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Photodynamic Diagnosis of Hepatocellular Carcinoma Using 5-Aminolevulinic Acid

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Abstract. *Backgtound/Aim: Since hepatocellular carcinoma (HCC) has a high recurrence rate, accurate diagnosis of its location and curative resection is important to improve survival. This study evaluated the utility of photodynamic diagnosis (PDD) using 5-aminolevulinic acid (5-ALA) for HCC. Materials and Methods: We used two human hepatoma cell lines (HuH-7 and Hep G2). Cells were treated with 5-ALA for 4 h. 5-ALA-induced fluorescence was then examined under a fluorescence microscope. We designed hepatoma mouse models, with mice receiving an intraperitoneal injection of 5-ALA. After 4 h, their liver tumors were removed and examined under a fluorescence microscope. We also analyzed 12 HCC patients who underwent curative liver resection. The patients were administered 5-ALA orally before surgery. The excised livers were sectioned and examined by fluorescence microscopy. Results: In vitro and in vivo, red fluorescence of protoporphyrin IX (PpIX) was observed in tumors. In 11 of 12 patients, red fluorescence was observed in their HCC. The tumor of only one patient did not exhibit red fluorescence because it had been necrosed by transcatheter arterial chemoembolization (TACE). Conclusion: Red fluorescence of PpIX was observed in hepatoma cells, tumors of HCC mouse models and HCC of patients. PDD of HCC using 5-ALA is simple and may be useful for real-time diagnosis during liver resection.*

Hepatocellular carcinoma (HCC) is a common malignant tumor in Asian countries (1). Despite recent improvements in diagnostic imaging and therapeutic strategies for HCC (2), the recurrence rate of HCC is still high. Several studies have reported that the recurrence rate of HCC after curative liver resection is 50-60% at 3 years and 70-100% at 5 years (3-9). For long-term survival, complete removal of the tumor is one of the most important factors in surgical oncology. Although the methods have progressed for diagnostic imaging, such as computed tomography (CT) and magnetic resonance imaging (MRI), it is difficult to accurately diagnose small and early-stage lesions preoperatively.

Fluorescence diagnosis has recently received a lot of attention as a powerful tool for early detection of malignant lesions. 5-aminolevulinic acid (5-ALA) is a precursor of heme and metabolized to fluorescent protoporphyrin IX (PpIX). Although PpIX is rapidly metabolized to non-fluorescent heme in normal cells, PpIX accumulates in malignant cells because of increased activity of porphobilinogen deaminase and decreased activity of ferrochelatase (10-14). PpIX emits strong red fluorescence at a peak of 635 nm with blue light excitation at 405 nm. Photodynamic diagnosis (PDD) using 5-ALA has been used clinically in neurosurgery and urology (15-18). We have previously reported that PDD using 5-ALA is useful to detect lymph node metastasis and peritoneal dissemination of colon and gastric cancers (19-22).

In this study, we evaluated the utility of PDD using orally administered 5-ALA for HCC.

Materials and Methods

Cell lines and cultures. Two human hepatoma cell lines (HuH-7 and Hep G2) from the Riken cell bank (Tokyo, Japan) were used in this study. The cells were cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere with 5% CO_2. HuH-7 cells, used for *in vivo* experiments, stably expressed enhanced green fluorescent protein (EGFP) by transient transfection of the *EGFP* gene and selection using 1 mg/ml G418 (Wako Pure Chemical Industries, Osaka, Japan).

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Key Words: Hepatocellular carcinoma, 5-aminolevulinic acid, photodynamic diagnosis, protoporphyrin IX.

Table I. *Patients' characteristics*.

Case	Age (years)	Gender	Virus infection	Pathological diagnosis	Serosal invasion	Vascular invasion	Background liver	Fluorescence
1	61	Male	C+	HCC	s0	vp0, vv0, va0	LC	+
2	71	Male	NBNC	HCC	s0	vp0, vv0, va0	CH	+
3	77	Male	NBNC	HCC	s0	vp0, vv0, va0	CH	+
4	66	Male	C+	Total necrosis	–	–	CH	–
5	52	Female	C+	HCC	s0	vp0, vv0, va0	LC	+
6	51	Male	B+	HCC	s0	vp0, vv0, va0	CH	+
7	68	Female	B+	HCC	s0	vp0, vv0, va0	CH	+
8	65	Male	C+	HCC	s0	vp1, vv0, va0	CH	+
9	48	Male	C+	HCC	s0	vp0, vv0, va0	LC	+
10	67	Male	C+	HCC	s0	vp0, vv0, va0	CH	+
11	66	Female	B+	HCC	s0	vp0, vv0, va0	CH	+
12	73	Male	NBNC	HCC	s0	vp0, vv0, va0	CH	+

NBNC, Non-B, non-C hepatitis; LC, liver cirrhosis; CH, chronic hepatitis; HCC, hepatocellular carcinoma; B+, B hepatitis-positive; C+, C hepatitis-positive.

Fluorescence microscopy analyses. Cells were plated at a density of 1×10^6 cells in 35-mm dishes. After incubation for 72 h, the cells were treated with 1 mM 5-ALA for 3 h and 5-ALA fluorescence (excitation: 440 nm; emission: 575-675 nm) was examined under an inverted fluorescence microscope (IX81; Olympus, Tokyo, Japan).

Animal model. All animal experiments were performed according to the standard guidelines for animal experiments of Kyoto Prefectural University of Medicine, Kyoto, Japan. Five-week-old female BALB/c nude mice were used in this study. EGFP-expressing HuH-7 cells (5×10^6) were injected into the spleen of nude mice under general anesthesia. After 4 weeks, nude mice received an intraperitoneal injection of 250 mg/kg 5-ALA. At 4 h after injection, the mice were sacrificed and their liver tumors were removed and observed by microscopy.

Patients. This study was approved by the institutional ethics committee and performed at the University Hospital of Kyoto Prefectural University of Medicine from November 2012 to April 2014. We analyzed 12 HCC patients who were preoperatively diagnosed by radiological examinations. All patients had undergone curative liver resection at our hospital. The patients provided informed consent preoperatively. The exclusion criteria included the presence of porphyria and a history of allergy.

5-ALA administration. 5-ALA hydrochloride (COSMO BIO Co., Ltd., Tokyo, Japan) at 15-20 mg per kg of body weight was administered orally to each patient 3 h before surgery. After surgery, the excised livers were sectioned and examined under a stereomicroscope.

Fluorescence observations and pathological examination. Fluorescence was observed under a stereoscopic microscope (SZX12; Olympus, Tokyo, Japan) equipped with a color charge-coupled digital (CCD) camera (DP71; Olympus) and mercury lamp (U-LH100HG; Olympus). Emitted light from the mercury lamp was filtered through a 405±10 nm band-pass filter (D405/20x; Chroma Technology Corp., Rockingham, VT, USA) to provide the excitation light. The fluorescent emission at wavelengths longer than 430 nm was transmitted through a long-pass filter (HQ430LP; Chroma Technology Corp.) and detected by the CCD camera. We used a spectral analytical system consisting of a stereoscopic microscope equipped with an intensified multichannel spectrophotometer (MCPD-7000; Otsuka Electronics, Osaka, Japan) for spectral analysis. The pathological diagnosis and classification of resected HCC tissues were performed according to the General Rules for the Clinical and Pathological Study of Primary Liver Cancer (23).

Results

Stereomicroscopic imaging analyses of hepatoma cell lines in vitro. All fluorescence images were obtained under identical conditions, including the photomultiplier voltage, acquisition time and excitation light intensity. As shown in Figure 1, red fluorescence of PpIX was observed in both hepatoma cell lines (HuH-7 and Hep G2) treated with 5-ALA.

Stereomicroscopic imaging analyses of the hepatoma mouse model. To determine whether 5-ALA administration can specifically visualize hepatoma, we employed a mouse model of human hepatoma. This model developed liver tumors that were microscopically visible within 4 weeks after injection of EGFP-expressing HuH-7 cells into the spleen of nude mice. At 4 h after intraperitoneal injection of 5-ALA, mice were sacrificed and their liver tumors were removed. The removed tumors were subjected to both white light and fluorescence imaging. Red fluorescence was observed in tumors with EGFP fluorescence (Figure 2). We confirmed that PpIX had accumulated in the liver tumors and red fluorescence were observed in the mouse model.

Analyses of 12 HCC patients who underwent curative liver resection. The characteristics of the 12 patients are provided in Table I. There were nine men and three women with a

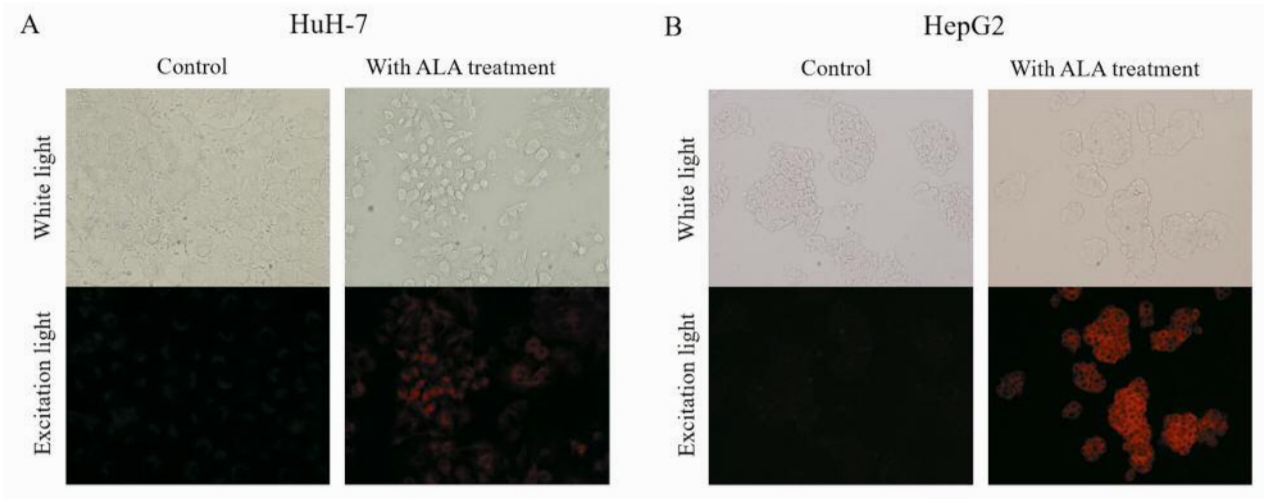

Figure 1. *Stereomicroscopic imaging analyses of hepatoma cell lines (HuH-7: (A); HepG2: (B)) in vitro. Imaging was performed under white light and excitation light (excitation: 440 nm; emission: 575–675 nm). Red fluorescence of protoporphyrin IX (PpIX) was observed in both hepatoma cell lines treated with 5-aminolevulinic acid (5-ALA) under excitation light.*

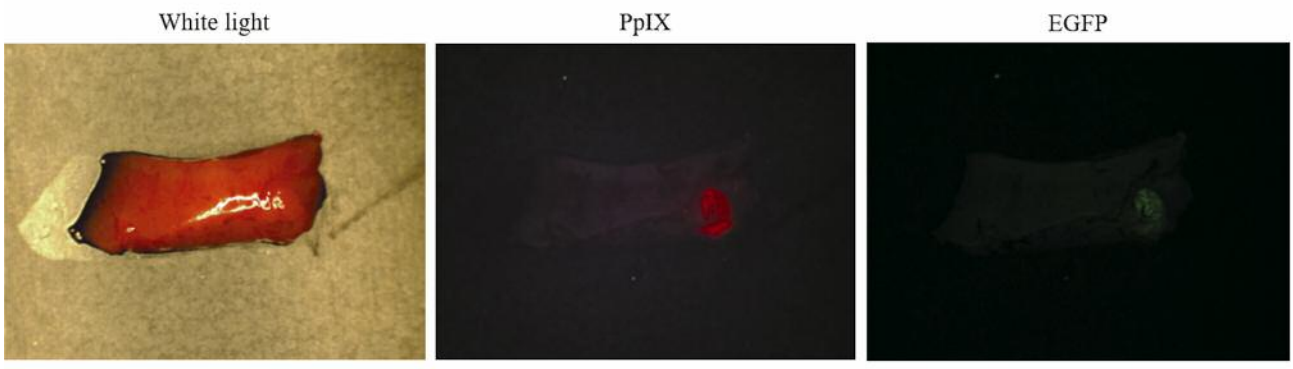

Figure 2. *Stereomicroscopic imaging analyses in the hepatoma mouse model. This model developed hepatomas that were microscopically visible within 4 weeks after injection of enhanced green fluorescent protein (EGFP)-expressing HuH-7 cells into the spleen. Red fluorescence was observed in tumors with EGFP fluorescence.*

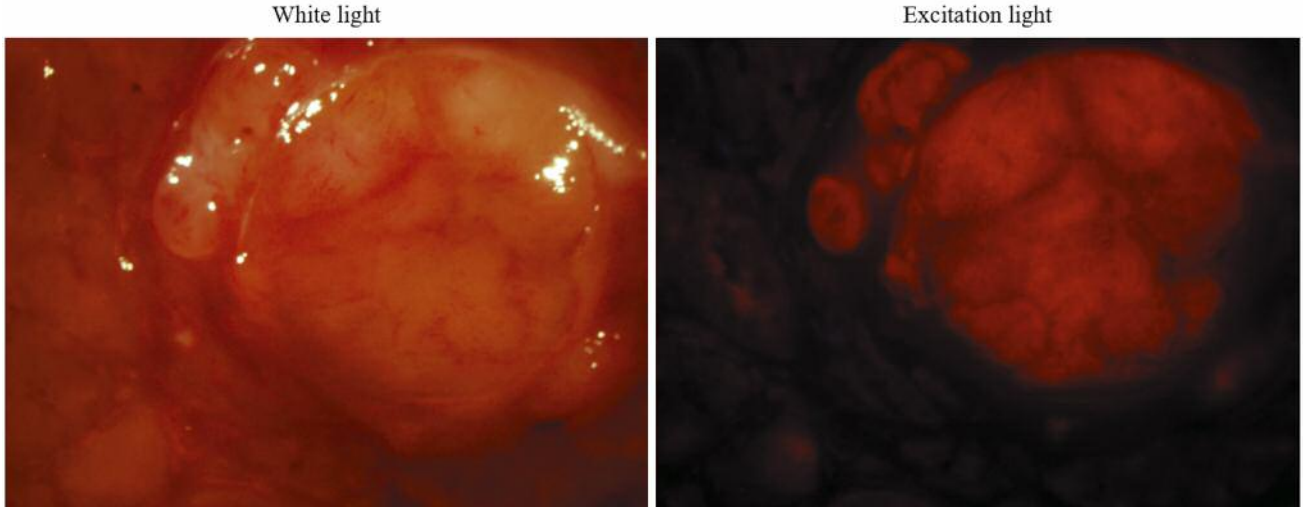

Figure 3. *Red fluorescence was observed at the incision surface of resected livers, which was parallel with the location of the tumor. Imaging under white light and excitation light.*

mean age of 63.8 years (range=51-77). Three patients had type B viral hepatitis and six patients had type C viral hepatitis. Except for one patient, all patients had undergone laparoscopic surgery. In 11 of the 12 patients, red fluorescence was observed at the surface of the incision in the resected liver, which was parallel with the location of the tumor (Figure 3). Fluorescence spectra with a peak of 635 nm were observed in these tumors using a spectral analytical system (Figure 4), which corresponds to the spectra of PpIX. Pathological diagnosis of the 11 patients who showed red fluorescence was HCC. The tumor of only one patient who underwent transcatheter arterial chemoembolization (TACE) did not show red fluorescence because their tumor had necrosed completely. Pathological examination revealed vascular and bile duct invasion in the tumor of one patient. Nine patients had chronic hepatitis and three patients had liver cirrhosis. Red fluorescence was detected regardless of vascular invasion, bile duct invasion and the liver background. Significant side-effects were not observed in this study.

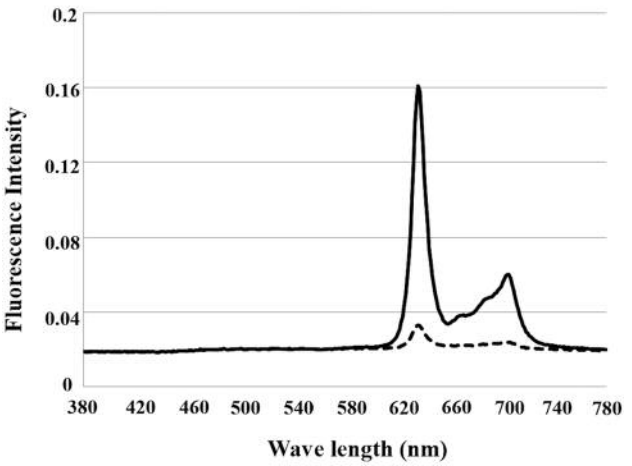

Figure 4. *Spectral analysis of the tumor and the background. Solid line indicates the fluorescence spectra in the tumor and dotted line indicates the background fluorescence spectra of the liver. Fluorescence with a peak of ~635 nm was also observed in these tumors using a spectral analytical system, which corresponds to the spectra of protoporphyrin IX (PpIX).*

Discussion

Recently, hepatic resection of HCC has improved significantly because of better surgical techniques and perioperative management. However, despite all these efforts, the long-term survival of HCC patients after surgery is still poor because of the high incidence of recurrence. The most common cause of the high recurrence rate after curative resection of HCC is intrahepatic recurrence. Whether intrahepatic recurrence after resection of HCC is the result of intrahepatic metastasis or multicentric occurrence of a new tumor in the liver remnant is under debate. In either case, complete removal of the tumor is one of the most important factors for long-term survival. To remove the tumor completely, correct and accurate diagnostic imaging technology is essential. Recently, the diagnosability of HCC has been improved by dynamic CT and gadoxetic acid-enhanced MRI. However, there is no consistent opinion of the efficacy of diagnostic radiologic technology for small and early-stage lesions.

Imaging methods using fluorescence are clinically useful because they are simple, rapid and easily applicable. Various fluorescent probes have been used in such methods. One of the most common and long established fluorescent probes is indocyanine green that has been particularly applied to detection of liver tumors during hepatectomy (24, 25). This probe has good depth resolution, light intensity and sufficient safety. However, it has the greatest disadvantage of not being specific for cancer. Furthermore, although various cancer-specific fluorescent probes have been developed, clinically useful fluorescent probes are limited because these probes are not safe for use in patients.

In this study, the red fluorescence of PpIX was observed in hepatoma cells *in vitro* and *in vivo*. Furthermore, we observed red fluorescence in HCC patients that had undergone hepatectomy. We demonstrated that 5-ALA is a sensitive and effective photosensitizer for HCC. This is the first report to evaluate the effectiveness of PDD using 5-ALA for HCC *in vivo*, *in vitro* and in HCC patients. Although some previous reports have demonstrated the effectiveness of 5-ALA-PDD for other organs, there is little evidence of the utility of ALA-PDD for liver tumors. Here, we have reported the potential of 5-ALA-PDD for HCC in hepatoma cell lines, animals and clinical specimens.

5-ALA has good tumor specificity and few side-effects. Previously, 5-ALA-PDD has been used to diagnose brain tumors, such as glioma and bladder cancer (15-18). In this study, we observed the red fluorescence of 5-ALA in the specimens of all HCC patients except for one patient with a completely necrotic HCC induced by TACE. The sensitivity of 5-ALA for HCC imaging was 100%.

A disadvantage of ALA-PDD is that the depth of excitation light is shallow and unsuitable for observations of deep tissue. The depth of light penetration into tissue at 630 nm, which is used for PpIX, ranges from 0.2 to 2 cm (26, 27). We have reported previously that diagnostic methods using this wavelength of light is suitable for diagnosis of peritoneal and lymph node metastasis (19-22). However, it is difficult to diagnose tumors in deep areas of parenchymatous organs.

The intrahepatic recurrence rate after resection of HCC, especially HCC complicated by cirrhosis, is extremely high.

Therefore, it is important to diagnose small and early-stage lesions (3). Furthermore, real-time diagnosis during liver resection plays a major role in complete curative resection because it is difficult to diagnose small and early-stage lesions preoperatively. Inoue *et al*. (28) detected 5-ALA fluorescence in all HCCs (100%) with serosa invasion (n=37) during liver resection. In addition, the positive microscopically margin rates in terms of the resection margin width using 5-ALA-mediated PDD were lower than those using the classical method. Thus, ALA-PDD may be useful to detect small and early-stage lesions at the liver surface during liver resection.

In conclusion, we observed the red fluorescence of PpIX in HuH-7 and Hep G2 cell lines, a hepatoma mouse model and specimens from HCC patients. PDD using 5-ALA for HCC is simple and may be useful for real-time diagnosis during liver resection.

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