Dynamic imaging in determining the optimum surgical time for near-infrared fluorescence image-guided surgery—a preliminary study

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Abstract

Introduction
Complete resection is essential for improved prognosis in head and neck cancer treatment. However, resection and organ preservation are difficult in advanced cases; particularly when the cancer has diffusely invaded deep organs. Tumours that cannot be touched or observed are often dealt with endoscopic or robotic surgery. In such cases, we propose using the indocyanine green (ICG) fluorescent method for navigation surgery in head and neck cancer.

Objective
The objective of this study was to use dynamic near-infrared fluorescent imaging to determine the optimum surgical time for tumour resection after ICG administration.

Materials and methods
Seven nude mice underwent dynamic ICG fluorescence imaging over 14 days after the implantation of KB cells. After intravenous administration of ICG, dynamic fluorescence images were investigated using an infrared camera. Surgical specimens were histologically evaluated using a laser scanning confocal microscope.

Results
Dynamic near-infrared fluorescence imaging indicated that the greatest contrast in fluorescent images between tumour and normal tissues was observed 6 h after ICG administration. The optimum surgical time was determined to be 3–24 h after ICG administration. Macroscopic imaging of the excise specimens with HEMS corresponded to the microscopic findings and near-infrared laser scanning confocal microscopic imaging.

Conclusion
ICG fluorescence imaging is effective in the detection of head and neck cancer in vivo. Preliminary findings suggest that the optimum surgical time is 3–24 h after ICG administration.

Introduction
Optical imaging using near-infrared (NIR) fluorescence is a new technique that can be utilized to visually observe the operation field in real time during surgery. NIR fluorophores have gained immense interest in various fields of biomedicine because the minimal interfering absorption and fluorescence from biological samples is excised by laser diode, minimal scattering, enhanced tissue penetration depth, and low autofluorescence, thereby providing high-quality contrast. Indocyanine green (ICG) is a water-soluble, anionic, amphiphilic, tricarbocyanine NIR probe with a hydrodynamic diameter of 1.2 nm and excitation and emission wavelengths in serum at 778 nm and 830 nm, respectively. It has been used in ophthalmic angiography and evaluation of cardiac output and hepatic function. More recently, ICG fluorescence imaging has been used for a variety of purposes in oncologic surgery. It can be potentially used for (1) detection of sentinel lymph nodes, (2) evaluation of lymphoedema, (3) microvascular circulation of free flaps in reconstructive surgery and (4) endoscopic marking of colorectal and gynaecological tumours.

The foremost goal of head and neck cancer surgery is the complete resection of the tumour with adequate surgical margins, while minimizing surgical morbidity and preserving organ function. After intravenous administration of ICG, in comparison with normal structures, the vascular hyperpermeability of cancer towards macromolecules results in a higher fluorescence intensity observed during the extravascular phase. In addition, ICG was found to be cleared from the vascular compartment by the liver. Herein, we used ICG with infrared camera imaging (Hyper Eye Medical System, HEMS, Mizuho Medical Co., Ltd., Tokyo, Japan) to observe the differences between normal and cancerous tissues. To our knowledge, this is the first study to describe in vivo application of ICG NIR dynamic imaging for examining its usefulness in head and neck cancer surgery.

Objective
To determine the optimum surgical time for tumour resection after ICG administration in vivo, dynamic ICG fluorescence imaging was used to observe the differences between normal and cancerous tissues.

Materials and methods

Experimental animals and implantation of cancer cells
Oral squamous cell carcinoma KB cell lines were used in this study. Seven female mice, aged 6 weeks (BALB/c A) were used in this study. Seven female mice, aged 6 weeks (BALB/c A), were used in accordance with the Institutional Animal Care and Use Committee guidelines.

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All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.
Materials and methods (Cont.)

KB cells were prepared to $1.0 \times 10^8$ cell/ml in 0.01 mol/L phosphate-buffered saline, and $1.0 \times 10^7$ viable cells were inoculated into the submental area subcutaneously, using a tuberculin syringe with a 27-gauge disposable needle (Terumo, Tokyo, Japan).

Real-time NIR fluorescence imaging

On the 14th day after implantation, the mice were injected with ICG (5 mg/kg, Diagnostgreen, Daiichi Sankyo, Tokyo, Japan) via the tail vein. Dynamic fluorescent images were observed with an infrared camera (HEMS). Fluorescent images were obtained at intervals of 10 min, 30 min, 1 h, 2 h, 3 h, 6 h, 12 h, 24 h and 48 h after the initial injection. All cases were scored according to the NIF imaging visibility score from 0 to 5: 0, lesion was not visible; 1, presence of a lesion that was hardly visible; 2, weak contrast, i.e. the lesion was detectable only when its exact location was known; 3, contrast of the lesion was clearly detectable yet inferior when compared with the contrast of other surrounding structures; 4, contrast of the lesion was similar to that of other surrounding structures and 5, contrast of the lesion dominated the image acquired. Each mouse was administered with ICG two times: at first, this was to evaluate real-time imaging and once the disappearance of fluorescent excitation was confirmed, a second time to excise the bright structures with peri-tumoural structures (e.g. mandibular bone, tongue) at the time of highest contrast.

Histological analysis and laser confocal microscopy

Each sample was immediately frozen after resection. Subsequently, each frozen section was placed on a microscopic slide and stained with haematoxylin and eosin (H&E) for pathological examination. The frozen samples were sectioned into 10 μm slices, and each slice was then counter-stained by VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, USA). Fluorescence images were obtained using a Leica TCS SP5 laser scanning confocal microscope (Leica, Exton, USA).

Results

Real-time NIR fluorescence imaging

On the 14th day after the implantation of KB cells, the developed tumour was detectable by inspection and palpation. After the intravenous administration of ICG under HEMS imaging, fluorescence emissions were immediately detected throughout the body of the mice, and no contrast was observed between the tumour and normal organs. Gradually, the fluorescence emissions lost brightness in normal structures. In our study, the greatest contrast between lesions and normal structures was observed approximately 6 h after ICG administration (Figure 1). All cases were examined according to the subjective visibility score. Scores were highest at 6 h following ICG administration. After 24 h, fluorescence emissions decreased in the tumours, and after 48 h, there was no contrast observable between the tumour and normal structures. The results of our data are summarized in Table 1.

Each tumour was resected under HEMS imaging 6 h after ICG administration. The skin was removed, and the subcutaneous tumour was then identified using HEMS imaging. All tumours displayed bright fluorescence emissions that clearly contrasted with the normal structures (Figure 2).

![Figure 1: Changes in ICG fluorescence images. The mice were injected with ICG (5 mg/kg) via the tail vein, and observation of dynamic fluorescence imaging was started with HEMS. At intervals of 10 min, 30 min, 1 h, 2 h, 3 h, 6 h, 12 h, 24 h, 36 h, and 48 h after injection, bright fluorescent spots were recorded in each period. The greatest fluorescence contrast between tumour and normal tissues was recorded after 6 h.](image1)

ICG fluorescence imaging of excised specimens

Figure 3 displays the gross examination of excised specimens. The tumour mass was saturated with ICG, which had bright fluorescent emissions (Figure 4). We evaluated the borders of both the tumour and normal tissues under light microscopic imaging. In our models, tumour masses and normal structures were clearly separated, as depicted by H&E staining. Following this, laser scanning confocal microscopic evaluation was performed.


Results (Cont.)

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Results of NIR fluorescence imaging as per the visibility score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time after ICG administration</td>
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<tr>
<td>Visibility score</td>
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<td>1</td>
<td>2</td>
</tr>
<tr>
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</tr>
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<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Total score</td>
<td>4</td>
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</tbody>
</table>

**NIR fluorescence imaging visibility score**

0: lesion was not visible
1: contrast indicated the presence of a lesion that was hardly visible
2: weak contrast, i.e. the lesion was detectable only when its exact location was known
3: contrast of the lesion was clearly detectable yet inferior compared with the other surrounding structures
4: contrast of the lesion was similar to that of the other surrounding structures
5: contrast of the lesion dominated the image

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Discussion

ICG is a widely used diagnostic reagent approved for use in the examination of hepatic function and cardiac output\(^7,^8\). The excitation wavelength of ICG is between 750 and 810 nm, and it generates a fluorescence peak wavelength at 845 nm. After intravenous injection of ICG, it quickly binds to globulins, preferentially to a1-lipoproteins, within 1–2 s\(^16\). ICG is not metabolized by the body, and hepatic elimination is the principal route of clearance.

In recent years, sentinel node biopsy with ICG NIR imaging has been extensively used in head and neck cancer treatment\(^17,^18\). Several studies have reported that sentinel node biopsy with ICG NIR imaging produces results that are as good as those of a radioactive tracer. However, there have been no reports concerning the use of fluorescence image-guided surgery combined with endoscopic and robotic surgery for the treatment of head and neck cancer. To our knowledge, this is the first study to describe in vivo application of ICG NIR dynamic imaging for examining its usefulness in head and neck cancer surgery.

Our evaluation of the dynamic imaging of ICG fluorescence emissions suggests that the ICG fluorescence contrast between tumours and normal tissues is maximum at 6 h after ICG administration. We determined that the optimum time for carrying out surgical procedures was between 3 and 24 h after ICG administration in head and neck cancer models, in vivo.

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Figure 2: Gross examination with ICG fluorescence images, and ICG fluorescence yielding visualization of tumour with HEMS. (A) Intra-operative view of oral cancer graft model under white LED light. A large solid mass presented at centre of the submental area. Tumour-invaded tongue (the wiggly circle). (B) Tumour showed strong fluorescence emission with HEMS.
Discussion (Cont.)

Figure 3: Cut surface of macroscopic image with HEMS in excised specimens. (A) Gross examination under white LED light. (B) Under HEMS imaging, tumour showed strong contrast against normal structure (i.e. tongue).

Figure 4: Microscopic features using laser scanning confocal microscope. The tumour masses were surrounded by normal striated muscle (H&E staining). (A) H&E, ×100 high power field (HPF) and (B) H&E, ×400 HPF. Laser scanning confocal microscopic imaging revealed fluorescent emission of tumour and normal structures. Bars = 50 μm. (C) Tumour was saturated with ICG and nuclear-counterstained with DAPI. (D) Tumour was clearly separated from normal tissue as depicted by laser scanning confocal microscope with ICG.

The vascular hyperpermeability of cancer towards macromolecules results in the saturation of fluorescence dyes in neoplastic lesions when compared with normal structures. Under laser scanning confocal microscopic imaging, we clearly observed an accumulation of ICG in tumour masses and no fluorescent emission in normal tissues. However, it was important for us to consider that ICG accumulation may not be limited to cancerous tissues. ICG would also be expected to accumulate in inflammatory tissues and areas of surgical trauma. Some reports have indicated that ICG NIR fluorescence imaging guided surgery is efficient in liver cancer surgery to detect not only large cancers but also micro-cancers. In this study, we have described the usefulness of ICG NIR fluorescence imaging not only in liver cancer surgery but also in head and neck cancer surgery.

In this study, we used a new HEMS hand-held device. HEMS can show ICG-enhanced images of tumours with vivid colour and can be used intra-operatively by surgeons. HEMS comprises a high-sensitivity charge-coupled device (CCD) camera with a custom-made optical filter and non-Bayer colour filter arrays. It can detect visible and NIR rays from 380 to 1200 nm without bias in colour balance at 30 frames per second. We have used HEMS for sentinel node biopsy and fluorescence angiography in daily surgical practice. HEMS is highly sensitive as a colour CCD camera is used for ICG fluorescence imaging.

Considering the high sensitivity, NIR imaging under HEMS is equal to laser scanning confocal microscopic imaging. We suggest that complete tumour resection for even advanced cancer can be conducted using ICG NIR fluorescence imaging. Macropscopic imaging with HEMS can secure adequate tumour-free margins while minimizing surgical...
Discussion (Cont.)
morbidity and preserving organ function. In this study, we demonstrated a successful method for distinguishing cancerous tissues from normal tissues and determining optimum surgical time with HEMS in animal models. These results demonstrate that this method can be applied to head and neck cancer surgery. The application of endoscopic and robotic surgery for oral, pharyngeal and skull base lesions enables minimally invasive surgery with superior results. We need to be able to detect tumours in deeper and invisible areas where palpation is not possible. ICG NIR fluorescence imaging proved effective in tumour detection, which in turn facilitates safe resection of tumours. Further investigations may lead to the development of a new minimally invasive surgical therapy that achieves better prognosis and organ function preservation in head and neck cancer treatment.

Acknowledgement
JY and MF contributed equally to this work. TA and SO conceived the study and participated in its design and coordination. MK and RY drafted the manuscript. JY and KI were involved in revising the manuscript. All authors read and approved the final manuscript. This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, and Technology (22591920) of Japan.

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Consent
Written informed consent was obtained from the patient for publication of this report and accompanying images.

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