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journal or publication title	International Journal of Clinical Oncology
volume	16
number	2
page range	109-117
year	2011-04-01
URL	http://hdl.handle.net/2297/28502

doi: 10.1007/s10147-011-0218-2

Cryoimmunology for malignant bone and soft-tissue tumors

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Key words: cryoimmunology, cryotreatment, bone and soft-tissue sarcoma, immunotherapy

Abstract

Several new methods have recently been developed for the treatment of malignant bone and soft tissue tumors, and many of these targeted therapies have yielded promising initial results in clinical settings. As more sarcomas become amenable to effective molecularly targeted therapy, the need to evaluate the synergistic effects of combination therapies with anticancer drugs will grow. Other immunologic therapies have also been reported, such as exogenous cytokines, dendritic cell (DC) therapy and peptide vaccines. Cryoimmunology has shown promising results in some malignant tumors after cryosurgery and is expected to influence the next generation of tumor immunotherapy. In this report, we describe the induction of a systemic antitumor immune response following liquid nitrogen cryotreatment of a destructive murine osteosarcoma. Combining tumor cryotreatment with DCs to promote tumor-specific immune responses enhanced systemic immune responses and inhibited metastatic tumor growth. We also describe the induction of a systemic antitumor immune response following reconstruction of malignant bone tumors using frozen autografts treated with liquid nitrogen.

Introduction

Promising developments building upon standard cancer therapies, such as chemotherapy, radiotherapy and surgery have dramatically improved patient survival for malignant bone and soft tissue tumors. For example, osteosarcoma is the most common primary malignant bone tumor. A standard protocol of neo-adjuvant chemotherapy, combined with wide local excision, improves five-year disease-free survival 60% to 90% [1-7]. However, some patients are refractory to these treatments, and lung metastasis is often associated with a fatal outcome. Thus, a more effective systemic treatment is called for in these patients [8].

1. Targeted therapies in bone and soft tissue sarcomas

Molecular targeting therapy is one of many novel therapies developed in the 1990's that is effective against some carcinomas. However, these drugs have been embraced slowly for clinical use in malignant bone and soft tissue tumors in the clinic. Recently, there have been some reports of clinical trials utilizing molecular targeting therapy.

Sorafenib targets a tyrosine kinase inhibitor. Pacey et al. reported a Phase II trial of sorafenib (400 mg twice daily for 12 weeks or more) that included patients with advanced soft tissue sarcoma. One patient achieved a partial response and three patients achieved minor responses, out of 26 patients [9]. The other

Phase II study of sorafenib in patients with metastatic or recurrent sarcomas reported that five of 37 patients with angiosarcoma had a partial response (response rate, 14%) [10]. As for antiangiogenic and immunomodulatory drugs, the most important regulator in angiogenesis is vascular endothelial growth factor (VEGF), which is targeted by several drugs. One such drug is the tyrosine kinase inhibitor semaxanib. Phase I data showed moderate drug-related toxicities. In 31 patients with soft tissue sarcoma, one minor response and five stable diseases (SD) were demonstrated [11, 12]. Another anti-VEGF agent being tested is bevacizumab. A Phase II trial in combination with doxorubicin at 75 mg/m^2 in 17 patients with metastatic soft tissue sarcoma was recently reported. Two partial responses (PR) were observed, 11 patients had SD for four or more cycles. The 12% response rate was no greater than that observed for single-agent doxorubicin therapy. However, the 65% SD in patients suggests further study is warranted for these tumors [13]. Chemotherapy combined with bevacizumab is effective for soft tissue sarcomas. Dramatic improvement was achieved with inoperable angiosarcoma with combination paclitaxel and bevacizumab chemotherapy [14]. Other combination therapies with sequential high-dose (HD) ifosfamide, carboplatin and etoposide refractory to standard chemotherapy in patients with sarcoma and germ cell cancer (GCC) have been reported. One almost complete response in a patient with GCC, previously progressive with three preceding protocols, was observed. Six patients had a partial response (sarcoma 4/13 patients; GCC 2/3 patients), and five patients achieved stable disease (sarcoma 5/13 patients) [15]. Of these, the most extensively studied is the tyrosine kinase inhibitor pazopanib. In a Phase II study,

pazopanib produced a progression rate arrest at three months sufficient to justify cohort expansion in patients with synovial sarcoma, leiomyosarcoma and a group of other tumors including those of vascular origin, but was relatively inactive against adipocytic tumors [16].

Imatinib is a molecularly targeted drug that inhibits tyrosine kinase, as well as type III tyrosine kinase receptors, such as platelet-derived growth factor receptor (PDGFR), KIT, colony-stimulating factor 1 receptor (CSF-1R), and discoidin domain receptor (DDR). KIT-positive gastrointestinal stromal tumors (GISTs), and PDGFR-positive dermatofibrosarcoma protuberans (DFSP) have been reported to be responsive to imatinib treatment. A Phase II trial of imatinib in patients with relapsed or refractory KIT-positive (excluding GISTs) or PDGFR-positive sarcomas was recently concluded. Twenty-two patients were evaluated for response. The response rate with a 600 mg/day dose of imatinib was 4.5% (0 complete responses, 1 partial response), however, this result did not indicate effectiveness of imatinib monotherapy in patients with relapsed or refractory KIT-positive (excluding GISTs) or PDGFR-positive sarcomas [17]. Neither long-lasting freedom from disease progression nor clinical benefit was observed for advanced chondrosarcoma in a Phase II trial[18].

2. Immunological treatment in bone and soft tissue sarcomas

Advances in basic tumor immunology and preliminary evidence for clinical activity of immunotherapy in

select settings have sustained optimism that immunotherapy will find an established place in cancer therapy. As for bone and soft tissue sarcoma, there are a number of clinical trials of immunotherapy currently, and we highlight some positive data from select studies in some detail below.

Cytokine therapy (interleukin-2: IL-2)

Luksch et al. have reported a clinical trial in osteosarcoma using IL-2, in which 18 children with localized osteosarcoma received four IL-2 courses ($9x10^6$ IU/ml/day x4), alternated with pre- and post-operative multiple chemotherapies. The results showed that intensive chemotherapies have no effect on IL-2-induced immune activation, and suggest a role for NK cells in the control of osteosarcoma [19].

Dendritic cell immunotherapy

Dendritic cells (DCs) are effective professional antigen presenting cells with the ability to prime both primary and secondary immune responses to tumor antigens. One Phase I clinical study using DCs against solid tumors in children, including osteosarcoma, has been reported. In this series, one patient with metastatic fibrosarcoma demonstrated a strong positive response without obvious toxic side effects [20]. The other clinical trial of DC vaccination was for chondrosarcoma. Immunotherapy with allogeneic DCs stimulated with tumor cell lysates was demonstrated to be feasible, safe and well tolerated. Unfortunately, this study did not observe any clinical or immune response following vaccination. $CD4^+$ and $CD8^+$ T regulatory cells could be responsible for the ineffectiveness of this particular immunotherapy

[21].

Tumor-specific peptide immunotherapy

Immunotherapy clinical trials are being conducted using a Wilms tumor (WT1) peptide for metastatic alveolar rhabdomyosarcoma. The patient received weekly intradermal injections of HLA-A*2404-restricted, 9-mer WT1 peptide against residual bone disease. After three months, her bone disease disappeared, concurrent with an increase in the frequency of WT1-specific cytotoxic T lymphocytes (CTLs) [22]. Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) antibodies have been employed in a clinical Phase II study coordinated by the Memorial Sloan-Kettering Cancer Centre in New York. The purpose of this clinical trial is to determine whether immune therapy with anti-CTLA-4 antibody is effective in patients with advanced synovial sarcoma. A total of 17 patients will be recruited. In this study, the investigators are trying to induce an immune response against synovial sarcoma with anti-CTLA-4 antibody in recurrent disease [23, 24].

Combination with immunotherapies

Mackall et al. reported a new immunotherapeutic regimen for recurrent or metastatic Ewing's sarcoma family of tumors or alveolar rhabdomyosarcoma. Combinations of autologous T cells, influenza vaccinations, and DCs pulsed with peptides derived from tumor-specific translocation breakpoints and E7 with or without recombinant human interleukin-2 showed minimal toxicity and favorable survival (43%

of five-year overall survival for immunotherapy patients compared with 15% for patients without immunotherapy) [25].

3. Cryosurgery in carcinomas

The first documented uses of cryosurgery in the treatment of malignancy dates back to the 1840s using crushed ice and salt to treat superficial cancers [26]. However, it was not until the 1960s that this technology was advanced enough to be used in the treatment of primary and secondary malignancies [27]. Recently, cryosurgery using liquid nitrogen has been successfully used for the ablation of tumors in the liver [28], prostate [29] and kidney [30], and the palliative treatment of locally advanced breast cancers [31].

Cryosurgery induces tumor cell death directly by causing damage to cell membranes and organelles and indirectly by causing vascular compromise through thrombosis of small vessels [32, 33]. With decreasing temperatures, the cells dehydrate and proteins are damaged because of high solute concentration, resulting in damage to the membranes and disruption of the enzymatic machinery of the cell. With faster cooling, ice crystals form inside the cells, damaging cell membranes and organelles by mechanical effects. With repeated freezing, thermal conductivity of tissue increases and spreads the damage. The indirect damage to cells occurs through vascular ischemia, resulting in cell death. Vessel wall damage can occur because

of perivascular cellular hydration, resulting in vessel distension and mechanical injury, or from direct cell damage to endothelial cells lining the vessels. Both pathways ultimately lead to increased permeability, edema, and a coagulation cascade, giving rise to microthrombi in vessels and tissue ischemia. Reperfusion injury is also proposed to play a role in the cell damage [34].

4. Cryosurgery in bone and soft tissue sarcomas

There have been some reports of cryosurgery for bone tumors. Marcove et al. reported the first cryosurgery for primary and metastatic bone tumors in 1968 [35]. They used cryosurgery as a therapy for benign bone tumors of Aneurysmal bone cyst [36], giant cell tumor [37], chondroblastoma [38], malignant bone tumor of chondrosarcoma [39] and osteogenic sarcoma [40]. The treatment protocols were that, following tumor curettage, liquid nitrogen was poured into the bone cavity and then allowed to thaw. Freeze-thaw cycles were repeated 2 to 3 times to obtain maximum tissue necrosis. This method was difficult for treating tumor margins. Tsuchiya et al. developed a new cryosurgery method for reconstruction for malignant bone tumors using tumor-bearing autografts treated with liquid nitrogen. The operative technique consisted of en bloc excision of the tumor, removal of soft tissue, curettage of the tumor, drilling and preparation for internal fixation or prosthetic replacement before incubation for 20 minutes in liquid nitrogen, thawing at room temperature for 15 minutes, thawing in distilled water for ten minutes, followed by internal fixation with an intramedullary nail, plate or composite use of prosthetic replacement [41] (Figure 1). A newly modified technique using pedicle frozen autografts to save the

continuity of anatomical structures was reported recently [42] (Figure 2).

5. Cryoimmunology in carcinomas

Previous reports have suggested that the immune system is activated by cryosurgery. The immunologic effects of cryosurgery [43] were first documented by Shulman et al. [44], who demonstrated the production of antibodies against rabbit male reproductive tissues after freezing. These antibodies, which reached a peak 7-10 days after cryoablation, were found to be tissue- and species-specific. Ablin et al. reported that spontaneous regression of metastases occurred after cryosurgery of a primary prostate tumor in clinical cases and coined the term “cryoimmunotherapy” [45, 46]. They found enhancement of immune responses after repeated in situ freezing, which they explained was due to multiple inoculations with the same antigen. These anecdotal reports of regression of metastases in patients, whose primary tumors were treated by cryosurgery, further stimulated interest in the immunostimulatory potential of cryoablation.

In animal models, cryosurgery of tumor tissue resulted in the rejection of tumor re-challenge and in inhibition of secondary and metastatic tumor growth. Neel et al. [47] showed increased resistance to tumor re-challenge in cryosurgically treated mice as compared to those mice whose tumors were amputated, ligated, or not treated. The mechanism of cryosurgery-induced immune responses was thought to be due to increased tumor-specific antibodies that arose after freezing. Bagley et al. [48] demonstrated

that lymphocytes from cryosurgically treated animals were cytotoxic against the tumor cells the animals had been inoculated with, thus showing cell-mediated immunity to be at least partially responsible for the antitumor effects observed following cryosurgery. Misao et al. [49] further characterized the kinetics of cryoablation-induced antitumor immunity using metastasizing rat mammary tumor No. 1 in Spague-Dawley rats. They observed an initial decrease in antitumor immunity after cryosurgery, which later increased gradually to reach a peak at 10 weeks after cryosurgery. The initial dip was considered as a result of the activation of suppressor cells due to of cryosurgical stress or slow and steady absorption of antigens. Joosten et al. [50] demonstrated that freezing of one of the tumors was superior to excision in retarding the growth of the untreated tumor. They also demonstrated decreased metastatic growth of the melanoma cell line MV3 in mice treated by cryoablation as compared with surgical resection. Within six hours of the procedure, mice whose tumors were frozen had higher interleukin-1 and tumor necrosis factor levels than mice whose tumors were excised. Sabel et al. [51] also examined the response triggered by cryosurgery in mammary adenocarcinoma (MT-901) in BALB/c mice. They found higher levels of the type 1 helper cytokines interleukin-2 and interferon gamma and higher tumor rejection rates in animals treated with cryoablation. Cryoablation also increased natural killer cell activity as compared with surgery alone. The many promising results lead to questions about the mechanism(s) of cryoimmunology. Cryoablation of tumor tissue leads to coagulative necrosis of cells. This causes a cellular breakdown and release of intracellular contents and proinflammatory cytokines. In addition to cytokines, the release of

heat shock proteins, DNA, RNA, or the chromosomal protein high mobility group box chromosomal protein 1 serves as a stimulus for the innate immune response and attracts granulocytes, macrophages, and natural killer (NK) cells. These cells further release cytokines and chemokines.

DCs are critical professional antigen-presenting cells (APCs), crucially important in the capture, processing, and presentation of tumor antigens to tumor-specific T cells [52]. Necrotic tumor debris contains endogenous "danger signals," such as high mobility group box chromosomal protein 1, required for the activation and maturation of APCs. DCs reach the damaged tissue, take up tumor antigens in the context of inflammation and abundant cytokines, and undergo a change in phenotype with the upregulation of cell surface markers, such as costimulatory, adhesion, and integrin molecules [53]. The activated APCs migrate to tumor-draining lymph nodes where they present tumor antigens on major histocompatibility complex molecules, as well as costimulatory signals, to tumor-specific T cells, inducing their activation. Activated, effector CD4 T cells can attack major histocompatibility complex Class II^+ tumors and provide help to a variety of antitumor effector cells, including $CD8^+$ cytotoxic T cells, B cells, and macrophages [34].

Adjuvant therapy has also been considered in order to enhance immunity after cryosurgery. Urano et al. described that cryosurgery combined with administration of the polysaccharide Krestin increased cytokine production and cytotoxic activity [54]. Lubaroff et al. reported that cryosurgery combined with bacillus Calmette-Guerin (BCG) was capable of producing antitumor immunity that rejected from

re-challenge [55]. Udagawa et al. recently reported that cryoablation combined with intra-tumoral administration of BCG-CWS and DCs induced tumor-specific T cells and reduced metastases [56]. Recently, there have been reports on the use of cryosurgery with injection of DCs as antigen presenting cells. Machilenkin et al. reported that intratumoral injections of DCs following cryosurgery led to local tumor-induced, tumor-specific cytotoxic T lymphocytes responses as well as reduced lung metastasis and a prolonged survival rate in mouse models of lung carcinoma and melanoma [57]. Den Brok et al. reported that the immatured DCs injection following cryoablation induced DC maturation and antitumour immunity in a mouse melanoma cell model [53]. Udagawa et al. reported that intratumoral administration of dendritic cells stimulated with Bacillus Calmette-Guerin cell wall components, which matures and activates DCs, following cryoablation induced tumor-specific $CD8^+$ T cells and increased the antitumor effect in a mouse colon cancer model [58]. In a clinical study, Osada et al. reported that patients with unresectable liver tumors treated with cryosurgery displayed tumor necrosis not only in the treated areas, but also outside of the treated areas, and had increased serum IL-6, TNF-alpha, and Th1/Th2 ratio. However, patients with a localized effect who did not show reduced satellite lesions did not have increased cytokines and required another immune activation method [59].

5. Cryoimmunology in bone and soft tissue sarcomas

Similar to other malignant tumors, cryoimmunology for malignant bone and soft tissue tumors has received a great deal of interest. We reported the possible induction of a systemic antitumor immune response induced by re-implantation of destroyed tumor tissue treated with liquid nitrogen in a murine osteosarcoma model [60]. Tissue from the tumor was frozen in liquid nitrogen, thawed in distilled water and re-implanted in the same animal. In addition, some mice received the immunological response modifier OK-432. Re-implantation of tumor tissue after cryotreatment activated immune responses and inhibited metastatic tumor growth, and OK-432 synergistically enhanced the antitumor effect after treatment. Kawano et al. reported combining tumor cryotreatment with DC immunotherapy in order to promote tumor-specific immune responses. They investigated whether cryotreatment with DCs could enhance systemic immune responses and inhibit metastatic tumor growth in a same model. In order to evaluate the activation of antitumor immunity, they prepared six groups of C3H mice: excision only, DCs without reimplantation of the cryotreated primary tumor, reimplantation of the cryotreated primary tumor alone, DCs combined with reimplantation of the cryotreated primary tumor, DCs exposed to cryotreated tumor lysates without reimplantation of the cryotreated primary tumor, and DCs exposed to cryotreated tumor lysates with reimplantation of the cryotreated primary tumor. They then compared antitumor immunity between the groups. Mice that received DCs exposed to cryotreated tumor lysates with reimplantation of the cryotreated primary tumor group had high serum interferon-γ (IFN-γ), reduced pulmonary metastases, and increased numbers of $CD8^+$ T lymphocytes in metastatic areas. Their

conclusion was that combining tumor cryotreatment with DCs enhanced systemic immune responses and inhibited metastatic tumor growth [61]. We also evaluated the possible induction of a systemic antitumor response in reconstructions of malignant bone tumors using frozen autografts treated with liquid nitrogen in clinical settings. We have treated 24 cases of malignant bone tumors by this treatment and studied the associated immune responses. Our study cohort consisted of 14 men and 10 women, with a mean age of 38.3 years (range: 12 to 73). The tumors were located in 12 femurs, 6 tibias, 3 pelves, 2 humorous and one radius. The diagnoses of malignant bone tumor were 14 osteosarcoma, 8 metastatic bone tumors (3 renal cell carcinoma, 1 breast cancer, osteosarcoma, hepatocellular carcinoma, colon cancer and ureter carcinoma) and 2 chondrosarcoma. Blood samples were collected from the patients before surgery and one and three months after surgery for the measurement of IFN-γ and IL-12 in order to assess immunity. At the final follow-up, five patients had died (mean follow-up, 13.8 months), 15 remained free from disease (mean follow-up, 19.9 months) and four patients were alive with disease. The mean INF-γ relative concentrations one and three months after surgery compared with before surgery were 155.1 and 268.3%, and mean IL-12 relative concentrations were 190.6 and 432.2%, respectively. Composite values for all 24 patients showed progressive increases in INF-γ and IL-12 levels one and three months after surgery (Table 1). We attempted adjuvant therapy cryotreatment with DC immunotherapy in clinical trials in order to develop cryoimmunological therapy for malignant bone and soft tissue tumors. Although preliminary, we have some initial results for liquid nitrogen cryotreatment combined with DC immunotherapy in an

osteosarcoma patient. This is a 17-year-old girl with osteosarcoma of distal femur with multiple metastases in pelvis, lumbar, humerus and lung. After the standard treatment of neoadjuvant chemotherapy, we performed wide excision with liquid nitrogen cryotreatment and composite reconstruction of the right femur, followed by adjuvant chemotherapy. We performed DC immunotherapy twice. After DC immunotherapy, the lung metastasis was stable for 12 months and pelvic metastases were partially reduced. The final follow-up was DOD two years after the initial treatment, at which time we considered the combination of liquid nitrogen cryotreatment and DC immunotherapy to have limited tumor growth at least partially.

Conclusions

Cryoimmunology continues to evolve, and is impacted by further investigations into cryoimmunological mechanisms and novel techniques to detect disease. The need for research is evident in several aspects of cryoimmunology, especially in better understanding how the immune system becomes activated, translating these discoveries to the clinic and applying cryoimmunology to malignant bone and soft tissue tumors. The benefits provided by cryoimmunology combined with adjunct chemotherapy require investigation. Preclinical work with experimental tumors shows cryo-immunologic responses, but, as yet, the means by which the responses can be reliably used in a clinical setting to treat human disease are still developing.

References

1. Bacci G, Longhi A, Versari M et al (2006) Prognostic factors for osteosarcoma of the extremity treated with neoadjuvant chemotherapy: 15-year experience in 789 patients treated at a single institution. Cancer 106: 1154-1161

2. Bielack SS, Kempf-Bielack B, Delling G et al (2002) Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 20: 776-790

3. Meyers PA, Schwartz CL, Krailo M et al (2005) Osteosarcoma: a randomized, prospective trial of the addition of ifosfamide and/or muramyl tripeptide to cisplatin, doxorubicin, and high-dose methotrexate. J Clin Oncol 23: 2004-2011

4. Provisor AJ, Ettinger LJ, Nachman JB et al (1997) Treatment of non-metastatic osteosarcoma of the extremity with preoperative and postoperative chemotherapy: a report from the Children's Cancer Group. J Clin Oncol 15: 76-84

5. Tsuchiya H, Yasutake H, Yokogawa A et al (1992) Effect of chemotherapy combined with caffeine for osteosarcoma. J Cancer Res Clin Oncol 118: 567-569

6. Tsuchiya H, Tomita K, Mori Y et al (1999) Marginal excision for osteosarcoma with caffeine assisted chemotherapy. Clin Orthop Relat Res 358: 27-35

7. Tsuchiya H, Tomita K, Mori Y et al (1998) Caffeine-assisted chemotherapy and minimized tumor excision for non-metastatic osteosarcoma. Anticancer Res 18: 657-666

8. Tsuchiya H, Kanazawa Y, Abdel-Wanis ME et al (2002) Effect of timing of pulmonary metastases identification on prognosis of patients with osteosarcoma: the Japanese Musculoskeletal Oncology Group study. J Clin Oncol 20: 3470-3477

9. Pacey S, Ratain MJ, Flaherty KT et al (2009) Efficacy and safety of sorafenib in a subset of patients with advanced soft tissue sarcoma from a Phase II randomized discontinuation trial. Invest New Drugs. [Epub ahead of print]

10. Maki RG, D'Adamo DR, Keohan ML et al (2009) Phase II study of sorafenib in patients with metastatic or recurrent sarcomas. J Clin Oncol 27: 3133-3140

11. Kuenen BC, Tabernero J, Baselga J et al (2003) Efficacy and toxicity of the angiogenesis inhibitor SU5416 as a single agent in patients with advanced renal cell carcinoma, melanoma, and soft tissue sarcoma. Clin Cancer Res 9:1648–1655

12. Stopeck A, Sheldon M, Vahedian M et al (2002) Results of a phase I dose-escalating study of the antiangiogenic agent, SU5416, in patients with advanced malignancies. Clin Cancer Res 8:2798–2805

13. D'Adamo DR, Anderson SE, Albritton K et al (2005) Phase II study of doxorubicin and bevacizumab for patients with metastatic soft tissue sarcomas. J Clin Oncol 23:7135–7142

14. Fuller CK, Charlson JA, Dankle SK et al (2010) Dramatic improvement of inoperable angiosarcoma with combination paclitaxel and bevacizumab chemotherapy. J Am Acad Dermatol. 63: 83-84.

15. Jordan K, Wolf HH, Voigt W et al (2010) Bevacizumab in combination with sequential high-dose chemotherapy in solid cancer, a feasibility study. Bone Marrow Transplant 45:1704-1709

16. Sleijfer S, Ray-Coquard I, Papai Z et al (2009) Pazopanib, a multikinase angiogenesis inhibitor, in patients with relapsed or refractory advanced soft tissue sarcoma: a phase II study from the European organisation for research and treatment of cancer-soft tissue and bone sarcoma group (EORTC study 62043). J Clin Oncol 27:3126-3132

17. Sugiura H, Fujiwara Y, Ando M et al (2010) Multicenter phase II trial assessing effectiveness of imatinib mesylate on relapsed or refractory KIT-positive or PDGFR-positive sarcoma. J Orthop Sci 15: 654-660

18. Grignani G, Palmerini E, Stacchiotti S et al (2010) A phase 2 trial of imatinib mesylate in patients with recurrent nonresectable chondrosarcomas expressing platelet-derived growth factor receptor-α or -β: an Italian Sarcoma Group study. Cancer [Epub ahead of print]

19. Luksch R, Perotti D, Cefalo G et al (2003) Immunomodulation in a treatment program including pre- and post-operative interleukin-2 and chemotherapy for childhood osteosarcoma. Tumori 3: 263-268

20. Geiger J, Hutchinson R, Hohenkirk L et al (2000) Treatment of solid tumours in children with

tumour-lysate-pulsed dendritic cells. Lancet 356: 1163-1165

21. Tabarkiewicz J, Radej S, Hus I et al (2008) Dendritic cells based immunotherapy of patient with chondrosarcoma--case report. Folia Histochem Cytobiol 46:165-170

22. Ohta H, Hashii Y, Yoneda A et al (2009) WT1 (Wilms tumor 1) peptide immunotherapy for childhood rhabdomyosarcoma: a case report. Pediatr Hematol Oncol 26:74-83

23. Ayyoub M, Taub RN, Keohan ML et al (2004) The frequent expression of cancer/testis antigens provides opportunities for immunotherapeutic targeting of sarcoma. Cancer Immun 4:7

24. Segal NH, Blachere NE, Guevara-Patino JA et al (2005) Identification of cancer-testis genes expressed by melanoma and soft tissue sarcoma using bioinformatics. Cancer Immun 5:2

25. Mackall CL, Rhee EH, Read EJ et al (2008) A Pilot Study of Consolidative Immunotherapy in Patients with High-Risk Pediatric Sarcomas. Clin Cancer Res 14: 4850

26. Gage A (1998) History of cryosurgery. Semin Surg Oncol 14: 99 –109

27. Flanders VL, Gervais DA (2010) Ablation of Liver Metastases: Current Status. J Vasc Interv Radiol 21: S214–S222

28. Huang A, McCall JM, Weston MD et al (2002) Phase I study of percutaneous cryotherapy for colorectal liver metastasis. Br J Surg 89: 303-310

29. Chin JL, Lim D, Abdelhady M (2007) Review of primary and salvage cryo-ablation for prostate cancer. Cancer Control 14: 231-237

30. Atwell TD, Farrell MA, Callstrom MR et al (2007) Percutaneous cryo-ablation of large renal masses: technical feasibility and short-term outcome. AJR Am J Roentgenol 188: 1195-1200

31. Sabel MS, Kaufman CS, Whitworth P et al (2004) Cryo-ablation of early-stage breast cancer: work-in-progress report of a multi-institutional trial. Ann Surg Oncol 11: 542-549

32. Karlsson JO, Cravalho EG, Borel Rinkes IH et al (1993) Nucleation and growth of ice crystals inside cultured hepatocytes during freezing in the presence of dimethyl sulfoxide. Biophys J 65:2524-2536

33. Hoffmann NE, Bischof JC (2002) The cryobiology of cryosurgical injury. Urology 60: 40-49

34. Sidana A, Chowdhury WH, Fuchs EJ et al (2010) Cryoimmunotherapy in urologic oncology. Urology 75: 1009-1014

35. Marcove RC, Miller TR, Cahan WC et al (1968) The treatment of primary and metastaticbone tumors by repetitive freezing. Acad Med 44: 532-544

36. Biesecker JL, Marcove RC, Huvos AG et al (1970) Aneurysmal bone cysts. A clinicopathologic study of 66 cases. Cancer 26: 615-625

37. Marcove RC, Weis LD, Vaghaiwalla MR et al (1978) Cryosurgery in the treatment of giant cell tumors of bone. A report of 52 consecutive cases. Cancer 41: 957-969

38. Huvos AG, Marcove RC (1973) Chondroblastoma of bone. A critical review. Clin Orthop Relat Res 95: 300-312

39. Marcove RC, Miké V, Hutter RV et al (1972) Chondrosarcoma of the pelvis and upper end of the femur. An analysis of factors influencing survival time in one hundred and thirteen cases. J Bone Joint Surg Am 54: 561-572

40. Marcove RC, Abou Zahr K, Huvos AG et al (1984) Cryosurgery in osteogenic sarcoma: report of three cases. Compr Ther 10: 52-60

41. Tsuchiya H, Wan SL, Sakayama K et al (2005) Reconstruction using an autograft containing tumour treated by liquid nitrogen. J Bone Joint Surg Br 87: 218-225

42. Tsuchiya H, Nishida H, Srisawat P et al (2010) Pedicle frozen autograft reconstruction in malignant bone tumors. J Orthop Sci 15: 340-349

43. Yantorno C, Soanes WA, Gonder MJ et al (1967) Studies in cryoimmunology. I. The production of antibodies to urogenital tissue in consequence of freezing treatment. Immunology 12: 395-410

44. Shulman S, Brandt EJ, Yantorno C (1968) Studies in cryo-immunology. II. Tissue and species specificity of the autoantibody response and comparison with iso-immunization. Immunology 14: 149-158

45. Ablin RJ (1972) Cryoimmunotherapy. Br Med J 3: 476

46. Soanes WA, Gonder MJ, Ablin RJ (1970) A possible immuno-cryothermic response in prostatic cancer. Clin Radiol 21: 253-255

47. Neel HB III, Ketcham AS, Hammond WG (1973) Experimental evaluation of in situ oncocide for

primary tumor therapy: comparison of tumor-specific immunity after complete excision, cryonecrosis and ligation. Laryngoscope 83: 376-387

48. Bagley DH, Faraci RP, Marrone JC et al (1974) Lymphocyte mediated cytotoxicity after cryosurgery of a murine sarcoma. J Surg Re 17: 404-406

49. Misao A, Sakata K, Saji S et al (1981) Late appearance of resistance to tumor rechallenge following cryosurgery. A study in an experimental mammary tumor of the rat. Cryobiology 18: 386-389

50. Joosten JJ, Muijen GN, Wobbes T et al (2001) In vivo destruction of tumor tissue by cryoablation can induce inhibition of secondary tumor growth: an experimental study. Cryobiology 42: 49-58

51. Sabel MS, Nehs MA, Su G et al (2005) Immunologic response to cryoablation of breast cancer. Breast Cancer Res Treat 90: 97-104

52. Steinman RM (2003) Some interfaces of dendritic cell biology. APMIS 111: 675-697

53. den Brok MH, Sutmuller RP, Nierkens S et al (2006) Efficient loading of dendritic cells following cryo and radiofrequency ablation in combination with immune modulation induces anti-tumour immunity. Br J Cancer 95: 896-905

54. Urano M, Tanaka C, Sugiyama Y et al (2003) Anti-tumor effects of residual tumor after cryo-ablation: the combined effect of residual tumor and a protein-bound polysaccharide on multiple liver metastases in a murine model. Cryobiology 46: 238-245

55. Lubaroff DM, Reynolds CM, Canfield L (1981) Immunologic aspects of the prostate. Prostate 23:

233-248

56. Udagawa M, Saito CK, Hasegawa G et al (2006) Enhancement of immunologic tumor regression by intra-tumoral administration of dendritic cells in combination with cryoablative tumor pretreatment and bacillus Calmette-Guerin cell wall skeleton stimulation. Clin Cancer Res 12: 7465-7475

57. Machlenkin A, Goldberger O, Tirosh B et al (2005) Combined dendritic cell cryosurgery of tumor induces systemic antimetastatic immunity. Clin Cancer Res 11: 4955-4961

58. Udagawa M, Kudo-Saito C, Hasegawa G et al (2006) Enhancement of immunologic tumor regression by intratumoral administration of dendritic cells in combination with cryoablative tumor pretreatment and bacillus calmette-guerin cell wall skeleton stimulation. Clin Cancer Res 12: 7465-7475

59. Osada S, Imai H, Tomita H et al (2007) Serum cytokine levels in response to hepatic cryoablation. J Surg Oncol 95: 491-498

60. Nishida H, Tsuchiya H, Tomita K (2008) Re-implantation of tumour tissue treated by cryotreatment with liquid nitrogen induces anti-tumour activity against murine osteosarcoma. J Bone Joint Surg Br 90: 1249-1255

61. Kawano M, Nishida H, Nakamoto Y et al (2010) Cryoimmunologic Antitumor Effects Enhanced by Dendritic Cells in Osteosarcoma. Clin Orthop Relat Res 468: 1373-1383.

Figure 1: Schematic diagram of cryotreatment using liquid nitrogen (free frozen method)

(a) Bone tumor in the ilium

(b) Internal hemipelvectomy and tumor curettage

(c) The specimen contained with tumor was frozen in liquid nitrogen

(d) Reconstruction using a plate with bone cement

Figure 2: Schematic diagram of cryotreatment using liquid nitrogen (pedicle frozen method)

(a) Bone tumor in the proximal femur

(b) Hip dislocation and tumor curettage

(c) Rotation of the bony lesion connecting with the limb, which is then frozen in liquid nitrogen

(d) Reconstruction using a hemiarthroplasty with bone cement

Figure 3: X-ray images, bone-scintigram and intraoperative photograph

(a) Preoperative radiography showing osteosarcoma of the distal femur

(b) Preoperative bone scintigram showing multiple bone metastases in pelvis, lumber, humerus and so on.

(c) Photograph of the cryotreatment method. The specimen was frozen in liquid nitrogen and reconstruction frozen autograft and total knee arthroplasty composite.

(d) Postoperative radiography showing composite reconstruction

Figure 4: Pelvic computed tomography (CT) images pre- and post- DC immunotherapy after cryptreatment

(a) Pelvis CT before DC immunotherapy, showing multiple bone metastases

(b) Pelvis CT 3 months after DC immunotherapy, demonstrating reduction of the pelvic bone metastases

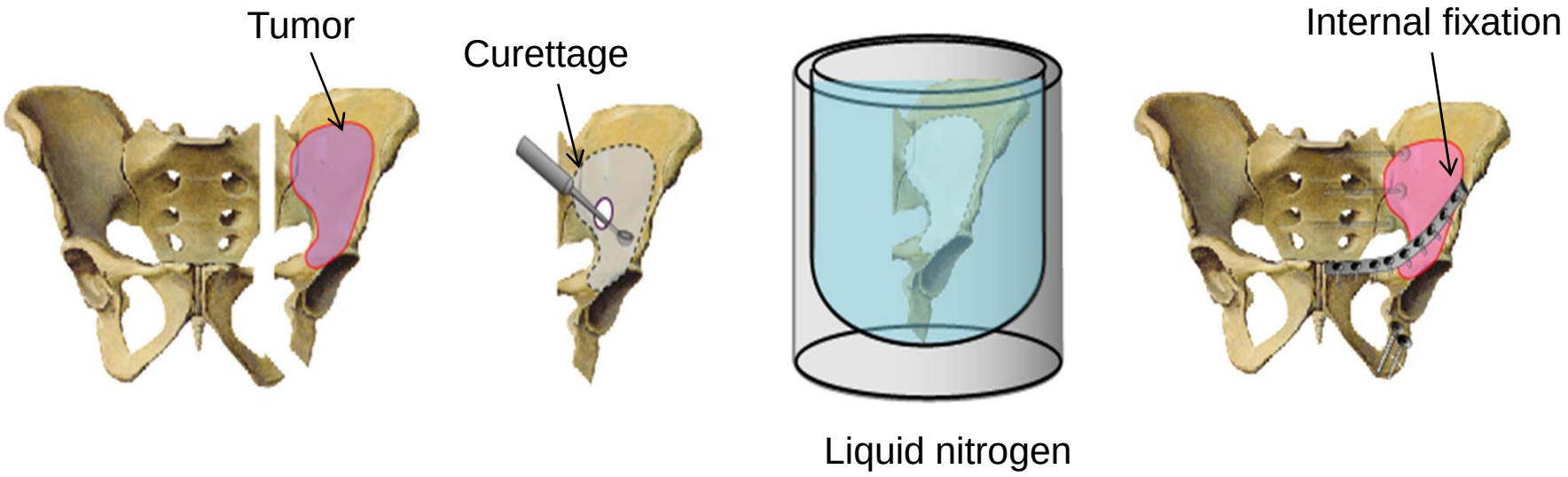


Fig. 1

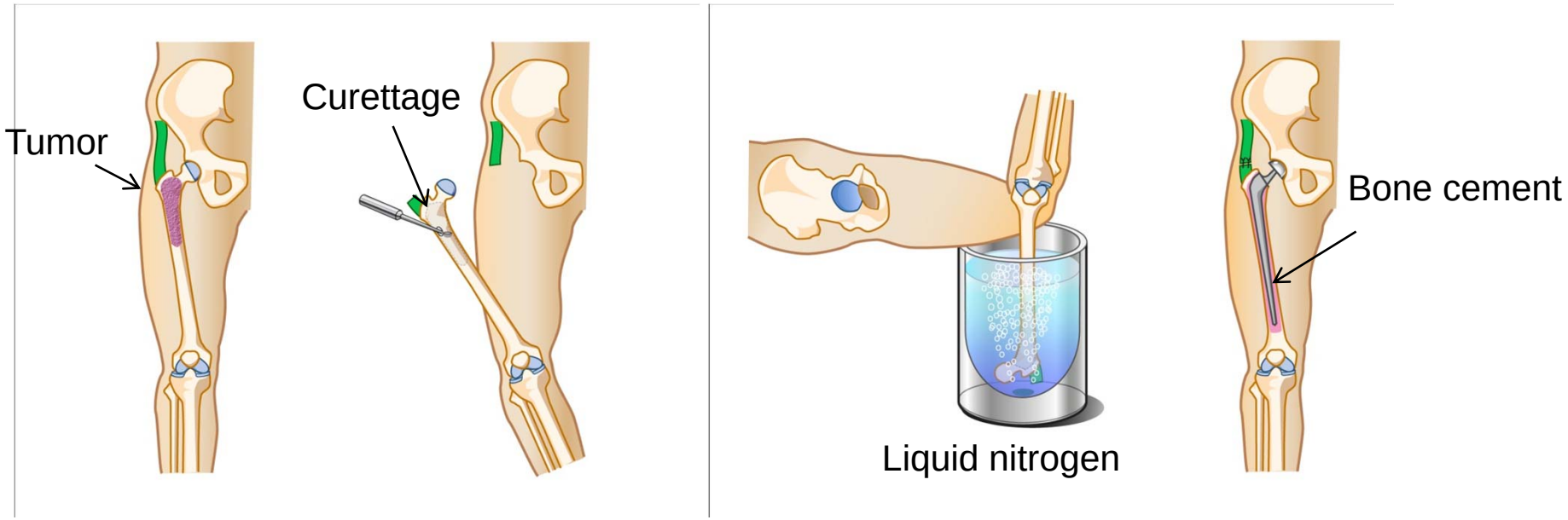
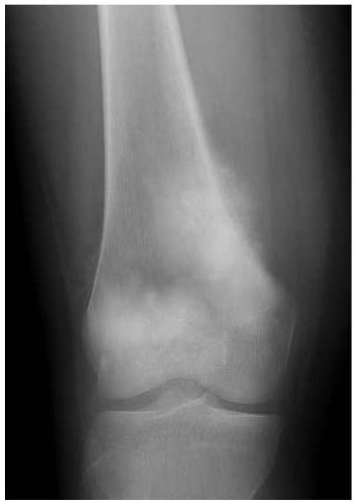


Fig. 2

Table 1. Immunological reaction of patients in reconstructions of malignant bone tumors using frozen autografts treated with liquid nitrogen

Case	Sex/age	Site	Diagnosis	Follow-up	IFN-gamma EIA					IL-12					Outcome
					pre-op.	post-op. 1M	concentrations 1M	post-op. 3M	concentrations 3M	pre-op.	post-op. 1M	concentrations 1M	post-op. 3M	concentrations 3M	
	(years)			(month)	(IU/ml)	(IU/ml)	(%)	(IU/ml)	(%)	(pg/ml)	(pg/ml)	(%)	(pg/ml)	(%)	
1	M/65	radius	metastasis of renal cell carcinoma	14	34.6	28.1	81.2	14.5	41.9	55.8	38.8	69.5	31.1	55.7	CDF
2	F/48	tibia	osteosarcoma	21	54.7	36.8	67.3	21.7	39.7	7.8	7.8	100.0	7.8	100.0	CDF
3	F/17	femer	osteosarcoma	18	8.3	6.3	75.9	8.7	104.8	7.8	7.8	100.0	7.8	100.0	CDF
4	F/71	femer	metastasis of ureter carcinoma	15	32.9	45.1	137.1	45.7	138.9	33.8	11.4	33.7	57.1	168.9	AWD
5	M/20	tibia	osteosarcoma	16	37.4	35.5	94.9	26.5	70.9	36.3	12.5	34.4	10.6	29.2	CDF
6	M/27	tibia	osteosarcoma	16	65.3	11.7	17.9	57	87.3	165	7.8	4.7	7.8	4.7	CDF
7	M/51	femer	osteosarcoma	16	123	163	132.5	58.1	47.2	34.1	296	868.0	7.8	22.9	CDF
8	M/14	tibia	osteosarcoma	27	2.8	8.7	310.7	7.3	260.7	7.8	7.8	100.0	7.8	100.0	CDF
9	M/20	tibia	osteosarcoma	14	10.7	5.1	47.7	28.5	266.4	7.8	7.8	100.0	51.2	656.4	CDF
10	M/18	femur	osteosarcoma	11	145	134	92.4	54.3	37.4	82.3	199	241.8	103	125.2	CDF
11	M/15	femur	osteosarcoma	8	2.5	21.2	848.0	67.9	2716.0	7.8	15.2	194.9	143	1833.3	CDF
12	F/49	femer	metastasis of brest cancer	33	15.9	23.7	149.1	14.7	92.5	30.5	7.8	25.6	7.8	25.6	NED
13	F/18	femer	metastasis of osteosarcoma	14	24.4	34.9	143.0	58.2	238.5	7.8	66	846.2	321	4115.4	DOD
14	F/12	humerous	osteosarcoma	6	6.7	3.7	55.2			7.8	7.8	100.0			DOD
15	F/22	femur	osteosarcoma	18	53.9	109	202.2	47.6	88.3	7.8	7.8	100.0	7.8	100.0	DOD
16	M/42	pelvis	osteosarcoma	14	127	139	109.4	47.2	37.2	35.8	19.5	54.5	13	36.3	DOD
17	M/34	femur	chondrosarcoma	29	14.4	42.9	297.9	47.6	330.6	9.9	15.1	152.5	8.7	87.9	CDF
18	M/62	femur	periosteal osteosarcoma	25	45.6	31.6	69.3	54.3	119.1	7.8	7.8	100.0	15.4	197.4	NED
19	F/76	humerous	metastasis of colon cancer	17	76.2	90	118.1			7.9	36.5	462.0			DOD
20	M/41	pelvis	chondrosarcoma	25	88.8	130	146.4			55.4	54.1	97.7			CDF
21	M/52	pelvis	metastasis of renal cancer	31	154	71.6	46.5			102	8.2	8.0	21.1	20.7	AWD
22	F/18	tibia	osteosarcoma	25	78	5	6.4	86.8	111.3	47.5	7.8	16.4			AWD
23	M/73	femer	metastasis of liver cell carcinoma	25	60	116	193.3			7.8	7.8	100.0			AWD
24	F/54	femer	metastasis of renal cell carcinoma	24	12	33.7	280.8			12	79.7	664.2			NED
average					53.1	55.3	155.1	41.5	268.3	32.8	39.1	190.6	46.1	432.2	

post-op. 1M, post -op. 1 month; post-op. 3M, post -op. 3 months;
concentrations 1M; concentrations of one month after compared with before surgery; concentrations 3M; concentrations of three months after compared with before surgery
AWD, alive with disease; CDF, continuously disease-free; DOD, died of disease; NED, no evidence of disease



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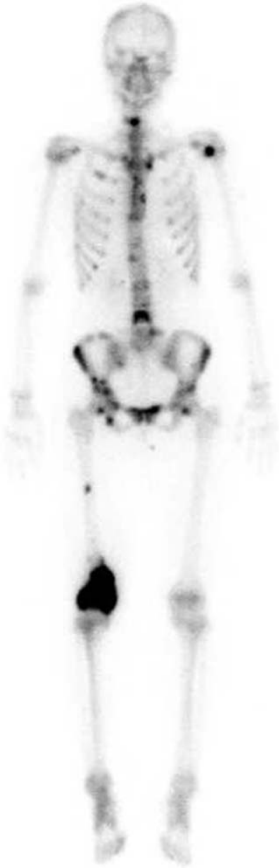

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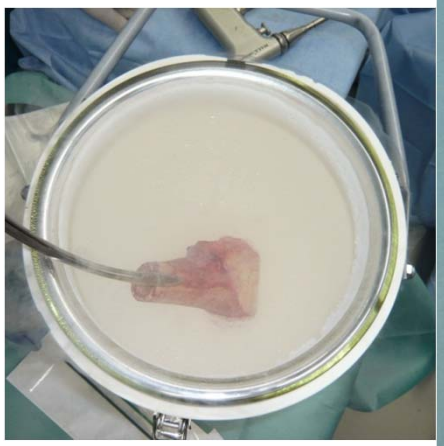
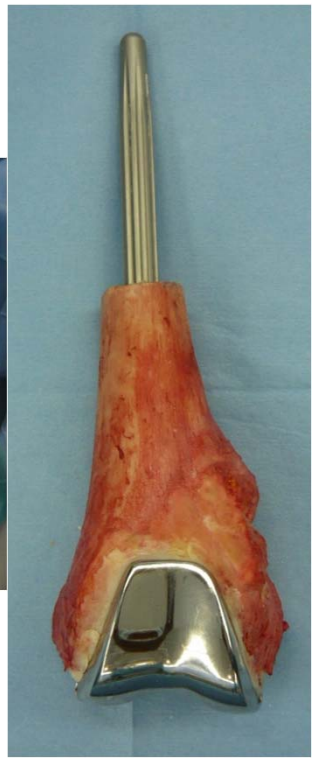
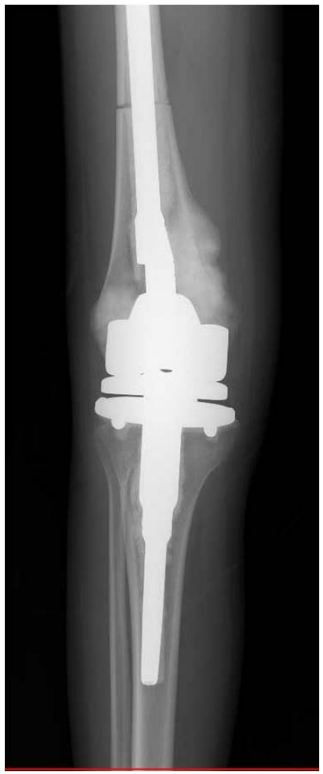


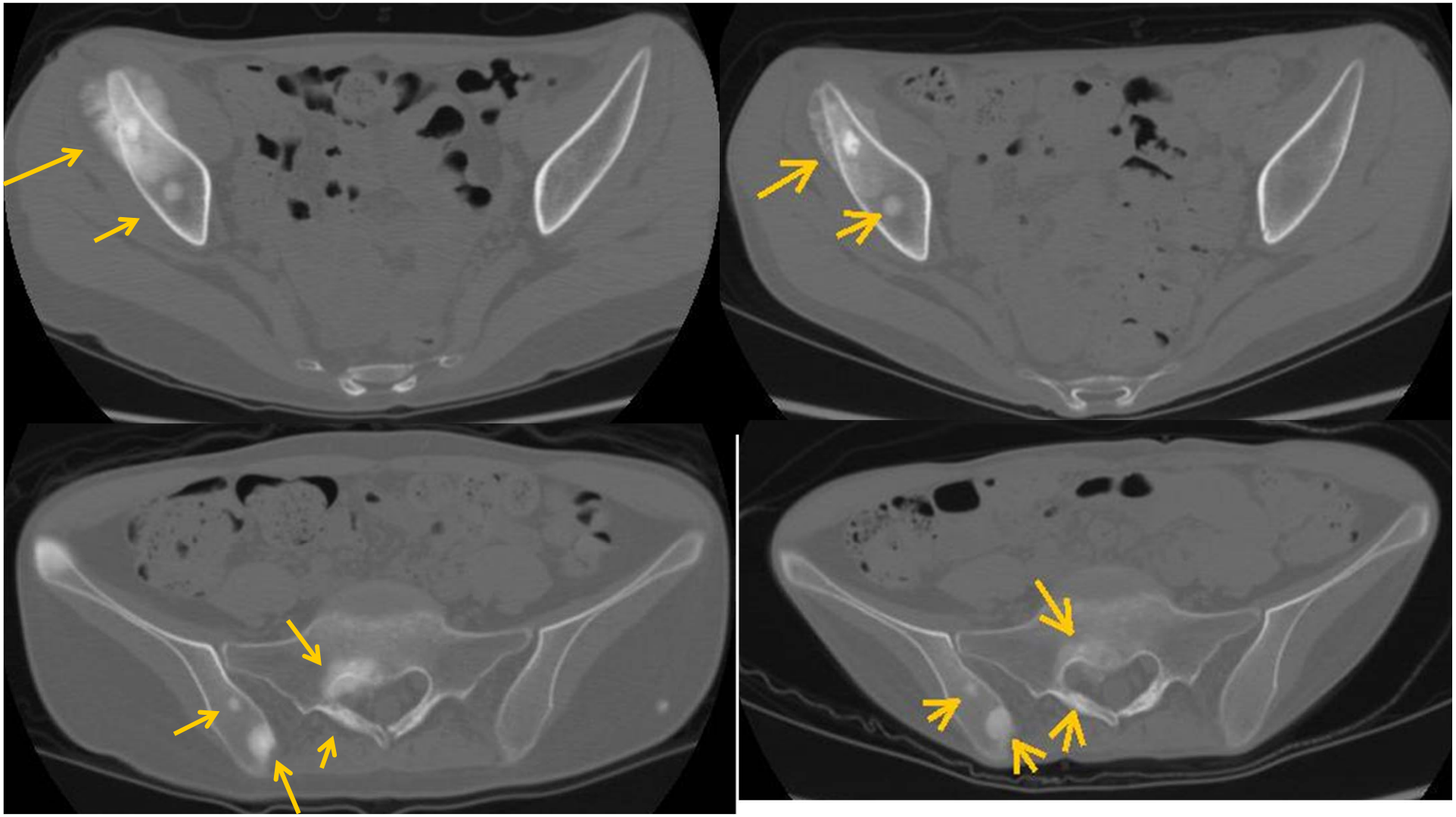


(c)



(d)

Fig. 3



(a) (b)

Fig. 4