is not strong enough to completely reject the tumor in the host. This means that further activation of immune responses by appropriate immunotherapy is essential for tumor rejection. Indeed, when these spontaneous immune responses were augmented by DNA vaccine, tumor growth was significantly inhibited. In contrast, we have reported that peptide vaccine using this NY-ESO-1 peptide enhanced tumor growth rather than inhibiting tumor growth unless it is vaccinated with proper adjuvants [13].

In fact, antigen-specific antibody may not play an important role for tumor rejection in our models, because 1) most tumor antigens including NY-ESO-1 and MAGE-A4 focused on this study, was exclusively expressed intracellularly by the tumor cells, thus not accessible for antibody [3] and 2) Ab responses were detected on day 21, namely later than $CD8^+$ T cell responses. Nevertheless, we have reported that injection of NY-ESO-1 mAb with chemotherapy, that can accentuate the release of intracellular tumor antigens to facilitate mAb access to intracellular target molecules, augmented anti-tumor effect in the same model system, though Ab administration alone did not inhibit tumor growth [18]. In our mouse model, spontaneously-primed anti-NY-ESO-1 Ab was detected when tumors reached a larger size. The level of spontaneously-primed antibody was, however, about 10-times lower than that achieved by mAb injection [18], suggesting that spontaneously-primed Ab responses may potentially have some anti-tumor effects, but the amount of Abs is too low to exhibit effective anti-tumor activity.

Since no NY-ESO-1 homologue is present in mice, the detected immune responses against NY-ESO-1 are considered to reflect a foreign antigen, rather than a self-antigen [3]. Whereas cancer-testis antigens like NY-ESO-1 are only expressed by cancer cells and testis, but not by normal somatic cells, mimicking foreign antigens, some cancer-testis antigens are reportedly expressed in medullary thymic epithelial cells under control of AIRE (Autoimmune regulator) [3,19]. It is plausible that cancer-testis antigens like NY-ESO-1 could be considered self-antigens during thymic selection, resulting in a repertoire of NY-ESO-1-specific T cells that are either subject to central or peripheral tolerance [3,20–22]. Thus, studies using mice in which NY-ESO-1 is a self-antigen should allow resolving this issue.

A unique finding of our study is that NY-ESO-1-specific $CD8^+$ T cell epitopes were present in an immunogenic part defined in humans [3]. This finding implies that immunogenicity may be characterized with similar components between humans and mice, further supporting the usefulness of our models. Our animal models provide important tools for the development of effective cancer vaccines.

In conclusion, we established animal models involving human tumor antigens, such as NY-ESO-1 or MAGE-A4 protein. These models allowed us to study the kinetics and distribution of antigen-specific immune responses in detail, and hence providing tools to optimize the efficacy of current cancer immunotherapy.

Acknowledgments

This article is dedicated to the memory of Lloyd J. Old, M.D. We thank Dr. J. Wing for critical reading and Ms. K. Mori and K. Sasada for excellent technical assistance.

This study was supported by Grants-in-Aid for Scientific Research (B) (HN) and Grants-in-Aid for Scientific Research on Priority Areas (HN and HS) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Cancer Research Institute Investigator Award (HN). Cancer Research Grant from Foundation of Cancer Research Promotion (HN), the Sagawa Foundation for Promotion of Cancer Research (HN) and Senri Life Science Foundation (HN).

Contributions: Designed the experiments: DM, HN, TK, HS. Performed the experiments: DM, HN, TN, LW, ES. Analyzed the data: DM, HN, NH, TK, HS. Contributed reagents/materials/analysis tools: IL, EN. Wrote the paper: DM, HN, TK. *Conflicts of interest:* D.M. and N.H. are employees of ImmunoFrontier, Inc. The other authors have no potential conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.02.056.

References

[1] Scanlan MJ, Gure AO, Jungbluth AA, Old LJ, Chen Y-T. Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. Immunol Rev 2002;188:22–32.
[2] Boon T, Coulie PG, Van den Eynde BJ, Human van der Bruggen P. T cell responses against melanoma. Annu Rev Immunol 2006;24:175–208.
[3] Gnjatic S, Nishikawa H, Jungbluth AA, Gure AO, Ritter G, Jager E, et al. NY-ESO-1: review of an immunogenic tumor antigen. Adv Cancer Res 2006;95:1–30.
[4] Belardelli F, Ferrantini M, Parmiani G, Schlom J, Garaci E. International meeting on cancer vaccines: how can we enhance efficacy of therapeutic vaccines? Cancer Res 2004;64(18):6827–30.
[5] Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med 2004;10(9):909–15.
[6] Chen Y-T, Scanlan MJ, Sahin U, Tureci O, Gure AO, Tsang S, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. Proc Natl Acad Sci USA 1997;94(5):1914–8.
[7] De Plaen E, De Backer O, Arnaud D, Bonjean B, Chomez P, Martelange V, et al. A new family of mouse genes homologous to the human MAGE genes. Genomics 1999;55(2):176–84.
[8] Duffour MT, Chaux P, Lurquin C, Cornelis G, Boon T, van der Bruggen P. A MAGE-A4 peptide presented by HLA-A2 is recognized by cytolytic T lymphocytes. Eur J Immunol 1999;29(10):3329–37.
[9] Miyahara Y, Naota H, Wang L, Hiasa A, Goto M, Watanabe M, et al. Determination of cellularly processed HLA-A2402-restricted novel CTL epitopes derived from two cancer germ line genes, MAGE-A4 and SAGE. Clin Cancer Res 2005;11(15):5581–9.
[10] Nishikawa H, Tanida K, Ikeda H, Sakakura M, Miyahara Y, Aota T, et al. Role of SEREX-defined immunogenic wild-type cellular molecules in the development of tumor-specific immunity. Proc Natl Acad Sci USA 2001;98(25):14571–6.
[11] Nishikawa H, Sato E, Briones G, Chen LM, Matsuo M, Nagata Y, et al. In vivo antigen delivery by a *Salmonella typhimurium* type III secretion system for therapeutic cancer vaccines. J Clin Invest 2006;116(7):1946–54.
[12] Griswold DP, Corbett TH. A colon tumor model for anticancer agent evaluation. Cancer 1975;36(6 Suppl):2441–4.
[13] Muraoka D, Kato T, Wang LA, Maeda Y, Noguchi T, Harada N, et al. Peptide vaccine induces enhanced tumor growth associated with apoptosis induction in $CD8^+$ T cells. J Immunol 2010;185(6):3768–76.
[14] Jager E, Stockert E, Zidianakis Z, Chen Y-T, Karbach J, Jager D, et al. Humoral immune responses of cancer patients against Cancer-Testis antigen NY-ESO-1: Correlation with clinical events. Int J Cancer 1999;84(5):506–10.
[15] Nishikawa H, Sakaguchi S. Regulatory T cells in tumor immunity. Int J Cancer 2010;127(4):759–67.
[16] Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;10(9):942–9.
[17] Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, et al. Intraepithelial $CD8^+$ tumor-infiltrating lymphocytes and a high $CD8^+$/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci USA 2005;102(51):18538–43.
[18] Noguchi T, Kato T, Wang LN, Maeda Y, Ikeda H, Sato E, et al. Intracellular tumor-associated antigens represent effective targets for passive immunotherapy. Cancer Res 2012;72(7):1672–82.
[19] Gotter J, Brors B, Hergenhahn M, Kyewski B. Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters. J Exp Med 2004;199(2):155–66.
[20] Danke NA, Koelle DM, Yee C, Beheray S, Kwok WW. Autoreactive T cells in healthy individuals. J Immunol 2004;172(10):5967–72.
[21] Nishikawa H, Jager E, Ritter G, Old LJ, Gnjatic S. $CD4^+CD25^+$ regulatory T cells control the induction of antigen-specific $CD4^+$ helper T cell responses in cancer patients. Blood 2005;106:1008–11.
[22] Nishikawa H, Qian F, Tsuji T, Ritter G, Old LJ, Gnjatic S, et al. Influence of $CD4^+CD25^+$ regulatory T cells on low/high-avidity $CD4^+$ T cells following peptide vaccination. J Immunol 2006;176(10):6340–6.

Hindawi Publishing Corporation
Journal of Skin Cancer
Volume 2013, Article ID 742925, 12 pages
http://dx.doi.org/10.1155/2013/742925

Review Article

Melanoma-Targeted Chemothermotherapy and *In Situ* Peptide Immunotherapy through HSP Production by Using Melanogenesis Substrate, NPrCAP, and Magnetite Nanoparticles

Kowichi Jimbow,[1,2] Yasue Ishii-Osai,[2] Shosuke Ito,[3] Yasuaki Tamura,[4] Akira Ito,[5] Akihiro Yoneta,[2] Takafumi Kamiya,[2] Toshiharu Yamashita,[2] Hiroyuki Honda,[6] Kazumasa Wakamatsu,[3] Katsutoshi Murase,[7] Satoshi Nohara,[7] Eiichi Nakayama,[8] Takeo Hasegawa,[9] Itsuo Yamamoto,[10] and Takeshi Kobayashi[11]

[1] *Institute of Dermatology & Cutaneous Sciences, 1-27 Odori West 17, Chuo-ku, Sapporo 060-0042, Japan*
[2] *Department of Dermatology, School of Medicine, Sapporo Medical University, South 1 West 16, Chuo-ku, Sapporo 060-8556, Japan*
[3] *Department of Chemistry, School of Health Sciences, Fujita Health University, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi 470-1192, Japan*
[4] *Department of Pathology 1, School of Medicine, Sapporo Medical University, South 1 West 16, Chuo-ku, Sapporo 060-8556, Japan*
[5] *Department of Chemical Engineering, Faculty of Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan*
[6] *Department of Biotechnology, School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan*
[7] *Meito Sangyo Co., Ltd., 25-5 Kaechi, Nishibiwajima-cho, Kiyosu, Aichi 452-0067, Japan*
[8] *Faculty of Health and Welfare, Kawasaki University of Medical Welfare, 288 Matsushimai, Kurashiki, Okayama 701-0193, Japan*
[9] *Department of Hyperthermia Medical Research Laboratory, Louis Pasteur Center for Medical Research, 103-5, Tanakamonzen-cho, Sakyo-ku, Kyoto 606-8225, Japan*
[10] *Yamamoto Vinita Co., Ltd., 3-12 ueshio 6, Tennoji-ku, Osaka 543-0002, Japan*
[11] *Department of Biological Chemistry, College of Bioscience and Biotechnology, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi 487-8501, Japan*

Correspondence should be addressed to Kowichi Jimbow; jimbow@sapmed.ac.jp

Received 9 August 2012; Revised 8 January 2013; Accepted 22 January 2013

Academic Editor: Mohammed Kashani-Sabet

Exploitation of biological properties unique to cancer cells may provide a novel approach to overcome difficult challenges to the treatment of advanced melanoma. In order to develop melanoma-targeted chemothermoimmunotherapy, a melanogenesis substrate, N-propionyl-4-S-cysteaminylphenol (NPrCAP), sulfur-amine analogue of tyrosine, was conjugated with magnetite nanoparticles. NPrCAP was exploited from melanogenesis substrates, which are expected to be selectively incorporated into melanoma cells and produce highly reactive free radicals through reacting with tyrosinase, resulting in chemotherapeutic and immunotherapeutic effects by oxidative stress and apoptotic cell death. Magnetite nanoparticles were conjugated with NPrCAP to introduce thermotherapeutic and immunotherapeutic effects through nonapoptotic cell death and generation of heat shock protein (HSP) upon exposure to alternating magnetic field (AMF). During these therapeutic processes, NPrCAP was also expected to provide melanoma-targeted drug delivery system.

1. Introduction

The incidence of melanoma is increasing worldwide at an alarming rate [1, 2]. As yet, management of metastatic melanoma is an extremely difficult challenge. Less than 10% with metastatic melanoma patients survive currently for five years because of the lack of effective therapies [3]. There is, therefore, an emerging need to

develop innovative therapies for the control of metastatic melanoma.

The major advance of drug discovery for targeted therapy to cancer cells can be achieved by exploiting their unique biological property. The biological property unique to the melanoma cell resides in the biosynthesis of melanin pigments, that is, melanogenesis occuring within specific compartments, melanosomes. Melanogenesis begins with the conversion of amino acid, tyrosine to dopa and subsequently to dopaquinone in the presence of tyrosinase. This pathway is uniquely expressed by all melanoma cells. It is well known that the clinically "amelanotic" melanoma tissues always have tyrosinase activity to some extent, and that "*in vitro* amelanotic" melanoma cells become "melanotic" ones when they are regrown in the *in vivo* condition. Melanin precursors are inherently cytotoxic through reacting with tyrosinase to form unstable quinone derivatives [4]. Thus, tyrosine analogues that are tyrosinase substrates can be good candidates for developing drugs to melanoma-targeting therapies [5]. N-propionyl and N-acetyl derivatives (NPr- and NAcCAP) of 4-*S*-cysteaminylphenol, that is, sulfur-amine analogue of tyrosine, were synthesized as possible melanoma-targeted drugs (Figure 1) and found to possess selective cytotoxic effects on *in vivo* and *in vitro* melanomas through the oxidative stress that derives from production of cytotoxic free radicals by interacting with tyrosinase within melanogenesis cascade [6–10].

Intracellular hyperthermia using magnetite nanoparticles (10–100 nm-sized Fe_3O_4) may be another choice to overcome the difficult challenges for melanoma treatment. It has been shown to be effective for treating cancers in not only primary but also metastatic lesions [11, 12]. Incorporated magnetite nanoparticles generate heat (thermotherapy) within the cells after exposure to AMF due to hysteresis loss [13]. In this treatment, there is not only the heat-mediated cell death but also immune reaction due to the generation of heat shock proteins (HSPs) [14–23]. HSP expression induced by hyperthermia has been shown to be involved in tumor immunity, providing the basis for developing a cancer thermoimmunotherapy.

Based upon these rationales, we now provide evidence that melanoma-targeted chemothermotherapy can be achieved by conjugating a chemically modified melanogenesis substrate, NPrCAP with magnetite nanoparticles, which then produce apoptotic and non-apoptotic cell death through interacting with tyrosinase and heat-mediated oxidative stress; hence, immunotherapy with production of *in situ* peptides is being established (Figure 2).

2. Melanogenesis Substrate as a Potential Candidate for Development of Selective Drug Delivery System and Cytotoxicity to Melanoma

2.1. Synthesis of Sulfur-Amine Analogues of Tyrosine, Cysteaminylphenols, and Their Selective Incorporation into Melanogenesis Cascade. With the interaction of melanocyte-stimulating hormone (MSH)/melanocortin 1 receptor (MC1R), the melanogenesis cascade begins from activation of microphthalmia transcription factor (MITF) for induction of either eu- or pheomelanin biosynthesis. Tyrosinase is the major player in this cascade. Tyrosinase is a glycoprotein, and its glycosylation process is regulated by a number of molecular chaperons, including calnexin in the endoplasmic reticulum [24, 25]. Vesicular transport then occurs to carry tyrosinase and its related proteins (TRPs) from trans-Golgi network to melanosomal compartments, which appear to derive from early and late endosomal compartments. In this process a number of transporters, such as small GTP-binding protein, adaptor proteins, and PI3-kinase, play important roles. Once melanin biosynthesis is completed to conduct either eu- or pheomelanogenesis within melanosomes, they then move along dendritic processes and are transferred to surrounding keratinocytes in normal skin [26–28]. In metastatic melanoma cells, however, there will be practically no melanosome transfer inasmuch as there will be no receptor cells such as keratinocytes. Thus melanosomes synthesized by melanoma cells are aggregated within autophagic vacuoles in which melanogenesis-targeted drugs will be retained. In order to utilize this unique melanogenesis pathway for developing melanoma-targeted drugs, N-acetyl and N-propionyl derivatives of cysteaminylphenols (NAc- and NPrCAPs) have been synthesized [8, 29] (Figure 1).

2.2. In Vivo and In Vitro Melanocyte Toxicity and Anti-Melanoma Effects of Cysteaminylphenols (CAPs). Both NPrCAP and NAcCAP were found to selectively disintegrate follicular melanocytes after single or multiple *ip* administration to newborn or adult C57 black mice, respectively [12, 30]. In the case of adult mice after repeated *ip* administration of NPrCAP, white follicles with 100% success rate can be seen at the site where hair follicles were plucked to stimulate new melanocyte growth and to activate new tyrosinase synthesis. A single *ip* administration of NPrCAP into a new born mouse resulted in the development of silver follicles in the entire body coat. The selective disintegration of melanocytes which is mediated by apoptotic cell death can be seen as early as in 12 hr after a single *ip* administration. None of surrounding keratinocytes or fibroblasts showed such membrane degeneration and cell death [31, 32] (Figure 3).

A high, specific uptake of NAcCAP was seen *in vitro* by melanoma cell lines compared to nonmelanoma cells [9]. A melanoma-bearing mouse showed, on the whole body autoradiogram, the selective uptake and covalent binding of NAcCAP in melanoma tissues of lung and skin [6]. The specific cytotoxicity of NPrCAP and NAcCAP was examined on various types of culture cells by MTT assay, showing that only melanocytic cells except HeLa cells possessed the low IC50 [8, 9]. The cytotoxicity on DNA synthesis inhibition was timedependent and irreversible on melanoma cells but was transient on HeLa cells [10].

The *in vitro* culture and *in vivo* lung metastasis assays showed the melanoma growth can be blocked by administration of NAcCAP combined with buthionine sulfoximide (BSO), which blocked the effect of antioxidants through reducing glutathione levels. There was a marked growth inhibition of cultured melanoma cells in the presence of BSO

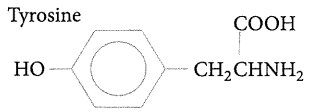
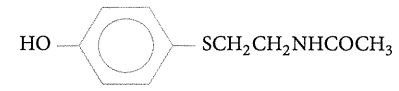
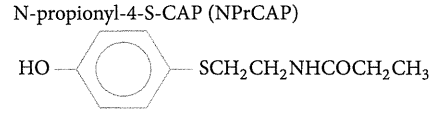
	Km (μM)	V_{max} (μmole/min/mg)
Tyrosine 	0.3	1.8
N-acetyl-4-S-CAP (NAcCAP) 	375	9.28
N-propionyl-4-S-CAP (NPrCAP) 	340.9	5.43

FIGURE 1: Synthesis and chemical structures of NAcCAP and NPrCAP and their tyrosinase kinetics.

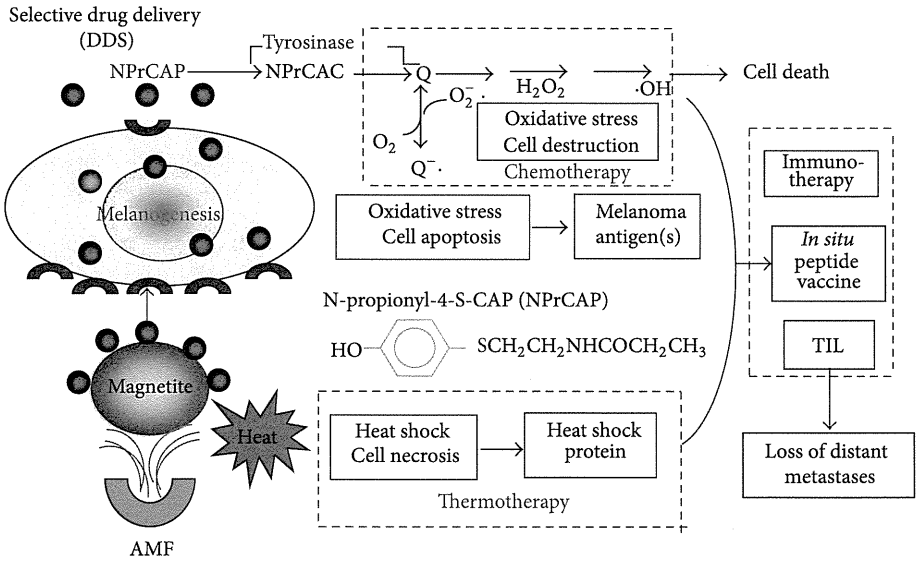


FIGURE 2: Strategy for melanogenesis-targeted CTI and *in situ* peptide vaccine therapy by conjugates of NPrCAP and magnetite nanoparticles with AMF exposure.

indicating that the selective cytotoxicity by CAP is mediated by the production of cytotoxic free radicals. The *in vivo* lung metastasis experiment also showed the decreased number of lung melanoma colonies [6]. The problem was, however, that a fairly large number of amelanotic melanoma lesions were seen to grow in the lung [6]. NPrCAP has been developed and conjugated with magnetite nanoparticles in the hope of increasing the cytotoxicity and overcoming the problem.

3. Conjugation of NPrCAP with Magnetite Nanoparticles and *In Vivo* Evaluation of Melanoma Growth Inhibition with/without Thermotherapy

3.1. Synthesis for Conjugates of NPrCAP with Magnetite Nanoparticles and Their Selective Aggregation in Melanoma for Development of Chemo-Thermo-Immunotherapy. Magnetite nanoparticles have been employed for thermotherapy in a number of cancer treatments including human gliomas and prostate cancers [33–35]. They consist of 10–100 nm-sized iron oxide (Fe_3O_4) with a surrounding polymer coating and generate heat when exposed to AMF [12]. We expected the combination of NPrCAP and magnetite nanoparticles to be a potential source for developing not only antimelanoma pharmacologic but also immunogenic agent. Based upon the melanogenesis-targeted drug delivery system (DDS) of NPrCAP, NPrCAP/magnetite nanoparticles complex was expected to be selectively incorporated into melanoma cells. It was also hypothesized that the degradation of melanoma tissues may occur from oxidative and heat stresses by exposure of NPrCAP to tyrosinase and by exposure of magnetite nanoparticles to AMF. These two stress processes may then produce the synergistic or additive effect for generating

tumor-infiltrating lymphocytes (TIL) by *in situ* formation of peptides that will kill melanoma cells in distant metastases (Figure 2).

In order to develop effective melanoma-targeted chemotherapy (by NPrCAP) and thermo-immunotherapy (by magnetite nanoparticles with HSP), hence providing a basis for chemo-thermo-immunotherapy (CTI therapy), we synthesized conjugates of NPrCAP and magnetite nanoparticles, on which NPrCAP is bound directly or indirectly on the surface of magnetite nanoparticles or magnetite-containing liposomes (Figure 4). Among these NPrCAP and magnetite complexes listed in Figure 4, NPrCAP/M and NPrCAP/PEG/M were chemically stable, did not lose biological property, and could be filtered as well as easily produced in large quantities. Most of the experiments described below were carried out by employing the direct conjugate of NPrCAP and magnetite nanoparticles, NPrCAP/M. A preliminary clinical trial, however, used NPrCAP/PEG/M to which polyethylene glycol (PEG) was employed to conjugate NPrCAP and magnetite nanoparticles.

In our studies, we found that NPrCAP/M nanoparticle conjugates were selectively aggregated in melanoma cells compared to non-melanoma cells [36]. The conjugates of NPrCAP and magnetite nanoparticles would be selectively aggregated on the cell surface of melanoma cells through still unknown surface receptor and then incorporated into melanoma cells by early and late endosomes. The conjugates were then incorporated into melanosomal compartment as the stage I melanosomes derive from late endosome-related organelles, to which tyrosinase was transported from the trans-Golgi network by vesicular transport [26].

3.2. In Vivo Growth Inhibition of Mouse Melanoma by Conjugates of NPrCAP and Magnetite Nanoparticles with/without Thermotherapy. The intracellular hyperthermia using magnetic nanoparticles is effective for treating certain types of primary and metastatic cancers [11, 12, 35–39]. Incorporated magnetic nanoparticles generate heat within the cells after exposure to the AMF due to hysteresis loss or relaxational loss [13, 40]. In our study of B16 melanoma cells using B16F1, B16F10, and B16OVA cells, we compared the thermotherapeutic protocols in detail by evaluating the growth of the rechallenge melanoma transplants as well as the duration and rates of survival of melanoma-bearing mice.

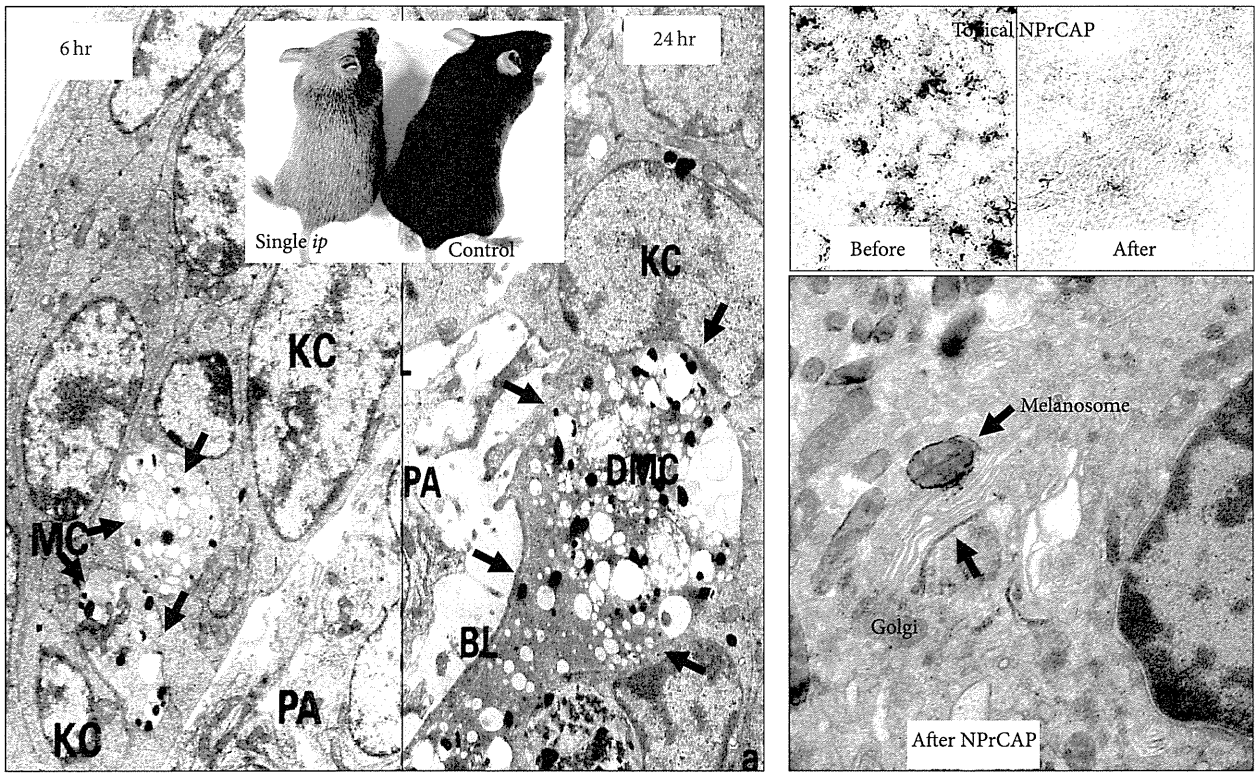
By employing B16F1 and F10 cells, we first evaluated the chemotherapeutic effect of NPrCAP/M with or without AMF exposure which generates heat. NPrCAP/M without heat inhibited growth of primary transplants to the same degree as did NPrCAP/M with heat, indicating that NPrCAP/M alone has a chemotherapeutic effect. However, there was a significant difference in the melanoma growth inhibition of re-challenge transplants between the groups of NPrCAP/M with and without heat. NPrCAP/M with AMF exposure showed the most significant growth inhibition in re-challenge melanoma transplants and increased life span of the host animals, that is, almost complete rejection of re-challenge melanoma growth, whereas NPrCAP/M without heat was much less, indicating that NPrCAP/M with heat possesses a thermo-immunotherapeutic effect (Figures 5(a), 5(b), and 5(c)).

Specifically our study indicated that the most effective thermoimmunotherapy for re-challenge B16F1 and F10 melanoma cells can be obtained at a temperature of 43°C for 30 min with the treatment repeated three times on every other day intervals without complete degradation of the primary melanoma [37]. This therapeutic approach and its biologic effects differ from those of magnetically mediated hyperthermia on the transplanted melanomas reported previously. In previous studies by Suzuki et al. [38] and Yanase et al. [39], cationic magnetoliposomes were used for B16 melanoma. They showed that hyperthermia at 46°C once or twice led to regression of 40–90% of primary tumors and to 30–60% survival of mice, whereas their hyperthermia at 43°C failed to induce regression of the secondary tumors and any increase of survival in mice [38, 39].

4. Production of Heat Shock Protein, Nonapoptotic Cell Death, and Tumor-Infiltrating Lymphocytes by Conjugates of NPrCAP and Magnetite Nanoparticles with Thermotherapy

4.1. Production of Heat Shock Protein and Non-Apoptotic Melanoma Cell Death by NPrCAP/Magnetite Nanoparticle Conjugates with Thermotherapy. It has been shown that hyperthermia treatment using magnetite cationic liposomes (MCLs), which are cationic liposomes containing 10-nm magnetite nanoparticles, induced antitumor immunity through HSP expression [12, 22, 41, 42]. In our studies using B16F1, F10, and OVA melanoma cells [43], the hyperthermia using NPrCAP/M with AMF exposure also showed antitumor immune responses via HSP-chaperoned antigen (Figure 6) [43]. It may be speculated that the HSPs-antigen peptide complex released from melanoma cells treated with this intracellular hyperthermia is taken up by dendritic cells (DCs) and cross-presented HSP-chaperoned peptide in the context of MHC class I molecules [44]. In our CTI therapy with AMF exposure, the heat-mediated melanoma cell necrosis was induced to NPrCAP/M-incorporated cells. In this group, we also found that repeated hyperthermia (3 cycles of NPrCAP/M administration and AMF irradiation) was required to induce the maximal antitumor immune response [37].

If melanoma cells escaped from this necrotic cell death, repeated hyperthermia should produce further necrotic cell death to the previously heat-shocked melanoma cells in which HSPs were induced. Our CTI therapy with AMF exposure using B16OVA cells showed that Hsp72/Hsc73, Hsp90, and ER-resident HSPs participated in the induction of $CD8^+$ T-cell response [43]. Different from the results of B16F1 and F10 cells, Hsp72 was largely responsible for the augmented antigen presentation to $CD8^+$ T cells. As Hsp72 is known to upregulate in response to hyperthermia or heat shock treatment [41], newly synthesized Hsp72 has a chance to bind to the heat-denatured melanoma-associated antigen.



(a) Depigmentation of C57 black mouse hair by a single *ip* administration of NPrCAP or NAcCAP

(b) Depigmentation of black skin by topical NPrCAP

FIGURE 3: Depigmenting effect of NPrCAP. (a) Depigmentation of C57 black mouse hair follicles by a single *ip* administration of NPrCAP or NAcCAP results in complete loss of melanin pigmentation. Entire coat color changes to silver from black. Electron microscopic observation reveals selective degradation of melanocytes and melanogenic organelles such as early-stage melanosomes at 6 hr after administration. At 24 hr after administration, these melanocytes reveal total degradation. (b) Depigmentation of black skin after topical application of NPrCAP. There is a marked decrease of melanocyte populations after topical application. Electron microscopic observation indicates selective accumulation of NPrCAP in the tyrosinase areas such as in melanosomes and Golgi apparatus as indicated by the deposition of electron dense materials (see arrows).

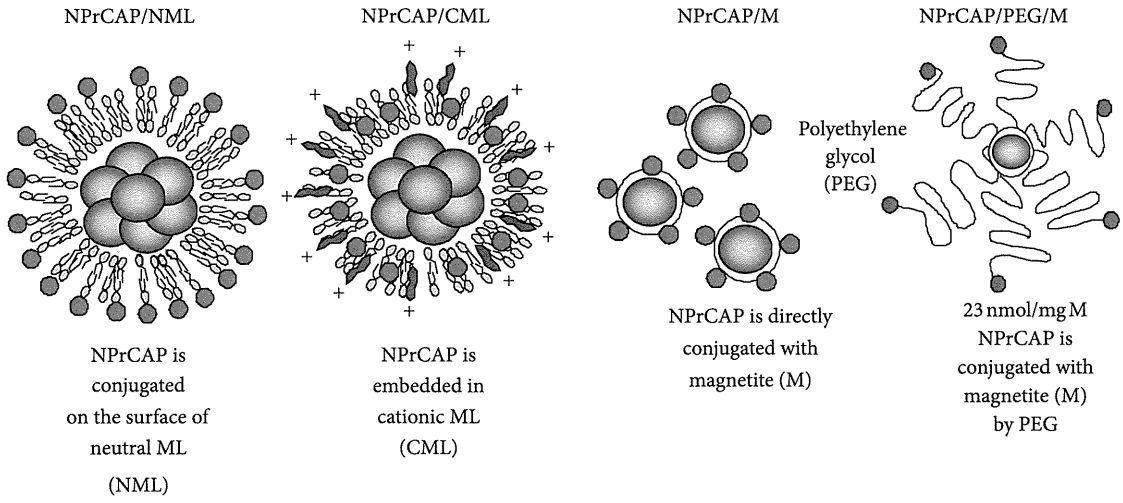
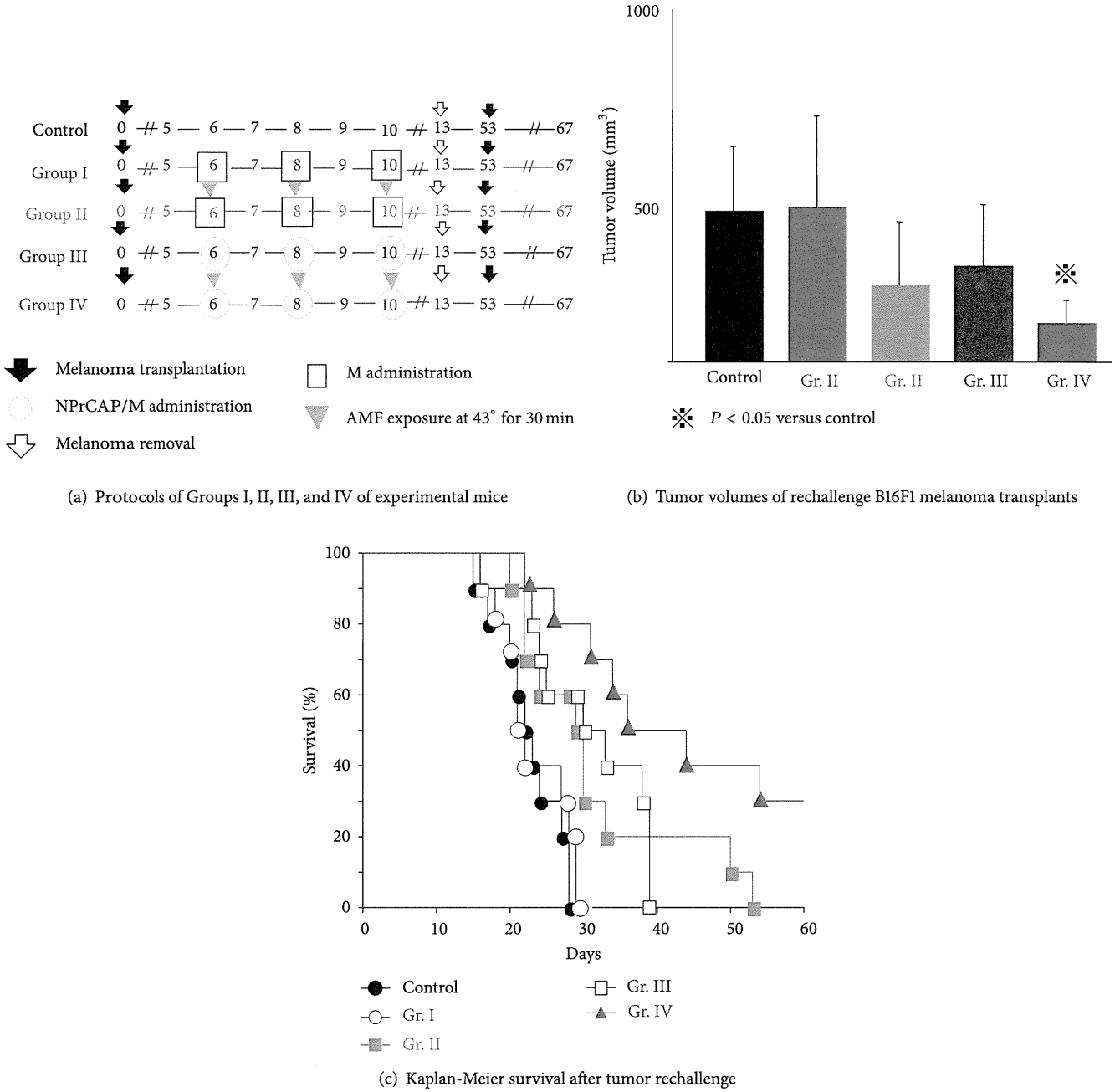


FIGURE 4: Conjugates of NPrCAP/magnetite nanoparticles for developing melanogenesis-targeted melanoma nanomedicine.



(a) Protocols of Groups I, II, III, and IV of experimental mice

(b) Tumor volumes of rechallenge B16F1 melanoma transplants

(c) Kaplan-Meier survival after tumor rechallenge

FIGURE 5: Melanoma growth and survival of melanoma-bearing mice by CTI therapy using NPrCAP/M with and without AMF exposure. (a) Experimental protocols. (b) Tumor volumes of rechallenge melanoma transplants on day 13 of after transplantation. (c) Kaplan-Meier survival of melanoma-bearing mice after treatment following experimental protocols of Figure 5(a).

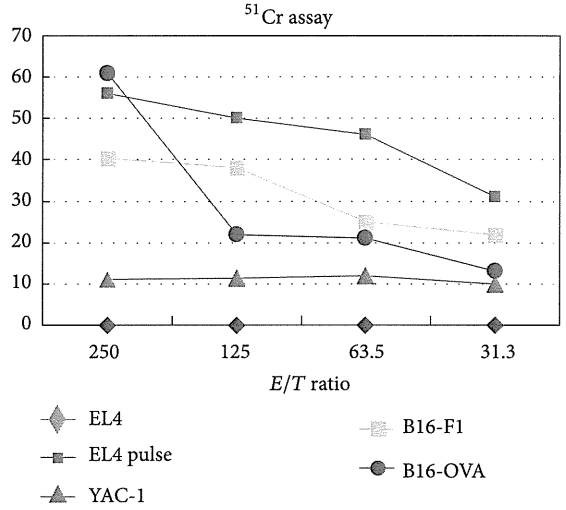


FIGURE 6: Hyperthermia of melanoma cells using B16OVA cells for induction of CTL in CTI therapy. Cytotoxic activity of spleen cells after CTI therapy against B16OVA cells, B16F1 cells, EL4 cells, EL4 cells pulsed with SL8 peptide (OVA-immunodominant peptide), or YAC-1 cells was determined by standard ^{51}Cr-release assay. B16OVA cells were subjected to hyperthermia using NPrCAP/M with AMF exposure *in vitro*.

4.2. T-Cell Receptor Repertoires of Tumor-Infiltrating Lymphocytes by Conjugates of NPrCAP and Magnetite Nanoparticles with Heat Exposure (Hyperthermia). It is clear now from our previous studies [22, 41] that conjugates of NPrCAP/magnetite nanoparticles (NPrCAP/M) with heat treatment (hyperthermia) can successfully induce the growth inhibition of primary and secondary melanoma transplants. It is also found that NPrCAP/M with hyperthermia elicited the response of cytotoxic T lymphocyte (CTL) via the release of HSP-peptide complex from degraded tumor cells [43] (Figure 6). In addition, $CD8^+$ T cells were observed within B16 melanoma nodules after hyperthermia using NPrCAP/M [37]. TIL reactivity to antigen is mediated via T-cell receptors (TCRs) consisting of α and β chains. We studied the TCR repertoire after hyperthermia using NPrCAP/M in order to further understand the T-cell response to melanoma after hyperthermia using NPrCAP/M [45]. We found that TCR repertoire was restricted in TILs, and the expansion of $V\beta 11^+$ T cells was preferentially found. DNA sequences of the third complementarity determining regions were identified. This approach is based on subcutaneous melanoma transplantation in the hind foot pad, which confines the DLN to the inguinal and popliteal lymph nodes. Melanoma growth was significantly suppressed by the treatment of NPrCAP/M-mediated hyperthermia. $CD8^+$ T cells were observed substantially around the tumor and slightly within the tumor, while few and no $CD8^+$ T cells were observed around and within the tumor of nontreated mice.

In addition, significant enlargement of inguinal DLNs was observed in all of tumor-bearing mice including nontreated mice and NPrCAP/M-injected mice. The number of $CD8^+$ T cells in inguinal DLNs increased significantly in the mice treated with NPrCAP/M-mediated hyperthermia.

5. Melanocytotoxic and Immunogenic Properties of NPrCAP without Hyperthermia

5.1. Induction of Apoptosis, Reactive Oxygen Species (ROS), and Tumor-Specific Immune Response by NPrCAP Administration Alone. In our animal study, those animals bearing B16F1 and B16F10 melanoma cells showed, to certain degree, rejection of second re-challenge melanoma transplantation by administration of both NPrCAP alone and NPrCAP/M minus AMF exposure [46]. Our working hypothesis for this finding is that there is a difference in the cytotoxic mechanism and immunogenic property of NPrCAP/M between experimental groups with and without hyperthermia by AMF exposure. The animals with NPrCAP/M without AMF exposure resulted in non-necrotic, apoptotic cell death. The animals with NPrCAP/M plus AMF exposure, on the other hand, resulted in nonapoptotic, necrotic cell death with immune complex production of melanoma peptide as well as Hsp70 and a small amount of Hsp 90.

To further examine the mechanism of the cell death induced by NPrCAP, those cells treated with NPrCAP alone were subjected to flow cytometric analysis, caspase 3 assay, and TUNEL staining [46]. The sub-G1 fraction was increased in the NPrCAP-treated B16F1 cells, comparable to TRAIL-exposed B16F1, but not in the NPrCAP-treated non-melanoma cells (NIH3T3, RMA) or nonpigmented melanoma cells (TXM18) (Figure 7). The luminescent assay detected caspase 3/7 activity in the NPrCAP-treated B16F1 cells remarkably increased (35.8-fold) compared to that in the nontreated cells. NIH3T3, RMA, and TXM18 cells treated with TRAIL showed 10.6-, 7.1-, and 5.8-fold increases of caspase 3/7 activation compared to the control, respectively, whereas those with NPr-4-*S*-CAP showed increases of 4.1-, 1.4-, and 1.8-fold, respectively. The number of TUNEL-positive cells was significantly increased only in the B16F1 tumor treated with NPrCAP. This increase was not observed in the B16F1 tumor without NPrCAP or in the RMA tumors with or without NPrCAP. The findings indicate that NPrCAP induces apoptotic cell death selectively in melanoma cells.

5.2. Melanocytotoxic and Immunogenic Properties of NPrCAP Compared to Monobenzyl Ether Hydroquinone. Monobenzyl ether of hydroquinone has long been known to produce the skin depigmentation at both the drug-applied area by direct chemical reaction with tyrosinase and the non-applied distant area by immune reaction with still unknown mechanism [43, 48–50]. The melanogenesis-related cytotoxicity primarily derives from tyrosinase-mediated formation of dopaquinone and other quinone intermediates, which produce ROSs such as superoxide and H_2O_2 [4, 31, 32, 51]. This unique biological property of melanin intermediates not only causes cell death, but also may produce immunogenic properties. We postulated that the cytotoxic action of NPrCAP appears to involve two major biological processes. One is

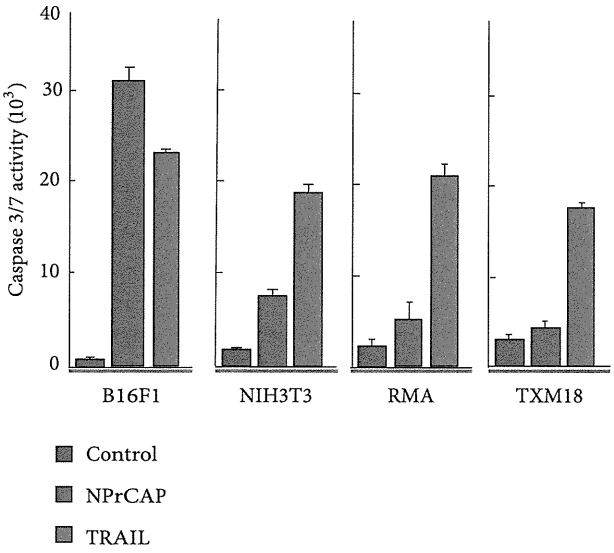


FIGURE 7: NPrCAP-mediated apoptotic cell death of B16F1 melanoma cells. Assay of caspase 3/7 in cells treated with NPrCAP or TRAIL. Cells were cultured in the presence of NPrCAP, TRAIL, or propylene glycol in 96-well plates and then processed for measurement of caspases 3 and 7 using a Caspase-Glo3/7 assay kit. From Ishii-Osai et al. [46].

cytostatic process which derives from the DNA synthesis inhibition through the interaction of quinone and free radicals with SH enzymes and thymidine synthase. Another is the cytocidal process by damage of DNA and mitochondrial ATP through oxidative stress and interaction with SH-enzyme [10]. They bind protein disulphide isomerase [52].

Monobenzyl ether form of hydroquinone was shown to produce a reactive *ortho*-quinone generated by tyrosinase-catalyzed oxidation and self-coupling and thiol conjugation reactions [53]. It was also shown to induce cell death without activating the caspase cascade or DNA fragmentation, indicating that the death pathway is non-apoptotic [53, 54]. It was further suggested that monobenzyl ether hydroquinone induced the immunogenicity to melanocytes and melanoma cells by forming quinone-haptens to tyrosinase protein and by inducing the release of tyrosinase and melanoma antigen recognized by T cells-1 (MART-1) containing $CD63^+$ exosomes following melanosome oxidative stress induction. The drug further augmented the processing and shedding of melanocyte differentiation antigens by inducing melanosome autophagy and enhanced tyrosinase ubiquitination, ultimately activating dendritic cells, which induced cytotoxic melanoma-reactive cells. These T cells eradicated melanoma *in vivo* [54, 55].

5.3. Development of Vitiligo during Melanoma Immunotherapy and Activation of NPrCAP by Tyrosinase to Form Possible Antigen Peptides. Advanced melanoma patients and melanoma patients treated by vaccine immunotherapy often reveal vitiligo-like changes of the skin. Interestingly, this vitiligo development is associated with a superior prognosis in melanoma patients [56]. Although there have been several separate theories for the pathogenesis of vitiligo, the haptenation theory has recently been put forth to explain the molecular mechanism of monobenzone-induced skin depigmentation [54, 57, 58]. Westerhof et al. proposed the haptenation theory in which increased intracellular H_2O_2 could trigger the increased turnover of elevated levels of surrogate substrates of tyrosinase, resulting in melanocyte-specific T-cell responses [57, 59]. According to this hypothesis, tyrosinase could be recognized as a melanoma-specific tumor antigen in relation to the systemic immune responses.

Phenolic substrates as prohaptens are oxidized by tyrosinase to produce *ortho*-quinones, which act as haptens that covalently bind to tyrosinase or other melanosomal proteins to generate possible neoantigens [44, 53, 54]. These neo-antigens, in turn, trigger an immunological response cascade that results in a melanocyte-specific delayed-type hypersensitivity reaction leading to melanocyte elimination to produce depigmentation in vitiligo and melanoma rejection. We examined the tyrosinase-mediated oxidation of NPrCAP and its subsequent binding to sulfhydryl compounds (thiols) in NPrCAP-treated melanoma tissues and demonstrated that NPrCAP is oxidized by tyrosinase to form a highly reactive *ortho*-quinone, (*N*-propionyl-4-*S*-cysteaminylcatechol, NPrCAQ; Figure 8), which then binds covalently to biologically relevant thiols including proteins through the cysteine residues. *In vitro* and *in vivo* studies were also conducted to prove the binding of the quinone-hapten NPrCAQ to proteins. The thiol adducts were analyzed after acid hydrolysis as 5-*S*-cysteaminyl-3-*S*-cysteinylcatechol (CA-CysC) (Figure 8). Our results specifically provided evidence that NPrCAP is oxidized by tyrosinase to an *ortho*-quinone, NPrCAQ, which is highly reactive yet stable enough to survive and then interact with biologically relevant thiols to form covalent adducts. The activation of NPrCAP to NPrCAQ by tyrosinase and the subsequent binding to proteins through cysteine residues were also demonstrated in the *in vitro* and *in vivo* experiments. Our finding was the first demonstration that the quinone-protein adduct formation actually takes place in melanoma cells and melanoma tissues through the tyrosinase-mediated mechanism. Furthermore, 60–80% of the NPrCAQ-thiol adducts were found in the protein fraction in melanoma cells and in the tumors. This is surprising when we consider the much lower reactivity of protein sulfhydryl groups compared with those in small thiols such as cysteine [60, 61]. The remaining nonprotein SH adducts were produced by the reaction of NPrCAQ with free cysteine or glutathione as a detoxifying mechanism. In this connection, it was previously shown that the depletion of glutathione augmented the melanocytotoxicity and antimelanoma effects of NAcCAP [62].

According to the potent melanoma immunotherapy theory using monobenzone [54, 55, 57–59], tyrosinase appears to trigger melanoma regression. Tyrosinase oxidation of monobenzone produces a highly reactive quinone-hapten [44, 54] and ROS concurrently [54]. The quinone-hapten binds to cysteine residues in tyrosinase or other melanosomal proteins thereby generating possible neoantigen, which activate hapten-reactive $CD8^+$ T-cells. The latter cells kill monobenzone-exposed melanocytes expressing haptenated

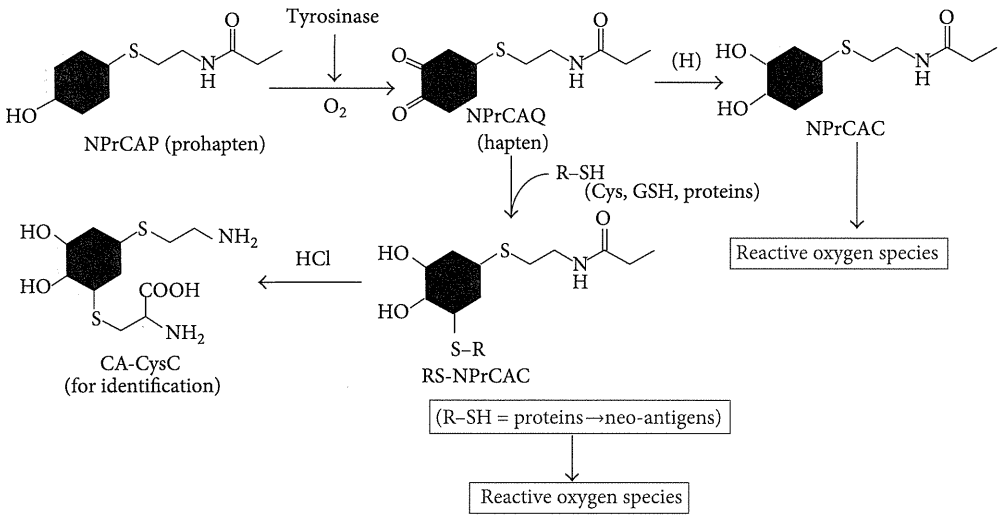


FIGURE 8: Tyrosinase activation of NPrCAP (prohapten) and binding of the quinone-hapten NPrCAQ with proteins thorough cysteine residues. Oxidation of NPrCAP with tyrosinase produces the quinone NPrCAQ, which is reduced to the catechol NPrCAC or binds to thiols (cysteine, glutathione, melanosomal proteins). The production of NPrCAQ-thiol adducts can be confirmed by the detection of CA-CysC after acid hydrolysis. NAcCys-NPrCAC is produced by the addition reaction of NAcCys (R-SH) with NPrCAQ. From Ito et al. [47].

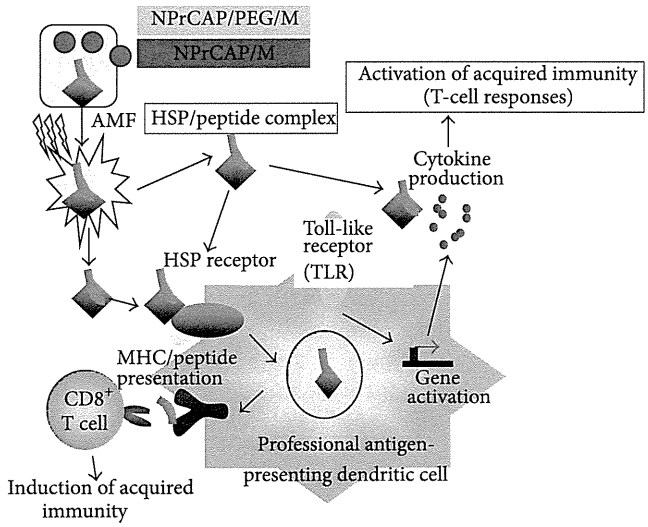


FIGURE 9: Scheme of intracellular hyperthermia using NPrCAP /PEG/M or NPrCAP/M with AMF exposure. NPrCAP/PEG/M nanoparticles are selectively incorporated in melanoma cells. Intracellular hyperthermia can induce necrotic cell death, and adjacent live melanoma cells suffer heat shock, resulting in increased level of intracellular HSP-peptide complexes. Repeated hyperthermia turns heat-shocked cells to necrotic cells, leading to the release of HSP-peptide complexes into extracellular milieu. The released HSPs-peptide complexes are taken up by dendritic cells (DCs). Then, DCs migrate into regional lymph nodes and cross-present HSP chaperoned antigenic peptides to $CD8^+$ T cells in the context of MHC class I molecules, thereby inducing antimelanoma cytotoxic $CD8^+$ T cells.

antigens on their surface, further liberating melanocyte antigens for presentation by dendritic cells. Finally, the antigen-specific T-cell response is induced and propagated [54, 57–59]. The ROS generated also causes damage to melanosomes leading to the presentation of melanosome-derived antigens and the induction of antigen-specific T-cell responses [58].

These immunological events can also be expected to occur for our NPrCAP because the involvement of $CD8^+$ T cells and the production of ROS in NPrCAP-treated melanoma cells were demonstrated in our previous study [46]. We expect the production of NPrCAC through redox exchange in melanoma cells and the subsequent production of ROS from the catechol because the closely related catechol, 4-*S*-cysteaminylcatechol, was shown to produce superoxide radicals (which are rapidly converted to hydrogen peroxide) [63]. The thiol adduct RS-NPrCAC, as a catechol, may also contribute to the production of ROS.

6. Summary and Conclusion

Several clinical trials using melanoma peptides or an antibody that blocks cytotoxic T-lymphocyte-associated antigen on lymphocytes have been shown to improve overall melanoma survival [64–66]. Promising oncogene-targeted melanoma therapy has also been successfully introduced recently [67].

Our study may however indicate that exploitation of a specific biological property to cancer cells can be another approach for developing novel melanoma-targeted drugs which can also trigger the production of melanoma-targeted *in situ* vaccine. Our approach using melanogenesis substrate and magnetite nanoparticles is based upon the expectation of (i) direct killing of melanoma cells by chemotherapeutic and thermo-therapeutic effect of melanogenesis-targeted drug (NPrCAP/M) and (ii) indirect killing by immune reaction (*in situ* peptide vaccine) after exposure to AMF. It is hoped from these rationales that a tumor-specific DDS is developed by NPrCAP, and selective cell death can be achieved by exposure of conjugates of NPrCAP/M nanoparticles to AMF. Hyperthermia increases the expression of intracellular HSPs which

is important in and necessary for the induction of antitumor immunity [41, 68]. Overexpression of HSPs increases tumor immunogenicity by augmenting the chaperoning ability of antigenic peptides and presentation of antigenic peptides in MHC class I molecules [39, 69]. In this process professional antigen-presenting dendritic cells play unique and important roles in taking up, processing, and presenting exogenous antigens in association with MHC class I molecules. Our study indicated that combination of melanogenesis substrate, NPrCAP, and local magnetite nanoparticles with hyperthermia could induce *in situ* a form of vaccine against tumor cells and may be effective not only for primary melanoma but also for distant secondary metastases (Figure 9).

Interestingly we found that NPrCAP by itself has potent chemotherapeutic and immune-adjuvant effects. It was demonstrated that the phenol NPrCAP, as a prohapten, can be activated in melanoma cells by tyrosinase to the reactive quinone-hapten NPrCAQ which binds to melanosomal proteins through their cysteine residues to form possible neo-antigens, thus triggering the immunological response (Figure 8).

Abbreviations

AMF: Alternating magnetic field
BSA: Bovine serum albumin
BSO: Buthionine sulfoximide
CA-CysC: 5-*S*-cysteaminyl-3-*S*-cysteinylcatechol
CAP: Cysteaminylphenol
CDR3: Third complementarity determining region
CML: Cationic magneto-liposome
CTI therapy: Chemothermoimmunotherapy
CTL: Cytotoxic T lymphocyte
DCs: Dendritic cells
DDS: Drug delivery system
DLNs: Draining lymph nodes
HSP/Hsp: Heat shock protein
IL: Interleukin
M: Magnetite nanoparticle
mAb: Monoclonal antibody
MART-1: Melanoma antigen recognized by T cells-1
MCLs: Magnetite cationic liposomes
MC1R: Melanocortin 1 receptor
MHC: Major histocompatibility complex
MITF: Microphthalmia transcription factor
ML: Noncationic magnetoliposome
MSH: Melanocyte stimulating hormone
NAcCAP: N-acetyl-4-S cysteaminylphenol
NDLN: Nondraining lymph node
NPrCAC: N-propionyl-4-S cysteaminylcatechol
NPrCAP: N-propionyl-4-S cysteaminylphenol
NPrCAQ: N-propionyl-4-S-cysteaminylquinine
Nrf2: NF-E2-related factor 2
OVA: Ovu-albumin
PEG: Polyethylene glycol
ROS: Reactive oxygen species
RT-PCR: Reverse transcription polymerase chain reaction
TILs: Tumor-infiltrating lymphocytes
TCRs: T-cell receptors
TRP-2: Tyrosinase-related protein-2.

Acknowledgments

This work was supported by a Health and Labor Sciences Research Grant-in-Aid for Research on Advanced Medical Technology from the Ministry of Health, Labor, and Welfare of Japan. The authors express their sincere appreciation to Mr. Noboru Minowa, Toray Industries, Inc., Tokyo, Japan, and Drs. Akiko Sato, Makito Sato, Tomoaki Takada, and Ichiro Ono, Sapporo Medical University, Sapporo, Japan, who have supported to carry out basic experiments and clinical trials of this study. The authors also express thier sincere appreciation to Ms. Makiko Jizou and Ms. Ikuko Ichimura for their secretarial help.

References

[1] E. de Vries, L. V. van de Poll-Franse, W. J. Louwman, F. R. de Gruijl, and J. W. W. Coebergh, "Predictions of skin cancer incidence in the Netherlands up to 2015," *The British Journal of Dermatology*, vol. 152, no. 3, pp. 481–488, 2005.

[2] C. M. Balch, J. E. Gershenwald, S. J. Soong et al., "Final version of 2009 AJCC melanoma staging and classification," *Journal of Clinical Oncology*, vol. 27, no. 36, pp. 6199–6206, 2009.

[3] C. M. Balch, A. C. Buzaid, S. J. Soong et al., "Final version of the American joint committee on cancer staging system for cutaneous melanoma," *Journal of Clinical Oncology*, vol. 19, no. 16, pp. 3635–3648, 2001.

[4] K. Reszka and K. Jimbow, "Electron donor and acceptor properties of melanin pigments in the skin," in *Oxidative Stress in Dermatology*, J. Fuchs and L. Packer, Eds., pp. 287–320, Marcel Dekker, New York, NY, USA, 1993.

[5] K. Jimbow, T. Iwashina, F. Alena, K. Yamada, J. Pankovich, and T. Umemura, "Exploitation of pigment biosynthesis pathway as a selective chemotherapeutic approach for malignant melanoma," *Journal of Investigative Dermatology*, vol. 100, no. 2, pp. s231–s238, 1993.

[6] F. Alena, T. Iwashina, A. Gili, and K. Jimbow, "Selective *in vivo* accumulation of N-acetyl-4-S-cysteaminylphenol in B16F10 murine melanoma and enhancement of its *in vitro* and *in vivo* antimelanoma effect by combination of buthionine sulfoximine," *Cancer Research*, vol. 54, no. 10, pp. 2661–2666, 1994.

[7] J. M. Pankovich and K. Jimbow, "Tyrosine transport in a human melanoma cell line as a basis for selective transport of cytotoxic analogues," *Biochemical Journal*, vol. 280, no. 3, pp. 721–725, 1991.

[8] M. Tandon, P. D. Thomas, M. Shokravi et al., "Synthesis and antitumour effect of the melanogenesis-based antimelanoma agent N-Propionyl-4-S-cysteaminylphenol," *Biochemical Pharmacology*, vol. 55, no. 12, pp. 2023–2029, 1998.

[9] A. Gili, P. D. Thomas, M. Ota, and K. Jimbow, "Comparison of *in vitro* cytotoxicity of N-acetyl and N-propionyl derivatives of phenolic thioether amines in melanoma and neuroblastoma cells and the relationship to tyrosinase and tyrosine hydroxylase enzyme activity," *Melanoma Research*, vol. 10, no. 1, pp. 9–15, 2000.

[10] P. D. Thomas, H. Kishi, H. Cao et al., "Selective incorporation and specific cytocidal effect as the cellular basis for the antimelanoma action of sulphur containing tyrosine analogs," *Journal of Investigative Dermatology*, vol. 113, no. 6, pp. 928–934, 1999.

[11] A. Ito, M. Shinkai, H. Honda, and T. Kobayashi, "Medical application of functionalized magnetic nanoparticles," *Journal of Bioscience and Bioengineering*, vol. 100, no. 1, pp. 1–11, 2005.

[12] N. Kawai, A. Ito, Y. Nakahara et al., "Anticancer effect of hyperthermia on prostate cancer mediated by magnetite cationic liposomes and immune-response induction in transplanted syngeneic rats," *Prostate*, vol. 64, no. 4, pp. 373–381, 2005.

[13] M. Shinkai, M. Yanase, H. Honda, T. Wakabayashi, J. Yoshida, and T. Kobayashi, "Intracellular hyperthermia for cancer using magnetite cationic liposomes: *in vitro* study," *Japanese Journal of Cancer Research*, vol. 87, no. 11, pp. 1179–1183, 1996.

[14] A. Ménoret and R. Chandawarkar, "Heat-shock protein-based anticancer immunotherapy: an idea whose time has come," *Seminars in Oncology*, vol. 25, no. 6, pp. 654–660, 1998.

[15] P. K. Srivastava, A. Ménoret, S. Basu, R. Binder, and K. Quade, "Heat shock proteins come of age: primitive functions acquired new roles in an adaptive world," *Immunity*, vol. 8, no. 6, pp. 657–665, 1998.

[16] Y. Tamura, N. Tsuboi, N. Sato, and K. Kikuchi, "70 kDa heat shock cognate protein is a transformation-associated antigen and a possible target for the host's anti-tumor immunity," *Journal of Immunology*, vol. 151, no. 10, pp. 5516–5524, 1993.

[17] Y. Tamura, P. Peng, K. Liu, M. Daou, and P. K. Srivastava, "Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations," *Science*, vol. 278, no. 5335, pp. 117–120, 1997.

[18] Y. Tamura and N. Sato, "Heat shock proteins: chaperoning of innate and adaptive immunities," *Japanese Journal of Hyperthermic Oncology*, vol. 19, pp. 131–139, 2003.

[19] P. K. Srivastava, "Immunotherapy for human cancer using heat shock protein-peptide complexes," *Current Oncology Reports*, vol. 7, no. 2, pp. 104–108, 2005.

[20] Y. Tamura, S. Takashima, J. M. Cho et al., "Inhibition of natural killer cell cytotoxicity by cell growth-related molecules," *Japanese Journal of Cancer Research*, vol. 87, no. 6, pp. 623–630, 1996.

[21] G. Ueda, Y. Tamura, I. Hirai et al., "Tumor-derived heat shock protein 70-pulsed dendritic cells elicit-tumor-specific cytotoxic T lymphocytes (CTLs) and tumor immunity," *Cancer Science*, vol. 95, no. 3, pp. 248–253, 2004.

[22] A. Ito, H. Honda, and T. Kobayashi, "Cancer immunotherapy based on intracellular hyperthermia using magnetite nanoparticles: a novel concept of "heat-controlled necrosis" with heat shock protein expression," *Cancer Immunology, Immunotherapy*, vol. 55, no. 3, pp. 320–328, 2006.

[23] H. Shi, T. Cao, J. E. Connolly et al., "Hyperthermia enhances CTL cross-priming," *Journal of Immunology*, vol. 176, no. 4, pp. 2134–2141, 2006.

[24] J. Dakour, T. Vinayagamoorthy, K. Jimbow et al., "Identification of a cDNA coding for a Ca^{2+}-binding phosphoprotein (p90) calnexin, on melanosomes in normal and malignant human melanocytes," *Experimental Cell Research*, vol. 209, no. 2, pp. 288–300, 1993.

[25] K. Toyofuku, I. Wada, K. Hirosaki, J. S. Park, Y. Hori, and K. Jimbow, "Promotion of tyrosinase folding in Cos 7 cells by calnexin," *Journal of Biochemistry*, vol. 125, no. 1, pp. 82–89, 1999.

[26] K. Jimbow, P. F. Gomez, K. Toyofuku et al., "Biological role of tyrosinase related protein and its biosynthesis and transport from TGN to stage I melanosome, late endosome, through gene transfection study," *Pigment Cell Research*, vol. 10, no. 4, pp. 206–213, 1997.

[27] K. Jimbow, J. S. Park, F. Kato et al., "Assembly, target-signaling and intracellular transport of tyrosinase gene family proteins in the initial stage of melanosome biogenesis," *Pigment Cell Research*, vol. 13, no. 4, pp. 222–229, 2000.

[28] K. Jimbow, C. Hua, P. F. Gomez et al., "Intracellular vesicular trafficking of tyrosinase gene family protein in Eu- and pheomelanosome biogenesis," *Pigment Cell Research*, vol. 13, no. 8, pp. 110–117, 2000.

[29] T. Miura, K. Jimbow, and S. Ito, "The *in vivo* antimelanoma effect of 4-S-cysteaminylphenol and its N-acetyl derivative," *International Journal of Cancer*, vol. 46, no. 5, pp. 931–934, 1990.

[30] S. Ito, T. Kato, K. Ishikawa, T. Kasuga, and K. Jimbow, "Mechanism of selective toxicity of 4-S-cysteinylphenol and 4-S-cysteaminylphenol to melanocytes," *Biochemical Pharmacology*, vol. 36, no. 12, pp. 2007–2011, 1987.

[31] Y. Minamitsuji, K. Toyofuku, S. Sugiyama, K. Yamada, and K. Jimbow, "Sulfur containing tyrosine analogs can cause selective melanocytotoxicity involving tyrosinase-mediated apoptosis," *Journal of Investigative Dermatology Symposium Proceedings*, vol. 4, no. 2, pp. 130–136, 1999.

[32] K. Jimbow, Y. Miyake, K. Homma et al., "Characterization of melanogenesis and morphogenesis of melanosomes by physicochemical properties of melanin and melanosomes in malignant melanoma," *Cancer Research*, vol. 44, no. 3, pp. 1128–1134, 1984.

[33] B. Thiesen and A. Jordan, "Clinical applications of magnetic nanoparticles for hyperthermia," *International Journal of Hyperthermia*, vol. 24, no. 6, pp. 467–474, 2008.

[34] M. Johannsen, U. Gneveckow, L. Eckelt et al., "Clinical hyperthermia of prostate cancer using magnetic nanoparticles: presentation of a new interstitial technique," *International Journal of Hyperthermia*, vol. 21, no. 7, pp. 637–647, 2005.

[35] A. Ito, M. Fujioka, T. Yoshida et al., "4-S-Cysteaminylphenol-loaded magnetite cationic liposomes for combination therapy of hyperthermia with chemotherapy against malignant melanoma," *Cancer Science*, vol. 98, no. 3, pp. 424–430, 2007.

[36] M. Sato, T. Yamashita, M. Ohkura et al., "N-propionyl-cysteaminylphenol-magnetite conjugate (NPrCAP/M) Is a nanoparticle for the targeted growth suppression of melanoma cells," *Journal of Investigative Dermatology*, vol. 129, no. 9, pp. 2233–2241, 2009.

[37] T. Takada, T. Yamashita, M. Sato et al., "Growth inhibition of re-challenge B16 melanoma transplant by conjugates of melanogenesis substrate and magnetite nanoparticles as the basis for developing melanoma-targeted chemo-thermo-immunotherapy," *Journal of Biomedicine and Biotechnology*, vol. 2009, Article ID 457936, 13 pages, 2009.

[38] M. Suzuki, M. Shinkai, H. Honda, and T. Kobayashi, "Anticancer effect and immune induction by hyperthermia of malignant melanoma using magnetite cationic liposomes," *Melanoma Research*, vol. 13, no. 2, pp. 129–135, 2003.

[39] M. Yanase, M. Shinkai, H. Honda, T. Wakabayashi, J. Yoshida, and T. Kobayashi, "Intracellular hyperthermia for cancer using magnetite cationic liposomes: an *in vivo* study," *Japanese Journal of Cancer Research*, vol. 89, no. 4, pp. 463–469, 1998.

[40] R. Hergt, S. Dutz, R. Müller, and M. Zeisberger, "Magnetic particle hyperthermia: nanoparticle magnetism and materials

development for cancer therapy," *Journal of Physics Condensed Matter*, vol. 18, no. 38, pp. S2919–S2934, 2006.

[41] A. Ito, M. Shinkai, H. Honda et al., "Heat shock protein 70 expression induces antitumor immunity during intracellular hyperthermia using magnetite nanoparticles," *Cancer Immunology, Immunotherapy*, vol. 52, no. 2, pp. 80–88, 2003.

[42] A. Ito, F. Matsuoka, H. Honda, and T. Kobayashi, "Antitumor effects of combined therapy of recombinant heat shock protein 70 and hyperthermia using magnetic nanoparticles in an experimental subcutaneous murine melanoma," *Cancer Immunology, Immunotherapy*, vol. 53, no. 1, pp. 26–32, 2004.

[43] A. Sato, Y. Tamura, N. Sato et al., "Melanoma-targeted chemo-thermo-immuno (CTI)-therapy using N-propionyl-4-S-cysteaminylphenol-magnetite nanoparticles elicits CTL response via heat shock protein-peptide complex release," *Cancer Science*, vol. 101, no. 9, pp. 1939–1946, 2010.

[44] P. Manini, A. Napolitano, W. Westerhof, P. A. Riley, and M. D'Ischia, "A reactive ortho-quinone generated by tyrosinase-catalyzed oxidation of the skin depigmenting agent monobenzone: self-coupling and thiol-conjugation reactions and possible implications for melanocyte toxicity," *Chemical Research in Toxicology*, vol. 22, no. 8, pp. 1398–1405, 2009.

[45] A. Ito, M. Yamaguchi, N. Okamoto et al., "T-cell receptor repertoires of tumor-infiltrating lymphocytes after hyperthermia using functionalized magnetite nanoparticles," *Nanomedicine*, 2012.

[46] Y. Ishii-Osai, T. Yamashita, Y. Tamura et al., "N-Propionyl-4-S-cysteaminylphenol induces apoptosis in B16F1 cells and mediates tumor-specific T-cell immune responses in a mouse melanoma model," *Journal of Dermatological Science*, vol. 67, no. 1, pp. 51–60, 2012.

[47] S. Ito, A. Nishigaki, Y. Ishii-Osai et al., "Mechanism of putative neo-antigen formation from N-propionyl-4-S-cysteaminylphenol, a tyrosinase substrate, in melanoma models," *Biochemical Pharmacology*, vol. 84, no. 5, pp. 646–653, 2012.

[48] K. Jimbow, H. Obata, M. A. Pathak, and T. B. Fitzpatrick, "Mechanism of depigmentation by hydroquinone," *Journal of Investigative Dermatology*, vol. 62, no. 4, pp. 436–449, 1974.

[49] J. J. Nordlund, B. Forget, J. Kirkwood, and A. B. Lerner, "Dermatitis produced by applications of monobenzone in patients with active vitiligo," *Archives of Dermatology*, vol. 121, no. 9, pp. 1141–1144, 1985.

[50] R. E. Boissy and P. Manga, "On the etiology of contact/occupational vitiligo," *Pigment Cell Research*, vol. 17, no. 3, pp. 208–214, 2004.

[51] C. J. Cooksey, K. Jimbow, E. J. Land, and P. A. Riley, "Reactivity of orthoquinones involved in tyrosinase-dependent cytotoxicity: differences between alkylthio- and alkoxy-substituents," *Melanoma Research*, vol. 2, no. 5-6, pp. 283–293, 1993.

[52] P. G. Parsons, D. Favier, M. McEwan, H. Takahashi, K. Jimbow, and S. Ito, "Action of cysteaminylphenols on human melanoma cells *in vivo* and *in vitro*: 4-S-cysteaminylphenol binds protein disulphide isomerase," *Melanoma research*, vol. 1, no. 2, pp. 97–104, 1991.

[53] V. Hariharan, J. Klarquist, M. J. Reust et al., "Monobenzyl ether of hydroquinone and 4-tertiary butyl phenol activate markedly different physiological responses in melanocytes: relevance to skin depigmentation," *Journal of Investigative Dermatology*, vol. 130, no. 1, pp. 211–220, 2010.

[54] J. G. van den Boorn, D. I. Picavet, P. F. van Swieten et al., "Skin-depigmenting agent monobenzone induces potent T-cell autoimmunity toward pigmented cells by tyrosinase haptenation and melanosome autophagy," *Journal of Investigative Dermatology*, vol. 131, no. 6, pp. 1240–1251, 2011.

[55] J. G. van den Boorn, D. Konijnenberg, E. P. Tjin et al., "Effective melanoma immunotherapy in mice by the skin-depigmenting agent monobenzone and the adjuvants imiquimod and CpG," *PloS ONE*, vol. 5, no. 5, Article ID e10626, 2010.

[56] P. Quaglino, F. Marenco, S. Osella-Abate et al., "Vitiligo is an independent favourable prognostic factor in stage III and IV metastatic melanoma patients: results from a single-institution hospital-based observational cohort study," *Annals of Oncology*, vol. 21, no. 2, pp. 409–414, 2010.

[57] W. Westerhof, P. Manini, A. Napolitano, and M. d'Ischia, "The haptenation theory of vitiligo and melanoma rejection: a close-up," *Experimental Dermatology*, vol. 20, no. 2, pp. 92–96, 2011.

[58] J. G. van den Boorn, C. J. Melief, and R. M. Luiten, "Monobenzone-induced depigmentation: from enzymatic blockage to autoimmunity," *Pigment Cell and Melanoma Research*, vol. 24, no. 4, pp. 673–679, 2011.

[59] W. Westerhof and M. D'Ischia, "Vitiligo puzzle: the pieces fall in place," *Pigment Cell Research*, vol. 20, no. 5, pp. 345–359, 2007.

[60] T. Kato, S. Ito, and K. Fujita, "Tyrosinase-catalyzed binding of 3,4-dihydroxyphenylalanine with proteins through the sulfhydryl group," *Biochimica et Biophysica Acta*, vol. 881, no. 3, pp. 415–421, 1986.

[61] S. Ito, T. Kato, and K. Fujita, "Covalent binding of catechols to proteins through the sulphydryl group," *Biochemical Pharmacology*, vol. 37, no. 9, pp. 1707–1710, 1988.

[62] F. Alena, K. Jimbow, and S. Ito, "Melanocytotoxicity and antimelanoma effects of phenolic amine compounds in mice *in vivo*," *Cancer Research*, vol. 50, no. 12, pp. 3743–3747, 1990.

[63] K. Hasegawa, S. Ito, S. Inoue, K. Wakamatsu, H. Ozeki, and I. Ishiguro, "Dihydro-1,4-benzothiazine-6,7-dione, the ultimate toxic metabolite of 4-S-cysteaminylphenol and 4-S-cysteaminylcatechol," *Biochemical Pharmacology*, vol. 53, no. 10, pp. 1435–1444, 1997.

[64] F. S. Hodi, S. J. O'Day, D. F. McDermott et al., "Improved survival with ipilimumab in patients with metastatic melanoma," *The New England Journal of Medicine*, vol. 363, no. 8, pp. 711–723, 2010.

[65] D. J. Schwartzentruber, D. H. Lawson, J. M. Richards et al., "gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma," *The New England Journal of Medicine*, vol. 364, no. 22, pp. 2119–2127, 2011.

[66] C. Robert, L. Thomas, I. Bondarenko et al., "Ipilimumab plus dacarbazine for previously untreated metastatic melanoma," *The New England Journal of Medicine*, vol. 364, pp. 2517–2526, 2011.

[67] P. B. Chapman, A. Hauschild, C. Robert et al., "Improved survival with vemurafenib in melanoma with BRAF V600E mutation," *The New England Journal of Medicine*, vol. 364, no. 26, pp. 2507–2516, 2011.

[68] K. Mise, N. Kan, T. Okino et al., "Effect of heat treatment on tumor cells and antitumor effector cells," *Cancer Research*, vol. 50, no. 19, pp. 6199–6202, 1990.

[69] A. Ito, F. Matsuoka, H. Honda, and T. Kobayashi, "Heat shock protein 70 gene therapy combined with hyperthermia using magnetic nanoparticles," *Cancer Gene Therapy*, vol. 10, no. 12, pp. 918–925, 2003.

FULL PAPER

BJC

British Journal of Cancer (2013) 108, 1119–1125 | doi: 10.1038/bjc.2013.51

Keywords: surgical treatment; detection marker; follow-up marker; recurrence; prognosis

NY-ESO-1 antibody as a novel tumour marker of gastric cancer

S Fujiwara[1], H Wada*[,1], J Kawada[1], R Kawabata[1], T Takahashi[1], J Fujita[2], T Hirao[3], K Shibata[3], Y Makari[4], S Iijima[4], H Nishikawa[5], A A Jungbluth[6], Y Nakamura[1], Y Kurokawa[1], M Yamasaki[1], H Miyata[1], K Nakajima[1], S Takiguchi[1], E Nakayama[7], M Mori[1] and Y Doki[1]

[1]Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita-city (E-2), Osaka 565-0871, Japan; [2]Surgery, Toyonaka Municipal Hospital, 4-14-1 Shibahara, Toyonaka-city, Osaka 560-0055, Japan; [3]Surgery, Ikeda City Hospital, 3-1-18 Jonan, Ikeda-city, Osaka 563-8510, Japan; [4]Surgery, Minoh City Hospital, 5-7-1 Kayano, Minoh-city, Osaka 562-0014, Japan; [5]Department of Experimental Immunology, Immunology Frontier Research Center, Osaka University, 3-1 Yamadaoka, Suita-city, Osaka 565-0871, Japan; [6]Ludwig Institute for Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA and [7]Faculty of Health and Welfare, Kawasaki University of Medical Welfare, 288 Matsushima, Kurashiki-city, Okayama 701-0193, Japan

Background: NY-ESO-1 antibodies are specifically observed in patients with NY-ESO-1-expressing tumours. We analysed whether the NY-ESO-1 humoral immune response is a useful tumour marker of gastric cancer.

Methods: Sera from 363 gastric cancer patients were screened by enzyme-linked immunosorbent assay (ELISA) to detect NY-ESO-1 antibodies. Serial serum samples were obtained from 25 NY-ESO-1 antibody-positive patients, including 16 patients with curative resection and 9 patients who received chemotherapy alone.

Results: NY-ESO-1 antibodies were detected in 3.4% of stage I, 4.4% of stage II, 25.3% of stage III, and 20.0% of stage IV patients. The frequency of antibody positivity increased with disease progression. When the NY-ESO-1 antibody was used in combination with carcinoembryonic antigen and CA19-9 to detect gastric cancer, information gains of 11.2% in stages III and IV, and 5.8% in all patients were observed. The NY-ESO-1 immune response levels of the patients without recurrence fell below the cutoff level after surgery. Two of the patients with recurrence displayed incomplete decreases. The nine patients who received chemotherapy alone continued to display NY-ESO-1 immune responses.

Conclusion: When combined with conventional tumour markers, the NY-ESO-1 humoral immune response could be a useful tumour marker for detecting advanced gastric cancer and inferring the post-treatment tumour load in seropositive patients.

Gastric cancer is the second most common cause of cancer-related death worldwide (Health and Welfare Statistics Association: Tokyo, 2006; Katanoda and Yako-Suketomo, 2009). Although complete removal of the tumour by surgical resection is an ideal treatment option for patients with gastric cancer, many patients with advanced-stage gastric cancer need to be treated with intensive chemotherapy. Gastric cancer patients exhibit high relapse rates even after curative surgery and unresponsiveness to chemotherapy, resulting in dismal survival rates (Sasako *et al*, 2011). Several methods for the prediction and early detection of subclinical 'minimal residual cancer' after surgery (Austrup *et al*, 2000; Klein *et al*, 2002) or relapse have been developed, for example, peritoneal lavage, positron emission tomography, gene profiling, and so on. (Motoori *et al*, 2006; Makino *et al*, 2010; Graziosi *et al*, 2011), reliable markers that can specifically reflect gastric cancer disease status have not been determined.

Analysing serum level of tumour markers is employed for cancer detection, monitoring patients' disease status, and prognosis prediction. Several organ-specific tumour markers are used in the clinic, for example, prostate-specific antigen and prostatic acid

*Correspondence: Dr H Wada; E-mail: hwada@gesurg.med.osaka-u.ac.jp

Received 29 June 2012; revised 9 January 2013; accepted 16 January 2013; published online 12 February 2013

phosphatase for prostate cancer (Seamonds *et al*, 1986; Ferro *et al*, 1987) and protein induced by vitamin K absence-II for liver cancer (Fujiyama *et al*, 1986). As no gastric cancer-specific markers have been determined, a combination of several nonspecific tumour markers, for example, carcinoembryonic antigen (CEA), CA19-9,and so on, is merely applicable for monitoring treatment efficacy, but not the diagnosis of gastric cancer (Takahashi *et al*, 1995, 2003). Carcinoembryonic antigen and CA19-9 are found in the sera of 20–60% of gastric cancer patients, and their expression levels in gastric cancer are related to clinical events, such as relapse (Kodera *et al*, 1996). Carcinoembryonic antigen value, in particular, is indicative of the formation of a large tumour, liver or peritoneal metastasis, and/or a high risk of relapse and poor prognosis (Ikeda *et al*, 1993; Yamamoto *et al*, 2004). However, as CEA, a cell surface-anchored glycoprotein, is expressed in normal cell membranes, 5% of CEA-positive cases are pseudopositives, that is, caused by heavy smoking, endometriosis, and ageing, and so on. (Alexander *et al*, 1976), suggesting the importance of novel markers for gastric cancer.

NY-ESO-1 antigen, a cancer/testis (CT) antigen, was originally identified in oesophageal cancer by serological expression cloning using autologous patient serum and has been shown to be strongly immunogenic. Spontaneous NY-ESO-1 antibody production is often observed in patients with NY-ESO-1-expressing tumours, for example, 9.4% of melanoma patients, 12.5% of ovarian cancer patients, 7.7–26.5% of breast cancer patients, 4.2–20.0% of lung cancer patients, and 52% of prostate cancer patients, but has not been detected in non-cancerous donors (Stockert *et al*, 1998; Nakada *et al*, 2003; Türeci *et al*, 2006; Chapman *et al*, 2007; Isobe *et al*, 2009; Gati *et al*, 2011). Thus, it is possible that the NY-ESO-1 humoral immune response could be used as a serological marker for detecting these cancers and to facilitate the clinical management of some patients with particular types of cancer (Gnjatic *et al*, 2006). Jäger *et al* (1999) found that the change in the NY-ESO-1 humoral immune response reflected the overall tumour load in 10 out of 12 patients with various cancers. However, there is ongoing controversy regarding the association between the NY-ESO-1 immune response and prognostic criteria (Yuan *et al*, 2011). To address these issues in gastric cancer, we investigated the clinical usefulness of the NY-ESO-1 humoral immune response for diagnosis, monitoring, and relapse prediction in gastric cancer patients.

MATERIALS AND METHODS

Serum sample and tissue specimen collection from gastric cancer patients. In all, 363 patients with histologically confirmed gastric cancer, who underwent surgical resection or chemotherapy at one of four institutions between 2004 and 2011, were included in this study after providing written informed consent. Serum samples were obtained from the 363 patients during their admission to hospital for surgical treatment and/or chemotherapy, and afterwards, serial serum samples were obtained at each follow-up visit from 25 patients who displayed NY-ESO-1 humoral immune responses. All serum samples were collected as surplus samples after routine blood tests and stored. Fixed and frozen gastric cancer tissue samples were obtained from 60 out of 363 patients during surgery and stored. The samples were subsequently subjected to expression analysis. Information regarding blood test results, tumour stage, histological type, depth of invasion, lymph node metastasis, and distant metastasis, which were obtained from pathological examinations and CT scans, were collected from the relevant patient databases. Serum samples obtained from 50 healthy donors were used as controls. This study was approved by the institutional review boards of Osaka University Hospital, Toyonaka Municipal Hospital, Ikeda City Hospital, and Minoh City Hospital.

Reverse transcription–polymerase chain reaction. Total cellular RNA was extracted from the frozen tissue using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The total RNA (1 μg) was subjected to the reverse transcription (RT) in 20 μl buffer with oligo-$(dT)_{15}$ primer using a RT system (Promega, Madison, WI, USA). Conventional polymerase chain reaction (PCR) was performed in a 25-μl reaction mixture containing 1 μl of cDNA template, 500 nM of each primer, and 1 U of *Taq* DNA polymerase (AmpliTaq Gold, Roche Molecular Systems, Pleasanton, CA, USA) in the following conditions: one cycle of 95 °C for 12 min; followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1.5 min; and then a final step of 72 °C for 10 min. The sequences of the primers for *NY-ESO-1* were as follows: ESO1-1, 5′-AGTTC TACCTCGCCATGCCT-3′; and ESO1-2, 5′-TCCTCCTCCAGC GACAAACAA-3′. The integrity of each RNA sample was verified by performing RT–PCR for *porphobilinogen deaminase* (*PBGD*). The PCR products were subjected to electrophoresis on a 2% agarose gel and visualised with ethidium bromide.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissues were used for the immunohistochemistry (IHC) analyses. Slides were incubated with the primary antibody overnight at 4 °C. The monoclonal antibody E978, which was previously generated by our group, was used to detect NY-ESO-1. The slides were then subjected to a heat-based antigen retrieval technique by immersing them in a preheated buffer solution (hipH solution; Dako, Carpinteria, CA, USA). A polymer-based antibody detection system (PowerVision; Leica Microsystems, Buffalo Grove, IL, USA) was used as the secondary reagent, and 3,3-diaminobenzidine tetrahydrochloride (Liquid DAB; Biogenex, San Ramon, CA, USA) was used as the chromogen. Normal adult testis tissue as a positive control and appropriate negative controls were included for each case.

Enzyme-linked immunosorbent assay. A measure of 100 μl of 1 μg ml^{-1} recombinant protein in coating buffer (pH 9.6) were added to each well of 96-well PolySorp immunoplates (Nunc, Roskilde, Denmark) and incubated overnight at 4 °C. The plates were then washed with PBS and blocked with 200 μl per well of 5% FCS/PBS for 1 h at room temperature. After being washed again, 100 μl of serially diluted serum were added to each well and incubated for 2 h at room temperature. Then, after extensive washing, goat anti-human IgG (Medical & Biological Laboratories, Nagoya, Japan) was added to the wells as a secondary antibody, and the plates were incubated for 1 h at room temperature. The plates were washed again, and the signals were developed with 100 μl per well of 0.03% *o*-phenylene diamine dihydrochloride, 0.02% hydrogen peroxide, and 0.15 M citrate buffer, and absorbance was read at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA, USA). Ovalbumin (OVA; Sigma, St Louis, MO, USA) was used as the control protein. Levels of NY-ESO-1 humoral response were assessed using optical density (OD) values.

CEA and CA19-9. Serum CEA and CA19-9 levels were measured at each hospital's clinical laboratory department. Carcinoembryonic antigen and CA19-9 positivity were defined as serum levels of CEA and CA19-9 of >5.0 ng ml^{-1} and >37 U ml^{-1}, respectively.

Statistical analysis. Fisher's exact test was used to assess the associations between NY-ESO-1 antibody expression and clinicopathological parameters. Kaplan–Meier curves were plotted to assess the effect of the NY-ESO-1 antibody on overall survival. Survival curves were compared using the log-rank test.

Table 1. Frequencies of NY-ESO-1 antibody, CEA, and CA19-9 in gastric cancer patients

Stage	NY-ESO-1 Ab	CEA	CA19-9	CEA and/or CA19-9	CEA and/or CA19-9 and/or NY-ESO-1 Ab
I	6/176 (3.4)	24/176 (13.6)	6/176 (3.4)	27/176 (15.3)	31/176 (17.6)
II	2/45 (4.4)	8/45 (17.8)	7/45 (15.6)	11/45 (24.4)	12/45 (26.6)
III	17/67 (25.3)	22/67 (32.9)	11/67 (16.4)	25/67 (37.3)	35/67 (52.2)
IV	16/75 (20.0)	23/75 (30.7)	30/75 (40.0)	40/75 (53.3)	46/75 (61.3)
I + II	8/221 (3.6)	32/221 (14.5)	13/221 (5.9)	38/221 (17.2)	43/221 (19.5)
III + IV	33/142 (23.2)	45/142 (31.7)	41/142 (28.9)	65/142 (45.8)	81/142 (57.0)
Total	41/363 (11.1)	77/363 (21.2)	54/363 (14.9)	103/363 (28.4)	124/363 (34.2)

Abbreviations: Ab = antibody; CA = carbohydrate antigen; CEA = carcinoembryonic antigen. Values within parentheses are percentages.

RESULTS

Determination of NY-ESO-1 humoral immune response positivity. We first determined the OD cutoff value for NY-ESO-1 humoral immune response positivity. When the serum samples from the 50 healthy donors were examined for reactivity to the NY-ESO-1 recombinant protein by ELISA, their OD values ranged from 0.08 to 0.20, and their mean and standard deviation values were 0.15 and 0.05, respectively, at a dilution of 1 : 200. Thus, NY-ESO-1 humoral immune response positivity was defined as an OD value of >0.25 at a dilution of 1 : 200 (95% accuracy level) and >3 times of the OD value against control protein (OVA).

NY-ESO-1 humoral immune responses of gastric cancer patients. Serum samples were obtained from 363 gastric cancer patients, including 176 stage I, 45 stage II, 67 stage III, and 75 stage IV patients at admission (Table 1). The NY-ESO-1 antibody was detected in 3.4% (6 of 176) of stage I, 4.4% (2 of 45) of stage II, 25.3% (17 of 67) of stage III, and 20.0% (16 of 75) of stage IV gastric cancer patients, resulting in an overall detection rate of 11.1% (41 of 363). An analysis of the gastric cancer patients' characteristics found that NY-ESO-1 antibody positivity was significantly correlated with gender (male > female) and tumour progression (Table 2). In particular, the patients with progressive gastric cancer involving deeper tumour invasion, positive lymph node metastasis, positive distant metastasis, or a higher clinical stage tended to produce the NY-ESO-1 antibody.

Analysis of NY-ESO-1 antigen expression. *NY-ESO-1* mRNA and NY-ESO-1 protein expression were analysed by RT–PCR and IHC, respectively, in gastric cancer tissues obtained from 60 patients for whom both frozen and formalin-fixed specimens were available, including 12 stage I, 12 stage II, 20 stage III, and 16 stage IV patients (Table 3). *NY-ESO-1* mRNA was detected in six specimens. NY-ESO-1 was immunohistochemically detected in 19 specimens, including 6 and 13 that were positive and negative for *NY-ESO-1* mRNA, respectively. Most of the specimens displayed a heterogeneous staining pattern (data not shown).

NY-ESO-1 antibody and antigen expression. We analysed the frequency of NY-ESO-1 antibody positivity in gastric cancer patients in whom NY-ESO-1 antigen expression was or was not detected by RT–PCR or IHC. As shown in Table 3, 9 out of the 60 gastric cancer patients whose specimens were available for expression analysis possessed the NY-ESO-1 antibody in their sera. The NY-ESO-1 antibody was detected in 8 of 19 (42.1%) patients with IHC-positive gastric cancer and 5 of 6 (83.3%) patients with RT–PCR (and IHC)-positive gastric cancer, whereas only 1 of 41 patients in whom both RT–PCR and IHC analysis produced negative results displayed an NY-ESO-1 humoral immune responses.

Table 2. Relationship between NY-ESO-1 antibody positivity and clinicopathological features in gastric cancer patients

Variable	NY-ESO-1 Ab		*P*-value*
	Negative	Positive	
Gender			
Male	223 (86.4)	35 (13.6)	0.04307
Female	99 (94.3)	6 (5.7)	
Age (years)			
>65	178 (88.6)	23 (11.4)	0.9209
<65	144 (88.9)	18 (11.1)	
Histological type			
Differentiated	143 (89.4)	17 (10.6)	0.5605
Undifferentiated	132 (87.4)	19 (12.6)	
Depth of tumour invasion			
cT1–T2	193 (92.8)	15 (7.2)	0.0044
cT3–T4	129 (83.2)	26 (16.8)	
Lymph node metastasis			
Negative	196 (97.0)	6 (3.0)	<0.001
Positive	126 (78.3)	35 (21.7)	
Distant metastasis			
Negative	277 (91.1)	27 (8.9)	<0.001
Positive	45 (76.3)	14 (23.7)	
Stage			
I–II	213 (96.4)	8 (3.6)	<0.001
III–IV	109 (76.8)	33 (23.2)	

Abbreviations: Ab = antibody. Fisher's exact test was used for the statistical analysis. Values within parentheses are percentages.

Frequencies of NY-ESO-1 humoral immune responses and conventional tumour markers in gastric cancer patients. The frequency of the NY-ESO-1 humoral immune response was compared with those of conventional tumour markers in gastric

Table 3. Frequency of NY-ESO-1 antibody positives in gastric cancer patients in whom the NY-ESO-1 antigen was or was not detected by IHC or RT–PCR

	IHC		
	Positive	**Negative**	**Total**
mRNA			
Positive	5/6 (83.3)	0/0 (0.0)	5/6 (83.3)
Negative	3/13 (23.1)	1/41 (2.4)	4/54 (7.4)
Total	8/19 (42.1)	1/41 (2.4)	9/60 (15.0)

Abbreviations: IHC = immunohistochemistry; RT–PCR = reverse transcription–polymerase chain reaction. Frozen and formalin-fixed tissue specimens from 60 patients, including 12 stage I, 12 stage II, 20 stage III, and 16 stage IV patients, were analysed. All stage IV patients had previously undergone surgical treatment. Values within parentheses are percentages.

cancer patients. The serum CEA and CA19-9 levels of 363 gastric cancer patients were measured at admission (Table 1). Carcinoembryonic antigen and CA19-9 positivity were observed in 21.2% (77 of 363) and 14.9% (54 of 363) of the gastric cancer patients, respectively, and, except for CA19-9 in the stage III patients, they displayed higher frequencies than the NY-ESO-1 humoral immune response in all stages of the disease. We then analysed whether the addition of the NY-ESO-1 humoral immune response to CEA and CA19-9 increased the diagnostic frequency of gastric cancer. The combined use of CEA and CA19-9 tests produced positivity rates of 15.3% (27 of 176) in stage I, 24.4% (11 of 45) in stage II, 37.3% (25 of 67) in stage III, and 53.3% (40 of 75) in stage IV gastric cancer patients, resulting in an overall positivity rate of 28.4% (103 of 363). When the NY-ESO-1 humoral immune response was added to these two conventional tumour markers, the positivity rates of all stages increased, resulting in information gains of 14.9% (from 25 to 35 patients; 10 of 67) in stage III and 11.2% (from 65 to 81 patients; 16 of 142) in stage III and IV gastric cancer patients.

Changes in the NY-ESO-1 humoral immune responses of the patients during their clinical courses. Serial serum samples were obtained from 25 gastric cancer patients who displayed positive NY-ESO-1 antibody at admission, and the changes in their NY-ESO-1 humoral immune responses were examined throughout their clinical courses. In all, 6 stage I, 2 stage II, and 8 stage III patients received curative surgical treatment, and 14 did not suffer recurrence. The NY-ESO-1 immune response levels of the patients who did not suffer recurrence decreased after treatment and had fallen below the cutoff level by 9 months after surgery in most cases and did not subsequently increase (Figure 1). The half-lives of their NY-ESO-1 humoral immune response levels were 1.5, 1.6, 2.1, 3.2, and 6.6 months in the stage I patients; 3.0 and 4.0 months in the stage II patients; and 1.6, 1.9, 2.3, 3.0, 3.2, 4.1, and 6.7 months in the stage III patients (mean: 3.0 months). On the other hand, the two patients who underwent curative surgery but subsequently suffered recurrence, M-2 (stage I) and M-11 (stage III), displayed not only incomplete decreases in their NY-ESO-1 humoral immune response levels but also their subsequent restoration to pretreatment levels (Figure 1 and Figure 2A and B). In a comparison between the patients' conventional tumour marker levels and their NY-ESO-1humoral immune response levels, we found that the changes in their CEA and CA19-9 levels were consistent with their NY-ESO-1 immune response levels in patient M-2, whereas patient M-11 was negative for both CEA and CA19-9 throughout their clinical course. Nine stage IV patients who received chemotherapy alone maintained high NY-ESO-1 humoral immune response levels throughout their clinical courses,

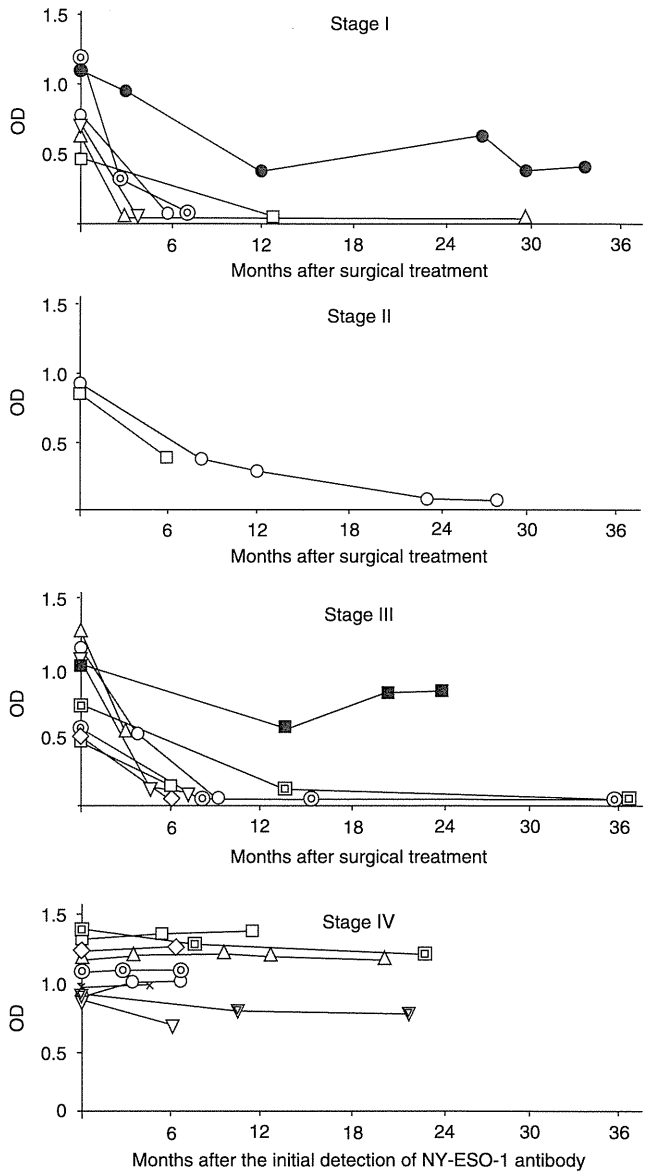


Figure 1. Change in the NY-ESO-1 humoral immune responses of gastric cancer patients after treatment. The serum NY-ESO-1 humoral immune responses of patients with stage I, II, III, or IV gastric cancer in whom NY-ESO-1 antibody production was detected before surgical treatment or chemotherapy were serially analysed. In all, 6 stage I, 2 stage II, and 8 stage III patients received curative surgery, and only 2 patients (●, ■) suffered recurrence. Other 14 patients did not suffer recurrence. Nine patients with stage IV gastric cancer received chemotherapy alone after the initial detection of NY-ESO-1 antibody. Each mark represents a patient. Optical density (OD) values were measured at a serum dilution of 1 : 200.

including some patients who achieved partial tumour responses after chemotherapy (Figure 1).

Prognostic value of the NY-ESO-1 humoral immune response in gastric cancer. The prognostic value of the NY-ESO-1 immune response was evaluated in gastric cancer patients. An analysis of the cumulative overall survival of the gastric cancer patients indicated that there was no difference in the survival rates of the patients who did and did not display positive NY-ESO-1 humoral immune responses (Figure 3A). However, among the patients with higher stage gastric cancer, overall survival was better in the patients in whom NY-ESO-1humoral immune responses were

detected, although the difference was not significant (Figure 3B). NY-ESO-1 protein expression, as detected by IHC, did not affect the overall survival rate (data not shown).

DISCUSSION

NY-ESO-1 antibody was detected in 23.2% of stage III and IV gastric cancer patients, and the combinatorial use of the NY-ESO-1 antibody with CEA and CA19-9 as tumour markers increase the percentage of tumour detection from 45.8 to 57.0%. As the frequency of NY-ESO-1 humoral immune response was relatively low in the patients with early-stage gastric cancer, analysing serum NY-ESO-1 antibody levels alone might not be useful for screening for early-stage gastric cancer. Nevertheless, the expression of NY-ESO-1, a CT antigen, is restricted to tumour tissues and NY-ESO-1 antibody is only detectable in patients with NY-ESO-1-expressing tumours (Stockert *et al*, 1998), indicating the highly specific nature of NY-ESO-1 humoral immune responses in cancer patients. Given that NY-ESO-1 expression by malignant cells is required for antibody induction (Stockert *et al*, 1998), the detection of NY-ESO-1 antibody would be helpful for diagnosing malignancy, although extensive analysis of serum samples from patients with non-cancerous disease, for example, liver or renal disorders, autoimmune diseases, and so on, would be necessary to confirm. In our expression analysis, more NY-ESO-1-positive cases were detected by IHC (19 of 60) than by RT–PCR (6 of 60). This was probably due to the heterogeneous expression of NY-ESO-1 in gastric cancer and the fact that a limited number of biopsy samples were used for the RT–PCR, whereas multiple slices from whole tumour specimens were used for the IHC. Extensive IHC analysis should be used for NY-ESO-1 expression studies of gastric cancer.

We detected a correlation between the NY-ESO-1 humoral immune response levels and the clinical outcome after therapy in gastric cancer patients. The patients who underwent surgery and did not suffer a subsequent relapse displayed consistent decreases in their NY-ESO-1 humoral immune response levels or even the complete disappearance of the NY-ESO-1 antibody from their sera. It is generally accepted that constant immunological stimulation is necessary to maintain a strong humoral immune response (Jager *et al*, 1999). Thus, reduction of antigen doses by the removal of NY-ESO-1-expressing tumour is one possible reason for the observed decreases in these patients' NY-ESO-1 humoral immune response levels after surgery. Patients M-2 and M-11, in whom NY-ESO-1 humoral immune responses remained high for 1 year after surgery and increased thereafter, may have a subclinical residual disease of the so-called 'minimal residual cancer' (Austrup *et al*, 2000; Klein *et al*, 2002) after curative surgery. Local recurrent tumours of 23 and 25 mm in diameter subsequently developed in M-2 and M-11, respectively, suggesting that even a small tumour burden is sufficient to stimulate antibody production. Patient M-2 showed a partial decrease in their NY-ESO-1 humoral immune response levels after the resection of the relapsed tumour, and we are carefully observing the progression of this tumour.

Nine patients with stage IV gastric cancer received chemotherapy alone. Among them, six patients displayed stable disease, two

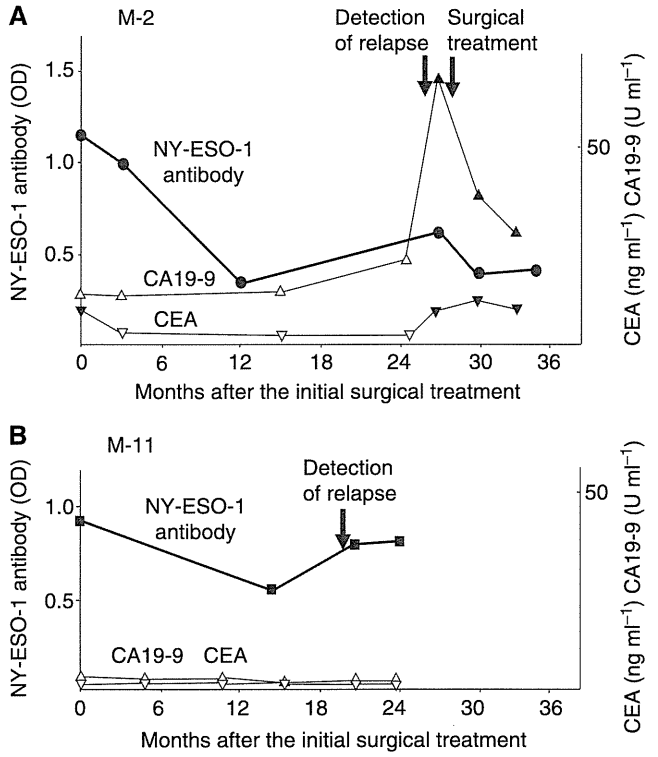


Figure 2. NY-ESO-1 humoral immune response, CEA, and carbohydrate antigen (CA)19-9 levels of patients who relapsed after curative surgery. The NY-ESO-1 humoral immune response (●, ■; Figure 1), CEA (▽), and CA19-9 (△) levels of two patients, M-2 (stage I) (**A**) and M-11 (stage III) (**B**), who underwent curative surgery but subsequently suffered recurrence, were serially analysed. OD values were measured at a serum dilution of 1 : 200. The closed marks indicate CEA or CA19-9 positivity.

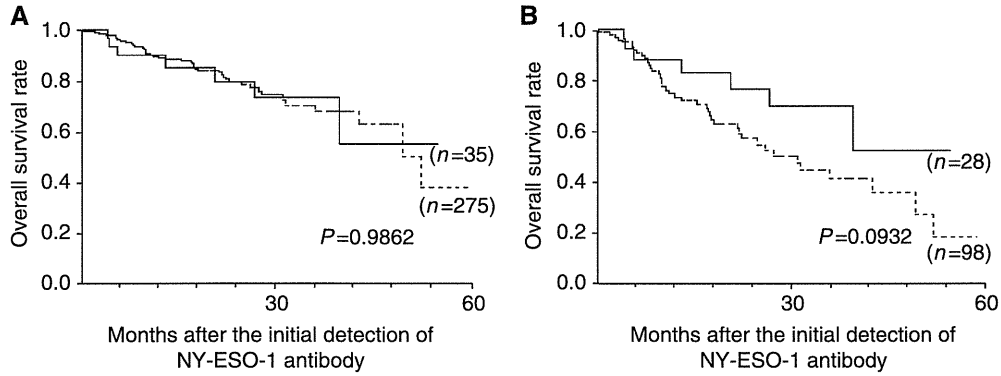


Figure 3. Prognostic role of NY-ESO-1 antibody in gastric cancer patients. The cumulative overall survival rate was analysed in all patients ($n=310$; **A**) and stage III and IV ($n=126$; **B**) gastric cancer patients in whom NY-ESO-1 antibodies were (continuous line) and were not detected (dotted line). The detection of NY-ESO-1 protein by IHC analysis did not affect the overall survival rate (data not shown). Survival curves were plotted using the Kaplan–Meier method. The log-rank test was used for comparisons between groups. *P*-values <0.05 were considered significant.

patients displayed progressive disease, and one patient (M-19) achieved a partial response. Serial analysis of the NY-ESO-1 humoral immune responses of these nine patients including M-19 showed that they barely changed throughout their clinical courses, suggesting that even small tumours are enough to provoke strong NY-ESO-1 humoral immune responses. In this regard, the NY-ESO-1 humoral immune response might not be suitable as a clinical marker for palliative therapy.

We have performed serial cancer vaccine clinical trials with NY-ESO-1 because of its strong immunogenicity and high specificity (Uenaka *et al*, 2007; Wada *et al*, 2008; Kakimi *et al*, 2011). The NY-ESO-1humoral immune response could be a reliable marker of the induction of immune response, as well as for predicting clinical responses in these trials. Furthermore, antibody-based examinations detected both intra- and intermolecular antigen spreading in the sera of patients who had been vaccinated with NY-ESO-1 protein (Kawada *et al*, 2012), suggesting the possible correlation of NY-ESO-1 humoral immne responses and clinical status. In addition, we have started a phase I study of vaccination with NY-ESO-1 protein mixed with Hiltonol (Poly ICLC), Picibanil (OK-432), and Montanide (ISA-51) in patients with NY-ESO-1-expressing cancers (UMIN000007954). Furthermore, NY-ESO-1 vaccine involving modulators of immune checkpoints, for example, anti-CTLA4 antibody and anti-PD-1 antibody, and reagents that are antagonistic to regulatory T cells, for example, anti-CCR4 antibody (Pardoll, 2012) should be considered.

Recently, the antibody against p53, another tumour antigen, has been recognised as a useful tumour marker (Lubin *et al*, 1995). Shimada *et al* (2000)) reported that p53 antibody was detected in 35% of serum samples from patients with *in situ* oesophageal cancer and that it disappeared after endoscopic mucosal resection, proposing that p53 antibody is useful for the early detection and subsequent monitoring of oesophageal cancer. In addition, Müller *et al* (2006) reported that p53 antibody was found in 23.4% of serum samples from cancer patients with 100% accuracy and was correlated with poor prognosis in hepatocellular carcinoma and breast cancer.

Here, we have demonstrated that the NY-ESO-1 humoral immune response could also be valuable as a marker for detecting advanced gastric cancer and inferring whether residual tumour cells remain after treatment, although its frequency in gastric cancer is not very high. We have started a prospective multi-institutional clinical study of NY-ESO-1 humoral immune responses in higher stage gastric cancer patients. In this new study, the NY-ESO-1 humoral immune responses of approximately 100 patients who relapsed after curative surgery will be serially analysed and then followed up. This trial has been registered as UMIN000007925 in Japan.

ACKNOWLEDGEMENTS

We thank Dr Lloyd J Old for his continuous encouragement and Dr K Kakimi for critically reviewing this manuscript.

REFERENCES

Alexander JC, Silverman NA, Chretien PB (1976) Effect of age and cigarette smoking on carcinoembryonic antigen levels. *JAMA* **235**: 1975–1979.

Austrup F, Uciechowski P, Eder C, Böckmann B, Suchy B, Driesel G, Jäckel S, Kusiak I, Grill HJ, Giesing M (2000) Prognostic value of genomic alterations in minimal residual cancer cells purified from the blood of breast cancer patients. *Br J Cancer* **83**: 1664–1673.

Chapman C, Murray A, Chakrabarti J, Thorpe A, Woolston C, Sahin U, Barnes A, Robertson J (2007) Autoantibodies in breast cancer: their use as an aid to early diagnosis. *Ann Oncol* **18**: 868–873.

Ferro MA, Barnes I, Roberts JB, Smith PJ (1987) Tumor markers in prostatic carcinoma. A comparison of prostate-specific antigen with acid phosphatase. *Br J Urol* **60**: 69–73.

Fujiyama S, Morishita T, Sagara K, Sato T, Motohara K, Matsuda I (1986) Clinical evaluation of plasma abnormal prothrombin (PIVKA-II) in patients with hepatocellular carcinoma. *Hepatogastroenterology* **33**: 201–205.

Gati A, Lajmi N, Derouiche A, Marrakchi R, Chebil M, Benammar-Elgaaied A (2011) NY-ESO-1 expression and immunogenicity in prostate cancer patients. *Tunis Med* **89**: 779–783.

Gnjatic S, Nishikawa H, Jungbluth AA, Güre AO, Ritter G, Jäger E, Knuth A, Chen YT, Old LJ (2006) NY-ESO-1: review of an immunogenic tumor antigen. *Adv Cancer Res* **95**: 1–30.

Graziosi L, Bugiantella W, Cavazzoni E, Cantarella F, Porcari M, Baffa N, Donini A (2011) Role of FDG-PET/CT in follow-up of patients treated with resective gastric surgery for tumour. *Ann Ital Chir* **82**: 125–129.

Health and Welfare Statistics Association: Tokyo (2006) Statistics and Information Department, Ministry of Health, Labour, and Welfare Vital Statistics of Japan 2004.

Ikeda Y, Mori M, Adachi Y, Matsushima T, Sugimachi K, Saku M (1993) Carcinoembryonic antigen (CEA) in stage IV gastric cancer as a risk factor for liver metastasis: a univariate and multivariate analysis. *J Surg Oncol* **53**: 235–238.

Isobe M, Eikawa S, Uenaka A, Nakamura Y, Kanda T, Kohno S, Kuzushima K, Nakayama E (2009) Correlation of high and decreased NY-ESO-1 immunity to spontaneous regression and subsequent recurrence in a lung cancer patient. *Cancer Immun* **9**: 8.

Jäger E, Stockert E, Zidianakis Z, Chen YT, Karbach J, Jäger D, Arand M, Ritter G, Old LJ, Knuth A (1999) Humoral immune responses of cancer patients against 'Cancer-Testis' antigen NY-ESO-1: correlation with clinical events. *Int J Cancer* **84**: 506–510.

Kakimi K, Isobe M, Uenaka A, Wada H, Sato E, Doki Y, Nakajima J, Seto Y, Yamatsuji T, Naomoto Y, Shiraishi K, Takigawa N, Kiura K, Tsuji K, Iwatsuki K, Oka M, Pan L, Hoffman EW, Old LJ, Nakayama E (2011) A phase I study of vaccination with NY-ESO-1f peptide mixed with Picibanil OK-432 and Montanide ISA-51 in patients with cancers expressing the NY-ESO-1 antigen. *Int J Cancer* **129**: 2836–2846.

Katanoda K, Yako-Suketomo H (2009) Comparison of time trends in stomach cancer incidence (1973–2002) in Asia, from Cancer Incidence in Five Continents, Vols IV–IX. *Jpn J Clin Oncol* **39**: 71–72.

Kawada J, Wada H, Isobe M, Gnjatic S, Nishikawa H, Jungbluth AA, Okazaki N, Uenaka A, Nakamura Y, Fujiwara S, Mizuno N, Saika T, Ritter E, Yamasaki M, Miyata H, Ritter G, Murphy R, Venhaus R, Pan L, Old LJ, Doki Y, Nakayama E (2012) Heteroclitic serological response in esophageal and prostate cancer patients after NY-ESO-1 protein vaccination. *Int J Cancer* **130**: 584–592.

Klein CA, Blankenstein TJ, Schmidt-Kittler O, Petronio M, Polzer B, Stoecklein NH, Riethmüller G (2002) Genetic heterogeneity of single disseminated tumour cells in minimal residual cancer. *Lancet* **360**: 683–689.

Kodera Y, Yamamura Y, Torii A, Uesaka K, Hirai T, Yasui K, Morimoto T, Kato T, Kito T (1996) The prognostic value of preoperative serum levels of CEA and CA19-9 in patients with gastric cancer. *Am J Gastroenterol* **91**: 49–53.

Lubin R, Schlichtholz B, Teillaud JL, Garay E, Bussel A, Wild CP (1995) P53 antibodies in patients with various types of cancer: assay, identification, and characterization. *Clin Cancer Res* **1**: 1463–1469.

Makino T, Fujiwara Y, Takiguchi S, Miyata H, Yamasaki M, Nakajima K, Nishida T, Mori M, Doki Y (2010) The utility of pre-operative peritoneal lavage examination in serosa-invading gastric cancer patients. *Surgery* **148**: 96–102.

Motoori M, Takemasa I, Doki Y, Saito S, Miyata H, Takiguchi S, Fujiwara Y, Yasuda T, Yano M, Kurokawa Y, Komori T, Yamasaki M, Ueno N, Oba S, Ishii S, Monden M, Kato K (2006) Prediction of peritoneal metastasis in advanced gastric cancer by gene expression profiling of the primary site. *Eur J Cancer* **42**: 1897–1903.

Müller M, Meyer M, Schilling T, Ulsperger E, Lehnert T, Zentgraf H, Stremmel W, Volkmann M, Galle PR (2006) Testing for anti-p53 antibodies increases the diagnostic sensitivity of conventional tumor markers. *Int J Oncol* **29**: 973–980.

Nakada T, Noguchi Y, Satoh S, Ono T, Saika T, Kurashige T, Gnjatic S, Ritter G, Chen YT, Stockert E, Nasu Y, Tsushima T, Kumon H, Old LJ, Nakayama E (2003) NY-ESO-1 mRNA expression and immunogenicity in advanced prostate cancer. *Cancer Immunol* **3**: 10.

Pardoll DM (2012) The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* **12**: 252–264.

Sasako M, Sakuramoto S, Katai H, Kinoshita T, Furukawa H, Yamaguchi T, Nashimoto A, Fujii M, Nakajima T, Ohashi Y (2011) Five-year outcomes of a randomized phase III trial comparing adjuvant chemotherapy with S-1 versus surgery alone in stage II or III gastric cancer. *J Clin Oncol* **29**: 4387–4393.

Seamonds B, Yang N, Anderson K, Whitaker B, Shaw LM, Bollinger JR (1986) Evaluation of prostate-specific antigen and prostatic acid phosphatase as prostate cancer markers. *Urology* **28**: 472–479.

Shimada H, Takeda A, Arima M, Okazumi S, Matsubara H, Nabeya Y, Funami Y, Hayashi H, Gunji Y, Suzuki T, Kobayashi S, Ochiai T (2000) Serum p53 antibody is a useful tumor marker in superficial esophageal squamous cell carcinoma. *Cancer* **89**: 1677–1683.

Stockert E, Jäger E, Chen YT, Scanlan MJ, Gout I, Karbach J, Arand M, Knuth A, Old LJ (1998) A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. *J Exp Med* **187**: 1349–1354.

Takahashi Y, Mai M, Kusama S (1998) Factors influencing growth rate of recurrent stomach cancer as determined by analysis of serum carcinoembryonic antigen. *Cancer* **75**: 1497–1502.

Takahashi Y, Takeuchi T, Sakamoto J, Touge T, Mai M, Ohkura H, Kodaira S, Okajima K, Nakazato H (2003) The usefulness of CEA and/or CA19-9 in monitoring for recurrence in gastric cancer patients: a prospective clinical study. *Gastric Cancer* **6**: 142–145.

Türeci O, Mack U, Luxemburger U, Heinen H, Krummenauer F, Sester M, Sester U, Sybrecht GW, Sahin U (2006) Humoral immune responses of lung cancer patients against tumor antigen NY-ESO-1. *Cancer Lett* **236**: 64–71.

Uenaka A, Wada H, Isobe M, Saika T, Tsuji K, Sato E, Sato S, Noguchi Y, Kawabata R, Yasuda T, Doki Y, Kumon H, Iwatsuki K, Shiku H, Monden M, Jungbluth AA, Ritter G, Murphy R, Hoffman E, Old LJ, Nakayama E (2007) T cell immunomonitoring and tumor responses in patients immunized with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein. *Cancer Immun* **7**: 9.

Wada H, Sato E, Uenaka A, Isobe M, Kawabata R, Nakamura Y, Iwae S, Yonezawa K, Yamasaki M, Miyata H, Doki Y, Shiku H, Jungbluth AA, Ritter G, Murphy R, Hoffman EW, Old LJ, Monden M, Nakayama E (2008) Analysis of peripheral and local anti-tumor immune response in esophageal cancer patients after NY-ESO-1 protein vaccination. *Int J Cancer* **123**: 2362–2369.

Yamamoto M, Baba H, Kakeji Y, Endo K, Ikeda Y, Toh Y, Kohnoe S, Okamura T, Maehara Y (2004) Prognostic significance of tumor markers in peritoneal lavage in advanced gastric cancer. *Oncology* **67**: 19–26.

Yuan J, Adamow M, Ginsberg BA, Rasalan TS, Ritter E, Gallardo HF, Xu Y, Pogoriler E, Terzulli SL, Kuk D, Panageas KS, Ritter G, Sznol M, Halaban R, Jungbluth AA, Allison JP, Old LJ, Wolchok JD, Gnjatic S (2011) Integrated NY-ESO-1 antibody and CD8+ T-cell responses correlate with clinical benefit in advanced melanoma patients treated with ipilimumab. *Proc Natl Acad Sci USA* **108**: 16723–16728.