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Cryimmunology for malignant bone and soft-tissue tumors

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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/m² 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 (9×10^6 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 immature DCs injection following cryoablation induced DC maturation and antitumor 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 pelvises, 2 humeri 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 IFN- 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 IFN- 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.

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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

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 (month)	IFN-gamma EIA						IL-12					Outcome
	(years)				pre-op. (IU/ml)	post-op. 1M (IU/ml)	concentrations 1M (%)	post-op. 3M (IU/ml)	concentrations 3M (%)	pre-op. (pg/ml)	post-op. 1M (pg/ml)	concentrations 1M (%)	post-op. 3M (pg/ml)	concentrations 3M (%)		
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	humorous	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	humorous	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

(a)

(b)

(c)

(d)

Fig. 3

(a)

(b)

Fig. 4