

## Clinical Trial of Photodynamic Therapy Using Acridine Orange with/without Low Dose Radiation as New Limb Salvage Modality in Musculoskeletal Sarcomas

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**Abstract.** Most patients with musculoskeletal sarcoma do not recover satisfactory limb function after limb salvage surgery. To achieve satisfactory improvement of limb function, we developed a unique surgical modality of photodynamic therapy using acridine orange (AO-PDT) and clinically applied it to patients with musculoskeletal sarcomas. Ten patients with primary musculoskeletal sarcomas were enrolled in the study. Of these, 6 had primary malignant soft tissue sarcoma and 4 had primary malignant bone tumor. In the AO-PDT procedure, intralesional or partially marginal tumor excision was initially conducted and microscopic curettage of the remnant tumor, which emitted green fluorescence under blue excitation after local administration of 1µg/ml AO solution, was performed using a fluorescence surgical microscope. Subsequently, blue light illuminated there for 10 minutes. The surgical wound was closed, followed by immediate X-ray irradiation of the resected area with 5 Gy in 5 out of 10 patients to enhance the effect of AO-PDT. The follow-up of the patients ranged from 24 to 48 months. All the patients (AO-PDT alone: 5, AO-PDT with 5-Gy radiation: 5) are alive; only one patient showed local recurrence of the tumor. The recurrence rate was 10%. None of the 5 patients treated by AO-PDT with radiation developed local tumor recurrence. The limb function in all the patients, except for one, recovered to the level before

surgery. None of the patients clinically showed any local or systemic complications. AO-PDT may be a promising new limb salvage modality for preservation of excellent limb function in patients with musculoskeletal sarcoma.

Limb salvage surgery with wide tumor resection followed by limb reconstruction using various types of endoprostheses, bone allograft or autograft for the treatment of musculoskeletal sarcomas has advanced remarkably over the last 30 years (1-5). However, recovery of limb function has not yet been satisfactorily achieved, and most of the treated patients are still not capable of running or swimming fast, jumping well, or throwing a ball far (6-8). Since these disabilities markedly interfere with the quality of life of the patients, methods for achieving satisfactory recovery of limb function after limb salvage surgery urgently need to be explored.

Recently, we reported that acridine orange (AO) had a strong cytotoxic effect after blue light illumination or low dose (5 Gy) of X-ray radiation, both *in vitro* and *in vivo* on mouse osteosarcoma cells (9-11), and suggested that AO might be useful for photodynamic therapy in musculoskeletal sarcomas. AO is also useful for microscopic curettage because of its tumor-specific accumulation and fluorescence emission (fluorovisualization effect) (12).

In this study, we investigated the usefulness of intralesional or partially marginal tumor excision supported by our unique surgical modality of the AO-PDT procedure including fluorescence-microscopic curettage using the fluorovisualization effect, with/without 5-Gy radiation, in patients with musculoskeletal sarcoma, to preserve, as far as possible, the functions of muscles, nerves, vessels and bones that are in close contact with the tumor, and obtain excellent recovery of limb function with a low risk of local recurrence and a good prognosis.

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Table I. Characteristics of patients.

Case No.	Diagnosis	Location Size (cm)	Age Sex	Stage	Follow-up period	Prognosis	Recurrence
1	Rhabdomyosarcoma	Plantar 6 X 5	11(M) F	III*	48(M)	NED	21(M)
2	Synovial sarcoma	Wrist 4 X 3	31(Y) F	IIC*	41(M)	CDF	no
3	Synovial sarcoma	Knee 3 x 3	22(Y) M	IIB*	37(M)	CDF	no
4	Synovial sarcoma	Thigh 8 X 4	38(Y) F	III*	35(M)	CDF	no
5	Ewing's sarcoma	Ilium 10 X 10	14(Y) M	IB**	30(M)	CDF	no
6	Parosteal osteosarcoma	Radius 8 X 6	25(Y) M	III**	28(M)	CDF	no
7	Synovial sarcoma	Thigh 6 X 6	7(Y) F	III*	28(M)	CDF	no
8	Osteosarcoma	Humerus 8 X 6	11(Y) F	IIB**	24(M)	CDF	no
9	Chondrosarcoma	Ilium 15 X 20	48(Y) F	IIB**	25(M)	CDF	no
10	Alveolar soft part sarcoma	Thigh 6 X 5	53(Y) F	III*	25(M)	CDF	no

\* GTNM staging system for soft tissue sarcoma

\*\* Enecking's surgical staging system for bone tumor

Cases 1, 3, 4, 5, 6: AO-PDT, Cases 2, 7, 8, 9, 10: AO-PDT with 5-Gy radiation.

Cases 1, 2, 3, 5, 9, 10: intralesional excision, Cases 4, 6, 7, 8: marginal resection.

## Materials and Methods

**Patients' profile.** From July 1999 to September 2001, 10 patients with primary malignant tumors of the bone or soft tissues who had no distant metastases, were recruited for this study at the Department of Orthopaedic Surgery of the University Hospital in Kyoto Prefectural University of Medicine, Japan (Table I). The patients comprised 4 males and 6 females, with an average age of 21 years (11 months to 53 years). Histologically, 6 of the 10 patients were diagnosed to have primary malignant soft tissue sarcomas, including 4 with synovial sarcomas, one with rhabdomyosarcoma, and one with alveolar soft part sarcoma, while 4 were diagnosed to have primary malignant bone tumors, including 2 with osteosarcoma (one conventional and one parosteal (grade 2) osteosarcoma), one with Ewing's sarcoma, and one with chondrosarcoma (grade 2). Three of the 6 soft tissue sarcomas arose from the thigh, one from the plantar aspect of the foot, one from the wrist, and one from the knee. Five of the 6 soft tissue sarcomas had a maximum diameter more than

5 cm and were deep-seated. Four were classified as stage III and 2 as stage IIB, IIC, according to the American Joint Commission on Cancer GTNM Classification and stage grouping of soft tissue sarcomas. Two of the 4 bone tumors arose from the ilium, one from the shaft of the radius, and one from the proximal metaphysis of the humerus. Three were classified as stage IIB, but one was IB by Enneking's surgical staging system for malignant bone tumors. The follow-up of the patients ranged from 24 to 48 months (average, 32 months). Of the total of 10 patients, 8 received pre- and post-operative chemotherapy (vincristin, adriamycin and cyclophosphamide (VAC) for rhabdomyosarcoma or Ewing's sarcoma; ifosphamide and adriamycin for synovial sarcoma; cisplatin and adriamycin for osteosarcoma), while the remaining 2, consisting of one with chondrosarcoma (Case 9) and the other with alveolar soft part sarcoma (Case 10), did not receive chemotherapy. All the lesions except for one were treated primarily at our hospital. Case 3 with synovial sarcoma had a recurrent lesion after intralesional tumor resection at another hospital.

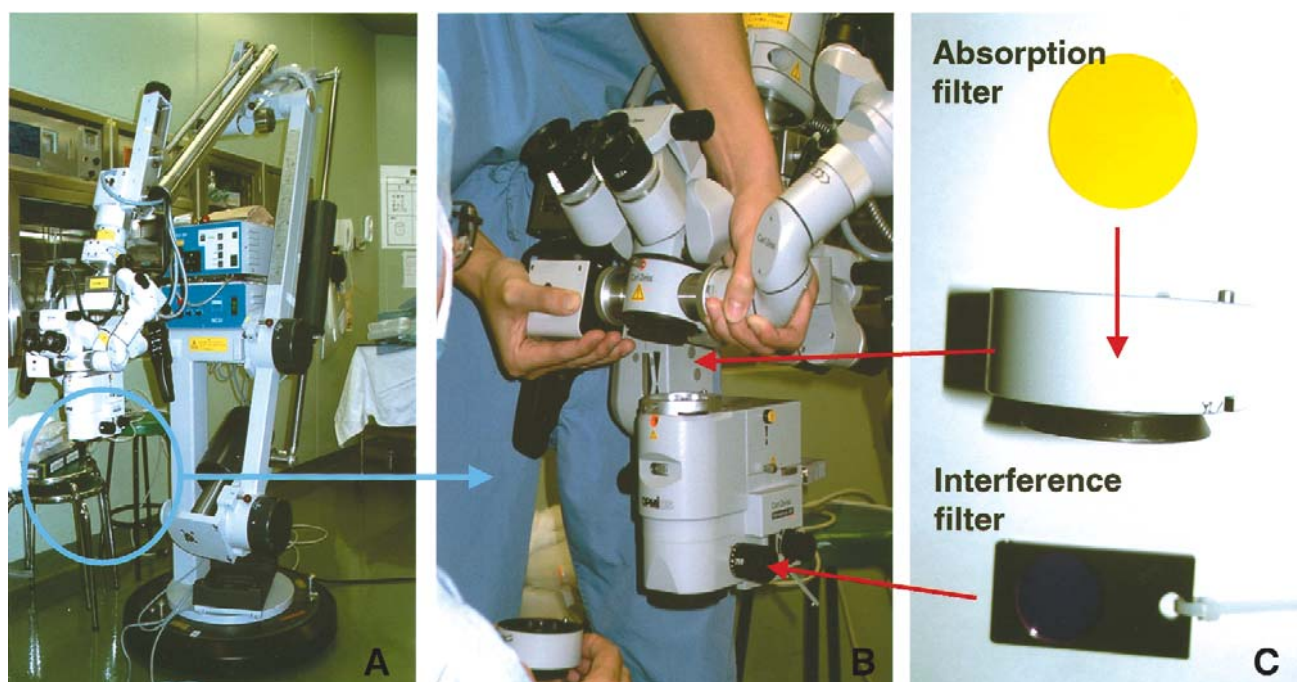


Figure 1. The fluorescence surgical microscope used in this study. A: The whole body of the fluorescence surgical microscope manufactured by Carl Zeiss Co. Ltd.. B: Filter insertion parts. C: Absorption (>520 nm) and interference (466.5 nm) filters.

**Procedure for AO-PDT with/without low dose radiation.** For Cases 1, 2, 3, 5, 9 and 10, intralesional tumor excision, similar to the conventional macroscopic curettage for benign bone or soft tissue tumors, was mainly performed, and for Cases 4, 6, 7 and 8, marginal resection of the tumor with partial intralesional excision was mainly performed. These procedures were applied, with the aim of minimizing, to the maximum extent possible, the damage to intact muscles, bones, or major nerves and vessels in close contact with the tumor, thereby obtaining good limb function after surgery. In the next step, microscopic curettage with an ultrasonic surgical knife (Olympus Co. Ltd., Tokyo, Japan) was performed additionally using a fluorescence surgical microscope, under observation of AO green fluorescence from remnant tumor fragment after local administration of 1  $\mu\text{g}/\text{ml}$  of AO solution (Sigma-Aldrich Co., St. Louis, MO, USA) for 5 minutes, followed by washing out of the excess AO solution with saline and by excitation with blue light (Figure 1). The microscope was equipped with an interference filter (466.5 nm) for selection of the blue beam from a Xenon lamp, and an absorption filter (> 520 nm) for observation of the green fluorescence of AO under an ordinary surgical microscope manufactured by Carl Zeiss Co., Ltd. (Oberkochen, Germany). After repeated sessions of microscopic curettage until complete disappearance of green fluorescence from the remnant tumor mass (Figure 2), AO-PDT was applied to the tumor curettage area by illumination with blue light (5000 lx) for 10 minutes, again using the fluorescence surgical microscope. After closure of the surgical wound without washing out the AO solution, X-ray irradiation of 5 Gy in a single session was applied to the resected area immediately in 5 patients (Cases 2, 5, 7, 8, 9) in the radiotherapy room. All the 5 patients gave their consent prior to the therapy. The surgical procedures are schematically summarized in Figure 3.

The conditions of AO concentration, light illumination time and lux, and the radiation dose were decided based on the data obtained from our basic studies using a mouse model (9-12). Noteworthy, irradiation with 5 Gy was proven to be sufficient to totally kill osteosarcoma cells *in vitro* (11).

**Ethical verification.** This clinical trial was officially certified by the ethical committee of the Kyoto Prefectural University of Medicine, Japan. Each patient and also a close family member gave their consent for the AO-PDT with/without 5-Gy radiation after a full explanation of the method and purpose of the study (informed consent).

**Study design.** Before the AO-PDT with/without 5-Gy radiation, we investigated the sensitivity of each of the sarcomas to AO by using fresh biopsy specimens, which were exposed *ex vivo* to 1  $\mu\text{g}/\text{ml}$  of AO solution. The therapy was administered only to those patients whose sarcoma specimens were sensitive to AO.

We principally applied this treatment to patients selected using the following criteria: patients expected to have serious deficit of limb function after wide tumor resection and limb reconstruction, or needing amputation; patients with a large tumor that could not be removed even by wide resection; patients with a high risk of death following wide tumor resection.

**Evaluation of the clinical outcome.** Local recurrence of tumors was evaluated by various imaging methods, such as computed tomography (CT), magnetic resonance imaging (MRI) and bone or thallium scintigraphy, and the local recurrence rate was calculated. Limb function after surgery was evaluated by the ISOLS criteria

(13). Additionally, local and systemic complications induced by AO administration, AO-PDT and AO-RDT were checked for by clinical symptoms and blood examination.

## Results

All the tumor specimens studied were determined to be sensitive to AO, based on their emitting green fluorescence after *ex vivo* exposure to AO solution and blue-light excitation.

Oncologically, all the patients enrolled in this study are alive at the time of writing, without any evidence of metastatic disease. Among all the patients who were followed up for more than 2 years, local recurrence of tumor was detected in only one patient with rhabdomyosarcoma arising from the plantar aspect of the foot (Case 1). The local recurrence rate was thus 10%. However, none of the 5 patients who received AO-PDT with 5-Gy radiation developed local recurrence. The limb function of all the patients recovered to the level before surgery, except in one patient with a humeral osteosarcoma (Case 8); the recovery was, therefore, evaluated to be 100% by the ISOLS criteria in which sports activity is not needed. Even the 2 patients with a malignant bone tumor of the pelvis are able to walk well without any support.

None of the patients clinically showed local or systemic complications that could be caused by AO administration, AO-PDT with 5-Gy radiation.

**Case presentation.** Case 1 was 11 months old and had a rhabdomyosarcoma arising from the plantar aspect of the foot. This was probably the first human case in the world to which AO-PDT was applied for treatment. MRI revealed a large tumor mass arising from the plantar aspect of the foot, invading the interphalangeal muscles (Figure 4A). However, intensive chemotherapy with multi-anticancer agents was effective, and caused a marked decrease in the size of the tumor (Figure 4B). Therefore, secondary biopsy was performed to histologically investigate the tumor cell viability in the shrunk tumor. Histopathological examination revealed the existence of living tumor cells and *ex vivo* AO exposure also showed viable tumor cells emitting green fluorescence between muscle bundles (Figure 5), indicating that the tumor cells were sensitive to AO. Since the parents of the infant stoutly rejected the suggestion of limb amputation, we conducted tumor curettage supported by AO-PDT, as described above. Low dose radiation was not applied, because irradiation induces growth inhibition of the foot bones. MRI at 12 months after surgery showed no evidence of local tumor recurrence (Figure 6A). Three months after AO-PDT, the infant started to walk without any complications and, later, she could also run well. However, MRI at 21 months unfortunately indicated a local tumor recurrence (Figure 6B). A below knee amputation had then to be conducted, followed by repeated intensive chemotherapy, and the child is now alive with no evidence of disease.

The next case (Case 2) was a 31-year-old female patient who had a synovial sarcoma arising from the wrist. Preoperative MRI revealed that the tumor mass was in close contact with the flexor tendons and ulnar nerve (Fig 7A). If wide tumor resection had been performed for this lesion, severe dysfunction of finger motion and sensation would have been unavoidable, even after reconstruction of the tendons and nerve. Therefore, we suggested tumor curettage supported by AO-PDT with 5-Gy radiation, to the patient and her family, for preserving finger and wrist joint function. After obtaining informed consent, AO-PDT with radiation was performed. Histopathological examination of the resected tumor revealed that there were many remnant tumor cells at the tumor margin (Figure 8). Because all of the flexor tendons and ulnar nerve were completely preserved, the motor function of all the fingers and wrist joint was normally maintained and no sensory disturbance of the hand was noted. Importantly, no tumor recurrence appeared even at 4 years after the surgery (Figure 7B).

The final case (Case 9) was a patient with a huge-sized chondrosarcoma arising from the iliac bone (Figure 9). Histological findings indicated grade 2 malignant chondrosarcoma. It is commonly accepted that the prognosis of pelvic chondrosarcoma is poor, even after wide resection, because tumor embolism in the vein frequently occurs, depending on the histological grade of the malignancy (14). In this case, to perform a wide tumor resection would have been difficult, even with hemipelvectomy and, had it been performed, the limb function would have been poor and the patient would not have been able to walk. Therefore, we recommended applying AO-PDT with 5-Gy radiation to this patient after a full explanation. Both the patient and the family agreed to the treatment. At 25 months after surgery, at the time of writing, the patient still remains without tumor recurrence (Figure 10), without any complications and with no evidence of disease whatsoever. She can walk fast at present and has been able to join the same office that she worked in before the surgery.

## Discussion

Acridine orange, AO, is a weak basic dye used for staining and has many unique biological activities, as previously reported, such as antitumor activity (15, 16), photosensitizing activity (17, 18), and toxic activity in bacteria, malarial parasites and fungi (19-22). It has a very low molecular weight (M.W. 463). It has also been reported that AO has the ability to rapidly flow into the cytoplasm through the cell membrane and bind to DNA (23), RNA (24) and lysosomes (25); however, our basic studies have revealed that AO binds mainly to RNA and not so avidly to DNA, emitting green fluorescence after blue light excitation in viable cultured mouse osteosarcoma cells, and also that it binds densely to lysosomes, emitting orange fluorescence (26). Because mouse osteosarcoma cells transplanted into the mouse emitted green



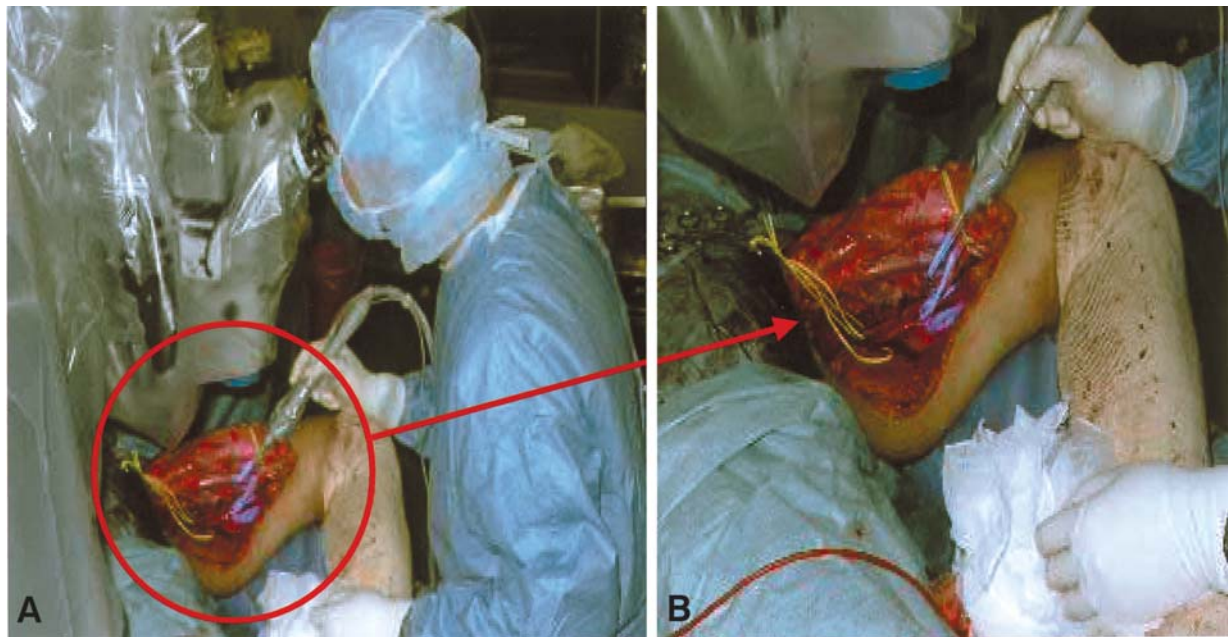


Figure 2. Microscopic curettage after AO exposure and blue light illumination with an ultrasonic surgical knife in a patient with synovial sarcoma involving the femoral artery and vein as well as the sciatic nerve, using the fluorescence surgical microscope shown in Figure 1. The tumor was marginally resected for the most part, but the portion in contact with vessels or nerves was curetted, and additional microscopic curettage was performed under fluorovisualization with AO, followed by AO-PDT.

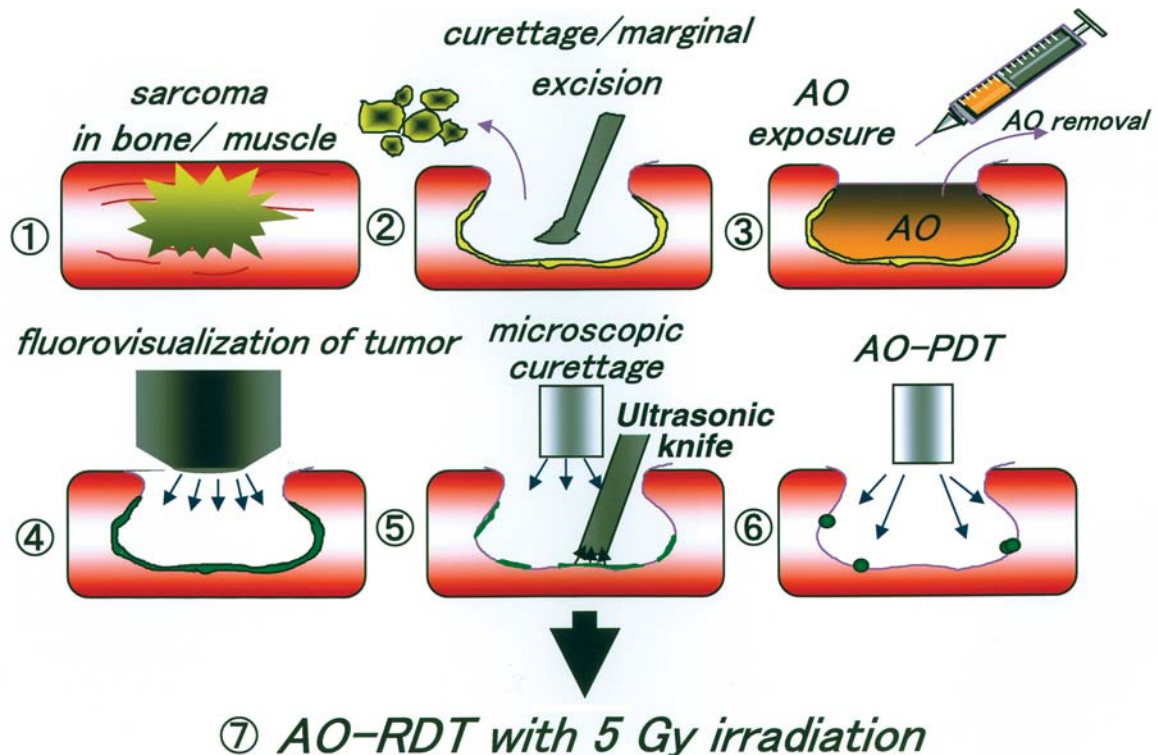


Figure 3. Schema showing the entire procedure of AO-PDT with AO-RDT, for clinical application to human musculoskeletal sarcomas. 1. Sarcoma localized in the muscle or bone. 2. Macroscopic curettage. 3. Local administration of 1  $\mu$ g/ml AO solution for exposure and removal of excessive AO solution, followed by washing out with saline. 4. Blue excitation of AO for fluorovisualization under a fluorescence surgical microscope. 5. Microscopic curettage using an ultrasonic surgical knife under visualization of green fluorescence from the remnant tumor. 6. AO-PDT by blue light illumination for 10 minutes. 7. AO-RDT by X-ray irradiation with 5 Gy.

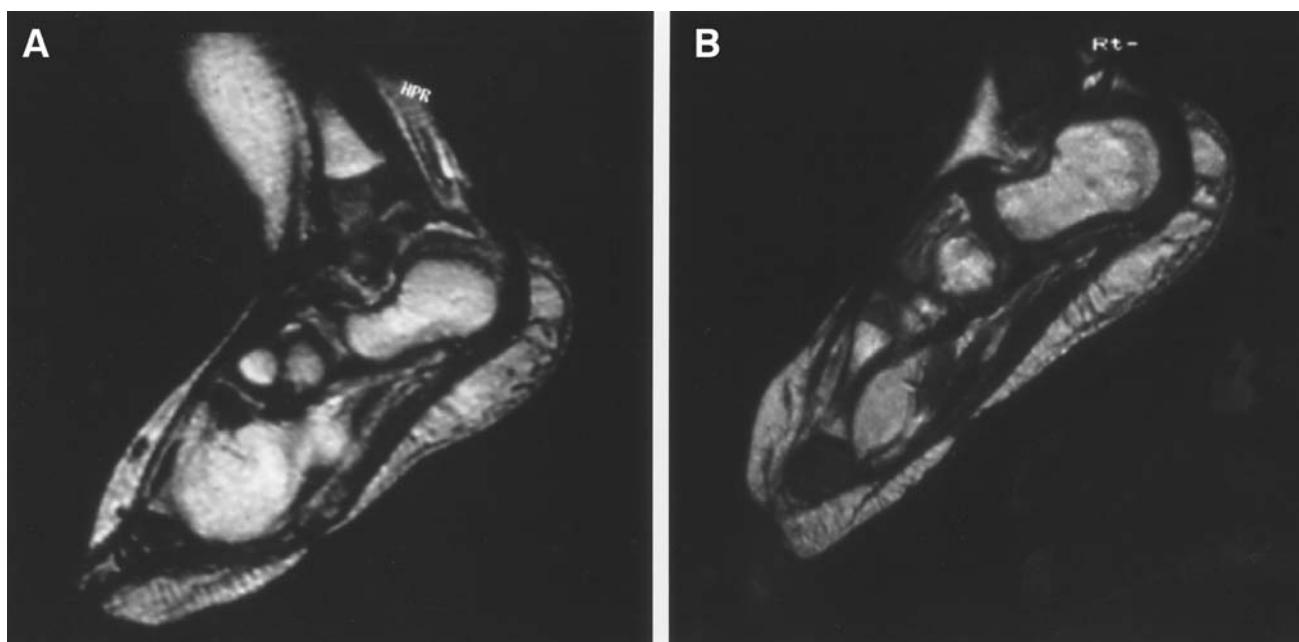


Figure 4. MRI findings of rhabdomyosarcoma (Case 1). A: Before preoperative chemotherapy, a sagittal T2-weighted image depicted the tumor as a high signal intensity localized in the plantar region of the foot, involving muscles and metatarsal bones. B: After preoperative chemotherapy, the tumor size was remarkably decreased.

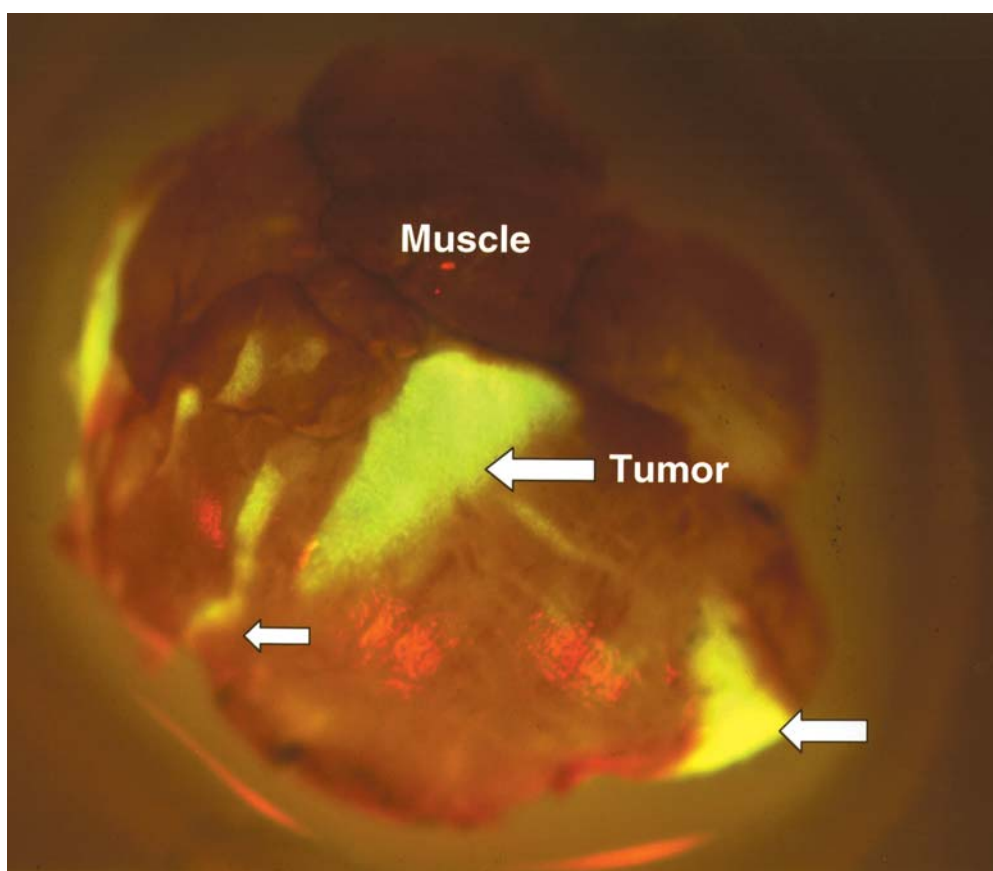


Figure 5. Fluorescence-view from fresh remnant tumor cells invading muscle bundles obtained from the shrunk tumor tissue of Case 1, after preoperative chemotherapy, under a fluorescence surgical microscope following AO exposure and blue excitation. The remnant rhabdomyosarcoma cells emit clear green fluorescence (arrows) between the muscle bundles.

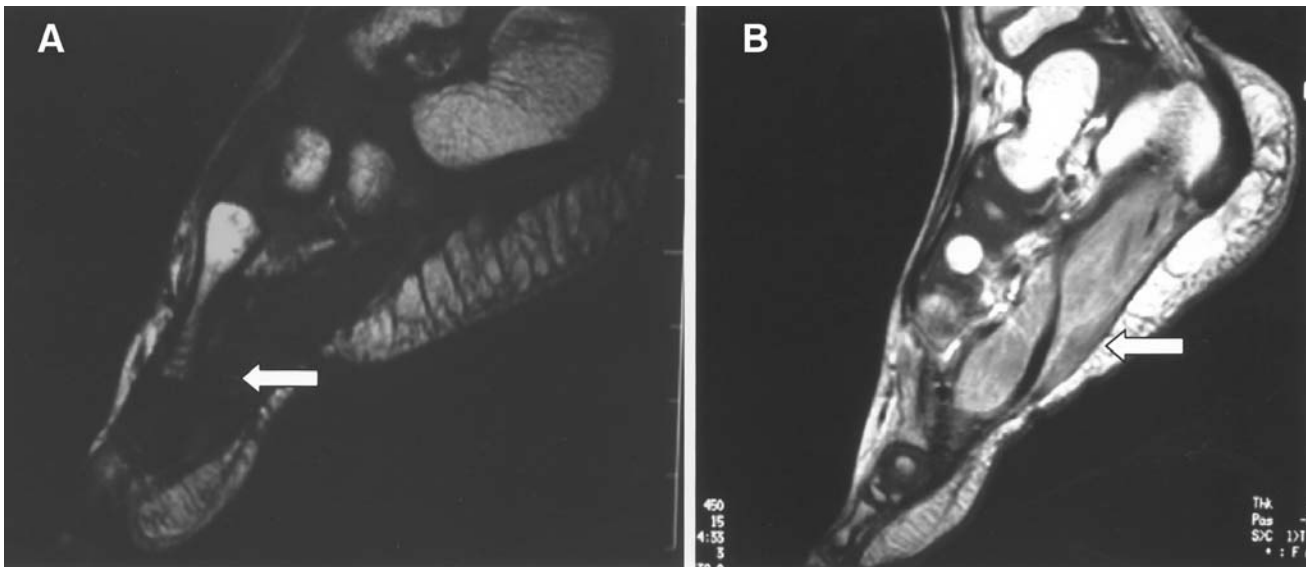


Figure 6. MRI findings after AO-PDT in Case 1. A: Sagittal T2-weighted image at 12 months after AO-PDT showing no recurrence of tumor (arrow). B: Sagittal T1-weighted image after enhancement with gadolinium demonstrates a recurrent tumor (arrow).

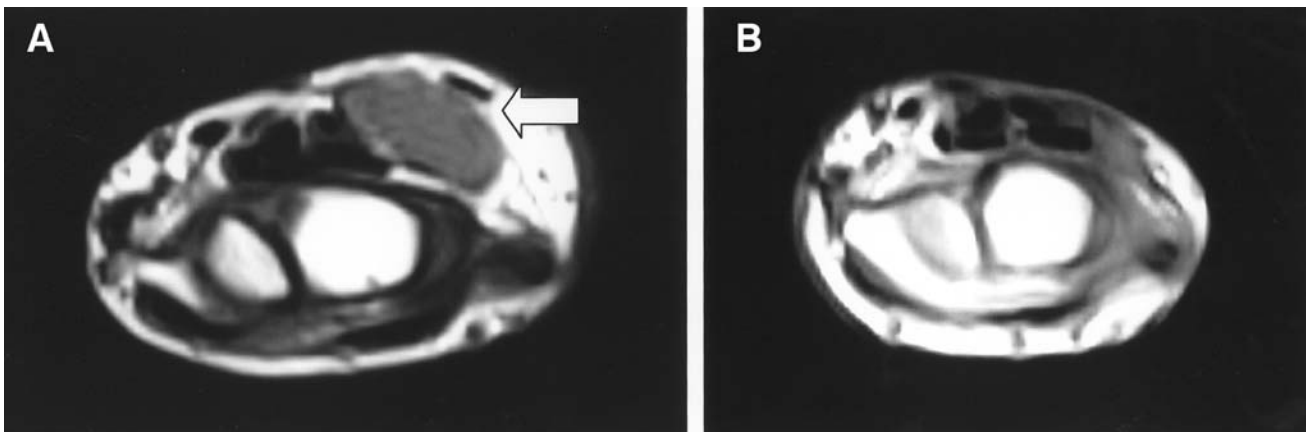


Figure 7. MRI findings of a patient with synovial sarcoma (Case 2) arising from the wrist. A: Coronal T1-weighted image at the wrist joint before surgery shows that the tumor is in close contact with the flexor tendons in the carpal tunnel, ulnar nerve, and blood vessels. B: Coronal T1-weighted image at 48 months after AO-PDT with AO-RDT indicates that there is no tumor recurrence.

fluorescence after intraperitoneal injection of AO followed by blue excitation, while normal muscle and adipose tissue cells did not, the tumor could be visually localized under the fluorescence surgical microscope (fluorovisualization effect) (12). We have confirmed that most human malignant bone and soft tissue tumors are sensitive to AO staining, because the surgically resected tumor specimens emit intense green fluorescence after exposure to AO solution and blue excitation. Although the mechanism underlying selective AO binding to musculoskeletal sarcomas is not clear yet, AO staining is useful for visual localization of the tumor during surgery under fluorescence microscope. We also found that AO had a strong cytotoxic effect on mouse osteosarcoma

cells after blue light illumination, both *in vitro* (9) and *in vivo* (10). This result suggested that AO might be useful for photodynamic therapy of musculoskeletal sarcomas. Many experimental studies previously reported that AO has properties as a photosensitizer and is useful for photodynamic therapy of cancer (27-31); however, there are, as yet, no reports of the clinical application of AO in cancer therapy. Although the reasons for this are not clearly understood, it is likely that investigators are wary of the potential toxic effects of AO, because AO has been reported to exert mutagenic activity on bacteria (19, 20). However, the carcinogenicity of AO has never yet been experimentally proven (32). An International Agency for Research on



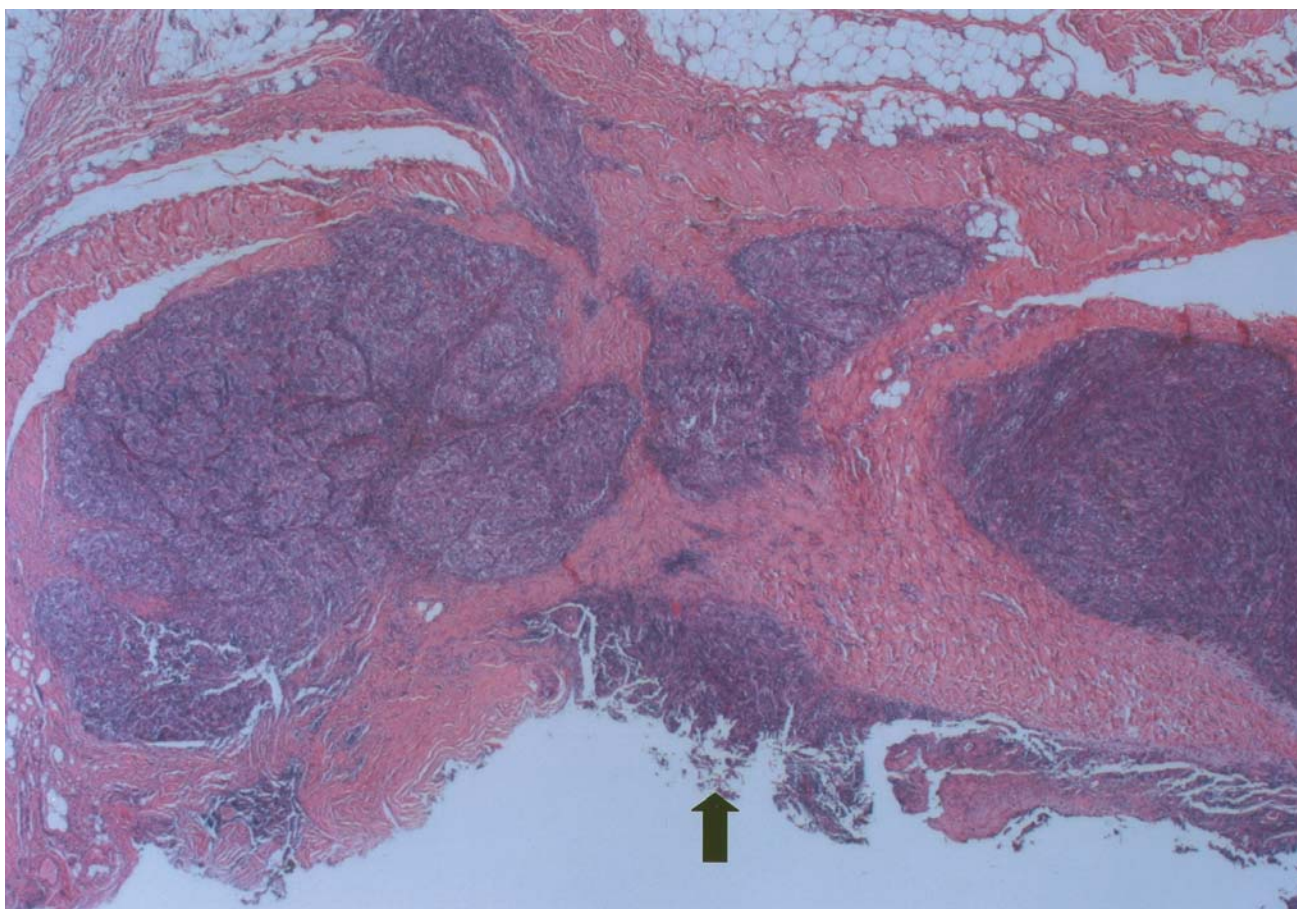


Figure 8. Histology of the resected tumor from Case 2 (H&E stain; objective lens x 4). Because many tumor cells were seen invading the fibrous tissue surrounding the tumor mass (arrow), the surgical margin of the tumor was histologically evaluated to be intralesional.

Cancer (IARC) report (33) in 1978 classified AO into Group 3, which means that the agent is not classifiable as to its carcinogenicity to humans. Local administration of AO to human subjects for gastric and cervical cancer screening has been reported (34), but none of the subjects have developed new cancer induced by AO. Since the concentration of AO solution used by us in this clinical study was very low, and AO was administered only locally, we believe that the risk of carcinogenesis induced by AO in our patients was probably significantly lower than that by most other known anticancer agents. Although for photodynamic therapy using porphyrin or its derivatives, which is commonly administered for various cancers (35), a laser beam which has high energy focused over a narrow area is commonly used as the excitation light source, we used a high-power Xenon lamp, because illumination of blue light over a wide area is necessary for the fluorovisualization effect of AO, as well as for a strong cytotoxic effect of AO-PDT on remnant tumor cells which are widely spread throughout the surgical field by curettage. In addition, a Xenon lamp is also much cheaper than a laser.

Our latest study (unpublished data) has also revealed that the cytotoxic effect of AO-PDT is dependent not only on the wavelength (blue light: 466.5 nm), but also on the lux value of the light. Therefore, while blue light needs to be used for microscopic curettage, for AO-PDT, full-wave light obtained from the Xenon lamp without an interference filter is more effective than blue light alone, because of the 10- to 100-fold higher lux of the former. Since the light emitted from a Xenon lamp contains a small amount of ultra-violet or ultra-red light, the cytotoxicity on normal tissues is minimized. We have, therefore, slightly modified the methodology of AO-PDT during the last 2 years by using full-wave light.

Before clinical application of AO-PDT to human sarcomas, we performed a simulation study of curettage supported by AO-PDT, using a mouse model (10). The results showed that AO-PDT after macroscopic and microscopic curettage of a mouse osteosarcoma significantly inhibited local tumor recurrence. While the recurrence rate was 80% in the control group, it was 23% in the treated group receiving AO-PDT. Furthermore, we also found that



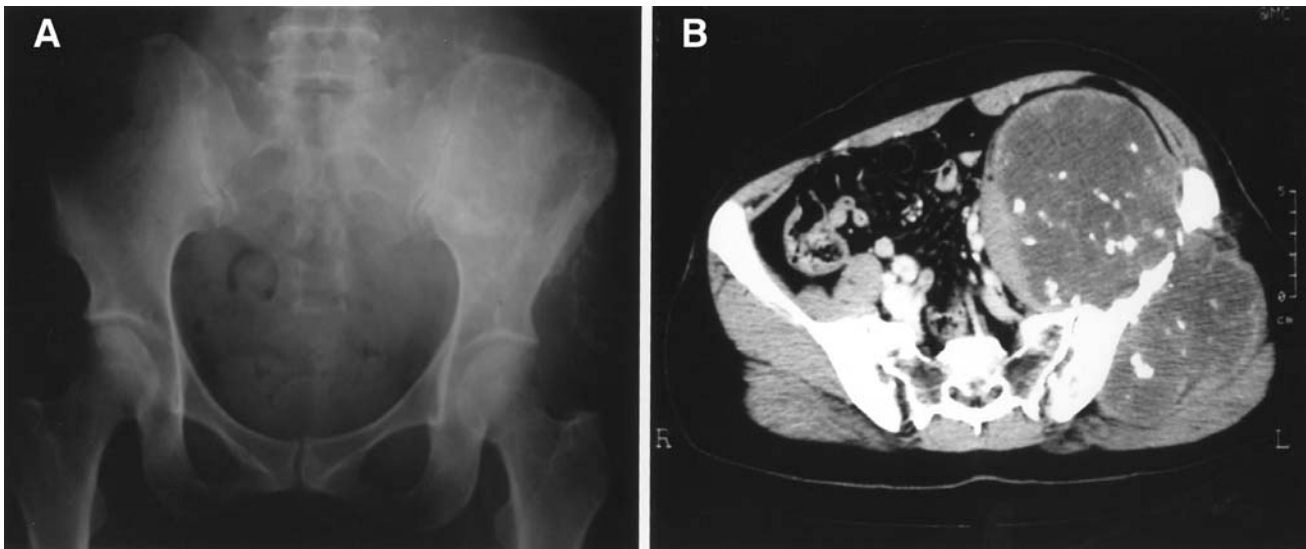


Figure 9. Preoperative plain X-ray (A) and CT (B) of Case 9 with a chondrosarcoma (grade 2) arising from the ilium. Huge tumor mass with calcification and bone destruction expands both towards the outside and internally.

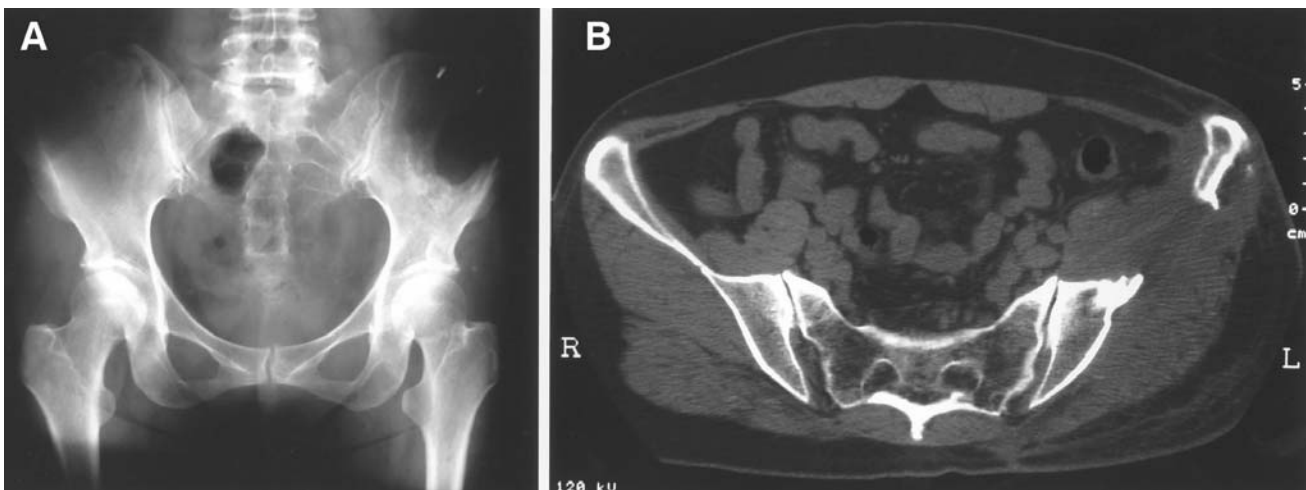


Figure 10. Postoperative plain X-ray (A) and CT (B) of Case 9 at 24 months after AO-PDT with AO-RDT. There is no evidence of local tumor recurrence around the ilium.

low dose X-ray irradiation with 5 Gy of a mouse osteosarcoma after exposure to AO showed the same strong cytotoxic effect as that of AO-PDT (11). X-ray irradiation has the advantage of reaching deeper areas of the human body than a light beam, even though it is more injurious to normal tissues. AO invades deeper tissues quickly at the rate of 1 cm per hour (unpublished data). These results of basic studies suggest that AO-PDT with low dose irradiation might be applicable for limb salvage in cases with malignant bone and soft tissue tumors. If it is effective, patients can have almost full recovery of normal limb function, with only a low risk of local recurrence.

Based on the evidence accumulated from these basic studies, after completely considering and clearing the ethical issues, we conducted a clinical study to determine the feasibility and usefulness of AO-PDT with/without low dose radiation in human musculoskeletal sarcoma patients. The results revealed an overall recurrence rate of 10% in the patients after surgery, which is almost the same as that after wide tumor resection (36-38). None of the five patients who received AO-PDT with radiation developed tumor recurrence during the follow-up period of more than 2 years. Even in the one case treated with AO-PDT alone who developed recurrence, the tumor did not recur until 21

months after the surgery, which is quite a long time when compared to that for recurrence after macroscopic curettage of high-grade malignant sarcoma. Although the follow-up duration in our study may not have been sufficient, we are convinced that the treatment protocol employed was beneficial and contend that AO-PDT with/without low dose radiation definitely has an inhibitory effect against local recurrence of musculoskeletal sarcomas, because, in general, most high-grade malignant sarcomas rapidly recur within 6 months after intralesional tumor excision (36-38). However, it still remains to be ascertained for how long this therapy might be effective.

The limb function of all patients, except for one, recovered to the baseline level after surgery, and all the patients were satisfied with this recovery. As compared with that following the presently employed limb salvage surgery with wide tumor resection followed by limb reconstruction, recovery of limb function after AO-PDT with/without low dose radiation in our study was superior. We believe that all of our patients in this study might spend the rest of their lives as normal and not handicapped people.

Local administration of AO, AO-PDT or 5-Gy radiation was not associated with any complications, such as skin hypersensitivity to light, which is often encountered with photodynamic therapy using porphyrin or its derivatives (35). Thus, with AO-PDT with/without low dose radiation, it may not be necessary to avoid exposure to the sun even in the early phase after surgery.

Finally, we conclude that AO-PDT with/without low dose radiation may be a promising new limb salvage modality for the preservation of limb function in musculoskeletal sarcoma cases, and may also be applicable to many other solid cancers, although studies on a larger number of patients with longer durations of follow-up are required.

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