

Tomohide Tsukahara and Takuro Wada

Abstract

Osteosarcoma is a highly malignant neoplasm occurring in young persons. The prognosis of nonresponders to chemotherapy is still poor and new treatment modalities are required. We have been working on the development and clinical application of immunotherapy for osteosarcoma since 1998. In this chapter, we describe our studies and discussed the future directions of immunotherapy for osteosarcoma.

Keywords

Tumor-associated antigen • Peptide vaccination • PBF • HLA-A24 • HLA-A2

3.1 Introduction

Osteosarcoma is a highly malignant neoplasm occurring primarily in young persons. The survival rate of patients was under 20 % before 1970. The introduction of chemotherapy raised the 5-year survival rate to 60–70 % in the 1970s [1]. Around the same time, pioneering adjuvant immunotherapy trials using autologous tumor vaccines for patients with osteosarcoma were conducted and surprisingly showed clinical benefits [2]. However, immunotherapy did not draw much attention because of the strong effects of chemotherapy. In the 2000s, the survival rate of patients with osteosarcoma has reached a plateau, and the limitations of chemotherapy have led to the spotlight focusing on immunotherapy again. In this chapter,

T. Tsukahara (✉)

Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo 060-8556, Japan

e-mail: tukahara@sapmed.ac.jp

T. Wada

Saiseikai Otaru Hospital, 10-1, Chikko, Otaru 047-9997, Japan

we describe and discuss our studies conducted for the development of peptide-based immunotherapy for osteosarcoma from basic experiments to clinical trials and the perspectives for the future.

3.2 Development of Immunotherapy for Osteosarcoma

3.2.1 Establishment of Osteosarcoma Cell Line OS2000 Showing Humoral and Cellular Immunogenicity

First, we established the new osteosarcoma cell line OS2000 from a biopsy specimen of osteosarcoma of the right distal femur of a 16-year-old female who underwent surgical resection of both eyes due to retinoblastoma because of the lack of the tumor suppressor gene Rb at infancy. Establishing the cell line took more than 3 years [3]. OS2000 cells showed *in vivo* tumorigenicity in nude mice and expressed osteocalcin mRNA when treated with 1,25(OH)₂D₃. These findings suggested that the characteristics of OS2000 were compatible with osteosarcoma. Using OS2000 and autologous sera, we performed serological identification using a recombinant expression cloning (SEREX) method to identify tumor-associated antigens recognized by the humoral immune system. As a result, we cloned cDNA coding smooth muscle myosin light chain and HLA-Cw*01:02 identical to the wild type. However, we could not detect any reactivity against these molecules in the sera of other patients with osteosarcoma.

Therefore, we next performed mixed lymphocyte-tumor cell culture using irradiated OS2000 cells as the antigenic stimulator and autologous peripheral blood mononuclear cells as the responder. The responder, TcOS2000, showed specific cytotoxicity against OS2000 cells. After limiting dilution of TcOS2000, we obtained three CTL clones specifically recognizing autologous OS2000 cells. Among them, we selected CTL clone TcOS cl-303 on the basis of the high cytotoxic ability. OS2000 expressed HLA-A*24:02, B*55:02, and Cw*01:02. TcOS cl-303 could recognize OS2000 and allogeneic osteosarcoma cell lines in the context of HLA-B*55:02.

These results suggested that human osteosarcoma could naturally prime humoral and cellular immunity. Moreover, the autologous pair of TcOS cl-303 and OS2000 could be useful as a probe to identify the antigenic peptide naturally presented by HLA-B*55:02 on osteosarcoma cells.

3.2.2 Identification of the Osteosarcoma Antigen, Papillomavirus Binding Factor (PBF)

Using the autologous pair of OS2000 and CTL clone TcOS cl-303, we performed cDNA library expression cloning. First, we constructed a cDNA library containing more than 100,000 clones from OS2000 mRNA. Next, we transiently transfected pools of library cDNA (80–120 clones per pool) into 293EBNA cells stably

expressing exogenous HLA-B*55:02, followed by coculture of TcOS cl-303. The cytotoxicity of TcOS cl-303 was measured by LDH release assay, detecting LDH released from dead cells in the supernatant. After screening approximately 1000 pools of library cDNA, we selected the cDNA pool 1B9. The cDNA pool was divided into single clones (approximately 400 clones) and screened again. Four cDNA clones were recognized by TcOS cl-303 and encoded the identical cDNA sequence. We designated the antigenic cDNA clone 1B9.1H4. cDNA clone 1B9.1H4 contained the 3' part of the open reading frame of papillomavirus binding factor (PBF). No mutations and no frameshift were identified. TcOS cl-303 could recognize the full length of PBF cDNA as well as the cDNA clone 1B9.1H4. Next, to identify CTL epitope of TcOS cl-303, we performed epitope mapping using truncated variants of PBF mRNA. We made more than 90 truncated variants. The C-terminus part of PBF was antigenic, and arginine at position 510 of 512 was the C-terminus amino acid of the CTL epitope. Subsequently, we synthesized peptides with various lengths and screened the response of TcOS cl-303 against 293EBNA cells expressing HLA-B*55:02 pulsed with each peptide. Finally, we identified a 12-mer peptide (CTACRWKACQR) as the epitope of TcOS cl-303 and simultaneously proved that PBF was the osteosarcoma antigen recognized by the autologous CTL clone [4].

3.2.3 Expression and Function of the Osteosarcoma Antigen PBF

mRNA of PBF was expressed in various sarcoma tissues, including osteosarcoma (79 %), Ewing's sarcoma (92 %), and synovial sarcoma (90 %). In addition, mRNA of PBF was expressed in epithelial cancer tissues such as lung cancer (90 %), gastric cancer (56 %), colon cancer (14 %), and breast cancer (80 %). On the other hand, mRNA of PBF was expressed in some normal organs, including the pancreas, ovary, and spleen. However, immunohistochemistry using an anti-PBF polyclonal antibody revealed that PBF protein was detected in tumors but not in normal organs expressing PBF mRNA [4]. As shown in Table 3.1, PBF protein was expressed in osteosarcoma (92 %), Ewing's sarcoma (90 %), and synovial sarcoma (100 %). Moreover, PBF expression was significantly correlated with poor prognoses for patients with osteosarcoma and Ewing's sarcoma [5]. These results suggested that PBF might be a good candidate molecule to target in an immunotherapeutic approach using antigenic peptide vaccination.

PBF is a transcription factor containing a Zn finger domain. It was first reported to regulate transcriptional activity in the human papillomavirus type 8 genome DNA [6]. However, osteosarcoma is not related to papillomavirus infection. Therefore, we hypothesized that PBF might regulate cell survival or apoptosis in osteosarcoma cells. To characterize the function of PBF, we performed yeast two-hybrid screening using a cDNA library of OS2000 and PBF as the bait to isolate the binding partners of PBF. As a result, we obtained cDNA clone 93 encoding Scythe/BAT3 (currently designated BAG6) as the associated molecule of PBF [7]. Scythe/BAT3 was reported to be an essential factor for cell proliferation [8] and inhibitor of

Table 3.1 Expression of PBF protein in bone and soft tissue sarcoma

	PBF status*			
	Number	Negative	Low	High
Bone tumors				
Osteosarcoma				
Primary lesion	83	7	27	49
Lung metastatic lesion	8	1	2	5
Chondrosarcoma	5	2	0	3
Malignant fibrous histiocytoma	2	1	0	1
Soft tissue tumors				
Malignant fibrous histiocytoma	15	2	7	6
Liposarcoma	5	1	0	4
Rhabdomyosarcoma	3	0	0	3
Clear-cell sarcoma	3	0	1	2
Ewing's sarcoma	2	0	0	2
Synovial sarcoma	2	0	1	1
Leiomyosarcoma	2	0	1	1
Alveolar soft-part sarcoma	2	0	0	2
MPNST	1	0	0	1
Total	133	14	39	80

MPNST malignant peripheral nerve sheath tumor

*Expression of PBF was graded according to the number of tumor cells stained positively with the anti-PBF antibody; negative (<5 % positive cells), low (5–50 % positive cells), or high (>50 % positive cells)

apoptosis [9]. Surprisingly, overexpression of PBF could induce apoptosis in 293EBNA cells and OS2000 cells. However, coexpression of Scythe/BAT3 could inhibit cell death induced by PBF. Moreover, PBF and Scythe/BAT3 colocalized in the nuclei of osteosarcoma cells but in the cytoplasm of normal tissues. These results suggested that colocalization of PBF and BAG6 in nuclei might be important for the survival of osteosarcoma cells. Recently, PBF was reported to be associated with innate immunity [10] and adipogenesis [11]. The function of PBF is variable and other characteristics might be revealed in the future.

3.2.4 Expression Status of HLA Class I in Osteosarcoma

For peptide-based immunotherapy for osteosarcoma, expression of target molecules in tumor cells is required. However, the expression of HLA class I molecules is also important to present antigenic peptides toward T-cell receptors of CTLs recognizing tumor cells. To assess this issue, we analyzed the expression status of HLA class I molecules on formalin-fixed paraffin-embedded sections of primary osteosarcoma tissues by immunohistochemistry using the pan-HLA class I monoclonal antibody EMR8-5 [12]. HLA class I molecules were detected as high grade (positive cells >50 %) in 48 %, low grade (5 ≤ positive cells ≤50 %) in 32 %, and

and negative (positive cells <5 %) in 20 % of primary lesions of osteosarcoma. Surprisingly, the overall survival and event-free survival of the patients with HLA class I-positive osteosarcoma were significantly better than those with HLA class I-negative osteosarcoma [13]. These findings suggested that osteosarcoma might be an immunogenic tumor surveyed by the human cellular immune system. In addition, peptide-based immunotherapy might be able to elicit the response of CTLs naturally reacting with tumor cells.

3.2.5 Identification of CTL Epitopes Presented by HLA-A24 and HLA-A2

As described above, we identified the antigenic peptide encoded by PBF in the context of HLA-B*55:02. However, this epitope is not adequate to clinically immunize patients with osteosarcoma because the frequency of the allele is too low (less than 2 %) in Japanese. Therefore, we identified the CTL epitopes of PBF in the context of HLA-A*24:02 and HLA-A*02:01. At first, ten candidate peptides each for HLA-A*24:02 and HLA-A*02:01 were selected using the peptide motif prediction system BIMAS. Using in vitro peptide binding assay, we selected peptides PBF A24.2 (AYRPVSRNI) and PBF A2.2 (ALPSFQIPV) as candidates to assess their immunogenicity. Next, we synthesized MHC/peptide tetramers (HLA-A*24:02/PBF A24.2 and HLA-A*02:01/PBF A2.2) and assessed the frequency of tetramer-positive cells in peripheral mononuclear cells of patients with osteosarcoma. We found that the frequencies of tetramer-positive cells ranged from 5×10^{-7} to 7×10^{-6} and from 2×10^{-7} to 5×10^{-6} in HLA-A*24:02-positive and HLA-A*02:01-positive patients, respectively [14]. These frequencies were compatible with that of anti-MAGE-3 peptide CTL in preimmunized HLA-A1 patients with melanoma ($<1.3 \times 10^{-6}$) [15]. Moreover, tetramer-positive cells could recognize allogeneic osteosarcoma cell lines in the context of HLA-A*24:02 or HLA-A*02:01. Therefore, peptides PBF A24.2 and PBF A2.2 were used for studies of clinical vaccination.

3.2.6 Clinical PBF-Derived Peptide Vaccination Study in HLA-A24-Positive and HLA-A2-Positive Patients with Osteosarcoma

Under approval by the IRB, we started a clinical phase I peptide vaccination trial in 2008. To date, five HLA-A24-positive patients and five HLA-A2-positive patients with osteosarcoma have been enrolled in the study. Three had shown stable disease (SD) and the other seven had progressive disease (PD). One HLA-A2-positive patient with metastatic lesions in the bilateral lungs and subcutaneous region of the right lower back vaccinated with peptide PBF A2.2 survived for 31 months without any other therapeutic intervention. The patient received the peptide PBF A2.2 vaccination under three protocols: Protocol 1 (six subcutaneous vaccinations with 1 mg of peptide PBF A2.2 mixed with incomplete Freund's adjuvant [IFA] at

14-day intervals), Protocol 2 (vaccination with 10 mg of the peptide mixed with IFA), and Protocol 3 (vaccination with 1 mg of the peptide mixed with IFA and subcutaneous injection of interferon- α on the same day and 3 days after the vaccination). Although Protocol 1 and Protocol 2 were successfully completed, Protocol 3 was discontinued after the 1st vaccination because of leukopenia that might have been a side effect of interferon- α . Vaccine peptide-specific immunological responses were observed by ELISpot assay [16] in Protocols 1, 2, and 3 (Fig. 3.1a). Protocol 2 showed the best immunological response. High-dose vaccination of the peptide seemed to be more important than combination with interferon- α . Although clinical responses were evaluated as PD due to the appearance of new metastatic lesions in the lung at the end of Protocol 1, a subcutaneous metastatic lesion showed marginal calcification (Fig. 3.1b, c). Such marginal calcification was occasionally observed after chemotherapy and considered to be a partial response. For Protocol 2 and Protocol 3, clinical responses were evaluated as SD. Considering the good immunological responses and the clinical observation of marginal calcification of the subcutaneous lesion, we investigated a resected specimen of the subcutaneous metastatic lesion after Protocol 1 (Fig. 3.1d). Microscopically viable tumor cells were observed. However, immunohistochemistry revealed CD8+ T-cell infiltration into the metastatic tumor, which was not observed in the primary biopsy specimen before the vaccination therapy. Obviously, a natural nonspecific response could not be denied. Nevertheless, we believe that the tumor-infiltrating lymphocytes were elicited by the peptide vaccination and contributed to killing the tumor for a long time. The clinical phase I trial is still continuing in our institute. After the trial, we are planning a vaccination trial for HLA-A24 patients with sarcoma with high risk for metastasis, including osteosarcoma, synovial sarcoma, and Ewing's sarcoma in the adjuvant setting using a peptide cocktail. In addition to peptide PBF, an inhibitor of apoptosis protein-derived peptide survivin 2B [16] is also used.

3.3 Future Perspectives of Immunotherapy for Osteosarcoma

Recently, monoclonal antibodies against immune checkpoint molecules (ipilimumab for CTLA-4 and nivolumab for PD-1) showed dramatic clinical responses in patients with melanoma [17, 18]. However, the response might be limited to some cancers having the characteristics of natural immunogenicity. In melanoma, many mutated antigens are recognized by tumor-infiltrating lymphocytes [19, 20]. In addition, melanoma antigens could be presented to and efficiently prime specific CTLs by Langerhans cells existing abundantly in the skin around the tumor. Indeed, ipilimumab did not show any clinical benefit for patients with synovial sarcoma [21]. However, we found that chromosomal translocation SYT-SSX-derived peptide vaccination could induce an immune response and provide clinical benefits including long SD (>20 months) [22]. On the other hand, adoptive cell transfer using engineered T lymphocytes expressing TCR directed to cancer-testis antigen NY-ESO-1 showed a clinical response in synovial

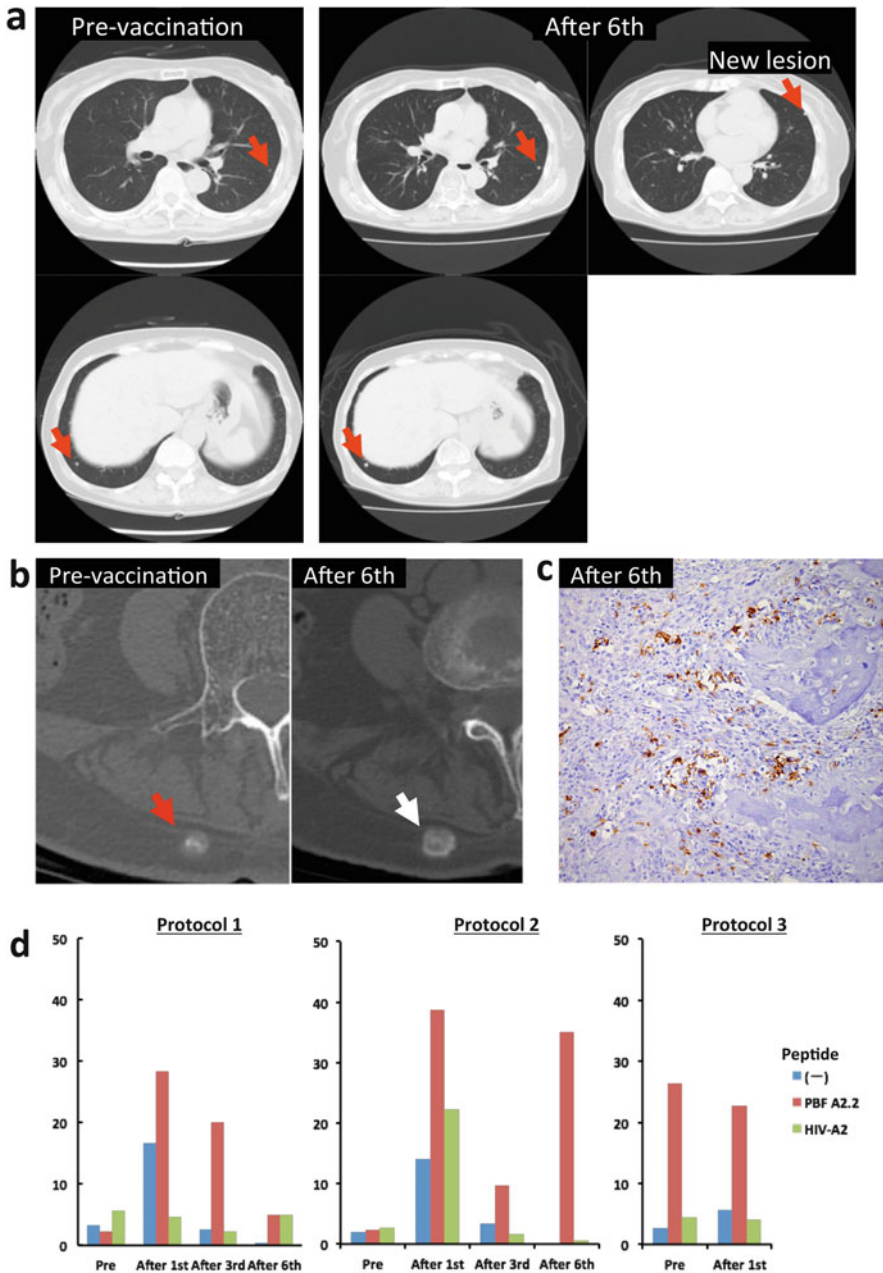


Fig. 3.1 Immunological and clinical responses against PBF peptide vaccination. (a) ELISpot assay. PBMC obtained from peripheral blood samples of the patient was in vitro stimulated with the peptide PBF A2.2 and used as responders for ELISpot assay. T2 cells pulsed with indicated peptides were used as stimulators. (b, c) Computed tomography of the bilateral lungs (b) and right lower back (c) before and after vaccination in Protocol 1. Metastatic lesions were indicated in *red arrows*. Marginal calcification appeared after 6th vaccination was indicated in a *white arrow*. (d) Immunohistochemistry of the resected subcutaneous lesion. Tumor-infiltrating CD8-positive lymphocytes were demonstrated. Original magnification was $\times 100$

sarcoma [23]. These findings suggest that synovial sarcoma cells are sensitive to CTL and also support the idea that specific antigenic stimulation is still required for the effectiveness of immune checkpoint antibodies, especially for sarcomas.

Since the first report of human CTL-defined tumor-associated antigen MAGE [24], many tumor-associated antigens and peptides have been reported in various tumors [25]. However, we believe that many other antigens that are more suitable for immunotherapy remain undiscovered. Suitable criteria for tumor-associated antigens in the next generation are (i) overexpression in tumor cells, (ii) regulation of proliferation, and (iii) undetectable expression in normal organs except for the testis lacking expression of HLA class I molecules. Recently, Rosenberg et al. wrote that “an ideal source of antigens to target using genetically modified lymphocytes are shared mutations that are unique to each cancer type and are not found on normal tissues” [26, 27]. Adoptive transfer therapy using lymphocytes expressing exogenous TCR or a chimeric antigenic receptor combined with lymphodepletion chemotherapy showed dramatic clinical responses [28, 29]; however, excessively boosted antigen-specific T-cell immunity also destroyed normal organs expressing the same or a mimicking tumor-associated antigen and caused serious adverse effects [30, 31]. Therefore, it is imperative to identify ideal tumor-associated antigens with ultrafine specificity. Since 2006 we have focused on cancer stemlike cells/cancer-initiating cells of bone and soft tissue sarcomas, “sarcoma stem cells (SSCs),” because SSCs might be rich sources of novel tumor-associated antigens conferring on SSCs higher tumorigenicity, proliferation ability, and mobility capacity. In addition, such SSC antigens might make it possible for the immune system to distinguish highly malignant tumor cells from normal cells. We identified SSCs in bone MFH cell line MFH2003 [32] using side population analysis based on the higher drug efflux ability of SSCs more than non-SSCs [33]. Subsequently, we established a CTL clone recognizing SSCs in the context of HLA class I, aiming to identify an autologous CTL-defined SSC antigen [34]. Next, we identified SSCs in epithelioid sarcoma using the ALDEFUOR assay based on the higher aldehyde dehydrogenase activity in SSCs and found that CD109 was highly expressed in SSCs compared to non-SSCs [35]. CD109 is also expressed in normal organs, including platelets, lung epithelium, and hematopoietic stem cells [36]. However, the glycosylation pattern of CD109 might be different in epithelioid sarcoma and normal cells. Therefore, we are trying to isolate monoclonal antibodies against CD109 with tumor-specific glycosylation. Moreover, we are currently isolating SSCs from a newly established osteosarcoma cell line based on sphere-formation ability and trying to identify SSC antigens.

On the other hand, sometimes it is very hard to activate CTLs recognizing antigenic peptides presented by HLA class I using peptide vaccination. To overcome this obstacle, we attempted to generate artificial monoclonal antibodies reacting with the HLA/peptide complex. We constructed a single-chain variable fragment (scFv) phage display library and isolated the scFv clone reacting with the HLA-A*02:01/peptide PBF A2.2 complex with high affinity ($K_D = 1.53 \times 10^{-9}$ M) [37]. Now we are trying to isolate more specific antibodies reacting with various tumor-associated antigens in the context of HLA class I with the aim of the development and clinical application of novel therapeutic antibodies.

References

1. Ferrari S, Palmerini E. Adjuvant and neoadjuvant combination chemotherapy for osteogenic sarcoma. *Curr Opin Oncol.* 2007;19:341–6.
2. Southam CM, Marcove RC, Levin AG, Buchsbaum HJ, Mike V. Proceedings: clinical trial of autogenous tumor vaccine for treatment of osteogenic sarcoma. *Proc Natl Cancer Conf.* 1972;7:91–100.
3. Nabeta Y, Kawaguchi S, Sahara H, Ikeda H, Hirohashi Y, Goroku T, Sato Y, Tsukahara T, Torigoe T, Wada T, Kaya M, Hiraga H, Isu K, Yamawaki S, Ishii S, Yamashita T, Sato N. Recognition by cellular and humoral autologous immunity in a human osteosarcoma cell line. *J Orthop Sci.* 2003;8:554–9.
4. Tsukahara T, Nabeta Y, Kawaguchi S, Ikeda H, Sato Y, Shimozawa K, Ida K, Asanuma H, Hirohashi Y, Torigoe T, Hiraga H, Nagoya S, Wada T, Yamashita T, Sato N. Identification of human autologous cytotoxic T-lymphocyte-defined osteosarcoma gene that encodes a transcriptional regulator, papillomavirus binding factor. *Cancer Res.* 2004;64:5442–8.
5. Yabe H, Tsukahara T, Kawaguchi S, Wada T, Sato N, Morioka H, Yabe H. Overexpression of papillomavirus binding factor in Ewing's sarcoma family of tumors conferring poor prognosis. *Oncol Rep.* 2008;19:129–34.
6. Boeckle S, Pfister H, Steger G. A new cellular factor recognizes E2 binding sites of papillomaviruses which mediate transcriptional repression by E2. *Virology.* 2002;293:103–17.
7. Tsukahara T, Kimura S, Ichimiya S, Torigoe T, Kawaguchi S, Wada T, Yamashita T, Sato N. Scythe/BAT3 regulates apoptotic cell death induced by papillomavirus binding factor in human osteosarcoma. *Cancer Sci.* 2009;100:47–53.
8. Desmots F, Russell HR, Lee Y, Boyd K, McKinnon PJ. The reaper-binding protein scythe modulates apoptosis and proliferation during mammalian development. *Mol Cell Biol.* 2005;25:10329–37.
9. Minami R, Shimada M, Yokosawa H, Kawahara H. Scythe regulates apoptosis through modulating ubiquitin-mediated proteolysis of the *Xenopus* elongation factor XEF1AO. *Biochem J.* 2007;405:495–501.
10. Jordanovski D, Herwartz C, Pawlowski A, Taute S, Frommolt P, Steger G. The hypoxia-inducible transcription factor ZNF395 is controlled by I κ B kinase-signaling and activates genes involved in the innate immune response and cancer. *PLoS One.* 2013;8:e74911.
11. Hasegawa R, Tomaru Y, de Hoon M, Suzuki H, Hayashizaki Y, Shin JW. Identification of ZNF395 as a novel modulator of adipogenesis. *Exp Cell Res.* 2013;319:68–76.
12. Torigoe T, Asanuma H, Nakazawa E, Tamura Y, Hirohashi Y, Yamamoto E, Kanaseki T, Hasegawa T, Sato N. Establishment of a monoclonal anti-pan HLA class I antibody suitable for immunostaining of formalin-fixed tissue: unusually high frequency of down-regulation in breast cancer tissues. *Pathol Int.* 2012;62:303–8.
13. Tsukahara T, Kawaguchi S, Torigoe T, Asanuma H, Nakazawa E, Shimozawa K, Nabeta Y, Kimura S, Kaya M, Nagoya S, Wada T, Yamashita T, Sato N. Prognostic significance of HLA class I expression in osteosarcoma defined by anti-pan HLA class I monoclonal antibody, EMR8-5. *Cancer Sci.* 2006;97:1374–80.
14. Tsukahara T, Kawaguchi S, Torigoe T, Murase M, Wada T, Kaya M, Nagoya S, Yamashita T, Sato N. HLA-A*0201-restricted CTL epitope of a novel osteosarcoma antigen, papillomavirus binding factor. *J Transl Med.* 2009;7:44.
15. Coulie PG, Karanikas V, Colau D, Lurquin C, Landry C, Marchand M, Dorval T, Brichard V, Boon T. A monoclonal cytolytic T-lymphocyte response observed in a melanoma patient vaccinated with a tumor-specific antigenic peptide encoded by gene MAGE-3. *Proc Natl Acad Sci U S A.* 2001;98:10290–5.
16. Kameshima H, Tsuruma T, Kutomi G, Shima H, Iwayama Y, Kimura Y, Imamura M, Torigoe T, Takahashi A, Hirohashi Y, Tamura Y, Tsukahara T, Kanaseki T, Sato N, Hirata K. Immunotherapeutic benefit of alpha-interferon (IFN α) in survivin2B-derived peptide vaccination for advanced pancreatic cancer patients. *Cancer Sci.* 2013;104:124–9.

17. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363:711–23.
18. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, Segal NH, Ariyan CE, Gordon RA, Reed K, Burke MM, Caldwell A, Kronenberg SA, Agunwamba BU, Zhang X, Lowy I, Inzunza HD, Feely W, Horak CE, Hong Q, Korman AJ, Wigginton JM, Gupta A, Sznol M. Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med*. 2013;369:122–33.
19. Robbins PF, El-Gamil M, Li YF, Kawakami Y, Loftus D, Appella E, Rosenberg SA. A mutated beta-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. *J Exp Med*. 1996;183:1185–92.
20. Robbins PF, Lu YC, El-Gamil M, Li YF, Gross C, Gartner J, Lin JC, Teer JK, Cliften P, Tycksen E, Samuels Y, Rosenberg SA. Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nat Med*. 2013;19:747–52.
21. Maki RG, Jungbluth AA, Gnjatich S, Schwartz GK, D'Adamo DR, Keohan ML, Wagner MJ, Scheu K, Chiu R, Ritter E, Kachel J, Lowy I, Old LJ, Ritter G. A pilot study of anti-CTLA4 antibody ipilimumab in patients with synovial sarcoma. *Sarcoma*. 2013;2013:168145.
22. Kawaguchi S, Tsukahara T, Ida K, Kimura S, Murase M, Kano M, Emori M, Nagoya S, Kaya M, Torigoe T, Ueda E, Takahashi A, Ishii T, Tatezaki S, Toguchida J, Tsuchiya H, Osanai T, Sugita T, Sugiura H, Ieguchi M, Ihara K, Hamada K, Kakizaki H, Morii T, Yasuda T, Tanizawa T, Ogose A, Yabe H, Yamashita T, Sato N, Wada T. SYT-SSX breakpoint peptide vaccines in patients with synovial sarcoma: a study from the Japanese Musculoskeletal Oncology Group. *Cancer Sci*. 2012;103:1625–30.
23. Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, Wunderlich JR, Nahvi AV, Helman LJ, Mackall CL, Kammula US, Hughes MS, Restifo NP, Raffeld M, Lee CC, Levy CL, Li YF, El-Gamil M, Schwarz SL, Laurencot C, Rosenberg SA. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol*. 2011;29:917–24.
24. Van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science*. 1991;254:1643–7.
25. Van Der Bruggen P, Stroobant V, Van Pel A, Van den Eynde B. Peptide database: T cell-defined tumor antigens. *Cancer Immun*. 2013;13:15.
26. Rosenberg SA. Finding suitable targets is the major obstacle to cancer gene therapy. *Cancer Gene Ther*. 2014;21:45–7.
27. Tran E, Turcotte S, Gros A, Robbins PF, Lu YC, Dudley ME, Wunderlich JR, Somerville RP, Hogan K, Hinrichs CS, Parkhurst MR, Yang JC, Rosenberg SA. Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. *Science*. 2014;344:641–5.
28. Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat Rev Cancer*. 2008;8:299–308.
29. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med*. 2011;365:725–33.
30. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther*. 2010;18:843–51.
31. Morgan RA, Chinnasamy N, Abate-Daga D, Gros A, Robbins PF, Zheng Z, Dudley ME, Feldman SA, Yang JC, Sherry RM, Phan GQ, Hughes MS, Kammula US, Miller AD, Hessman CJ, Stewart AA, Restifo NP, Quezado MM, Alimchandani M, Rosenberg AZ, Nath A,

- Wang T, Bielekova B, Wuest SC, Akula N, McMahon FJ, Wilde S, Mosetter B, Schendel DJ, Laurencot CM, Rosenberg SA. Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. *J Immunother.* 2013;36:133–51.
32. Tsukahara T, Kawaguchi S, Ida K, Kimura S, Tamura Y, Ikeda T, Torigoe T, Nagoya S, Wada T, Sato N, Yamashita T. HLA-restricted specific tumor cytotoxicity by autologous T-lymphocytes infiltrating metastatic bone malignant fibrous histiocytoma of lymph node. *J Orthop Res.* 2006;24:94–101.
 33. Murase M, Kano M, Tsukahara T, Takahashi A, Torigoe T, Kawaguchi S, Kimura S, Wada T, Uchihashi Y, Kondo T, Yamashita T, Sato N. Side population cells have the characteristics of cancer stem-like cells/cancer-initiating cells in bone sarcomas. *Br J Cancer.* 2009;101:1425–32.
 34. Kano M, Tsukahara T, Emori M, Murase M, Torigoe T, Kawaguchi S, Wada T, Yamashita T, Sato N. Autologous CTL response against cancer stem-like cells/cancer-initiating cells of bone malignant fibrous histiocytoma. *Cancer Sci.* 2011;102:1443–7.
 35. Emori M, Tsukahara T, Murase M, Kano M, Murata K, Takahashi A, Kubo T, Asanuma H, Yasuda K, Kochin V, Kaya M, Nagoya S, Nishio J, Iwasaki H, Sonoda T, Hasegawa T, Torigoe T, Wada T, Yamashita T, Sato N. High expression of CD109 antigen regulates the phenotype of cancer stem-like cells/cancer-initiating cells in the novel epithelioid sarcoma cell line ESX and is related to poor prognosis of soft tissue sarcoma. *PLoS One.* 2013;8:e84187.
 36. Hwang SM, Kim MJ, Chang HE, Hong YJ, Kim TS, Song EY, Park KU, Song J, Han KS. Human platelet antigen genotyping and expression of CD109 (human platelet antigen 15) mRNA in various human cell types. *Biomed Res Int.* 2013;2013:946403.
 37. Tsukahara T, Emori M, Murata K, Hirano T, Muroi N, Kyono M, Toji S, Watanabe K, Torigoe T, Kochin V, Asanuma H, Matsumiya H, Yamashita K, Himi T, Ichimiya S, Wada T, Yamashita T, Hasegawa T, Sato N. Specific targeting of a naturally presented osteosarcoma antigen PBF peptide using an artificial monoclonal antibody. *J Biol Chem.* 2014;289:22035.