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Abstract. Conjunctival squamous cell carcinoma (SCC) is an uncommon disease. However, SCC has recently become an important clinical problem due the identification of a significantly high incidence of SCC among a group of black African patients with AIDS. However, basic research concerning SCC, including both intraepithelial and invasive squamous neoplasia, is limited due to the lack of an ocular tumor animal model. Specifically, current ocular imaging and treatment modalities are insufficient for investigating currently available small animal models, because the conjunctival space is not comparable to that of humans. We describe the development of a reproducible model of subconjunctival squamous carcinoma in moderate-sized immunocompetent rabbits. Under optical coherence tomography guidance, 1×10^7 VX2 carcinoma cells are inoculated into the subconjunctival space of 3 to 4-kg New Zealand white rabbits. Malignant tumor involvement developed on the subconjunctival space after an average of 1 to 2 weeks. This subconjunctival tumor model induction method will likely facilitate a broad range of investigation of subconjunctival cancer diagnostics and therapeutics. © 2013 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: [10.1117/1.JBO.18.7.070501](https://doi.org/10.1117/1.JBO.18.7.070501)]

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1 Introduction

The incidence of conjunctival squamous cell carcinoma (SCC) varies geographically and ranges from 0.02 to 3.5 cases per 100,000 individuals.¹ Even before the term ocular surface squamous neoplasia was introduced to encompass both the spectrum of conjunctival and corneal intraepithelial neoplasia and SCC,² published series often included both intraepithelial and invasive squamous neoplasia.³

There have been many reports concerning the increased incidence of conjunctival tumors in patients with HIV/AIDS, who live in central, south, and east Africa. These patients exhibit a more aggressive and faster growing form of conjunctival tumors, which may suggest that the traditional behavior of conjunctival tumors need to be re-evaluated and requires a new treatment model^{4,5}; however, the mechanism of this increased incidence remains unknown.

There is currently a reproducible rabbit model for hematogenous spread of metastases of the VX2 tumor to the CNS or the inner eye.⁶ Besides, there are many previous studies that use

VX2 tumor cells to be inoculated into many organs such as the liver,⁷ pleura,⁸ colon,⁹ and kidney¹⁰ to make monitoring and therapeutic models. However, there is currently no reproducible model of the advanced conjunctival tumor relevant for developing monitoring and treatment modalities. The subconjunctiva has excellent accessibility under direct visualization, which makes it easier to follow-up tumor development from tumor nest to angiogenesis. Therefore, we developed a subconjunctival tumor model using skin VX2 tumor cells. This model may ultimately become a good tool to study *in vivo* tumor behavior.

Optical coherence tomography (OCT)¹¹ is a noninvasive imaging modality that permits high resolution (2 to 10 μm) cross-sectional imaging of scattering media in real time, and can be applied easily to visualize numerous biological tissues. Although OCT has a limited imaging depth of 1 to 2 mm in scattering tissues, this range is sufficient to visualize from the conjunctival epithelium to the sclera. Thus, OCT may be useful for evaluating tumor development through time sequences with an aid of ultrasound (US).

In this study, we evaluated a new method of direct inoculation of VX2 tumors within the subconjunctival area for the noninvasive evaluation of tumor growth using OCT and US imaging.

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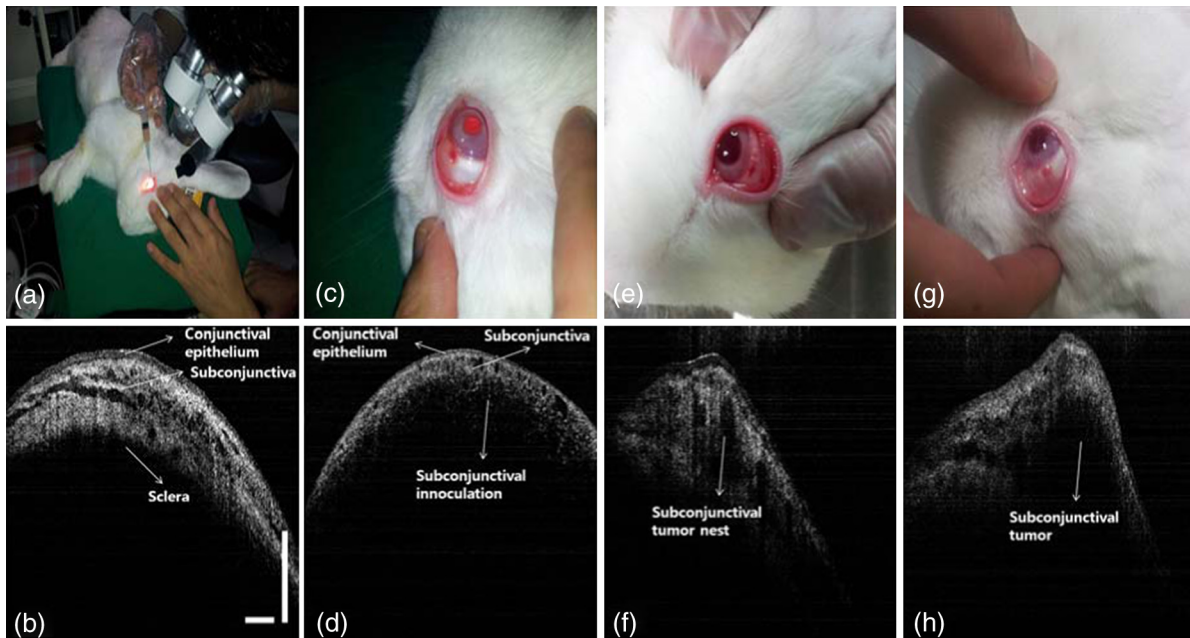


Fig. 1 Photographic and optical coherence tomographic (OCT) confirmation for VX2 tumor inoculation. (a) VX2 tumor inoculation, (b) OCT image of a normal conjunctiva before inoculation, (c) photo right after inoculation, (d) OCT image right after inoculation, (e) photo after three days, (f) OCT image after five days, (g) photo after seven days, and (h) OCT image after seven days. OCT images were taken by a system built in-house.

2 Methods

2.1 Animals and Tumor Cells

Five male New Zealand white rabbits weighing 3.0 to 4.0 kg were used in the present study, which was approved by the Animal Care and Use Committee of Kosin University. Animals were housed in separate cages at a controlled temperature of $22 \pm 2^\circ\text{C}$ with $55 \pm 5\%$ humidity. VX2 cells derived from rabbit skin SCC were used for tumor inoculation. All cells were obtained as a subculture from the femoral muscle of a study rabbit. Briefly, a thin (30 g) section of a tumor was cut, washed, suspended in Eagle's Minimum Essential Medium, and centrifuged at $220 \times g$ for 5 min.

2.2 Operative Procedures

After the injection of general anesthesia (ketamine 10 mg/kg, xylazine 3 mg/kg, subcutaneously), rabbits were secured in a supine position, and their conjunctival areas were exposed under visual guidance [Fig. 1(a)]. Using a 27-gauge needle, 0.4 ml of a suspension of VX2 cells (1×10^7) was injected into the subconjunctival space directly over the pars plana in the five rabbits [Fig. 1(c)] located 2.75 mm from the 9 o'clock limbus of the right eye. OCT evaluation was performed before tumor injection [Fig. 1(b)] and after tumor injection [Fig. 1(d)], respectively. Antibiotics were administered during all procedures.

After the implantation, a slit lamp examination (SLE) [Fig. 1(e)] coupled with OCT were used to confirm initial findings and evaluate tumor nest formation [Fig. 1(f)]. On the seventh day after implantation, a second SLE [Fig. 1(g)] and OCT were performed [Fig. 1(h)]. At this point, if a tumor islet was found, the tumor was evaluated every three days thereafter. When tumors grew beyond a size of 5 mm, and if we were unable to evaluate OCT image of tumor due to the limited

imaging depth, ultrasonographic examination was performed to measure tumor size and depth of tumor invasion. Animals were euthanized for histologic characterization of the tumor and implantation site. If no tumor cells were detected, animals were allowed to recover from anesthesia. SLE and OCT were repeated after 2 weeks, and animals were euthanized at this point, regardless of whether or not a tumor was detected. Two ophthalmologists, who were not aware of the previous tumor inoculation, conducted a blind examination of rabbit eyes using OCT and slit lamp independently.

2.3 OCT System

We built an 850-nm spectrometer-based OCT system. A broadband light source (Broadlighter D855, Superlum, Cork, Ireland) with a center wavelength of 850 nm and a full-width-at-half-maximum of 100 nm was used. Interference fringe pattern was collected by a line scan camera (Sprint sPL4096-140 km, Basler, Exton, Pennsylvania) with a line rate of 140 kHz and 4096 pixels. A two-axis scanner was customized using two galvanometers (6220H, Cambridge Technology, Cambridge, Massachusetts). B-mode images were acquired at 10 fps for 1024-lateral pixels. Further, a point spread function was measured and demonstrated a depth resolution of $4 \mu\text{m}$ in air, a roll-off of 12 dB/mm, and a signal-to-noise ratio of 103 dB. We also used a commercial OCT system (Spectralis, Heidelberg Engineering, Heidelberg, Germany).

2.4 US System

We used a US system with a high-frequency linear transducer (40 to 8 MHz) (Sonix Touch, Ultrasonix, British Columbia, Canada). The depth range was 0.2 to 3 cm, and geometric focus was 6 mm.

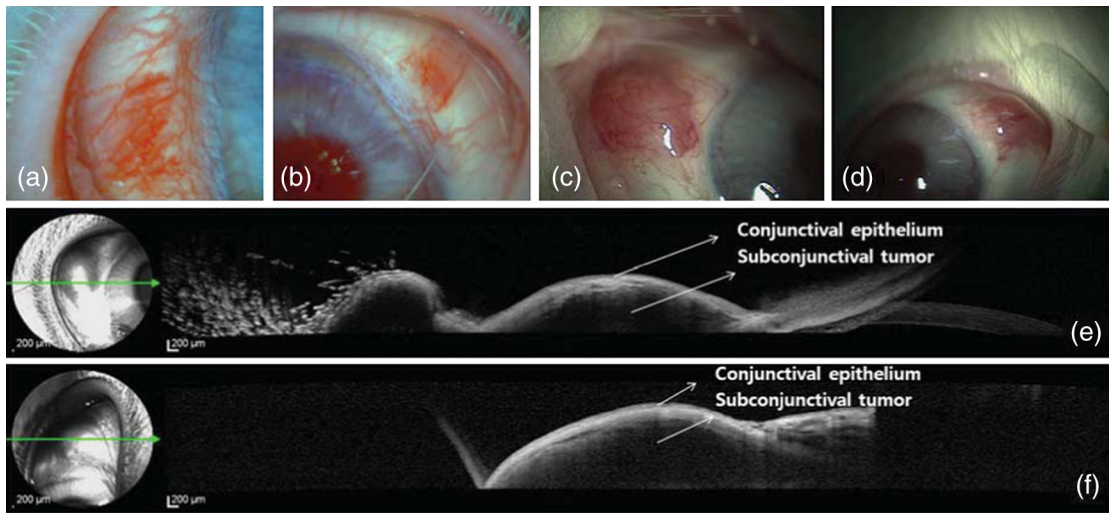


Fig. 2 (a) Photo of left conjunctiva after seven days, (b) photo of right conjunctiva after seven days, (c) photo of left conjunctiva after 14 days, (d) photo of right conjunctiva after 14 days, (e) OCT image of left conjunctiva after 14 days, and (f) OCT image of right conjunctiva after 14 days. OCT images were taken by Spectralis, Heidelberg Engineering.

2.5 Pathological Evaluation

After the death of animals, both eyes were harvested together. We performed exenteration to harvest the eye ball. All tumors were not extended to the fornix and the palpebral subconjunctiva at the day of harvest. The resulting tissues were then fixed with formalin, embedded in paraffin, sliced at 5- μm intervals, stained with hematoxylin-eosin, and examined microscopically. Bulbar conjunctivas were evaluated for abnormalities by gross inspection, and pathological examination was performed as needed. Formation of a viable VX2 tumor was defined pathologically as follows: infiltration of lymphocytes beneath the conjunctival epithelium at the site of inoculation and surrounding mass of tumor cells visualized as clear images. This mass was considered to be a viable tumor in the subconjunctival space.

3 Results

3.1 Subconjunctival Tumor Formation Under Slit Lamp Examination

Tumor cells were injected into the subconjunctival space of five rabbits. Of the treated rabbits, all five successfully formed tumors over an average period of 14 days prior to euthanization [Fig. 2(c) and 2(d)]. Likewise, of the five rabbits, small nodular lesions were first found in three rabbits (60%) at day 7 and in two rabbits by 14 days, respectively.

3.2 OCT Examination

Of the five rabbits used in this study, five (100%) revealed a small low scattering area of the subconjunctiva at day 7, resulting in a mass in the next 14 days [Fig. 2(e)]. The average size of tumor at the seventh day was $100 \pm 50 \mu\text{m}$. After that the measurement of tumor size was impossible because OCT had limited imaging depth [Fig. 2(e) and 2(f)].

3.3 Ultrasonographic Examination

All rabbits showed well-marginated hyperechoic nodules of the subconjunctiva by US, which were confirmed by pathology. The average size of tumors at day 14 was $5 \pm 3 \text{ mm}$ [Fig. 3(a)].

3.4 Pathologic Finding

Pathologic examination of VX2 tumors in subconjunctiva revealed characteristics typical of carcinomas. The tumor characteristics ranged from small clusters of malignant cells infiltrating the conjunctival epithelium and underlying sclera to macroscopically visible nodules as large as 5 mm. Histologically, tumors consisted of epithelioid cells arranged in sheets, solid nests, and glandular patterns. All cells had a high nuclear:cytoplasmic ratio with an eosinophilic cytoplasm. The nuclei had small to inconspicuous nucleoli, and were very

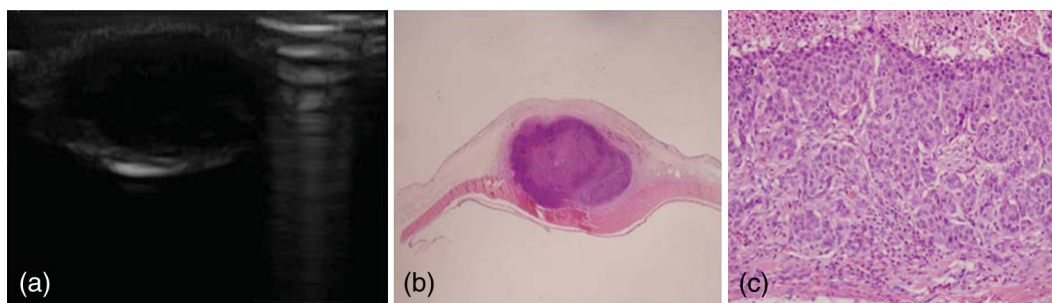


Fig. 3 (a) High frequency ultrasound (US) image (20 MHz, 1 cm of image depth), (b) a pathology of conjunctival tumor, and (c) enlarged pathologic image.

mitotically active with mitotic counts as high as 5 to 6 per high-power field [Fig. 3(b) and 3(c)].

4 Discussion

At present, the majority of advanced stage conjunctival tumors ultimately require subtotal or total exenteration depending on the extent of tumor invasion for definitive treatment.² Therefore, early diagnosis and evaluation of the treatment response of conjunctiva or subconjunctival tumors have become more important than ever to save the eyeball.

This study evaluated the successful development of a reproducible subconjunctival tumor model in rabbits, a model which envisioned several useful areas including cancer detection and treatment investigation. The tumor cell line used in these studies originated from a cultured rabbit VX2 tumor, which shared similar characteristics of a SCC from a conjunctival tumor. The clinical presentation also shared many characteristics of primary conjunctival squamous carcinoma including tumor involvement of the subconjunctiva as well as the sclera.¹² In the five rabbits, clinical deterioration occurred rapidly, after which all animals were euthanized.

The model described in this paper has several advantages. First, site selectiveness of a lesion is achieved by inoculating cell suspension through a subcutaneous injection. Thus, the inoculation is directed to an intended site in the subconjunctiva under OCT guidance, thereby enabling a highly defined tumor location. Second, repeated measurement of tumor size by OCT and US in the present study was important, primarily due to the correlation between tumor size on OCT (US) and the time after inoculation. Therefore, it was possible to evaluate the efficacy of a multimodal method using OCT and US monitoring the invasion of locally advanced conjunctival tumor by measuring the change of tumor nodules without sacrificing treated animals. It was approximately seven days after inoculation that the tumors remained solitary within the subconjunctiva. This result suggested that early detection of early subconjunctival tumors using OCT can be used to study cancer microenvironments like early angiogenesis, early translational processes, and for early therapeutic modalities such as photodynamic therapy and cryotherapy.

In the present study, the observation that OCT has a better detection rate on day 7 than SLE suggested the clinical implication of OCT in detecting early subconjunctival cancer. The assessment of advanced tumor lesion favors the US utility. The penetration depth of OCT was limited to 1 to 2 mm, but the resolution is very high up to 4 μm in air. On the contrary, US had a deeper penetration depth (up to 3 cm with the current setup), but limited resolution. Therefore, the two modalities are

complementary. Based on the above consideration of our study, further evaluation is required to combine the two diagnostics modalities.

5 Conclusion

A solitary subconjunctival tumor model in rabbits was established by subcutaneous administration of cancer cells. This model enables evaluation of serial development of subconjunctival tumors. Further studies are required to determine the optimum amount of inoculated cells and the extent of the experimental period after inoculation.

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