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Case Study

Delayed and Aberrant Presentation of VX2 Carcinoma in a Rabbit Model of Hepatic Neoplasia

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A socially-housed New Zealand white rabbit presented with a large subcutaneous mass on the ventral thorax approximately 11 mo after the intrahepatic delivery of a suspension of VX2 carcinoma cells to induce hepatocellular carcinoma as part of a nanoparticle study. The mass and closely associated axillary lymph node were removed en bloc. Immunohistochemical staining identified the mass as an undifferentiated carcinoma. The rabbit demonstrated no appreciable pathology at the study end point at 16 mo after VX2 inoculation. An additional rabbit from the same VX2 injection cohort was found at necropsy to have an unanticipated intraabdominal mass, also identified as an undifferentiated carcinoma. This case report summarizes the molecular analysis of both tumors through a novel PCR assay, which identified the delayed and aberrant onset of VX2 carcinoma in an extended timeframe not previously reported.

Abbreviation: CRPV, cottontail rabbit papillomavirus.

The VX2 squamous cell carcinoma cell line was developed in 1938 from cells isolated from a domestic rabbit with a Shope papillomavirus-induced skin papilloma.[11,16] The inoculation of allogenic adult rabbits with the VX2 tumor cells results in a wholly anaplastic carcinoma that grows rapidly and forms frequent metastases.[7,11] Molecular analysis of the VX2 carcinoma has revealed multiple integrated copies of highly methylated cottontail rabbit papilloma virus (CRPV) genomic material.[5-7,17] For decades, the rabbit VX2 carcinoma has supported oncology research by providing a large animal model that supports the investigation of neoplasms of diverse tissues including liver, lung, pleural space, muscle, and bone.[9,12,15,18,19] Benefits of this model include relatively simple inoculation, rapid growth with local tissue invasion, predictable metastases, and reproducibility.[3,4,13,14] In the current report, we describe the use of VX2 cells intended to induce a hepatic carcinoma model in rabbits and the unexpected outcomes associated with the model.

Case Report

Adult New Zealand white rabbits (*Oryctolagus cuniculus*; male, $n = 6$; Harlan Laboratories, Indianapolis, IN) were procured to investigate the effectiveness of a radioactive nanoparticle agent against inoperable hepatic neoplasia. Rabbits were sedated with acepromazine (0.7 mg/kg; Vedco, St Joseph, MO) and glycopyrrolate (0.02 mg/kg; West-Ward, Eatontown, NJ) followed by anesthesia induced with ketamine (16 mg/kg; Butler Schein Animal Health, Dublin, OH) and xylazine (3.4 mg/kg; Lloyd, Shenandoah, IA) intramuscularly. Rabbit livers were injected by using ultrasound-guided delivery of 1×10^7 VX2 carcinoma cells (vial designation G050867; originally obtained from the NCI–Frederick Cancer DCT Tumor Repository). After injection, tumor growth in rabbits was monitored by weekly transcutaneous ultrasonography. Nanoparticle therapy would have been initiated once hepatic tumors had reached 2 cm in diameter. All activities associated with this study were approved by the University of Missouri IACUC and conducted in accordance with recommendations in the *Guide for the Care and Use of Laboratory Animals*.[1,2,10] As required by the USDA and the *Guide*, rabbits were housed in stainless steel cages with slatted floors in an environmentally controlled animal facility at 61 to 72 °F, 30% to 70% relative humidity, and a 12:12-h light:dark cycle. Rabbits were provided with 130g pelleted feed (5326 Rabbit Diet, Purina, St Louis, MO) supplemented with grass hay and potable water daily. The University of Missouri is USDA-licensed and AAALAC-accredited.

Throughout the planned postinjection period of 120 d, ultrasound monitoring revealed no evidence of tumor development. Approximately 11 mo after VX2 cell injection, 1 of the 6 rabbits that recently had begun playpen socialization presented with a firm, round (diameter, 8 cm) subcutaneous mass overlying the ventral thorax just caudal to the right axilla. The skin overlying the mass was grossly normal. The rabbit did not resent manipulation of the mass and exhibited no ambulatory deficits. An abscess was suspected initially, as a sequela to rough play during socialization. Fine-needle aspiration yielded approximately 175 mL of dark brown to red, nonviscous fluid. Cytology was interpreted as minimally diagnostic, suggestive of cystic fluid with evidence of necrosis, and rare extracellular bacteria of unknown significance.

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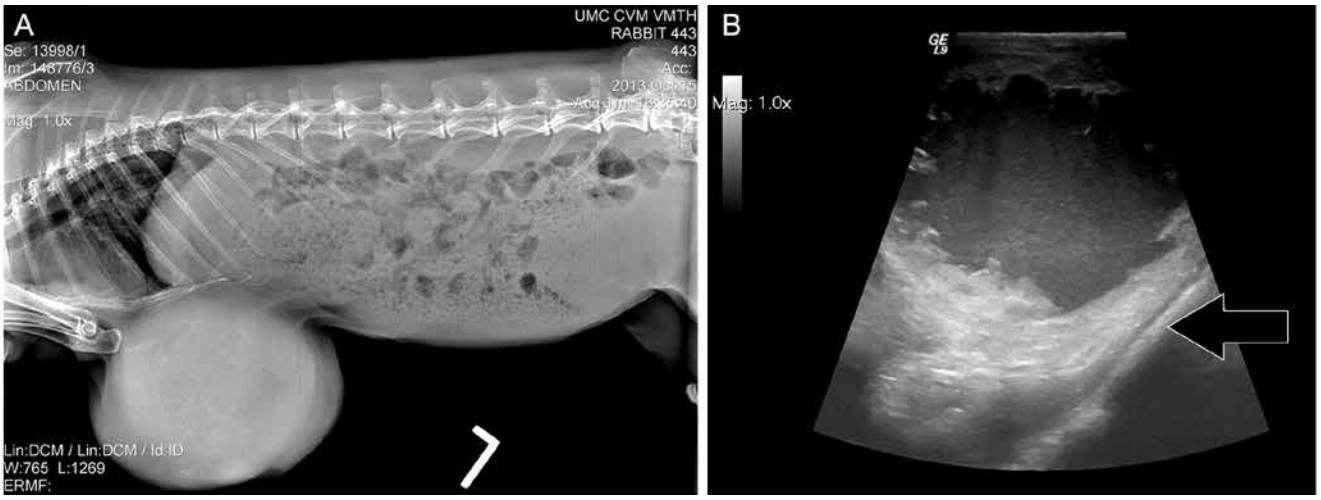

Figure 1. Imaging of the subcutaneous mass. (A) Left lateral radiograph showing the ventral thoracic mass of soft tissue opacity. (B) Ultrasound image revealing cavitary nature of the mass, with no communication with the pleural space (arrow indicates thoracic wall).

Low numbers of coagulase-negative *Staphylococcus* spp. were cultured from the fluid aspirate. Radiographic and ultrasonographic examination revealed a single cystic structure with no evidence of thoracic or abdominal communication (Figure 1). Surgical removal of the mass was elected.

The rabbit was anesthetized with ketamine (25 mg/kg IM; Butler Schein Animal Health) and xylazine (5 mg/kg IM; Lloyd), intubated, and provided isoflurane (MWI, Boise, ID) at 2%. The ventral thoracic and abdominal skin was clipped (Figure 2 A), aseptically prepped, and draped for surgery. After an initial incision in the center of the mass to allow for suction-assisted drainage, the core was identified as necrotic (Figure 2 B) and the skin tightly adhered, preventing subcutaneous dissection from this initial incision. The mass and surrounding skin were removed en bloc by using a separate incision made at the base. A nearby axillary lymph node was found to be enlarged and removed intact (Figure 2 C). The excision site was closed with 3-0 polydiaxanone suture (PDS, Ethicon, Somerville, NJ) in 2 layers, with a deep simple-interrupted subcutaneous pattern and overlying simple-interrupted subcuticular pattern (Figure 2 D). The rabbit was given buprenorphine (0.03 mg/kg IM; Reckitt Benckiser Pharmaceuticals, Richmond, VA) prior to extubation, as well as flunixin meglumine (1 mg/kg SC; Phoenix, St Joseph, MO) and enrofloxacin (5 mg/kg SC; Bayer, Shawnee Mission, KS). Recovery from anesthesia was unremarkable. The rabbit was monitored daily postoperatively for incision-site healing, subjective pain evaluation, food and water consumption, and defecation. Healing was complete and uneventful.

Representative sections of the mass and the entire lymph node were fixed in 10% neutral-buffered formalin for 24 h and sectioned for histopathology (IDEXX BioResearch). The pathology report revealed that the surgically excised and drained subcutaneous mass with necrotic center had neoplastic cells extending to the cut margins of submitted tissues. The tissue was a densely cellular and well-demarcated, nodular tumor partially surrounded by a moderately dense capsule with minimal cellular infiltrates beyond capsule borders. Vascular invasion by neoplastic cells was observed in all sections. Neoplastic cells were closely packed, with various patterns of organization including sheets, nests, and acinar structures with a moderate amount of fibrous stroma (Figure 3). Cells were pleomorphic and exhibited anisocytosis with distinct borders and a scant amount of lightly basophilic, granular cytoplasm. Nuclei were pleomorphic, eccentric, displayed a finely stippled and hypochromatic chromatin pattern, and contained 1 to 3 basophilic nucleoli. Mitoses ranged from 3 to 9 (average, 6) per 400× field. Bizarre mitoses were present in some fields. Approximately 50% of the regional axillary lymph node was replaced by unencapsulated densely packed neoplastic cells organized in nests and acini with central necrosis. Mitosis ranged from 9 to 12 per 400× field. Immunohistochemical assays revealed that the tumor tissue was strongly positive for the cytoplasmic keratins AE1 and AE3, and the capsule was positive for vimentin. The mass was identified as an epithelial carcinoma with local lymph node metastasis.

At 16 mo after injection, all rabbits remained clinically normal. Rabbits were removed from the study, euthanized, and underwent postmortem evaluation for the presence of gross abnormalities. Another rabbit (different from the animal with the subcutaneous mass) had a round (diameter, 8 cm), encapsulated cystic-like mass in the region of the gastric limb of the pancreas. In addition, the rabbit with the previous subcutaneous mass appeared grossly normal, with no evidence of tumor recurrence. No abnormalities were noted in the remaining rabbits.

The intraabdominal mass from the second rabbit was formalin-fixed for 24 h before being sectioned for histopathology and immunohistochemistry. This mass was histologically similar to the subcutaneous mass, with epithelioid tumor cells forming sheets, nests, and acini (Figure 4 A). As with the subcutaneous mass from the first rabbit, immunohistochemical assays of the intraabdominal mass demonstrated tumor tissue that was strongly positive for cytokeratins AE1 and AE3 (Figure 4 B) and a capsule positive for vimentin (Figure 4 C).The intraabdominal tumor was identified as an epithelial carcinoma.

Although immunohistochemistry identified both tumors as carcinomas, the morphologic patterns did not reveal the tissue of origin, because the lesions were atypical of tumors that arise in those regions. Given the history of experimentation and to identify a possible source of the masses, we sought to compare our findings with the morphologic appearance of previously reported tumors that developed after VX2 cell inoculation. Independent

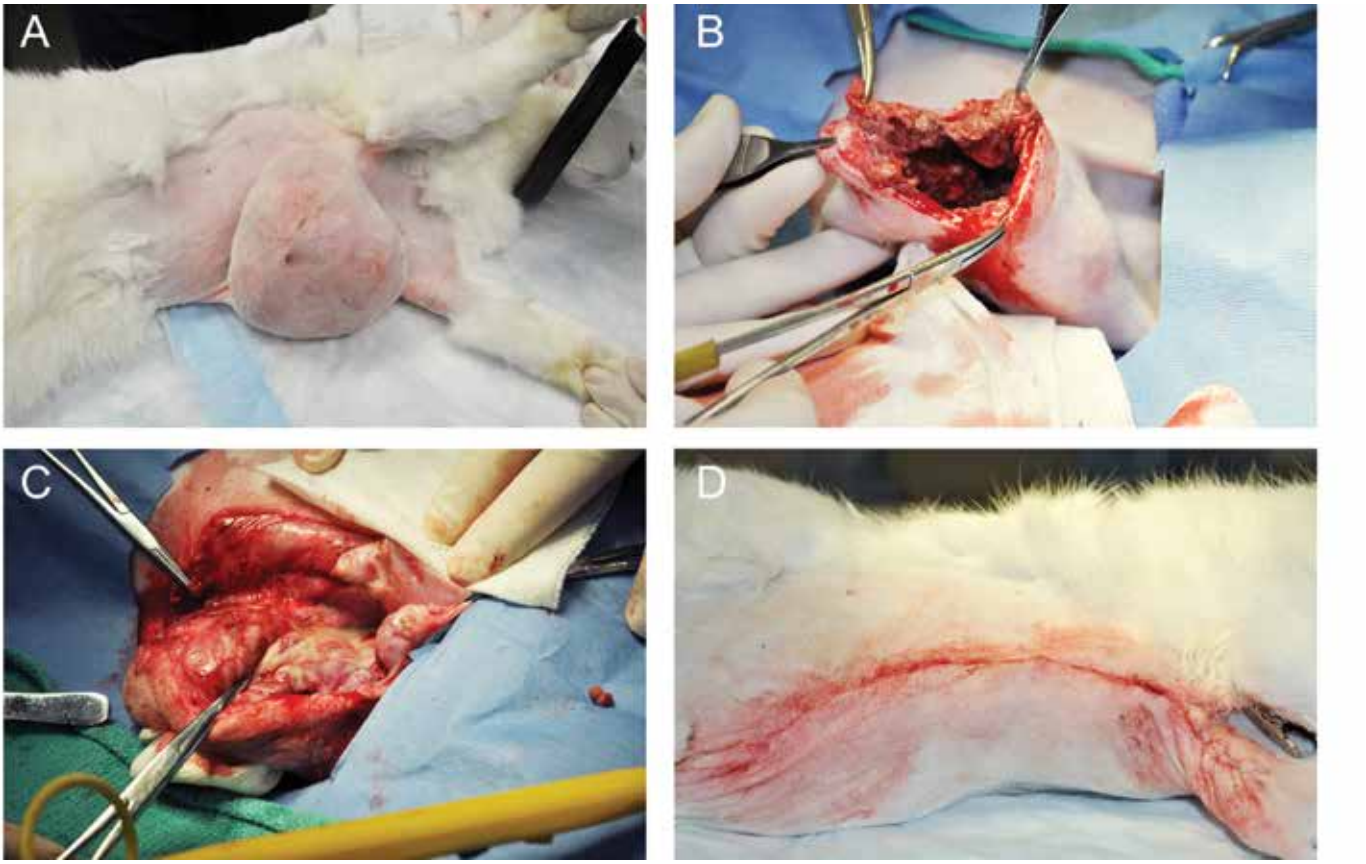

Figure 2. Surgical mass excision. (A) Intact subcutaneous mass, with hair clipped. (B) Initial incision to allow for suction-assisted drainage, exposing cavitary nature and necrotic core of mass. (C) Incision along base of mass near thoracic wall to allow subcutaneous dissection and removal of mass with minimal skin attached. Note enlarged axillary lymph node (arrow). (D) The 2-layer closure, resulting in skin apposition.

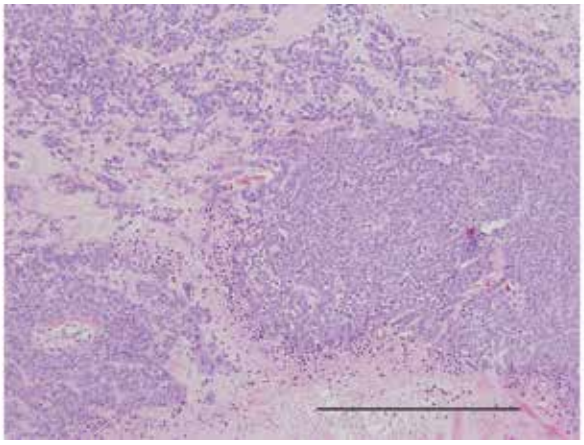

Figure 3. Representative section of subcutaneous mass exhibiting nests of cells and acinar structures, with moderate amounts of fibrous stroma. Hematoxylin and eosin stain; scale bar, 500 µm.

of the tumor location, previously reported VX2 carcinomas are similar in appearance to the tumors these rabbits developed, with cells arranged in sheets, nests, and acini, and atypical mitoses of a similar number. In addition, tumor cells in the previous studies were strongly positive for cytokeratins AE1 and AE3.[12,18]

Literature reports confirm mRNA expression of the CRPV in transplantable VX2 carcinoma.[7] Because the VX2 cell line was developed from a skin mass induced by infection with CRPV, we developed a PCR assay to identify the presence of the CRPV genome in the cell line and tumor tissue. The genomic structure of the CRPV was determined in 1985.[8] Primers specific for the E2 encoding region were selected by using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized (forward, 5′ TGT GGA CTT CTC AAC CGA CG 3′; reverse, 5′ TGA CCT TGA CCT TCG TCT GC 3′; Integrated DNA Technologies, Coralville, IA) to amplify a 224-bp product. DNA was extracted from formalin-fixed paraffin-embedded blocks of both tumor tissues (subcutaneous and intraabdominal) and the affected axillary lymph node as well as heart tissue from the rabbit with the intraabdominal mass by using the QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA) and an input of 5 sections, each with a thickness of 10 µm, as directed in the manufacturer's instructions. The same VX2 cell line that was used for inoculation was obtained from the University of Missouri Cell and Immunology Core and cultured as previously described.[9] Thigh muscle and liver biopsies from 2 healthy, experimentally naïve New Zealand white rabbits were used as negative-control samples. DNA was extracted from the VX2 cell line and control rabbit tissue by using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. DNA quantity and purity was measured by using Nanodrop technology (Thermo Scientific, Wilmington, DE). Each 25-µL reaction contained 25 ng template DNA. PCR cycling conditions were 35 cycles of denaturation at

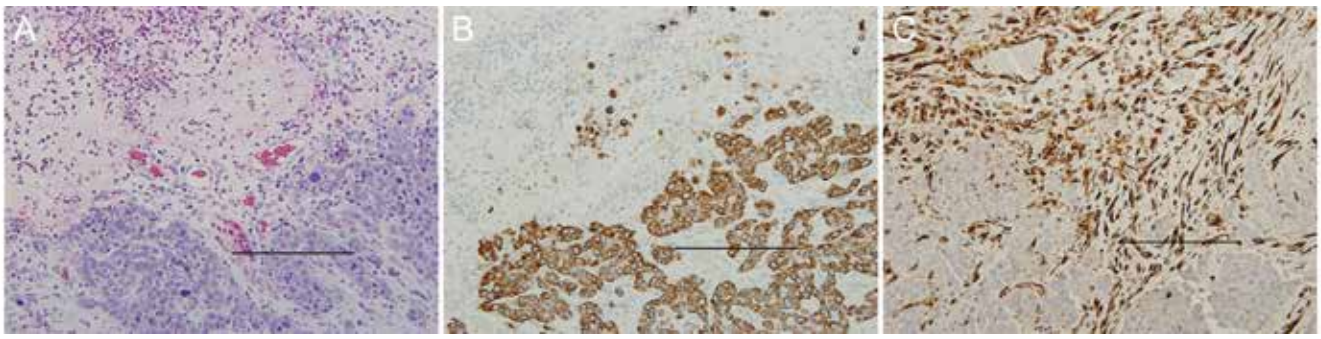

Figure 4. Histopathology and immunohistochemistry of the intraabdominal mass from the second rabbit. (A) The mass exhibited epithelioid neoplastic cells forming sheets, nests, and acini surrounded by a dense capsule. Hematoxylin and eosin stain. (B) Tumor tissue stained positive for cytoplasmic keratins AE1 and AE3; capsule was negative. (C) Tumor tissue was negative for vimentin, but the capsule stained positive. Scale bars, 200 µm.

98 °C for 15 s, annealing at 55 °C for 30 s, and synthesis at 72 °C for 30 s. PCR products were visualized by using QIAxcel capillary electrophoresis (Qiagen) and then were processed through the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) according to the manufacturer's instructions. Purified PCR products were submitted to the University of Missouri DNA Core for Sanger sequencing.

A distinct and prominent band representing a 224-bp amplicon was present in the VX2 cell line, as well as the tumor tissue obtained from both rabbits. The tissues obtained from control rabbits and no-template control lanes were negative for this product, as was DNA extracted from heart tissue from the rabbit with the intraabdominal mass (Figure 5). A BLAST homology search[8] using the sequencing results as a query string revealed an identical match to the known CRPV sequence deposited in GenBank, except for one single-nucleotide polymorphism (A to G) at position 3769. The results of the molecular diagnostics confirmed that both tumors originated from the inoculated VX2 cell line.

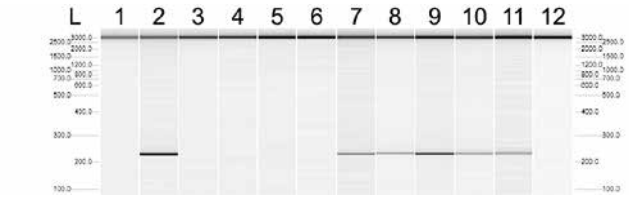

Figure 5. Capillary electrophoresis of amplified genomic DNA extracted from tissue and paraffin blocks. L, molecular-weight ladder; lane 1, no-template control; lane 2, VX2 cell line; lanes 3 and 4, liver from negative-control rabbits 1 and 2, respectively; lanes 5 and 6, muscle from negative-control rabbits 1 and 2, respectively; lane 7, axillary lymph node; lanes 8 and 9, subcutaneous mass; lanes 10 and 11, intraabdominal mass; and lane 12, heart muscle from the rabbit with the intraabdominal mass.

Discussion

Among the 6 rabbits that received intrahepatic injections of VX2 tumor cell suspensions, none developed hepatic tumors, and 2 developed tumor masses in extrahepatic locations (subcutis and parapancreatic) at 11 and 16 mo after inoculation, respectively. Subsequent histopathologic examinations and PCR-based amplification of CRPV from the cultured VX2 cells confirmed that the masses stemmed from the VX2 cell inoculation. The development of extrahepatic tumors and the markedly prolonged latency to tumor development were surprising complications of the procedure.

One particular previous study[3] best illustrates the events that occurred in our index rabbit case. In that report, rabbits were split into groups to compare the efficacy of 2 methods of intrahepatic tumor inoculation using VX2 cells and were euthanized at 2 wk after inoculation.[3] In one group, each rabbit received a hepatic subcapsular inoculation of a 1 mm^3 VX2 tumor fragment; this procedure resulted in 95% of the group developing intrahepatic tumors, with no evidence of leakage (defined as tumor development beyond the liver). In the other group, rabbits received an intrahepatic injection of a VX2 cell suspension (1×10^6 in a volume of 0.1 mL), after which as many as 50% of rabbits had evidence of tumor cell leakage, and only 35% developed intrahepatic tumors. Extrahepatic tumors developed in either the peritoneal cavity or surrounding organs.[3]

In our current report, we describe the clinical case study of a subcutaneous mass in a rabbit recently introduced to social housing. Consultation with the investigator revealed that the entire cohort of rabbits had received percutaneous injections of VX2 tumor cells into the liver approximately 11 mo prior to presentation and that tumor development had not been identified during serial ultrasonographic evaluations. Histopathology and immunohistochemical assays of the excised subcutaneous mass revealed it to be an undifferentiated carcinoma. At study conclusion, an additional rabbit was found to harbor an unanticipated undifferentiated carcinoma. Despite the size of tumor and involvement of the local axillary lymph node, the rabbit with the subcutaneous mass showed no gross recurrence of neoplasia. This event was surprising given the invasive nature of the primary tumor and vascular infiltration of neoplastic cells identified on histopathology. Molecular testing was developed to definitively link the intrahepatic injection of VX2 cell suspension to the extrahepatic development of carcinomas in both rabbits, despite a markedly protracted timeframe previously unreported in the VX2 model.

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