

## Cryoablation for Malignant Bone and Soft Tissue Tumors and Histological Assessment of Ablated Tumors

KUNIHIRO ASANUMA<sup>1</sup>, ATSUHIRO NAKATSUKA<sup>2</sup>, TOMOKI NAKAMURA<sup>1</sup>, MASASHI FUJIMORI<sup>2</sup>, TAKASHI YAMANAKA<sup>2</sup>, TOMOHITO HAGI<sup>1</sup>, YUMI MATSUYAMA<sup>1</sup>, TAKAHIRO IINO<sup>1</sup> and MASAHIRO HASEGAWA<sup>1</sup>

<sup>1</sup>Department of Orthopedic Surgery, Mie University School of Medicine, Tsu, Japan;

<sup>2</sup>Department of Radiology, Mie University School of Medicine, Tsu, Japan

**Abstract.** *Background/Aim:* Cryoablation is a new, minimally invasive option for local tumor therapy that is attracting attention due to its potential interactions with the immune system. The purpose of this study was to evaluate the efficacy of cryoablation for local control of bone and soft tissue lesions, to elucidate risk factors for recurrence, and to clarify histological changes. *Patients and Methods:* Participants comprised 25 patients who underwent cryoablation for 53 discrete lesions of bone or soft tissue recurrence after resection or as metastases of cancer or sarcoma. Local progression-free survival was evaluated after completion of cryoablation. The histology of tumor tissues resected after cryoablation was assessed for seven cases. *Results:* Local progression-free survival rates were 88.1% at 1 year and 79.7% at 2 and 3 years. Risk of local progression was significantly higher for recurrent lesions after resection, and for lesions  $\geq 4.0$  cm in diameter than for metastatic lesions, or lesions  $< 4.0$  cm, respectively ( $p < 0.05$  each). In a subgroup analysis of bone lesions, lesions with an extraskeletal component tended to be associated with worse local recurrence-free survival than those without an extraskeletal component. On histological examination, tissue in the ablated area was completely necrotic. In the border area between ablated and non-ablated areas, CD68-positive

cells including CD16-M1-like and CD204-positive M2-like cells were more frequently observed than T cells. *Conclusion:* Cryoablation has shown good anti-tumor efficacy across various tumor types, including those affecting the bone. However, local control was inadequate for recurrent lesions and tumors larger than 4.0 cm in diameter. Further analysis of the relationship between macrophages and cryoablation is needed and may provide critical insights into achieving a more effective anti-tumor response.

Malignant tumors of the bone and soft tissue comprise primary bone or soft tissue sarcomas and metastases from sarcomas and cancers. Recurrent or metastatic disease after the completion of initial therapy for primary sarcomas is a substantial problem, along with cancer metastasis. In epidemiologic analyses of cancer, bone metastasis was observed in 5.1% of patients with cancer according to the surveillance, epidemiology and end results (SEER) database (1) and soft tissue metastasis was detected in 1.8% of cancer patients (9/500) using FDG-PET/computed tomography (CT) (2). In epidemiologic analyses of sarcoma, the local recurrence rate was 18% in 2,084 localized primary soft tissue sarcomas (3) and 2.2% in 8,234 soft tissue sarcomas presenting with bone metastasis according to the SEER database (4). In a study of the SEER database, 4.8% of 4,785 primary malignant bone neoplasms developed bone metastases (5). For these kinds of relapsed lesions, local control is a critical issue in planning the therapeutic strategy.

Surgical resection is commonly the most reliable treatment for achieving local control. However, in cases of recurrent or systemically disseminated disease (stage IV), resection does not always lead to improvement. Systemic chemotherapy is usually adapted for stage IV disease. In such cases, oligometastasis, characterized by a limited metastatic burden, has been reported to benefit from local control, which may improve survival (6). As a result, oligometastasis should be actively treated with curative intent. Even if multiple metastatic lesions are observed, partial local control can provide great benefits by avoiding severe symptoms, such as pathological fracture, nerve palsy, unendurable pain, and organ failure.

*Correspondence to:* Kunihiro Asanuma, Department of Orthopedic Surgery, Mie University School of Medicine, 2-174 Edobashi, Tsu City, Mie 514-8507, Japan. Tel: +81 592315022, Fax: +81 592315211, e-mail: kasanum@gmail.com

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Radiofrequency ablation (RFA) and cryoablation are new options for local antitumor therapy using minimally invasive devices. For bone and soft tissue tumors, the most common indications for ablation are local control and pain palliation (7). Our group has already reported the efficacy of RFA against recurrent soft tissue sarcoma (8). Compared with RFA, cryoablation can visualize the treatment zone as an ice ball on CT, with the cytotoxic margin estimated as 5–10 mm behind the leading edge. This may provide benefits for better local control than RFA (9). Previous reports have demonstrated particularly good local control with cryoablation (10–13). In addition, cryoablation has attracted attention regarding its relationship with the immune system. Studies into abscopal effects from combined therapy with cryoablation are increasing (14–16).

The purpose of this study was to evaluate the efficacy of cryoablation for local control of bone and soft tissue lesions, to elucidate risk factors for local progression, and to clarify histological changes of necrosis and immune cell invasion after cryoablation.

## Patients and Methods

A retrospective review of all patients was performed using data collected from hospital records and follow-up information. This study was approved by the Ethics Committee of the Mie University Graduate School of Medicine (approval no. H2020-224). All procedures performed in studies involving human participants were undertaken in accordance with the ethical standards of the Ethics Committee of Mie University and with the 1975 Declaration of Helsinki. The requirement for informed consent was waived by the Ethics Committee of Mie University because of the retrospective nature of the study.

**Patients.** Twenty-seven patients who underwent a total of 55 sessions of percutaneous cryoablation for the treatment of bone and soft tissue tumors between 2012 and 2017 at Mie University Hospital were enrolled in this study. Patients were followed-up or treated in the Department of Orthopedic Surgery for malignant tumors in the bone or soft tissue. All cryoablation procedures were performed in the Department of Interventional Radiology with the aim of achieving local control.

**Cryoablation.** Cryoablation was performed using an argon gas-based system (CryoHit, Galil Medical Ltd., Yokneam, Israel) under CT-fluoroscopic guidance (Aquilion ONE, Canon Medical Systems Corporation, Otawara, Japan).

Seventeen-gauge cryoprobes were placed into the tumor according to their size and shape under local anesthesia with 1% lidocaine (Xylocaine; Astellas Pharma Inc., Tokyo, Japan). CT was performed to evaluate the entire tumor, with the ice ball extending at least 5 mm beyond the tumor margin. Two freeze-thaw cycles of cryoablation were performed. If the ice ball did not sufficiently cover the tumor margins, an additional cryoprobe was considered for complete coverage, depending on tumor size and shape. After completion of cryoablation, non-enhanced CT was performed to screen for immediate complications. In bone lesions, an extraskeletal lesion was defined as one involving the formation of soft tissue lesion by expansion of the tumor from inside of the bone following cortical bone destruction.

**Follow-up.** Contrast-enhanced MRI was performed within one month after cryoablation to assess margin coverage based on the area showing loss of enhancement. If margin coverage was insufficient, additional cryoablation or wide resection was performed. Local progression-free survival (LPFS) was defined as the time from cryoablation to the date of clinically documented local progression.

**Sample collection from ablated tumor tissues.** Seven patients (26.9%, 7/26) underwent additional surgery after cryoablation. Samples from the ablated tumor were obtained during these operations. Four patients (15.4%, 4/26) underwent curettage of a bone lesion and plate fixation to prevent pathological fracture from mechanical weakness due to bone resorption by the tumor and bone necrosis following cryoablation. Two patients (7.7%, 2/26) underwent wide resection to remove residual or locally progressed tumor after cryoablation. The remaining patient (3.8%, 1/26) developed infection at the site of cryoablation and underwent surgical debridement.

**Histological analysis.** Tissues were fixed with 10% neutral-buffered formalin and embedded in paraffin according to conventional methods, and then sectioned at a thickness of 4 µm using a microtome. Sections were stained with hematoxylin and eosin staining. For immunohistochemistry, sections were subjected to paraffin removal. Then, antigen retrieval was conducted by heating sections for 10 min in 10 mM citrate buffer (pH 6.0) at 120°C using a pressure cooker (CLIPSO 4L; Tefal, Rumilly, France). Slides were then incubated overnight at room temperature with primary antibodies specific for CD68 (monoclonal mouse anti-CD68, diluted 1:50, catalog M0876; Dako, Glostrup, Denmark), CD4 (monoclonal mouse anti-CD4, diluted 1:50, catalog M7310; Dako), CD8 (monoclonal mouse anti-CD3, diluted 1:50, catalog N1592; Dako), CD16 (polyclonal rabbit anti-CD16, diluted 1:300, catalog 16559-1-AP; Proteintech, Rosemont, IL, USA), CD204 (monoclonal mouse anti-CD204, diluted 1:200, catalog KMUMA01; COSMO BIO Co., Tokyo, Japan), indoleamine 2,3-dioxygenase (IDO) (monoclonal mouse anti-IDO, diluted 1:250, catalog MAB60302; R&D Systems, Minneapolis, MN, USA), and CD47 (polyclonal sheep anti-CD47, diluted 1:200, catalog AF4670; R&D Systems). CD68, CD16, and CD204 were used as markers of macrophages, M1 macrophages, and M2 macrophages, respectively. After washing, sections were incubated for 30 min at 24°C in methanol containing 0.3% H<sub>2</sub>O<sub>2</sub> to eliminate endogenous peroxidase activity. After washing, slides were incubated for 30 min at 24°C with anti-mouse immunoglobulin that had been conjugated with horseradish peroxidase using an immuno-enzyme polymer (Histofine® Simple Stain MAX PO; Nichirei, Tokyo, Japan). All specimens were visualized in 3,3'-diaminobenzidine tetrahydrochloride solution containing H<sub>2</sub>O<sub>2</sub>. After washing in water, sections were counterstained with hematoxylin.

**Statistical analysis.** Statistical analyses were performed to compare various parameters using the Mann–Whitney test. Kaplan–Meier survival plots and log-rank tests or Bonferroni tests were used to assess differences in time to local progression in each cryoablated lesion. To evaluate the threshold for detecting local progression, receiver operating characteristic (ROC) curves were analyzed. The ROC curves were created by plotting sensitivity on the y-axis and the false-positive rate (1 - specificity) on the x-axis. The area under the curve (AUC) was assessed to evaluate the effectiveness of the size of tumor lesion for predicting local progression. LPFS was defined as the time from cryoablation to the date of clinically documented local progression. Values of *p*<0.05 were considered

Table I. Histological diagnosis.

	Patients	Sessions
Total	25	53
Bone and soft tissue tumor	11	32
Chordoma	2	6
MPNST	1	7
Extraskeletal myxoid chondrosarcoma	1	6
SFT	1	5
Synovial sarcoma	2	4
Leiomyosarcoma	2	2
GIST	1	1
Chondrosarcoma	1	1
Cancer	14	21
Renal carcinoma	4	10
Hepatocellular carcinoma	4	5
Rectal cancer	1	1
Breast cancer	1	1
Lung cancer	1	1
Thyroid cancer	1	1
Cholangiocellular carcinoma	1	1
Cancer of unknown primary origin	1	1

MPNST: Malignant peripheral nerve sheath tumors; SFT: solitary fibrous tumor; GIST: gastrointestinal stromal tumors.

statistically significant. EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria), was used for all statistical analyses (17).

## Results

**Patient and tumor characteristics.** Mean age of the 25 patients (19 males, 6 females) was 60.9 years (range=29-83 years). The underlying pathology was sarcoma in 11 patients (44%, 11/26) and cancer in 14 patients (56.0%, 14/25). Histopathological diagnoses are shown in Table I.

A total of 53 lesions were targeted for cryoablation. Fifty sessions were performed for 47 metastatic lesions. One lesion needed two sessions, and another lesion required three sessions. For the six recurrent lesions, seven sessions were performed. One lesion needed two sessions. All six recurrences were sarcomas. Three recurrent lesions developed after surgical resection and four recurrent lesions developed after carbon ion radiotherapy. Mean tumor size was 3.8 cm (range=0.6-12.4 cm).

Among bone lesions, the spine, rib, and pelvis were the most frequent sites, while among soft tissue lesions, the retroperitoneum was the most frequent (Table II). Mean duration of follow-up was 20 months (range=0.5-77.3 months).

**Three-year LPFS.** Total LPFS rate was 88.1% at 1 year, 79.7% at 2 and 3 years (Figure 1A). Risk of tumor progression was the same for sarcoma lesions as for cancer lesions (3-year LPFS rate: sarcoma 81.4%, cancer 74.1%;  $p=0.97$ ) (Figure 1B). Risk of local progression tended to be higher for soft tissue lesions

Table II. Location of target lesion.

Bone	40	Soft tissue	13
Spine	15	Retroperitoneum	3
Rib	10	Buttock	2
Pelvis	8	Thigh	2
Femur	3	Chest wall	2
Sternum	2	Abdominal wall	1
Scapula	1	Abdominal cavity	1
Patella	1	Neck	1
		Back	1

than for bone lesions (3-year LPFS rate: soft tissue lesion 72.5%; bone lesion 82.3%;  $p=0.173$ ) (Figure 1C). Risk of local progression was significantly higher for cryoablation of a recurrent lesion than for a metastatic lesion (3-year LPFS rate: recurrent lesion 50.0%, metastatic lesion 84.7%;  $p<0.00176$ ) (Figure 1D). Given these results, bone and metastatic lesions were considered good targets for local control by cryoablation.

**Lesion size.** Lesion size was evaluated in various ways. Cancer lesions and soft tissue lesions were significantly larger than sarcoma lesions and bone lesions, respectively (Figure 2A and B). No significant difference in size was seen between metastasis and recurrent lesions (Figure 2C). Lesions that showed local progression after cryoablation were larger than lesions without local progression after cryoablation ( $p=0.0163$ ) (Figure 2D). In ROC analysis of size, AUC was evaluated to determine the diagnostic accuracy for identifying local progression within one year after cryoablation.

Using a threshold of 4.0 cm, sensitivity and specificity for identifying 1-year local progression were 100% and 70.8%, respectively (Figure 3A). A threshold of 4.0 cm was adopted using the Youden index and patients were divided into a large group (diameter  $\geq 4.0$  cm) and a small group (diameter  $<4.0$  cm). The large group showed a significantly higher local progression rate (3-year LPFS rate: 53.7%) than the small group (3-year LPFS rate: 94.4%;  $p=0.00168$ ) (Figure 3B). Lesions  $\geq 4.0$  cm in diameter were at high risk of local progression after cryoablation.

By subgroup analysis based on size and the nature of lesions as metastatic or recurrent, recurrent lesions  $\geq 4.0$  cm in diameter showed significantly poorer local control than others (Figure 3C). In addition, extraskeletal lesions caused by bone destruction were observed in 18 of 40 bone lesions. Lesions with extraskeletal lesions tended to show a worse 3-year LPFS rate (68.2%) than those without extraskeletal lesions (91.7%;  $p=0.135$ ) (Figure 3D).

**Histological evaluation of tumors resected after cryoablation.** Tissues from seven lesions resected after cryoablation were investigated (Table III). Planned surgery to prevent pathological

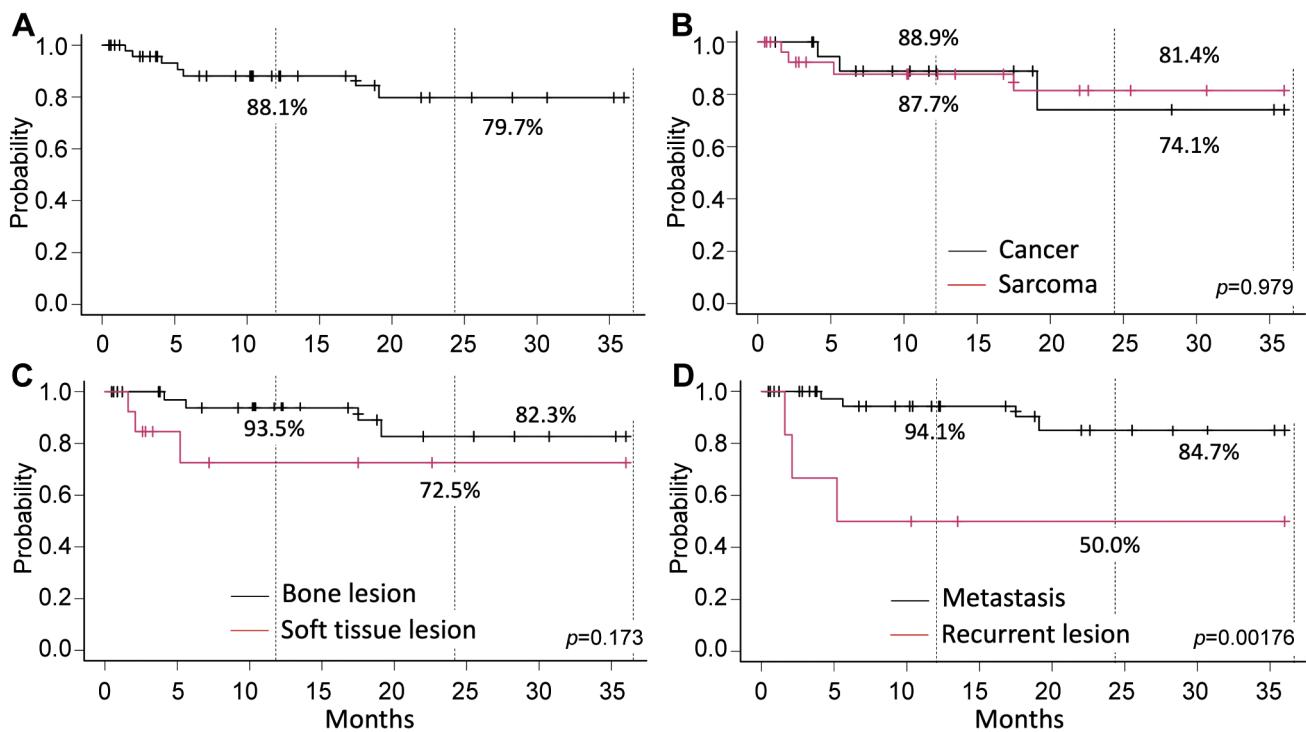


Figure 1. Local recurrence-free survival (LRFS) shown by Kaplan-Meier curves. Statistical differences were analyzed by using log-rank testing. A) Total. B) Cancer vs. sarcoma. C) Bone vs. soft tissue. D) Metastatic lesion vs. recurrent lesion.

fracture was performed for four lesions (57.1%, 4/7) within 14 days after cryoablation (Patients 1-4). In these cases, histology showed completely necrotic tissues in three lesions (Patients 1, 2, and 4). Tumor cells remained in one lesion (Patient 3). After curettage, recurrence was not detected in any of these four lesions (Patients 1-4). Wide resection was performed for one lesion (14.3%, 1/7) to resect residual tumor 47 days after cryoablation (Patient 6). Pathologically, viable tumor cells remained in the residual tumor. Wide resection was performed for another lesion (14.3%, 1/7) due to recurrence >200 days after cryoablation (Patient 5). In that pathological evaluation, tumor cells were again observed in resected tissues. Sufficient time was considered to have elapsed since cryoablation to allow tumor regrowth and the ablated area was decreased or lost. Debridement was performed in one case (14.3%, 1/7) due to infection after cryoablation (Patient 7). Histologically, no viable tumor cells remained, and necrosis was observed. As invasion by neutrophils was rarely observed, necrosis was considered to have resulted from cryoablation rather than infection.

**Representative cases.** Representative cases (Patients 1, 2, and 6 in Table III) are described.

**Patient 1.** A 49-year-old woman presented with a growing tumor on the right thigh, for which she had undergone needle biopsy at a previous hospital. Malignant tumor was suspected,

and she was referred to our hospital. Leiomyosarcoma was diagnosed by pathologists at our hospital, and she underwent wide resection. One year later, X-ray, MRI, and positron emission tomography-computed tomography showed lung metastases and bone metastases in the right femur (Figure 4A-E). Fifteen months after primary resection, lung resection was performed. After another three months, she received cryoablation for the right femur (Figure 4G), with curettage and plate fixation performed eight days after ablation (Figure 4F). She then experienced no recurrences for nine years. Tissue from curettage showed viable and necrotic areas (Figure 4H). CD4- and CD8-positive cells were rarely observed (Figure 4I and J). CD68-positive cells were observed more frequently in non-necrotic areas and less frequently in necrotic areas (Figure 4K). CD204-positive cells (Figure 4M) tended to be more evident than CD16-positive cells (Figure 4L). IDO was not observed (Figure 4N). No CD47-positive cells were observed (data not shown).

**Patient 2.** A 61-year-old man suffered from leiomyosarcoma of the left leg (Table III, Patient 2). After neoadjuvant chemotherapy, above-the-knee amputation was performed. Two months later, multiple lung metastases were seen to be gradually increasing in size and RFA was performed. At 1.5 years after amputation, bone metastasis was detected in the trochanteric region of the left femur (Figure 5A and B).

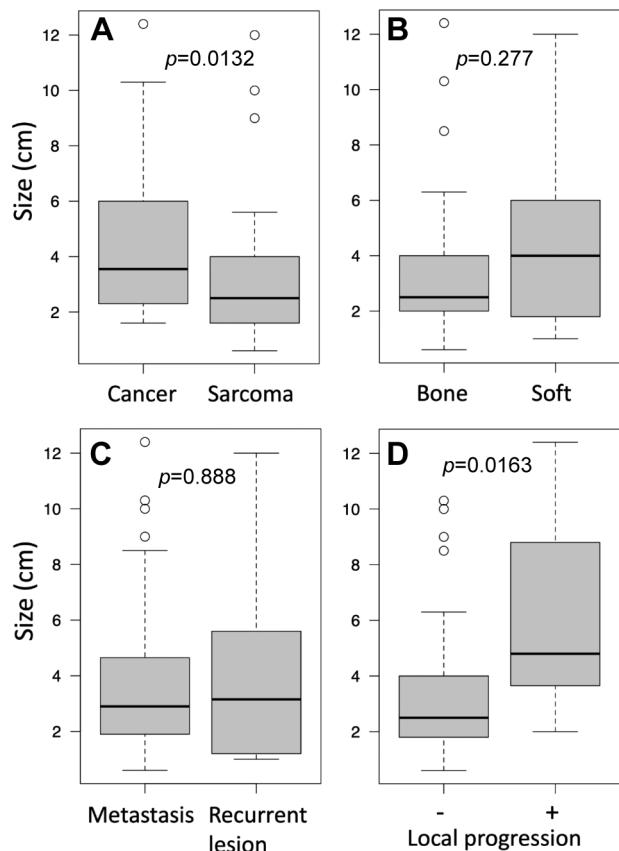


Figure 2. *Lesion size comparisons. A) Cancer vs. sarcoma. B) Bone vs. soft tissue. C) Metastatic lesion vs. recurrent lesion. D) Lesion without local progression vs. with local progression. Statistical differences were analyzed using the Mann-Whitney test.*

Cryoablation was performed for local control (Figure 5E) and contrast enhancement of the tumor lesion disappeared (Figure 5C). Nine days after cryoablation, he underwent tumor curettage, cement filling, and plate fixation (Figure 5D). No local progression was observed approximately 17 months after cryoablation.

Tissues from curettage appeared necrotic (Figure 5F). No CD4- or CD8-positive cells were observed (Figure 5G and H). CD68-positive cells were apparent in necrotic tissues (Figure 5I) and numbers of CD16- (Figure 5J) and CD204-positive cells (Figure 5K) did not appear significantly different. IDO was not observed (Figure 5L). CD47-positive cells were not observed (data not shown).

**Patient 6.** A 49-year-old woman was diagnosed with neurofibromatosis type 1. One year before presentation to our hospital, she received wide resection for malignant peripheral nerve sheath tumor (MPNST) of the scapular region at another hospital. The tumor recurred and multiple lung metastases developed. Chemotherapy was performed and the

lung metastases showed no increase in size, whereas the axial tumor continued to grow. She was introduced to our hospital for ablation of lung metastasis and recurrent MPNST.

A 12-cm recurrent tumor was identified in the scapular region (Figure 6A and E). Cryoablation was performed (Figure 6B and F). Five days after ablation, the high-intensity lesion at the center of the tumor on T2WI had shrunk (Figure 6C and D). The ablated area was evaluated by contrast-enhanced MRI as demonstrated by disappearance of contrast-enhancement (Figure 6D and H). Wide resection was performed 47 days after cryoablation (Figure 6I and J). Residual tumor was observed (Figure 6H and J; blue line). White line in Figure 6C and D and red line in Figure 6G and H corresponded to the yellowish tissue area on gross examination of the tumor (Figure 6I: white line; Figure 6J: red line).

The ablated area was histologically necrotic (Figure 6K: red line). Further histological evaluation was performed in seven areas of necrotic tissue, border and viable areas (Figure 6K, Areas 1-7). Areas 1 and 4 were necrotic areas. Areas 2, 5, and 6 represented border areas between necrotic and viable areas. Areas 3 and 7 were viable areas. No viable cells were observed in necrotic areas 1 (Figure 7A, D, G, J, M, P, and S) or 4 (Figure 8A, E, I, M, Q, U). In the upper line of Figure 6K (Areas 1-3), a few CD4-positive cells were observed in the viable area (Figure 7C), but not in necrotic or border areas (Figure 7A and B). CD8-positive cells were observed in the viable area (Figure 7F), but not in necrotic or border areas (Figure 7D and E). CD68-positive cells were observed in border and viable areas (Figure 7H and I). CD16- and CD204-positive cells were seen in border and viable areas (Figure 7K, L, N, O), but not in necrotic areas (Figure 7J and M). IDO-positive staining was seen in viable areas (Figure 7Q and R). In the lower line of Figure 6K (Areas 4-7), more CD68-positive cells (Figure 8J-L) with few CD4 (Figure 8B-D) and CD8 cells (Figure 8F-H) were observed in the border area (Areas 5 and 6) and alive area (Area 7). CD204-positive cells (Figure 8R-T) were more frequent than CD16-positive cells (Figure 8N-P). Overall, CD68-positive cells were evident and cryoablation may induce M2 macrophages more than M1 macrophages. IDO was positive in tumor cells (Figure 8V-X). No CD47-positive cells were observed (data not shown).

## Discussion

Cryoablation is adopted for local control of malignant tumors developing in various organs, such as the lungs, liver, and kidneys. Bone and soft tissue malignant tumors represent additional attractive targets for cryoablation. Previous studies of bone lesions treated using cryoablation accumulated good outcomes for local control. In various tumor types, local control rates for bone metastasis were over 90% at around 1 year, about 68-84.6% at around 2 years, and 76.9-82% at

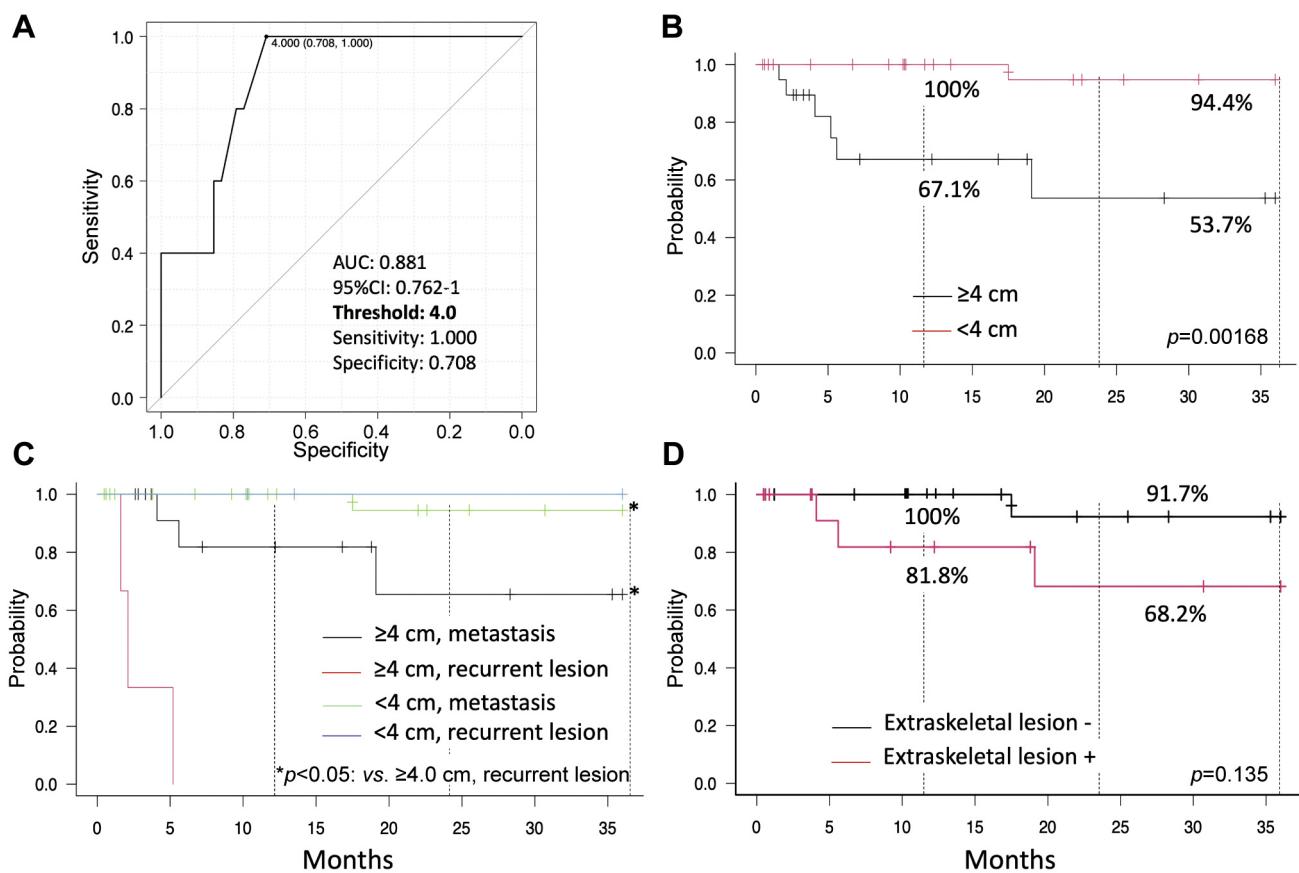


Figure 3. Sub-group analysis for local progression-free survival (LPFS). ROC analysis was performed for detection of recurrence within one year. A) Based on the Youden index, 4.0 cm was used as the threshold for dividing patients into two groups. B) LPFS for tumor diameter <4.0 cm and ≥4.0 cm by Kaplan-Meier curves. C) Patients were divided into four groups according to bone or soft tissue and tumor diameter <4.0 cm or ≥4.0 cm. D) LPFS was compared with or without extraskeletal lesions from destroyed bone using Kaplan-Meier curves.

Table III. Clinical data of patients with tumor resection after cryoablation.

	No	Diagnosis	Location	Size (cm)	Duration of cryo-operation (days)	Reason for operation	Operation	Histology
Bone	1	Leiomyosarcoma	Femoral diaphysis	3	8	Prevention of fracture after cryoablation	Curettage + plate	Necrosis
	2	Leiomyosarcoma	Femoral neck	4	9		Curettage + CHS	Necrosis
	3	SFT	Femoral diaphysis	2.5	10		Curettage + CHS	Tumor cells remaining
	4	Breast cancer	Patella	3	13		Curettage + cement	Necrosis
	5	Rectal cancer	Ischium	4.8	222		Wide	Invasive tumor cells remaining
Soft tissue	6	MPNST	Scapular region	12	47	Wide resection for residual tumor after cryoablation	Wide	Tumor cells remaining
	7	Chordoma	Thigh	9	106		Debridement	Necrosis

CHS: Cannulated hip screws; MPNST: malignant peripheral nerve sheath tumor; SFT: solitary fibrous tumor.

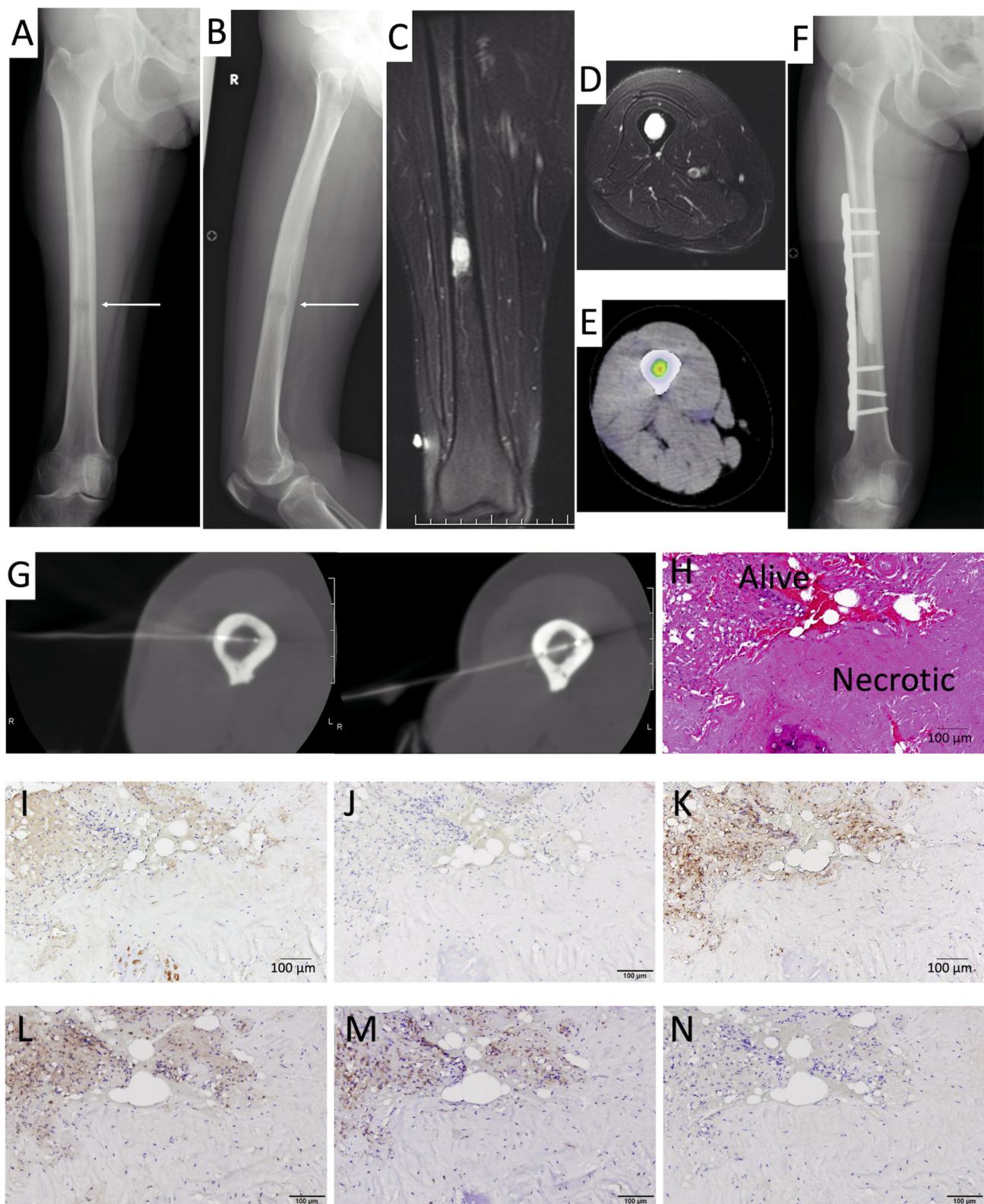


Figure 4. Patient 1 in Table III. A) Frontal x-ray. B) Lateral x-ray. C) T2-weighted fat-suppressed coronal magnetic resonance imaging (MRI). D) Axial MRI. E) Positron emission tomography-computed tomography. F) Postoperative x-ray. G) CT-guided cryoablation. H) H&E stain. I-N) Immunostaining for anti-CD4 (I), anti-CD8 (J), anti-CD68 (K), anti-CD16 (L), anti-CD204 (M), and anti-IDO (N).

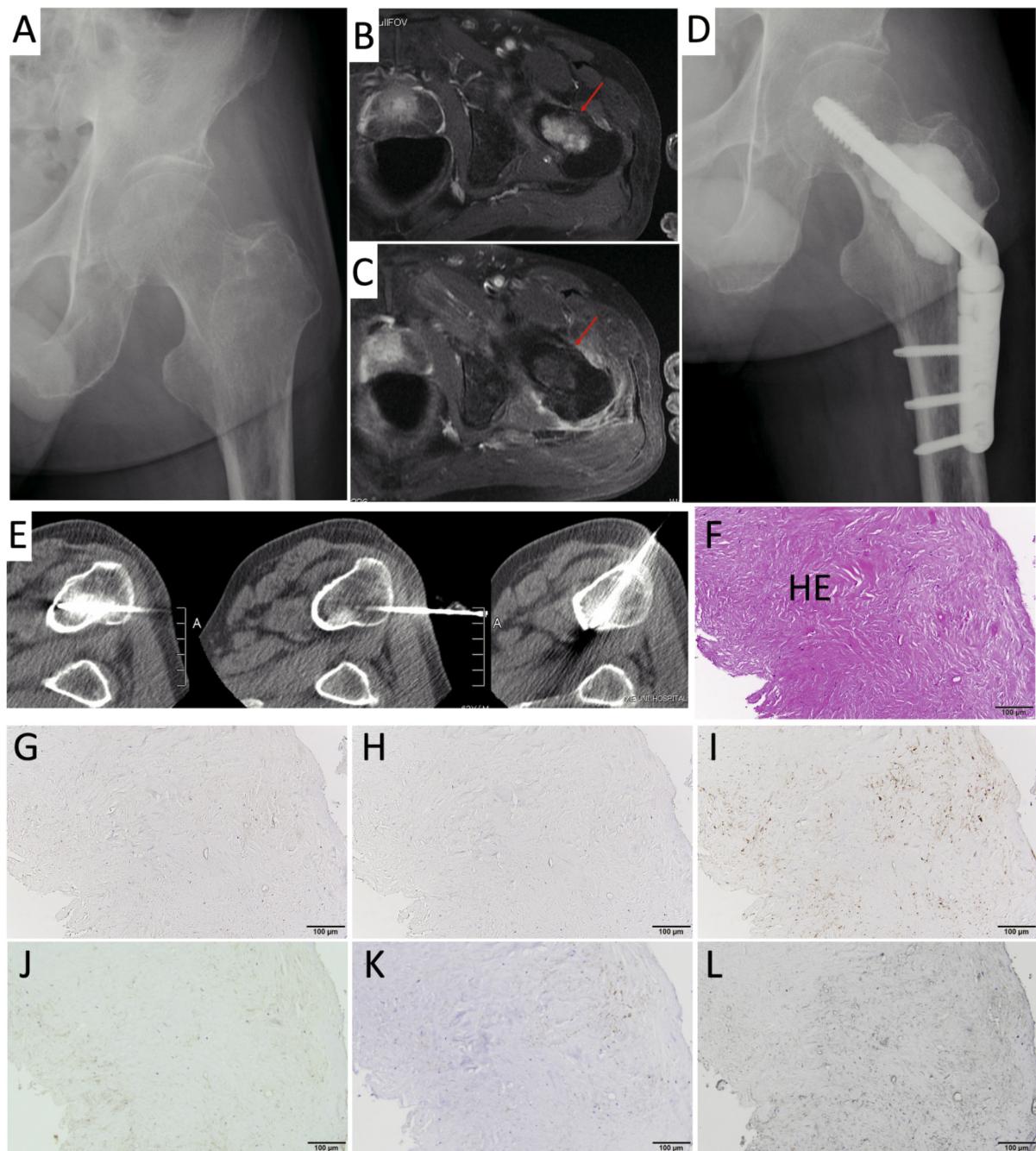
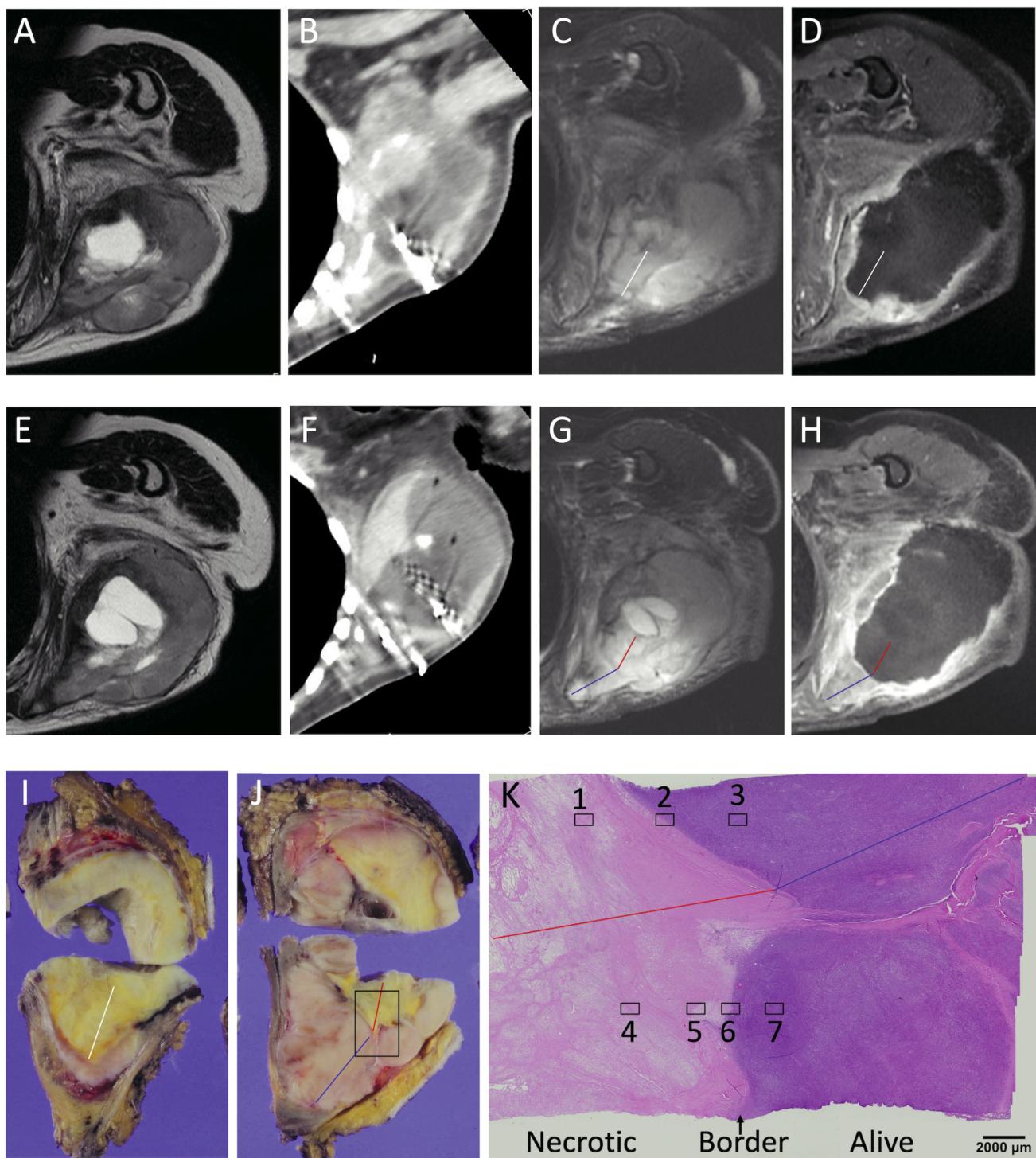


Figure 5. Patient 2 in Table III. A) Frontal x-ray. B) Dynamic contrast-enhanced T1-weighted axial magnetic resonance imaging (MRI) before cryoablation. C) MRI after cryoablation. D) Postoperative x-ray. E) Computed tomography-guided cryoablation. F) H&E stain. G-L) Immunostaining for anti-CD4 (G), anti-CD8 (H), anti-CD68 (I), anti-CD16 (J), anti-CD204 (K), and anti-IDO (L).

around 3 years (10-12, 18, 19). In total, cryoablation has demonstrated anti-tumor efficacy without distinction between tumor cell types or bone sites. In our study, local control rates for bone lesions were 93.5% at 1 year, and 82.3% at 2 and 3 years. Our outcome was equivariant to those of previous studies.

Fewer reports have described cryoablation for soft tissue metastasis than for bone metastasis. Local control rates for soft tissue lesions in various malignant neoplasms were 90-100% at 11-21 months (10, 20, 21). For sarcomas, LPFS rates for recurrent or metastatic lesions at 1, 2, and 3 years were 82.09%, 59.70%, and 46.29%, respectively (22). In



**Figure 6.** Patient 6 (images and HE-stained sections) in Table III. A) Proximal slice from T2-weighted axial magnetic resonance imaging (MRI). B) Proximal slice from computed tomography-guided cryoablation. C) Dynamic contrast-enhanced T1-weighted axial MRI before cryoablation. D) Proximal slice from dynamic contrast-enhanced T1-weighted axial MRI after cryoablation. E) Distal slice from T2-weighted axial MRI. F) Distal slice from CT-guided cryoablation. G) MRI after cryoablation. H) Distal slice from dynamic contrast-enhanced T1-weighted axial MRI after cryoablation. I) Proximal slice from tumor picture. J) Distal slice from tumor picture. K) H&E staining of tissue from the square area in J. Immunostaining of squares in K is shown in Figures 7 and 8. White, red, and blue lines correspond to D, H, I, J, and K, according to the matching colors. White and red lines show the ablated area. Histologically, the tumor was necrotic (white and red line), and residual tumor cells were observed (blue line) (D, H, I, J, K).

Area No.

1

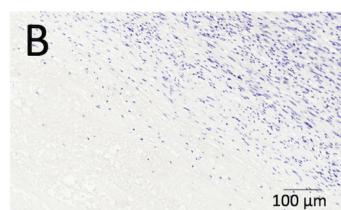
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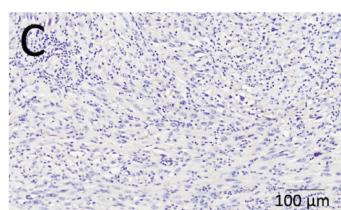
CD4



100 µm

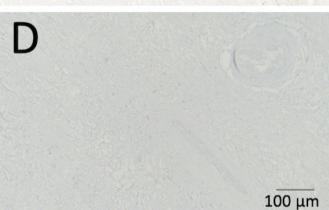


100 µm

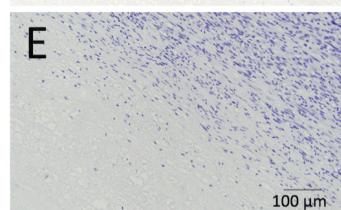


100 µm

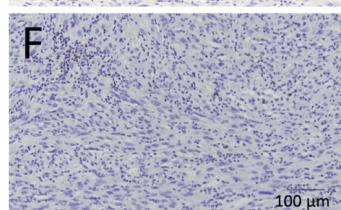
CD8



100 µm



100 µm

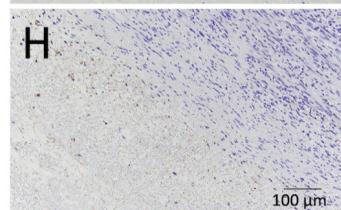


100 µm

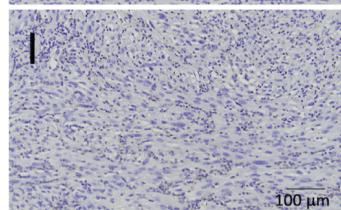
CD68



100 µm

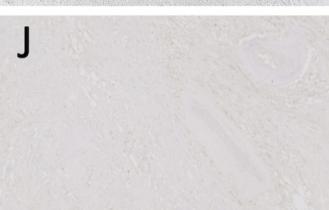


100 µm

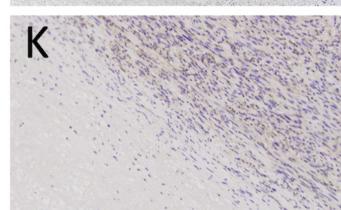


100 µm

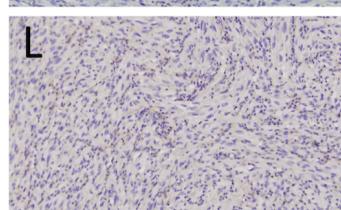
CD16



100 µm



100 µm

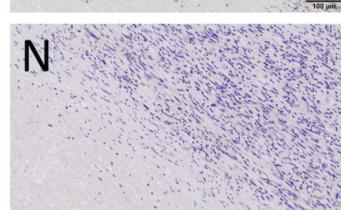


100 µm

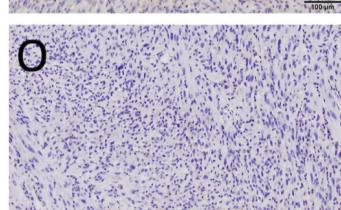
CD204



100 µm

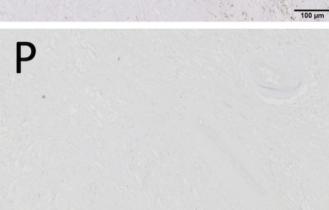


100 µm

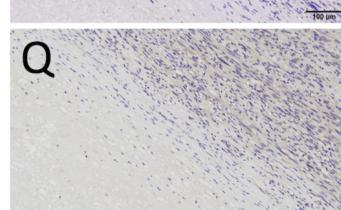


100 µm

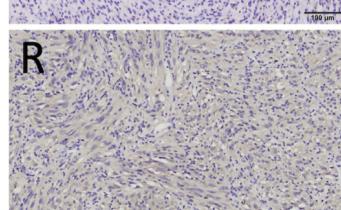
IDO



100 µm

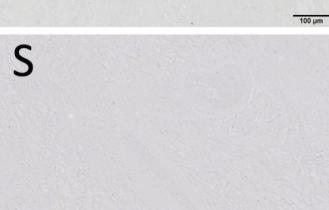


100 µm

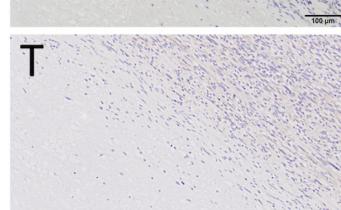


100 µm

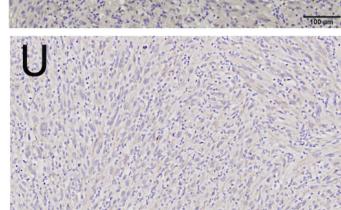
Beta catenin



100 µm



100 µm



100 µm

Figure 7. Patient 6 (immunostaining: areas 1, 2, 3). Numbers correspond to squares in Figure 6K. Area 1 is necrotic. Area 2 is the border. Area 3 is remnant tumor. Immunostains: A-C, anti-CD4; D-F, anti-CD8; G-I, anti-CD68; J-L, anti-CD16; M-O, anti-CD204; P-R, anti-IDO; S-U, anti-beta-catenin.

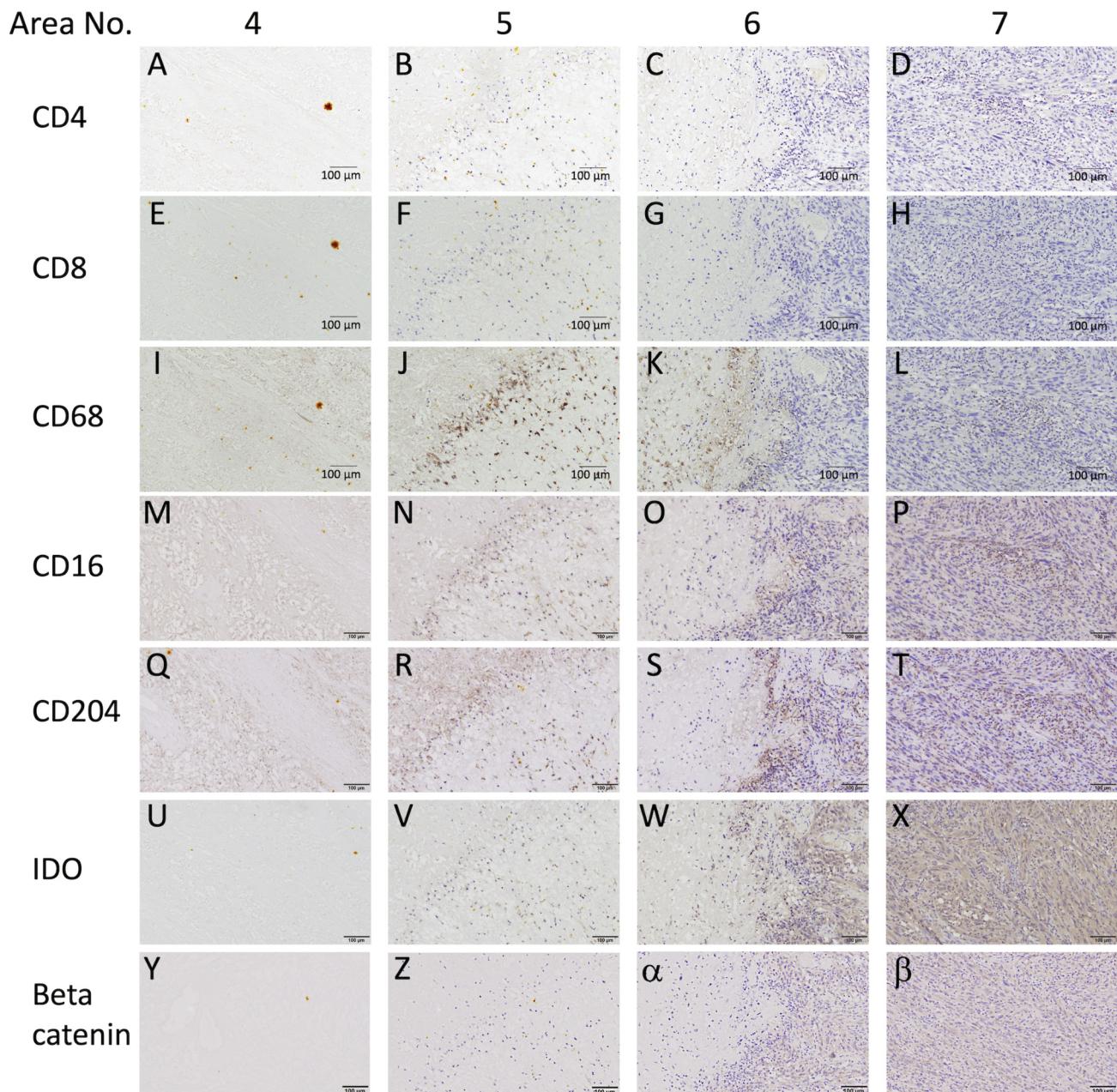


Figure 8. Patient 6 (immunostaining: areas 4-7). Numbers correspond to squares in Figure 6K. Area 4 is necrotic. Area 5 is the border on the necrotic side. Area 6 is the border on the tumor side. Area 7 is remnant tumor. Immunostains: A-D, anti-CD4; E-H, anti-CD8; I-L, anti-CD68; M-P, anti-CD16; Q-T, anti-CD204; U-X, anti-IDO; Y, Z,  $\alpha$ ,  $\beta$ , anti-beta catenin.

particular, local control is difficult to achieve for recurrent retroperitoneal soft tissue sarcoma, resulting in LPFS rates of 94.9%, 56.4%, and 7.7% at 6 months, 1 year, and 2 years, respectively (23). In our study, local control rates with soft tissue lesions were 72.5% at 1-3 year. Our outcomes for soft tissue lesions were not inferior to those of previous studies.

Several studies have mentioned risk factors for local recurrence after cryoablation. Fan *et al.* demonstrated significantly poorer prognosis for large tumors ( $\geq 10$  cm) than for small tumors ( $< 10$  cm) in a study of recurrent retroperitoneal soft tissue sarcoma (23, 24). Oligometastasis and a larger difference between maximum cryoablation diameter and tumor diameter were significant predictors of

good control (19). Bone cortical erosion, bone lesions >2 cm in diameter, and synchronous bone metastasis were significant risk factors for local failure (25). In our study, recurrent lesions and tumor diameter  $\geq 4$  cm were associated with poorer LPFS than metastatic lesions or diameter  $\leq 4$  cm, respectively. In particular, recurrent lesions  $\geq 4$  cm in diameter showed extremely poor LRFS.

Histological changes after cryoablation were precisely reported in a study of breast malignancies. Necrosis and inflammatory cell infiltration were seen within three days after cryoablation. Shadows of tumor cells were observed until two weeks. After three weeks, fibrosis had progressed (26). From tissues resected within 28 days after cryoablation for 87 breast cancers, viable cancer cells were not seen within the cryoablation zone in any cases (27). In our study, tumors were resected after cryoablation in seven cases. Four cases (Patients 1, 2, 4, 7) showed necrotic tissues. One case (Patient 3) showed remnant tumor cells, but no recurrence was observed during curettage. In Patient 6, the ablated area showed complete necrosis. Cryoablation appears markedly effective as a tool for killing tumor cells.

In addition, cryoablation has been attracting attention due to its relationship with the immune system. At present, cryoablation has shown to produce both immune stimulation and immunosuppression. The mechanisms underlying these two effects have not yet been clearly elucidated (28). The mode of cell death (necrosis or apoptosis) based on the freezing temperature is considered to be important for the kinds of cytokines released, and additionally, antigen presentation and the ratio of necrosis to apoptosis has strong impacts on immune stimulation (29). The inner zone reaches  $-40^{\circ}\text{C}$ , leading to the formation of lethal intracellular ice. Subsequent thawing then results in coagulative necrosis of tumor cells. From these broken cells, intracellular contents are released (30, 31) and the resultant antigens are processed and expressed on major histocompatibility complex (MHC) Class I cells, inducing cytotoxic T-cell responses (15). The cytokine profile produced is based on T helper-type 1 cells, such as interleukin (IL)-2, interferon  $\gamma$ , tumor necrosis factor  $\alpha$ , and IL-12 (31). The outer zone experiences extracellular freezing and results in apoptosis of tumor cells through the freezing of extracellular fluid (32, 33). Apoptosis does not release DNA, RNA, or heat shock protein and the dendritic cells remain immature (34). The cytokine profile in the outer zone is based on Th2 cells, such as IL-4, IL-5, and IL-10 (35). In total, the increase in CD4+ and CD8+ T cells and the decrease in Treg cells are critical for tumor regulation (36). Enhancement of the immune response continues until four weeks and anti-tumor cytolytic effects from T cells work for 2-4 weeks after ablation (37). However, in our study, CD4+ or CD8+ T cells were rarely observed. In our representative cases, only a few T cells were observed from tumor specimens obtained surgically at 8, 9, and 47 days after cryoablation. The small number of T cells

in our cases compared to previous reports may be due to differences in the timing of tissue harvesting. Another reason for the small number of T cells identified was thought to be immune checkpoint proteins. The IDO pathway suppresses T-cell proliferation and induces apoptosis (38). Further, a recent study demonstrated that activation of the WNT-beta-catenin pathway is related to T-cell exclusion, with sarcomas as a highly affected category of malignant neoplasm (39). From two case analyses of recurrent melanoma after adaptive immune therapy, resistance to immune checkpoint inhibitors was attributed to T-cell exclusion with involvement of the WNT-beta-catenin pathway (40). In our study, positive results were obtained for IDO and beta-catenin in tumor tissues. IDO and beta-catenin may exclude T cells from tumor tissues and around cryoablated tissues.

On the other hand, CD68-positive macrophages aggregated in the border area (Figure 8). The same phenomena of macrophage infiltration of the border area from cryoablation were previously reported by Gu *et al.* (41). In a breast cancer study, macrophages were observed two weeks after cryoablation (26), but the influence of macrophages on tumors after cryoablation was not remarked upon in that study. In addition, the distribution of M1 and M2 macrophages in tumor tissues represents a critical issue in terms of tumor behavior (42). In our study, M1- and M2-like cells were observed in the tissues after cryoablation. However, the roles of M1 and M2 macrophages after cryoablation require further investigation. Recently, an attractive study that combined radiotherapy and CD47 blockade induced macrophage-mediated abscopal effects (43). This brings about an expectation that the combination of cryoablation and immune checkpoint inhibitors against macrophages may induce superior anti-tumor effects.

**Study limitations.** This study was a retrospective analysis conducted at a single institution. The number of patients and incidence of each subtype were small, thus statistical analyses could not be performed for each specific pathological subtype. However, we nevertheless report our experience with the treatment of malignant bone and soft tissue tumors by cryoablation and histological evaluations, and we believe that this study provides worthwhile additional information regarding cryoablation of malignant tumors.

## Conclusion

Cryoablation is a new, minimally invasive option for local antitumor therapy against recurrent or metastatic disease. Total LRFS rates were 88.1% at 1 year, and 79.7% at 2 and 3 years. However, risk of recurrence was significantly higher with recurrent lesions and with lesions  $\geq 4.0$  cm in diameter. Histologically, macrophages were the main infiltrating immune cells observed after cryoablation. A combination of cryoablation

and additional treatments such as immune checkpoint inhibitors against macrophages may produce superior anti-tumor effects; however, this requires further study.

## Conflicts of Interest

The Authors declare no competing interests in relation to this study.

## Authors' Contributions

K.A.: Conceptualization, Writing - original draft. A.N.: Resources. T.N.: Formal analysis. M.F.: Review & editing. T.Y.: Resources. T.H.: Data curation. T.I.: Visualization. A.S.: Supervision.

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