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(Received on January 10, 2017, accepted on January 24, 2017)

ABSTRACT

Patient-derived xenograft (PDX) models are useful for preclinical evaluation of anticancer agents. However, establishing PDX models of cervical cancers are known to be challenging. We modified a protocol from the existing literature and established a model of an HPV16-positive squamous cell carcinoma on scid mice. The xenograft was positive for $p16^{INK4a}$ even after a passage, indicating the continued involvement of the E7 viral oncoprotein in abnormal cell growth. After 24 days of treatment with a nucleoside analog, gemcitabine, tumor growth was found to be suppressed in a dose-dependent manner, and the tumor became undetectable after high-dose treatment. Cediranib, an orally bioavailable inhibitor of neovascularization, reversed tumor growth until it was barely detectable. A hydrophobic cancer stem cell inhibitor, salinomycin, did not show any significant effect when used alone, but showed a tendency to act synergistically with low-dose gemcitabine. Although further procedural refinements are required, the model appeared to be useful for preclinical evaluation of various anticancer agents, including novel ones that target specific molecules. *Ryukyu Med. J., 36 (1, 2) 25~28, 2017*

Key words: cervical cancer, patient-derived xenograft, gemcitabine, cediranib, salinomycin

INTRODUCTION

An increasing number of anticancer agents that target cancer-specific molecular aberrations, rather than universal cellular machinery, are undergoing clinical trials (https://clinicaltrials.gov/). Patient-derived xenograft (PDX) models are useful for predicting the performance of anticancer agents in clinical trials, and such models are available for tumors of skin, brain, head/neck, breast, lung, liver, pancreas, stomach, colon, kidney, bladder, prostate, and ovary, but not for those of the uterine cervix (http://tumor.informatics.jax.org/). Although the generation of PDX for cervical cancers is notoriously difficult, one report with a realistic success rate has been published[1]. The study involved 10 patients with cervical cancer and attained 7/10 successful engraftments onto mice, followed by 4/7 successful passages to the second mice. The authors concluded that the injection of tissue fragments is superior to the injection of dissociated cells or the transplantation of a tissue cube. Based on this report, we have developed a modified protocol and tested its utility through evaluation of candidate agents for cervical cancer treatment.

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MATERIALS and METHODS

Engraftment experiments

The study was performed with the approval of the Clinical Study Review Board and the Animal Care and Use Committee of our university. Eight patients with stage IB1 cervical cancer were recruited, and written informed consent was obtained from all of them. HPV genotyping and immunohistochemistry were performed as described previously[2,3]. For engraftment experiments, surgically-removed cancer tissues were washed in normal saline, and their portions (approximately 125 mm^3) were collected and kept in 50-ml tubes containing RPMI-1640 medium supplemented with 10% fetal calf serum. Efforts were made to collect tissues from appropriate parts so as not to interfere with pathological diagnosis. Under sterile conditions, tissues were transferred into 100-mm culture dishes containing PBS, minced into pieces, centrifuged briefly in 50-ml tubes, resuspended in 200 μl of PBS, and subcutaneously injected into the shaved backs of 7-week-old female CB-17 scid mice using 18-gauge needles. Both the RPMI medium and the PBS contained 300 μg/ml of tetracycline. Once tumor engraftment occurred, the tumor was serially passaged using the same technique.

Evaluation of anticancer agents

Tumor length (L) and width (W) were measured with a caliper, and the volume (V) was calculated using the formula $V = L \times W^2 \times 0.5$[4]. Treatment with the anti-cancer agents were initiated when L reached 4 mm[5] ($V = 23 \pm 2\ mm^3$, mean $\pm$ SE). Gemcitabine hydrochloride (Eli Lilly, Indianapolis, IN) and salinomycin (Sigma-Aldrich, St. Louis, MO) were dissolved in normal saline and 25% (v/v) dimethylsulfoxide, respectively, and administered intraperitoneally. Cediranib (AZD2171, LKT Laboratories, St. Paul, NM) was dissolved in 1% (w/v) Tween 80 and administered through an oral gavage. The doses and administration schedules as well as the observation period of 24 days, were adopted from published studies[5,6]. Difference between tumor growth curves were analyzed by two-factor repeated measures analysis of variance. A p value <0.05 was considered significant. Analyses were performed using JMP® Pro 12.1.0 software (SAS Institute, Cary, NC).

RESULTS

The tumor tissue was not collected from one patient, because her tumor was found to be too small upon operation. Successful engraftment was achieved in 1 out of 7 experiments. The successfully engrafted tumor was from a patient with HPV16-positive squamous cell carcinoma (SCC). Unsuccessful experiments involved 2 SCCs, 1 adenosquamous carcinoma, and 3 adenocarcinomas. After the engraftment, passaging was performed without any major problems. The xenograft contained clusters of SCC-like cells that were positive for $p16^{INK4a}$, which accumulated as a consequence of viral E7-mediated Rb inhibition[7], indicating the continued expression of functional E7 even after the passage (Fig. 1).

The utility of this model was tested using three anti-cancer agents: gemcitabine, a nucleoside analog inhibitor of DNA replication; cediranib, an orally bioavailable inhibitor of tumor-induced neovascularization; and salinomycin, a hydrophobic compound toxic to cancer stem cells (CSCs). Gemcitabine suppressed the growth of tumors in a dose-dependent manner (Fig. 2A). Tumors became undetectable after high-dose treatments. Cediranib reversed the growth of tumors to a barely detectable size (Fig.

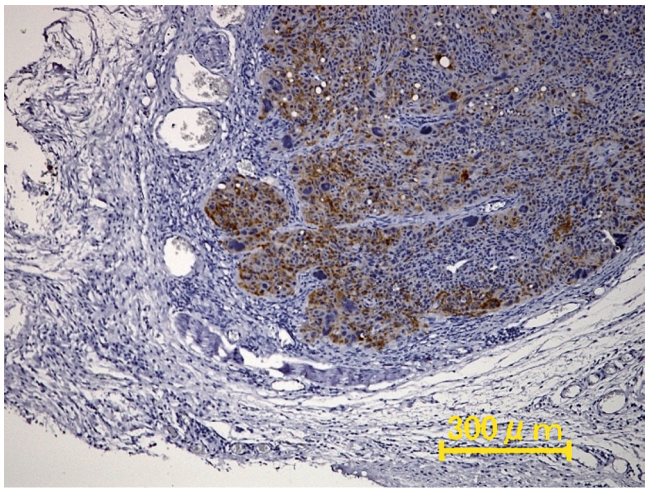

Fig. 1 **$p16^{INK4a}$ expression in PDX** An HPV16-positive cervical SCC tissue was engrafted on scid mice and passaged once. Formalin-fixed tumor tissues from a passaged mouse were examined for the expression of p16INK4a by immunohistochemistry. Anti-human $p16^{INK4a}$ (clone E6H4) was from Roche. Avidin-biotin/3,3'-diaminobenzidine and hematoxylin were used for $p16^{INK4a}$ detection and counterstaining, respectively.

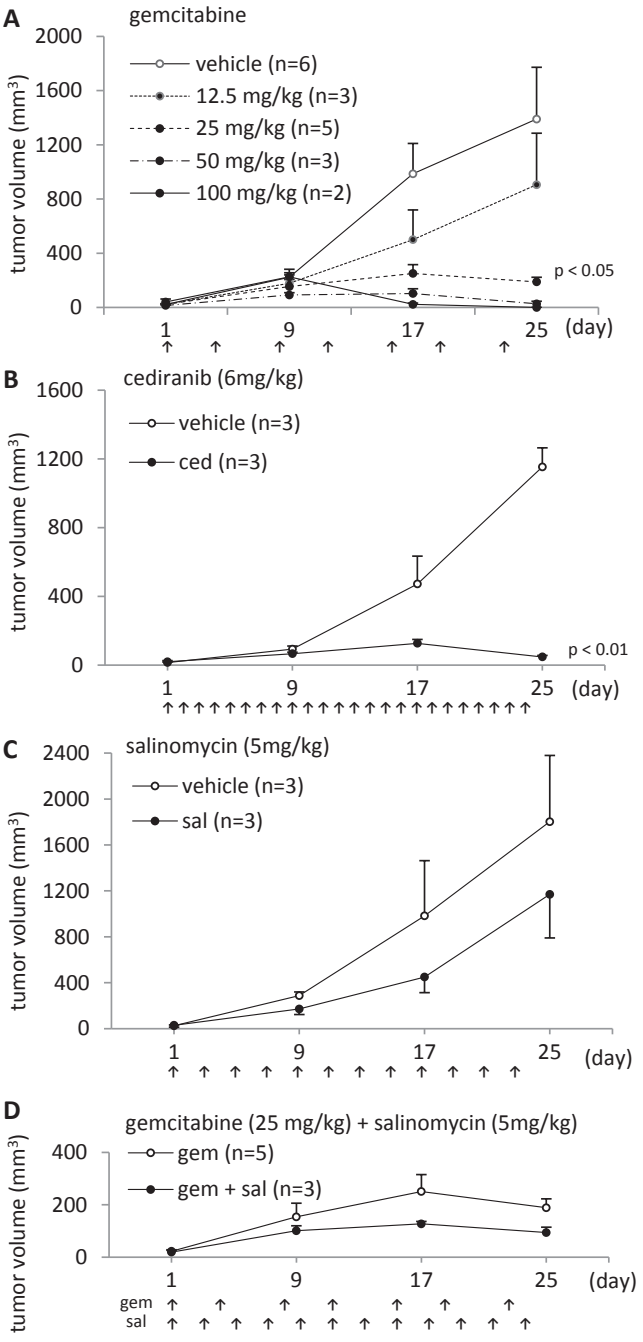

Fig. 2 **Effects of various agents on PDX growth** Starting on the day when PDX diameter reached 4 mm (day 1), anti-cancer agents were administered as indicated by arrows. Doses and numbers of tested mice are also indicated. Tumor volumes (mean and SE) during the 24-day treatments are shown. For gemcitabine experiments (100 mg/kg), values shown are mean and variation of data from two mice. Tumors disappeared from both of them and from one mouse that received 50 mg/kg gemcitabine (day 25). Values from the 25-mg/kg gemcitabine experiments in (A) were compared with those from the combination therapies in (D).

2B). Salinomycin showed no significant effect when used alone (Fig. 2C) but tended to act synergistically with low doses of gemcitabine (Fig. 2D).

DISCUSSION

In the present study, we developed a protocol for the generation of PDX models of cervical cancer. The success rate for engraftment was low (1/7), probably due to the use of scid mice instead of the more immunocompromised scid beige mice[1]. However, a study that used less immunocompromised nude mice for developing a model was reported recently, although only 4 lines were described, and no information regarding the initial engraftment success rate was provided[8]. Inadequate technical proficiency was probably not a reason, as we succeeded in the second experiment. Success may depend on cancer cell type. However, SCCs appeared to be relatively difficult to engraft (only 2/5)[1]. In future experiments, histopathological examination of collected cancer tissues may provide explanation for successful engraftments. Also, it would be interesting to further analyze this particular xenograft to check for any genetic or epigenetic aberrations that confer it the exceptional vitality we observed.

One useful feature of PDX is that any agent or combination of any agents can be tested as a first-line therapy for previously untreated human cancers. In the present study, mice were treated with agents that were not approved for cervical cancer treatments in Japan at this point. Although comparisons with current first-line agents have not been made, gemcitabine performed well at doses close to clinical doses, suggesting its potential to serve as a first-line agent. Gemcitabine exhibited only minimal efficacy as a single agent in a phase-II clinical trial of cervical SCC, but this may have been because subjects had already undergone radiotherapy and/or chemotherapy[9]. Cediranib, with doses slightly above clinical dose, also performed well as a single agent. Although this study was performed on mouse vascular endothelial cells and not on human cancer cells, these results indicated a major role of tumor-derived VEGF in tumor growth[10]. In contrast, salinomycin showed no significant effect as a single agent. However, it appeared to potentiate the effect of low-dose gemcitabine. Although the effectiveness of combined salinomycin and gemcitabine has been reported in a previous study on pancreatic cancer

xenografts, the xenograft used was cell-line based and was generated by injecting a mixture of sorted CSC and non-CSC populations[5]. Thus, despite limitations such as small number of subject mice and lack of appropriate vehicle control mice, this study might be the first PDX study to show the potential of a combination therapy of conventional agents and anti-CSC agents.

The further usefulness of PDX is that a cancer tissue from a single patient can be tested in many experiments. In the era of molecularly targeted anticancer agents, numerous experiments are necessary because aberrations of different molecules can give rise to the same cancer, and different agents can target the same molecule through different mechanisms. For example, the activation of the HER2 gene by mutation, amplification, or HPV integration was found in a subgroup of cervical cancers through massively parallel DNA and RNA sequencing[11]. Similarly, among 9 pairs of cervical cancer and PDX, which were molecularly profiled, one pair was found to have aberrant HER2 amplification[12]. Treatment of the PDX tumors with a combination of an anti-HER2 antibody, trastuzumab, and a HER2 tyrosine kinase inhibitor, lapatinib, led to 50% reduction of tumor weight. In addition, the authors noted that their earlier PDX experiments using trastuzumab or lapatinib as a single agent were both unsuccessful. Thus, they performed 3 experiments using 2 agents to successfully target a single molecular aberration. With further identification of key molecular aberrations for cervical cancer, more targeted agents will be developed and needed to be tested. PDX models of cervical cancer will provide means to search for the most promising molecularly targeted therapy to be tested in clinical trials.

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