implanting the xenograft in the abdominal wall. The animals were humanely sacrificed through inhalation of CO_2 when they reached the following end-points: (tumor diameter reached 20 mm), prostration, obvious weight loss, difficulty breathing and rapid drop in body temperature. Both caliper methods along with ultrasound were used in order to calculate tumor volume. Calculations were obtained using the formula $V = (W(2) \times L)/2$ for caliper measurements and the

formula V = (4/3) × π × (L/2) × (L/2) × (D/2) for ultrasonography measurements, where V is tumor volume, W is tumor width, L is tumor length and D is tumor depth.

The PDOX mice were divided into two arms once tumor volume exceeded 50 mm³. G1: untreated control; G2: irinotecan (IRT) plus temozolomide (TEM) (IRT: i.p., 4 mg/kg, daily intraperitoneal infusion for 14 days, TEM: p.o., 25 mg/kg, daily for 14 days as well).

Results and Discussion

Compared to the untreated group, the combination chemotherapy demonstrated a remarkable decrease in tumor size on day 10 (p < 0.001). Tumor volume ratios on day 10 compared to day 1 were as follows: untreated control group (G1) combination of IRT with TEM (G2) (0.38 ± 0.14) as illustrated in figure 3. The comparison showed relative tumor volume shrinkage with the IRT-TEM combination group indicating tumor regression, which is an important indicator of IRT-TEM efficacy in clinical application [11].
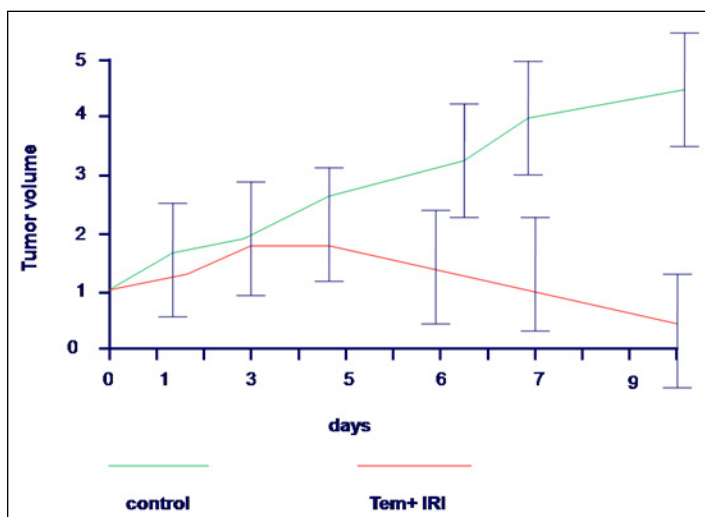
Figure 3: Tumor volume change in control group in green vs treatment group in red. Temozolomide (Tem), Irinotecan (IRI). Red line shows a decrease in tumor volume in response to the combination chemotherapy over 10 days.

PDOX models showed histological similarity compared with the primary [10]. A biopsy was taken from the primary tumor before and after treatment and figure 4 illustrates the difference between the two samples. Necrosis rate and fibrosis is observed in (B) compared with no necrosis in (A) which reflects good response to treatment.
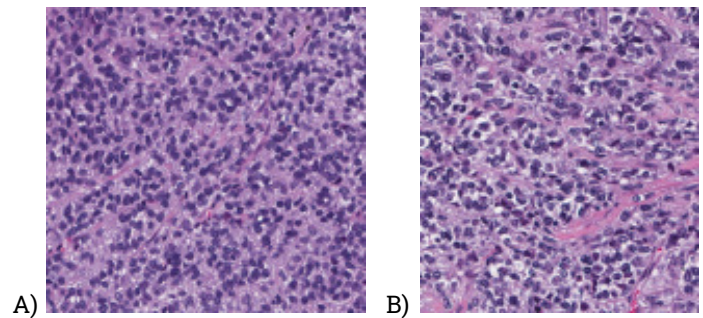
Figure 4: Shows a comparison between control groups on the left (A) vs treated group with slight necrosis on the right (B)

Previous Establishment of the ES PDOX Model

Patients with relapsed ES in the left chest wall received neoadjuvant chemotherapy using combination induction chemotherapy. Those patients were subjected to a wide surgery performed in the Department of Surgery at Al Bairouni University Cancer Center, Damascus, Syria. A tiny tumor tissue used to establish a PDOX model in the abdominal wall of a nude mouse. Informed consent obtained from all patients included in the study from the very beginning. The ES PDOX established after being implanted in the abdominal wall of nude mice [10].

The PODX model can help both researchers and oncologist to better design new combination chemotherapies for ES patients in relapse or even test new treatment strategies. According to this finding, we have decided to conduct a study using the same combination on a group of patients diagnosed with relapsed ES and to find the degree of concordance between the laboratory and the clinical response. All patients have signed an informed consent before enrollment in the study. The study was also approved by the local and national committee of ethics covering both the animal model and the human cohort.

Our Study Design in Real Life Practice

The study was carried out at Al Bairouni University Cancer Center and Centre Hospitalierde chicas, France between September 2012 and February 2016. We have recruited 35 patients diagnosed with Ewing sarcoma treated with Doxorubicine containing regimens developing resistant or progressive disease after two lines of chemotherapy. The age of patients was between 14 and 32 years (18 in median). 9 females and 26 males. Ten of them received one line chemotherapy before inclusion while the remaining 25 received two lines. All patients presented with performance status (PS) of 0, 1, 2

all of them demonstrated normal renal and hepatic functions. Table 1 shows patients' characteristics. Every patient included in the study signed an informed consent. Statistical analysis and Kaplan-Meier plot performed by SPSS program.

| Age (Median)	14-32 (18)
Sex	
Females	9 (25.71%)
males	26 (74.28%)
Lines received	
1 line	10 (28.57%)
2 lines	25 (71.4%)
Primary site	
Ribs	17 (48%)
Humerus	2 (5.7%)
Femur	7 (20%)
Tibia	2 (5.7%)
pelvis	7 (20%)
Metastatic site	
Bone	5 (14.2%)
Liver	5 (14.2%)
Lung	12 (34.2%)
skull	13 (37.1%)
Bone marrow involvement	8 (22.8)

Table 1: Patients' characteristics.

Treatment Protocol

The ES PDOX was established for all patients recruited in the study and as illustrated at the beginning of this article. The treatment protocol showed response in comparison with the control in the 35 patients' specimens implanted in the abdominal wall of the nude mice. Patients received oral temozolomide 100mg/m² on days 1 through 5 plus IV irinotecan 10-20 mg/m²/day on days 1 through 5 and day 8 through 12 (repeated every 3-4 weeks) which are equivalent to the dose used in the mice model. GCS-f support was given in the light of white blood cell count after the completion of the first cycle. Patients evaluated by CT-scan and MRI according to the site of involvement and a PET-CT after the completion of the 3rd cycle and 6th one.

RESULTS

The overall objective response was documented in 27 patients (77%) (10 complete responders and 17 partial responders). Complete responders were those with rib involvement (7), humerus (1), femur (1) and tibia (1), where the other 17 partial responders allocated as follows: rib (6), humerus (1), femur (5), and pelvis (5). Regarding response on metastatic sites, complete response was documented in two patients with hepatic metastasis, 6 patients with pulmonary metastasis and 1 patient with bone localization. However, no response observed in those with skull disease.

The maximum response seen was after the completion of three months of treatment as documented by clinical examination and radiologic studies, and then it began to decline to reach a nadir after seven month from the beginning of treatment. The eight non-responders progressed while on treatment during the first three months. Among the 27 responders, only 10 kept the response between 3-9 months (7 months in median). Nine patients progressed while on treatment between the 3rd and 5th month, while the remaining eight patients progressed (three on the 6th month, two in the 7th month, one in the 8th month and two in the 9th month). Therefore, the progression free survival at 1 year was 22.9% [0.229 (0.108-0.376)] as illustrated in figure 5.
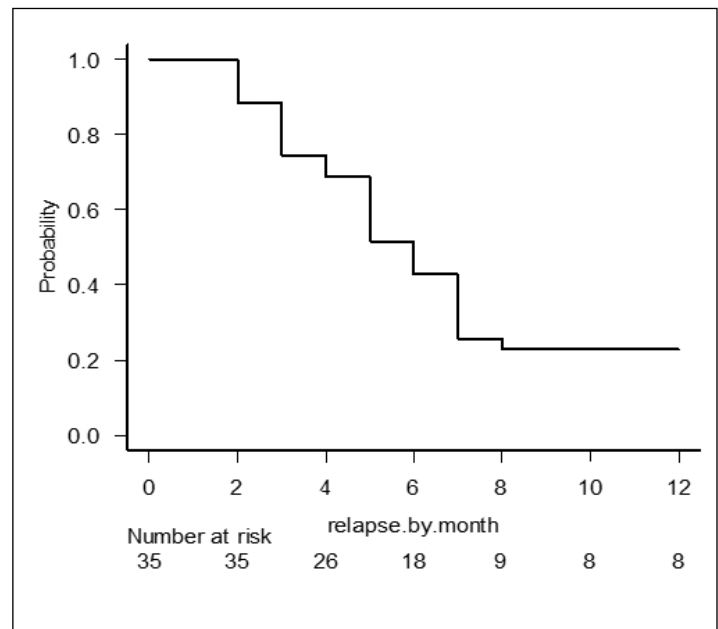
Figure 5: Progression free survival curve at 12 months: event free-survival [EFS 0.229 (0.108-0.376)].

The first reported death was at five months from the base line, with another three deaths reported between 6 and 9 months. The study reached the end of the first year with another nine deaths reported between 9 and 12 months with a one year survival of 58.2% [0.582 (0.398-0.728)] as illustrated in figure 6.
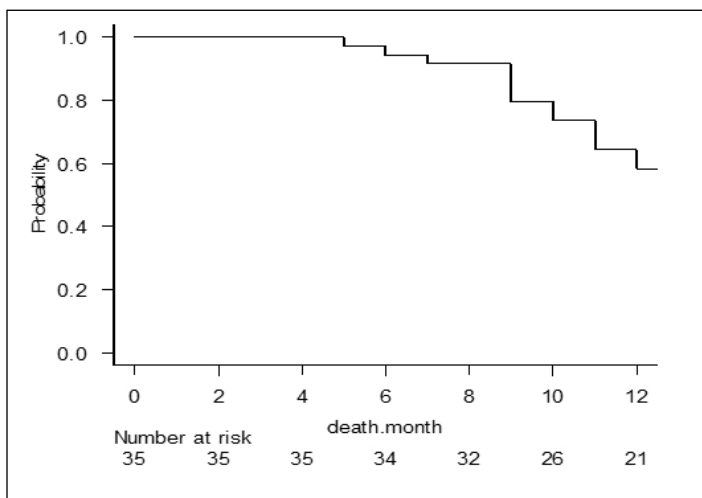
Figure 6: Illustrates the overall survival: OS: 0.582 (0.398-0.728).

Toxicity profile was fine enough to complete treatment for all recruited patients. The most frequent side effect was grade I and II neutropenia in nine patients (25%), mucositis in 29 patients (82%) and diarrhea in 7 patients (20%) as illustrated in table 2. The 12 patients with thrombocytopenia with different grades were able to continue treatment after a rest between 7-15 days; however, no one needed platelet transfusion. Regarding those four patients with acute renal failure, they were admitted to hospital and we found that it was attributed to a severe dehydration; therefore, they were kept for a median of 5 days and discharged without complications.

Side Effect	Percentage
Neutropenia	9 (25%)
mucositis	29 (82%)
diarrhea	7 (20%)
Skin rash	3 (8%)
thrombocytopenia	12 (34%)
Acute renal failure	4 (11%)

Table 2: Toxicity profile.

DISCUSSION

In order to improve results of relapsed and progressed patients of ES, emerging of genetic profile played an important role in revealing oncogenes laying behind both progression and resistance to chemotherapy in ES cases. Further, new genes discovery may help us suggest new targeted therapies and others using modulation of the immune response. Based on the former idea, Miyake K et al, have conducted a study combining both irinotecan and temozolomide in ES Doxorubicin-resistant cases using PDOX nude mice model [18]. They found that TMZ-IRI was an effective combination compared to the untreated control group (p = 0.022). Gemcitabine plus docetaxel was also found to be effective (p = 0.026). However, Pazopanib and yondelis did not show to be promising agents. A similar study conducted by Takashi M et al, used the same methods (PDOX) and recruited patients with both FUS-ERG fusion and CDKN2A/B loss which is a rare abnormality in the same patient [19,20]. CDKN2A, which is a tumor suppressor gene, can play a major role in CDK4/6 inhibition. Therefore, CDKN2A loss can lead to a cancer-cell progression in the cell cycle. Frequency of CDKN2A loss in ES reported to be near 11.2%. Palbociclib (PD0332991), a CDK4/6 inhibitor, demonstrated good efficacy in many types of cancers including ovarian, glioblastoma, and chordoma cell lines with CDKN2A loss [21-23]. Same results were obtained from patients with liposarcoma [24]. Data showed significant improvement from CDK4/6 and IGF-1R inhibitors in some rare cases of ES with FUS-ERG fusion and CDKN2A/B using PODX [25]. In our study, we have managed to create a mice model for every patient; however, the response was not the same when compared with the patients in real life, the thing that opens a way for a serious investigation about the genetic and non-genetic factors implicated in resistance and progression of ES patients. We should stress that working on mice model is a double edged method since the cancer environment is different between mice and humans, further, the nude mice have no cell mediated immunity which is not the case in healthy human which forms a complete bias. In the present and future practice, genetic profile may play an important role in guiding our treatment plan in the precision medicine era.

DISCLOSURE

All authors declare no conflict of interest.

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