References

1. Nakatsura T, Yoshitake Y, Senju S, Monji M, Komori H, Motomura Y, et al. Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun 2003; 306:16-25; PMID:12788060; http://dx.doi.org/10.1016/S0006-291X(03)00908-2.
2. Nakatsura T, Komori H, Kubo T, Yoshitake Y, Senju S, Katagiri T, et al. Mouse homologue of a novel human oncofetal antigen, glypican-3, evokes T-cell-mediated tumor rejection without autoimmune reactions in mice. Clin Cancer Res 2004; 10:8630-40; PMID:15623647; http://dx.doi.org/10.1158/1078-0432.CCR-04-1177.
3. Shirakawa H, Kuronuma T, Nishimura Y, Hasebe T, Nakano M, Gotohda N, et al. Glypican-3 is a useful diagnostic marker for a component of hepatocellular carcinoma in human liver cancer. Int J Oncol 2009; 34:649-56; PMID:19212669.
4. Shirakawa H, Suzuki H, Shimomura M, Kojima M, Gotohda N, Takahashi S, et al. Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma. Cancer Sci 2009; 100:1403-7; PMID:19496787; http://dx.doi.org/10.1111/j.1349-7006.2009.01206.x.
5. Komori H, Nakatsura T, Senju S, Yoshitake Y, Motomura Y, Ikuta Y, et al. Identification of HLA-A2- or HLA-A24-restricted CTL epitopes possibly useful for glypican-3-specific immunotherapy of hepatocellular carcinoma. Clin Cancer Res 2006; 12:2689-97; PMID:16675560; http://dx.doi.org/10.1158/1078-0432.CCR-05-2267.
6. Motomura Y, Ikuta Y, Kuronuma T, Komori H, Ito M, Tsuchihara M, et al. HLA-A2 and -A24-restricted glypican-3-derived peptide vaccine induces specific CTLs: preclinical study using mice. Int J Oncol 2008; 32:985-90; PMID:18425324.
7. Sawada Y, Yoshikawa T, Nobuoka D, Shirakawa H, Kuronuma T, Motomura Y, et al. Phase I trial of glypican-3-derived peptide vaccine for advanced hepatocellular carcinoma: immunological evidence and potential for improving overall survival. Clin Cancer Res 2012; •••: http://dx.doi.org/10.1158/1078-0432.CCR-11-3044.
8. Yoshikawa T, Nakatsugawa M, Suzuki S, Shirakawa H, Nobuoka D, Sakemura N, et al. HLA-A2-restricted glypican-3 peptide-specific CTL clones induced by peptide vaccine show high avidity and antigen-specific killing activity against tumor cells. Cancer Sci 2011; 102:918-25; PMID:21281401; http://dx.doi.org/10.1111/j.1349-7006.2011.01896.x.
9. Suzuki S, Yoshikawa T, Hirosawa T, Shibata K, Kikkawa F, Akatsuka Y, et al. Glypican-3 could be an effective target for immunotherapy combined with chemotherapy against ovarian clear cell carcinoma. Cancer Sci 2011; 102:1622-9; PMID:21668581; http://dx.doi.org/10.1111/j.1349-7006.2011.02003.x.
10. Rosenberg SA, Dudley ME. Adoptive cell therapy for the treatment of patients with metastatic melanoma. Curr Opin Immunol 2009; 21:233-40; PMID:19304471; http://dx.doi.org/10.1016/j.coi.2009.03.002.

Identification of an H2-K^b or H2-D^b restricted and glypican-3-derived cytotoxic T-lymphocyte epitope peptide

TATSUAKI IWAMA[1,2], KAZUTAKA HORIE[1], TOSHIAKI YOSHIKAWA[1], DAISUKE NOBUOKA[1], MANAMI SHIMOMURA[1], YU SAWADA[1] and TETSUYA NAKATSURA[1,2]

[1]Division of Cancer Immunotherapy, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa, Chiba 277-8577; [2]Research Institute for Biomedical Sciences, Tokyo University of Science, Japan

Received November 15, 2012; Accepted December 28, 2012

DOI: 10.3892/ijo.2013.1793

Abstract. Glypican-3 (GPC3) is overexpressed in human hepatocellular carcinoma (HCC) but not expressed in normal tissues except for placenta and fetal liver and therefore is an ideal target for cancer immunotherapy. In this study, we identified an H2-K^b or H2-D^b restricted and murine GPC3 (mGPC3)-derived cytotoxic T-lymphocyte (CTL) epitope peptide in C57BL/6 (B6) mice, which can be used in the design of preclinical studies of various therapies with GPC3-target immunotherapy *in vivo*. First, 11 types of 9- to 10-mer peptides predicted to bind with H2-K^b or H2-D^b were selected from the mGPC3 amino acid sequence based on the binding score as calculated by the BIMAS software. We evaluated the peptide-binding affinity and confirmed that all peptides were able to bind to H2-K^b or H2-D^b by *in vitro* cellular binding assay. Subsequently, a mixed peptide vaccine and single peptide vaccine were given to B6 mice to evaluate immunogenic potential of the 11 selected peptides. Using the splenocytes from peptide-vaccinated mice, interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assays showed that mGPC3-1$_{127\text{-}136}$ (AMFKNNYPSL) peptide was the most efficient for inducing CTLs among the 11 peptides. Next, we demonstrated that the mGPC3-1 peptide-specific CTL line could recognize mGPC3-expressing cancer cells, suggesting that mGPC3-1 peptide was an endogenously presented peptide. In conclusion, we identified mGPC3-1 as an H2-K^b or H2-D^b restricted, mGPC3-derived CTL epitope peptide.

Introduction

Liver cancer ranks fifth in frequency in the world and is the third most common cause of lethal cancer (1). Liver cancer consists of hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC), with HCC as the most common. Regarding HCC therapy, hepatectomy, percutaneous local therapy and transcatheter arterial embolization (TAE) are common, but the recurrence rate with conventional therapies for advanced HCC patients is still high (2). Therefore, developing a novel curative therapy or an effective adjuvant therapy for HCC is important.

Recently, immunotherapy, which consists of a peptide vaccine, protein vaccine, or DNA vaccine, has become a potentially promising option for HCC (3,4). Many tumor antigen-derived peptides recognized by cytotoxic T-lymphocyte (CTL) have been identified (5). However, to date, vaccine therapy using these peptides has not proven adequate antitumor efficacy in clinical trials for advanced HCC patients (6-8).

In HCC, glypican-3 (GPC3) is overexpressed and is not expressed in normal tissues except for the placenta and embryonic liver (9). Hence, GPC3 is a novel target molecule in HCC patients. GPC3 is a member of the heparan sulfate proteoglycan family and the glypican family regulates cell growth and division through Wnt signaling, Hedgehogs, fibroblast growth factors and bone morphogenetic proteins (10-12). We previously identified HLA-A*24:02-restricted GPC3$_{298\text{-}306}$ (EYILSLEEL) and HLA-A*02:01-restricted GPC3$_{144\text{-}152}$ (FVGEFFTDV) peptides and showed that both peptides can induce GPC3-specific CTLs without an autoimmune response (13,14). Clinical trials of a GPC3-derived peptide vaccine for HCC patients are currently in progress. The phase I clinical trial of a GPC3-derived peptide vaccine for advanced HCC showed safety as well as immunological evidence and potential for improving overall survival (15-17). The phase I clinical trial suggested that the GPC3-derived peptide vaccine could be an attractive approach for treatment of HCC, however, the effect of tumor reduction was limited. Therefore, further studies are needed to enhance the effect of GPC3-targeted immunotherapy and to establish a GPC3-specific CTL-inducible mouse model. We previously conducted a preclinical study of the GPC3-derived peptide vaccine using HLA-A2.1 transgenic mice (18). The treatment model experiment using HLA transgenic mice is limited.

Mice with the C57BL/6 (B6) background have been reported to spontaneously develop liver cancer (19,20). Recently, the NASH mouse model (named STAM mice C57BL/6N-NASH), which had a B6 background and spontaneously developed liver

Correspondence to: Dr Tetsuya Nakatsura, Division of Cancer Immunotherapy, Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan
E-mail: tnakatsu@east.ncc.go.jp

Key words: glypican-3, cytotoxic T-lymphocyte epitope, hepatocellular carcinoma, mouse

cancer, was exploited by Stelic Institute & Co. In this mouse model, the cancer incidence rate is high and cancer incident time is short, thus, STAM mice C57BL/6N-NASH is an attractive model for studying GPC3-targeted therapy for HCC. Therefore, identification of a mouse major histocompatibility complex (MHC) class I epitope peptide to induce GPC3-specific CTL was needed for establishment of the appropriate mouse model.

Strategies to identify epitope peptides have previously been reported (21-24). A summary of our strategy follows. First, peptides binding MHC class I epitope were predicted from antigen amino acid sequences *in silico* by prediction software and the ability of the predicted peptides to bind MHC class I was confirmed *in vitro* by a binding assay. Then, the immunogenic potential of the predicted peptides was examined by *in vivo* immunization or *in vitro* stimulation. Lastly, whether peptides that have immunogenic potential are presented by cells endogenously expressing the antigen was confirmed. In summary, we identified peptides with immunogenic potential that were presented by cells endogenously expressing the antigen. We attempted to identify H2-K^b or H2-D^b restricted, GPC3-derived CTL epitope peptides in C57BL/6 mice based on the above strategy.

Materials and methods

Mice. C57BL/6 (B6) mice were purchased from Charles River Laboratories Japan, Inc. and STAM mice C57BL/6N-NASH were a gift from this company. Mice were maintained under the institutional guidelines set by the Animal Research Committee of the National Cancer Center Hospital East. Mice were housed in specific pathogen-free (SPF) conditions with a 12-h light cycle and food and water at *ad libitum*. Six to eight-week-old female B6 mice were used in all experiments and STAM mice C57BL/6N-NASH were provided with a very high-fat rodent diet (rodent diet with 60% kcal% fat, Research Diet Inc.). All animal procedures were performed according to the guidelines for Animal Research Committee of the National Cancer Center, Japan.

Cell lines and transfection. B6 thymoma RMA and RMA-S cell lines, which have H2-K^b and -D^b as MHC class I epitopes, were maintained in our laboratory. RMA-S is an antigen processing-defective cell line and the cells cannot present endogenous antigens with MHC class I epitopes (25). To obtain RMA transiently expressing murine GPC3 (RMA-GPC3-puro), RMA (GPC3-negative) was transfected with pCAGGS-mGPC3-internal ribosomal entry site (IRES)-puromycin-resistant (puro-R) using Lipofectamine 2000 reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's protocols. As negative control, RMA, which was transfected with pCAGGS-IRES-puro-R in a similar way, was named RMA-puro. Expression of murine GPC3 (mGPC3) in RMA-GPC3-puro or RMA-puro was confirmed by reverse transcription polymerase chain reaction (RT-PCR). All cells were cultured in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco).

RT-PCR. Total ribonucleic acid was isolated from RMA-GPC3-puro or RMA-puro homogenized with the TRIzol Reagent (Life Technologies, Inc., Rockville, MD, USA) according to the manufacturer's protocols. The first-strand complementary deoxyribonucleic acid (cDNA) was synthesized with a PrimeScript® II 1st strand cDNA Synthesis kit (Takara Bio Inc., Japan), then mGPC3 was amplified using a Takara PCR Amplification kit (Takara Bio Inc.). The amplification protocol was as follows: 150 sec at 94˚C for initial denaturation, 35 amplification cycles at 58˚C for 40 sec and 72˚C for 40 sec, followed by a final extension at 72˚C for 5 min. The primer sequences for mGPC3 were as follows: sense, 5'-ACGGGATGGTGAAA GTGAAGA-3' and antisense, 5'-GAAAGAGAAAAGAGGGA AACA-3'. The primer sequences for β-actin were as follows: sense, 5'-GAGCAATGATCTTGATCTTCAT-3' and antisense, 5'-TCCATCATGAACTGTGACGT-3'. PCR products were visualized by ethidium bromide staining after separation on a 1% agarose gel. After normalization using β-actin messenger ribonucleic acid (mRNA) as a control, we compared the expression of mGPC3 mRNA.

Generation of bone marrow-derived dendritic cells (BM-DCs) from BM cells. BM cells (4x10^6) from B6 mice were cultured in RPMI-1640 containing FBS (10%), 2-mercaptoethanol (2-ME, 50 μM) and murine granulocyte macrophage colony-stimulating factor (mGM-CSF, 20 ng/ml) for 1 week.

Peptides. Eleven types of 9- to 10-mer peptides predicted to bind with H2-K^b or H2-D^b were selected from mGPC3 amino acid sequences (accession code AAH36126) based on the binding score as calculated by BIMAS software (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD, USA) and 11 synthetic peptides (custom ordered) were purchased from Scrum Inc. (Tables I and II). The 11 amino acid sequences were as follows: mGPC3-1, AMFKNNYPSL; mGPC3-2, SLFPVIYTQM; mGPC3-3, LFPVIYTQM; mGPC3-4, KSFINFYSAL; mGPC3-5, LTARLNMEQL; mGPC3-6, LGSDINVDDM; mGPC3-7, QYVQKNGGKL; mGPC3-8, YVQKNGGKL; mGPC3-9, DTLCWNGQEL; mGPC3-10, RNGMKNQFNL; mGPC3-11, MKNQFNLHEL. Each peptide was dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries, Japan) and each peptide's density was 10 mg/ml.

H2-K^b or H2-D^b binding assay. To evaluate the binding affinity of the predicted peptides to H2-K^b or H2-D^b molecules, an *in vitro* cellular binding assay was performed as previously reported (23,26). Briefly, after incubation of RMA-S cells in culture medium at 26˚C overnight, cells (1x10^6) were washed with PBS and suspended in 100 μl Opti-MEM® (Invitrogen) with or without 10 μg peptide, followed by incubation at 26˚C for 3 h and then at 37˚C for 3 h. After washing with PBS, H2-K^b or H2-D^b expression was measured with a BD FACSCanto™ II flow cytometer (BD) using FITC-conjugated H2-K^b (BioLegend Inc., AF6-88.5) or H2-D^b (BioLegend Inc., KH95) specific monoclonal antibody and mean fluorescence intensity (MFI) was recorded. Percent MFI increase was calculated as follows: percent MFI increase = (MFI with the given peptide - MFI without peptide)/(MFI without peptide) x 100.

Vaccination. The mixed peptide vaccine per mouse consisted of 5 μl mGPC3-1 to mGPC3-11 solution, 55 μl sodium bicar-

Table I. Synthetic peptides predicted to bind with H2-K^b.

	Peptide sequence (position)	Binding score[a]
mGPC3-1	AMFKNNYPSL (127-136)	52.8
mGPC3-2	SLFPVIYTQM (172-181)	44
mGPC3-3	LFPVIYTQM (173-181)	66
mGPC3-4	KSFINFYSAL (395-404)	40

[a]Binding scores were estimated by using BIMAS software (http://www-bimas.cit.nih.gov/molbio/hla_bind/).

Table II. Synthetic peptides predicted to bind with H2-D^b.

	Peptide sequence (position)	Binding score[a]
mGPC3-5	LTARLNMEQL (82-91)	200
mGPC3-1	AMFKNNYPSL (127-136)	343.2
mGPC3-6	LGSDINVDDM (156-165)	260
mGPC3-7	QYVQKNGGKL (331-340)	720
mGPC3-8	YVQKNGGKL (332-340)	240
mGPC3-9	DTLCWNGQEL (418-127)	600
mGPC3-10	RNGMKNQFNL (437-446)	200
mGPC3-11	MKNQFNLHEL (440-449)	288

[a]Binding scores were estimated by using BIMAS software (http://www-bimas.cit.nih.gov/molbio/hla_bind/).

bonate solution and 110 μl incomplete Freund's adjuvant (IFA). Single peptide vaccine per mouse consisted of 5 μl peptide, 45 μl sodium bicarbonate solution and 50 μl IFA. Each vaccine solution was emulsified. The mice were immunized by intradermal injection at the base of the tail every 7 days for a total of two vaccinations. Similarly, STAM mice C57BL/6N-NASH were immunized seven times with the mGPC3-1 peptide vaccine.

Restimulation of splenocytes obtained from immunized mice. Seven days after the last immunization, splenocytes were collected and cluster of differentiation 8 (CD8) positive splenocytes were isolated by positive selection with anti-CD8 microbeads (Miltenyi Biotec) according to the manufacturer's protocol. CD8-positive splenocytes were cocultured with BM-DCs pulsed with each peptide as previously described (13). Seven days after coculture, the detection of antigen-specific T cells producing interferon (IFN)-γ was performed using the BD ELISPOT kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer's protocols.

Establishment of GPC3-1-specific CTL line. The GPC3-1-specific CTL line was established as previously described (27). Splenocytes (1x10^4) derived from B6 mice immunized with the GPC3-1 peptide vaccine were cocultured with B6-derived and irradiated (35 Gy) splenocytes (5x10^4) in RPMI-1640 contained with FBS (10%), sodium pyruvate (1 mM, Gibco), MEM non-essential amino acid solution (1X, Gibco) and 2-ME (50 μM). Seven days later, recombinant interleukin-2 (rIL-2, 50 U/ml, Nipro, Osaka, Japan) was added to the culture medium.

IFN-γ enzyme-linked immunospot (ELISPOT) analysis. IFN-γ ELISPOT assay was performed according to the manufacturer's protocols. Briefly, restimulated CD8-positive splenocytes (5x10^4) as target cells were added to the plate and then BM-DCs (5x10^4) pulsed with each peptide (10 μg/ml) as effector cells or non-pulsed BM-DCs (5x10^4) as control and target cells were added to the plate, which was then incubated for 20 h at 37˚C, 5% CO_2. Using the GPC3-1-reactive CTL line (1x10^5) as effector cells, RMA-S (5x10^4) pulsed with each peptide (10 μg/ml) as target cells and non-pulsed RMA-S as control and target cells (5x10^4), the plate was incubated for 20 h at 37˚C, 5% CO_2. Using the mGPC3-1-reactive CTL line (1x10^5) as effector cells, RMA-GPC3-puro as target cells (5x10^5) and RMA-puro (5x10^5) as control and target cells, the plate was incubated for 48 h at 37˚C, 5% CO_2. The number of spots was automatically counted using the Eliphoto system (Minerva Tech, Tokyo, Japan).

Cytotoxicity assay. Cytotoxic activity against target cells was analyzed using the Terascan VPC system (Minerva Tech) as previously described (28). Target cells were incubated with calcein AM (Dojindo, Kumamoto, Japan) solution for 30 min at 37˚C and labeled. Then the labeled cells were incubated with effector cells for 4 h. Fluorescence intensity was measured before and after the culture and specific cytotoxic activity was evaluated using the following formula: % cytotoxicity = {1- [(average fluorescence of the sample wells - average fluorescence of the maximal release control wells) - (average fluorescence of the minimal release control wells - average fluorescence of the maximal release control wells)]} x 100%.

Statistical analysis. Statistical analyses were performed with a Mann-Whitney U test (n=3). Significant differences were defined as $^*p<0.05$ or $R^2>0.5$.

Results

Evaluation of selected peptide-binding affinity to H2-K^b or H2-D^b. The selected 11 peptides derived from mGPC3 by the BIMAS software were evaluated by an *in vitro* binding assay to determine each peptide's binding affinity to H2-K^b or H2-D^b. The peptide with the highest binding affinity for H2-K^b was mGPC3-2 (percent MFI, 376.6%), followed by the mGPC3-3 peptide (128.0%) and the mGPC3-1 peptide (72.7%) (Fig. 1A). That for H2-D^b was mGPC3-10 peptide (539.1%) followed by the mGPC3-1 peptide (298.2%) and the mGPC3-8 peptide (191.1%) (Fig. 1B). These results show that all 11 peptides could bind H2-K^b or H2-D^b, although the binding score calculated by the BIMAS software did not correlate with the actual binding affinity (Fig. 1C and D).

Induction of CTL response against mGPC3-derived peptides in B6 mice. The vaccine schedule was performed as follows (Fig. 2A): At days 0 and 7, peptide vaccine was given. At day 14, primed mice were sacrificed and CD8-positive

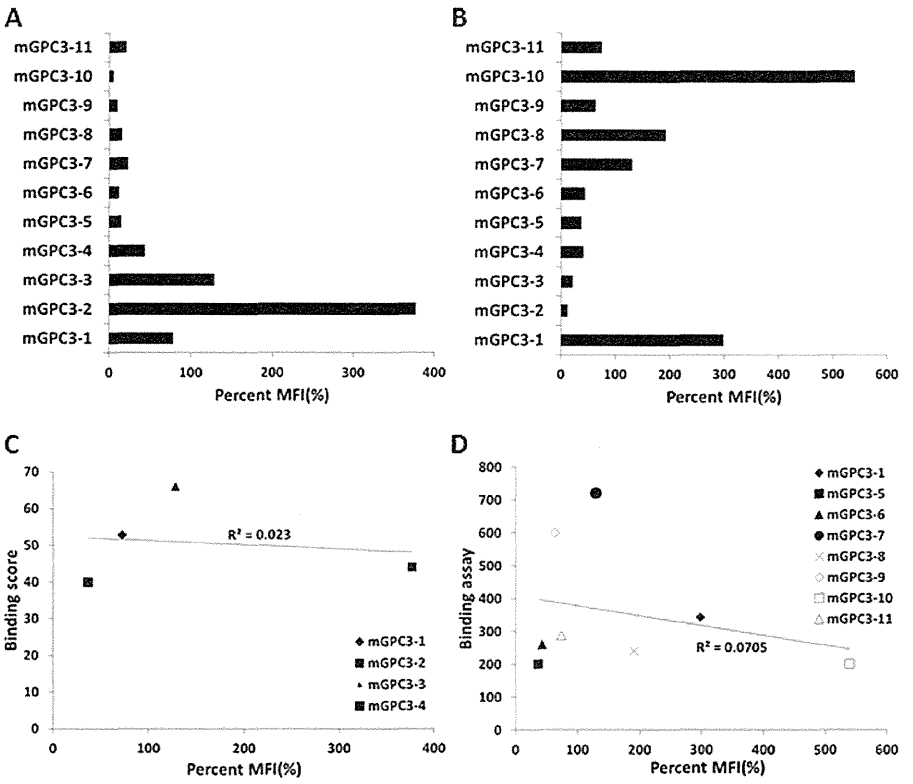

Figure 1. *In vitro* cellular peptide binding assays to H2-K^b (A) or H2-D^b (B) were performed using a FACS system. Comparison of BIMAS binding score with percent MFI for H2-K^b (C) or H2-D^b (D). Percent MFI increase = (MFI with the given peptide - MFI without peptide)/(MFI without peptide) x 100.

splenocytes were collected. CD8-positive splenocytes were restimulated with BM-DCs pulsed with each peptide. At day 21, the peptide's immunogenic potential was evaluated by IFN-γ ELISPOT assay.

The mixed peptide vaccination was performed to evaluate immunogenic potential of the 11 peptides and IFN-γ ELISPOT assays were performed using BM-DCs pulsed with each peptide and non-pulsed BM-DCs as target cells. The CD8-positive splenocytes from mice primed with the mixed vaccine released more IFN-γ to BM-DCs pulsed with mGPC3-1 peptide (average number of spots, 44.3±15.3) and mGPC3-4 peptide (average number of spots, 7.6±3.2) than to non-pulsed BM-DCs (average number of spots, 0.3±0.5). These results suggest that the mGPC3-1 and mGPC3-4 peptides had immunogenic potential and were able to induce peptide-specific CTLs in B6 mice primed by the mixed vaccine system (Fig. 2B and C).

Next, to confirm whether the peptides are CTL-inducible peptides, a single peptide vaccine was given and IFN-γ ELISPOT assays were performed using BM-DCs pulsed with either peptide and non-pulsed BM-DCs as target cells. The CD8-positive cells from mice immunized with mGPC3-1 peptide released more IFN-γ to BM-DCs pulsed with mGPC3-1 peptide (average number of spots, 101.0±33.2) than to non-pulsed BM-DCs (average number of spots, 2.1±3.7) (Fig. 2D and E). The CD8-positive cells from mice immunized with mGPC3-4 peptide released more IFN-γ to BM-DCs pulsed with mGPC3-4 peptide (average number of spots, 5.3±4.0) than to non-pulsed BM-DCs (average number of spots, 1.8±0.7), but no significant differences were observed (Fig. 2F and G). These results suggest that mGPC3-1 peptide is more efficient for inducing CTLs than the mGPC3-4 peptide in a single peptide vaccine system.

Taken together, the above results suggest that mGPC3-1 peptide is the most efficient peptide for inducing CTLs among the 11 peptides.

mGPC3-1 peptide-specific CTL line recognition of target cells endogenously expressing mGPC3. To further investigate the ability of mGPC3-1 peptide-specific CTLs induced by peptide vaccination, we established a CTL line from immunized mice according to the above described protocol. IFN-γ ELISPOT assays were performed using RMA-S pulsed with mGPC3-1 peptide and non-pulsed RMA-S to confirm whether the CTL line had mGPC3-1 peptide specificity. The CTL line clearly released more IFN-γ to RMA-S pulsed with mGPC3-1 peptide than to non-pulsed RMA-S, which suggests that the CTL line is the mGPC3-1 peptide-specific CTL (Fig. 3A).

Subsequently, a cytotoxicity assay was performed to confirm whether the mGPC3-1-specific CTLs could kill RMA-S pulsed with mGPC3-1 peptide. The CTLs killed RMA-S pulsed with the mGPC3-1 peptide (16.4%) better than non-pulsed RMA-S (2.2%), suggesting that the mGPC3-1-specific CTL line could specifically recognize and kill RMA-S pulsed with the mGPC3-1 peptide (Fig. 3B).

Finally, we examined whether the CTL line could recognize RMA GPC3-puro endogenously expressing mGPC3. Expression of mGPC3 in RMA-GPC3-puro and RMA-puro

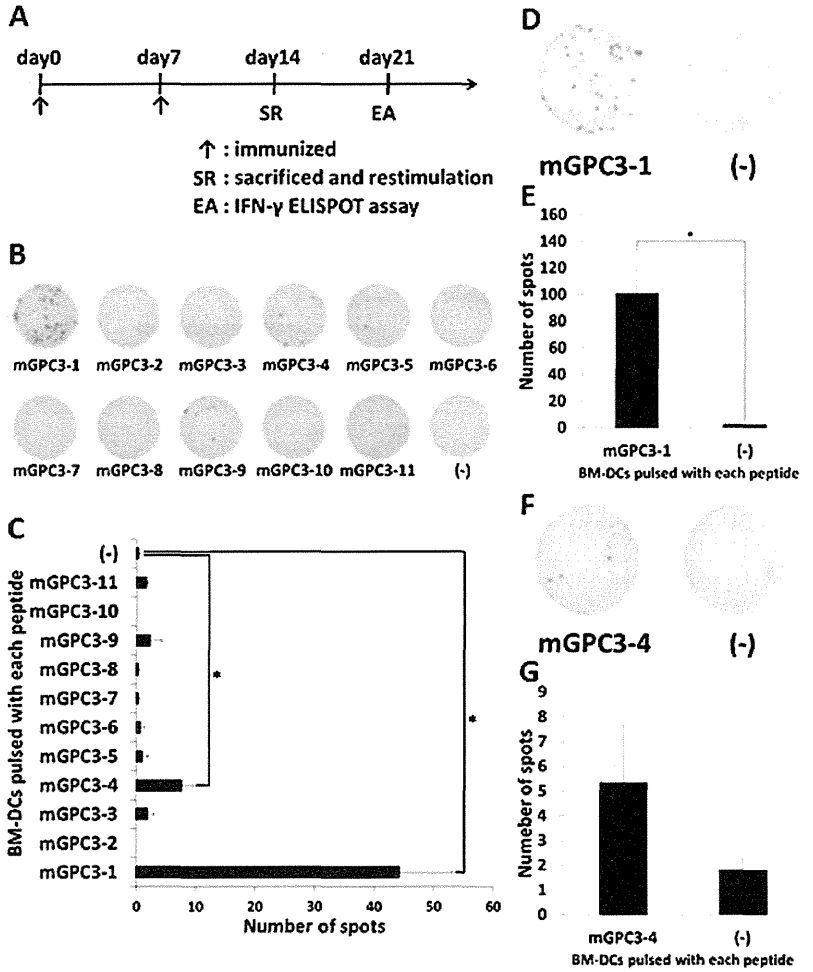

Figure 2. *In vivo* mixed peptide vaccine and single peptide vaccine. Analysis was performed for each vaccine. (A) Schedule of mixed peptide vaccine and single peptide vaccine. (B and C) The mixed peptide vaccine was given to mice and the responses of CD8-positive cells to the 11 peptides were examined. IFN-γ ELISPOT assays were performed using BM-DCs pulsed with each peptide and non-pulsed BM-DCs as target cells (n=3, $^{*}p<0.05$). Representative data are shown (B). To confirm whether mGPC3-1 or mGPC3-4 was a CTL-inducible peptide, the single peptide vaccine was given. (D and E) mGPC3-1 peptide vaccine was given and IFN-γ ELISPOT assays were performed using BM-DCs pulsed with mGPC3-1 and non-pulsed BM-DCs as target cells (n=3, $^{*}p<0.05$). Representative data are shown D). (F and G) mGPC3-4 peptide vaccine was given and IFN-γ ELISPOT assays were performed using BM-DCs pulsed with mGPC3-4 and non-pulsed BM-DCs as target cells (n=3). Representative data are shown (F).

was confirmed by RT-PCR. The results showed that RMA-GPC3-puro expressed mGPC3 and RMA-puro did not express mGPC3 (Fig. 3C). IFN-γ ELISPOT assays were performed using RMA-GPC3-puro and RMA-puro as target cells to investigate whether the CTL line could recognize RMA-GPC3-puro expressing endogenous mGPC3. The CTL line released more IFN-γ to RMA-GPC3-puro (average number of spots, 32.2±5.0) than to RMA-puro (average number of spots, 18.2±6.2). This result suggests that the mGPC3-1 peptide is an endogenously presented peptide (Fig. 3D).

CTL response against the mGPC3-derived peptides induced in STAM mice. Previously, the NASH mouse model (named STAM mice C57BL/6N-NASH) was exploited by Stelic Institute & Co. and STAM mice with a B6 background spontaneously developed liver cancer. We observed that liver cancer developed in 18-week-old STAM mice (Fig. 4A and B). Furthermore, to verify whether mGPC3-1 peptide-specific CTLs were induced in STAM mice C57BL/6N-NASH, a mGPC3-1 peptide vaccine was given and an IFN-γ ELISPOT assay was performed using RMA-S pulsed with mGPC3-1 peptide or non-pulsed RMA-S. The CD8-positive cells derived from immunized mice released IFN-γ only to pulsed RMA-S (average number of spots, 100±74.3), not to non-pulsed RMA-S (average number of spots, 0.0±0.0) (Fig. 4C and E). However, the CD8-positive cells derived from unimmunized mice did not release IFN-γ to either pulsed (average number of spots, 0±0.0 or non-pulsed (average number of spots, 0.0±0.0) RMA-S (Fig. 4D and E). These results suggest that peptide-specific mGPC3-1 could be induced in STAM mice C57BL/6N-NASH immunized with the mGPC3-1 peptide vaccine but could not be induced in un-immunized STAM mice C57BL/6N-NASH.

Discussion

HCC is the most common liver cancer and the recurrence rate for treated HCC patients is high, thus establishment of an effective preventative method, such as a vaccination to prevent

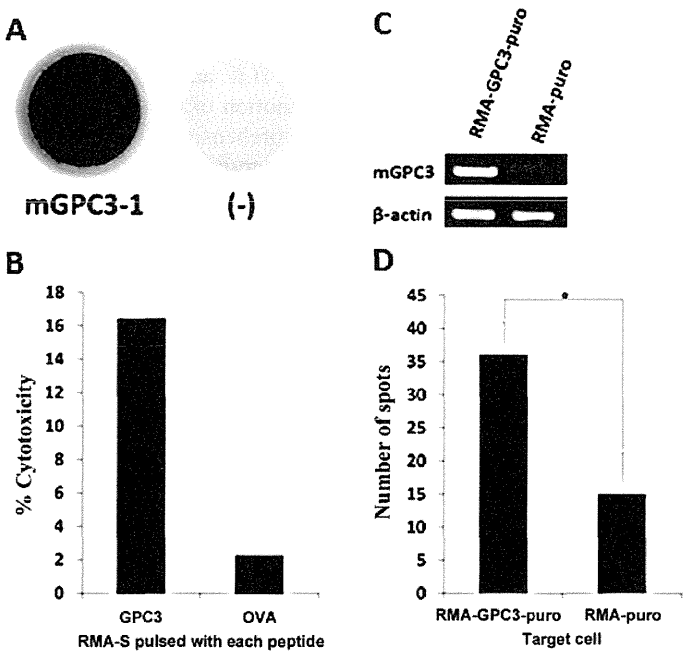

Figure 3. Analysis of established CTL line. (A) IFN-γ ELISPOT assays were performed using GPC3-1 pulsed or non-pulsed RMA-S as target cells. (B) Cytotoxicity assays were performed using GPC3-1 pulsed or unpulsed RMA-S as target cells. Percent cytotoxicity = {1- [(average fluorescence of the sample wells - average fluorescence of the maximal release control wells) - (average fluorescence of the minimal release control wells - average fluorescence of the maximal release control wells)]} x 100%. (C) mGPC3 expression of RMA-GPC3-puro and RMA-GPC3 by RT-PCR. (D) IFN-γ ELISPOT assays were performed using RMA-GPC3-puro and RMA-puro as target cells (n=3, $^*p>0.05$).

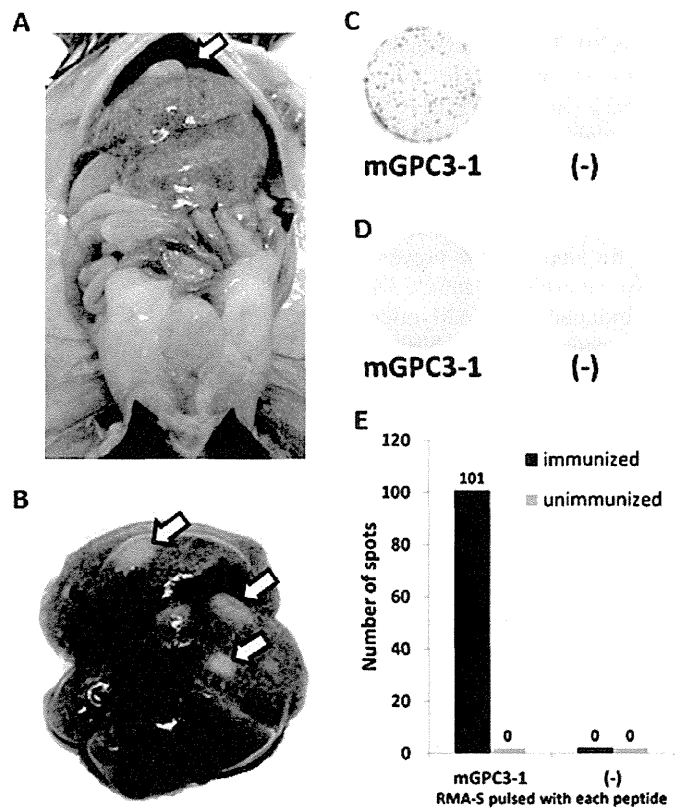

Figure 4. Analysis of STAM mice C57BL/6N-NASH. (A and B) Liver cancer was observed in 18-week-old STAM mice C57BL/6N-NASH. (C-E) To confirm whether mGPC3-1 peptide-specific CTL was induced, the mGPC3-1 peptide vaccine was given to STAM mice C57BL/6N-NASH and the IFN-γ ELISPOT assay was performed. Arrow indicates the area of developing cancer (C and E). The CD8-positive cells derived from immunized mice released IFN-γ to RMA-S pulsed with mGPC3-1 peptide (n=3). Representative data are shown (C). (D and E) As a control, IFN-γ ELISPOT assays were performed using the CD8-positive cells derived from unprimed mice. Representative data are shown (D).

the occurrence and recurrence of HCC, is needed. GPC3 is overexpressed in HCC and is not expressed in normal tissue except for the placenta and embryonic liver. Clinical trials of a GPC3-derived peptide vaccine for HCC have been performed and a phase I clinical trial has shown the safety and immunological and clinical potential of the vaccine (15,16). Moreover, to study the preventive effect as a potential of the GPC3-derived peptide vaccine, we attempted to establish a mouse model to induce GPC3-specific CTLs by the peptide vaccine.

First, mGPC3-derived peptides binding to H2-K^b or H2-D^b were determined *in silico* using BIMAS software. Moreover, a binding assay was performed *in vitro* and showed that all peptides predicted by the BIMAS software could bind H2-K^b and H2-D^b. However, the BIMAS score did not correlate with the actual binding affinity.

Peptides that can bind to MHC class I are not always able to induce peptide-specific CTLs (21,29). Therefore, to investigate actual CTL-inducible peptides among the 11 selected peptides, a mixed peptide vaccine and single peptide vaccine were given to mice. These results (Fig. 2) suggested that mGPC3-1 could induce peptide-specific CTLs. In addition, antigen-derived and CTL-inducible peptides are not necessarily presented by cancer cells endogenously expressing the antigen (23,30). Hence, we confirmed whether the mGPC3-1 peptide-specific CTL line could recognize RMA-GPC3-puro endogenously expressing mGPC3 (Fig. 3D). Furthermore, confirming whether the mGPC3-1 peptide-specific CTL line killed cancer cells presenting the mGPC3-1 peptide is important, thus a cytotoxicity assay was performed (Fig. 3B).

Mice with a B6 background that spontaneously develop liver cancer have been reported (19,20). These mice enable investigations as to whether a peptide vaccine for GPC3 has a preventive capability. Recently, the STAM mice C57BL/6N-NASH was established as a non-alcoholic-steatohepatitis (NASH) mouse model by Stelic Institute & Co. STAM mice C57BL/6N-NASH are drug-treated B6 mice and liver cancer occurs spontaneously and early in NASH mice. Therefore, this mouse is an attractive model for studying the preventive effects of a cancer vaccine. We showed that mGPC3-1 peptide-specific CTL could be induced in STAM mice C57BL/6N-NASH (Fig. 4E). Simultaneously, we established a liver cancer cell line derived from STAM mice C57BL/6N-NASH and observed the cancer cell line expressed mGPC3 (data not shown).

However, the GPC3 peptide vaccine did not prevent the occurrence of liver cancer in STAM mice C57BL/6N-NASH (data not shown). Therefore, further research to develop strong GPC3-specific immunotherapies or combinational approaches in an appropriate mouse model is needed. Identification of an H2-K^b or H2-D^b restricted, GPC3-derived peptide is the first step. The established cell line from STAM mice C57BL/6N-NASH, which show GPC3 expression, may help us to develop a new mouse model system for a GPC3-targeted therapy.

In conclusion, mGPC3-$1_{127-136}$ AMFKNNYPSL was identified as an H2-K^b or H2-D^b restricted, GPC3-derived CTL most-inducible epitope peptide and mGPC3-1 peptide-specific CTL can kill RMA-S pulsed with the mGPC3-1 peptide. Furthermore, we established an mGPC3-1-specific CTL-inducible model in B6 mice using an mGPC3-1 peptide vaccine.

Acknowledgements

D.N., H.K. and Y.S. would like to thank the Foundation for Promotion of Cancer Research (Japan) for the Third-Term Comprehensive Control Research for Cancer for awarding them a research resident fellowship. This study was supported in part by Health and Labor Science Research Grants for Clinical Research and Third Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor and Welfare, Japan and the National Cancer Center Research and Development Fund.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Yamamoto J, Okada S, Shimada K, *et al*: Treatment strategy for small hepatocellular carcinoma: comparison of long-term results after percutaneous ethanol injection therapy and surgical resection. Hepatology 34: 707-713, 2001.
3. Greten TF, Manns MP and Korangy F: Immunotherapy of hepatocellular carcinoma. J Hepatol 45: 868-878, 2006.
4. Motomura Y, Senju S, Nakatsura T, *et al*: Embryonic stem cell-derived dendritic cells expressing glypican-3, a recently identified oncofetal antigen, induce protective immunity against highly metastatic mouse melanoma, B16-F10. Cancer Res 66: 2414-2422, 2006.
5. Mizukoshi E, Nakamoto Y, Arai K, *et al*: Comparative analysis of various tumor-associated antigen-specific T-cell responses in patients with hepatocellular carcinoma. Hepatology 53: 1206-1216, 2011.
6. Butterfield LH, Ribas A, Dissette VB, *et al*: A phase I/II trial testing immunization of hepatocellular carcinoma patients with dendritic cells pulsed with four alpha-fetoprotein peptides. Clin Cancer Res 12: 2817-2825, 2006.
7. Greten TF, Forner A, Korangy F, *et al*: A phase II open label trial evaluating safety and efficacy of a telomerase peptide vaccination in patients with advanced hepatocellular carcinoma. BMC Cancer 10: 209, 2010.
8. Butterfield LH, Ribas A, Meng WS, *et al*: T-cell responses to HLA-A*0201 immunodominant peptides derived from alpha-fetoprotein in patients with hepatocellular cancer. Clin Cancer Res 9: 5902-5908, 2003.
9. Nakatsura T, Yoshitake Y, Senju S, *et al*: Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun 306: 16-25, 2003.
10. Capurro MI, Xiang YY, Lobe C and Filmus J: Glypican-3 promotes the growth of hepatocellular carcinoma by stimulating canonical Wnt signaling. Cancer Res 65: 6245-6254, 2005.
11. Filmus J: Glypicans in growth control and cancer. Glycobiology 11: R19-R23, 2001.
12. Filmus J, Capurro M and Rast J: Glypicans. Genome Biol 9: 224, 2008.
13. Komori H, Nakatsura T, Senju S, *et al*: Identification of HLA-A2- or HLA-A24-restricted CTL epitopes possibly useful for glypican-3-specific immunotherapy of hepatocellular carcinoma. Clin Cancer Res 12: 2689-2697, 2006.
14. Nakatsura T, Komori H, Kubo T, *et al*: Mouse homologue of a novel human oncofetal antigen, glypican-3, evokes T-cell-mediated tumor rejection without autoimmune reactions in mice. Clin Cancer Res 10: 8630-8640, 2004.
15. Sawada Y, Sakai M, Yoshikawa T, Ofuji K and Nakatsura T: A glypican-3-derived peptide vaccine against hepatocellular carcinoma. Oncoimmunology 1: 1448-1450, 2012.
16. Sawada Y, Yoshikawa T, Nobuoka D, *et al*: Phase I trial of a glypican-3-derived peptide vaccine for advanced hepatocellular carcinoma: immunologic evidence and potential for improving overall survival. Clin Cancer Res 18: 3686-3696, 2012.
17. Nobuoka D, Yoshikawa T, Takahashi M, *et al*: Intratumoral peptide injection enhances tumor cell antigenicity recognized by cytotoxic T lymphocytes: a potential option for improvement in antigen-specific cancer immunotherapy. Cancer Immunol Immunother: Nov 11, 2012 (Epub ahead of print).

18. Motomura Y, Ikuta Y, Kuronuma T, *et al*: HLA-A2 and -A24-restricted glypican-3-derived peptide vaccine induces specific CTLs: preclinical study using mice. Int J Oncol 32: 985-990, 2008.
19. Koike K, Moriya K, Iino S, *et al*: High-level expression of hepatitis B virus HBx gene and hepatocarcinogenesis in transgenic mice. Hepatology 19: 810-819, 1994.
20. Moriya K, Yotsuyanagi H, Shintani Y, *et al*: Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol 78: 1527-1531, 1997.
21. Ikuta Y, Hayashida Y, Hirata S, *et al*: Identification of the H2-K^d-restricted cytotoxic T lymphocyte epitopes of a tumor-associated antigen, SPARC, which can stimulate antitumor immunity without causing autoimmune disease in mice. Cancer Sci 100: 132-137, 2009.
22. Wu X, Xu X, Gu R, *et al*: Prediction of HLA class I-restricted T-cell epitopes of islet autoantigen combined with binding and dissociation assays. Autoimmunity 45: 176-185, 2012.
23. Nakatsugawa M, Horie K, Yoshikawa T, *et al*: Identification of an HLA-A*0201-restricted cytotoxic T lymphocyte epitope from the lung carcinoma antigen, Lengsin. Int J Oncol 39: 1041-1049, 2011.
24. Hofmann UB, Voigt H andersen MH, Straten PT, Becker JC and Eggert AO: Identification and characterization of survivin-derived H-2Kb-restricted CTL epitopes. Eur J Immunol 39: 1419-1424, 2009.
25. Zhou X, Glas R, Momburg F, Hammerling GJ, Jondal M and Ljunggren HG: TAP2-defective RMA-S cells present Sendai virus antigen to cytotoxic T lymphocytes. Eur J Immunol 23: 1796-1801, 1993.
26. Stuber G, Leder GH, Storkus WT, *et al*: Identification of wild-type and mutant p53 peptides binding to HLA-A2 assessed by a peptide loading-deficient cell line assay and a novel major histocompatibility complex class I peptide binding assay. Eur J Immunol 24: 765-768, 1994.
27. Tsukahara T, Kawaguchi S, Torigoe T, *et al*: HLA-A*0201-restricted CTL epitope of a novel osteosarcoma antigen, papillomavirus binding factor. J Transl Med 7: 44, 2009.
28. Yoshikawa T, Nakatsugawa M, Suzuki S, *et al*: HLA-A2-restricted glypican-3 peptide-specific CTL clones induced by peptide vaccine show high avidity and antigen-specific killing activity against tumor cells. Cancer Sci 102: 918-925, 2011.
29. Yamazoe S, Tanaka H, Iwauchi T, *et al*: Identification of HLA-A*0201- and A*2402-restricted epitopes of mucin 5AC expressed in advanced pancreatic cancer. Pancreas 40: 896-904, 2011.
30. Guo Y, Zhu Y and Sun S: Identification and functional studies of HLA-A0201 restricted CTL epitopes in the X protein of hepatitis B virus. Acta Virologica 55: 107-115, 2011.

Cancer Chemother Pharmacol (2012) 70:391–397
DOI 10.1007/s00280-012-1920-0

ORIGINAL ARTICLE

Comparison of dose intensity of vincristine, *d*-actinomycin, and cyclophosphamide chemotherapy for child and adult rhabdomyosarcoma: a retrospective analysis

Yuki Kojima · Kenji Hashimoto · Masashi Ando · Kan Yonemori · Harukaze Yamamoto · Makoto Kodaira · Mayu Yunokawa · Chikako Shimizu · Kenji Tamura · Ako Hosono · Atsushi Makimoto · Yasuhiro Fujiwara

Received: 3 April 2012 / Accepted: 25 June 2012 / Published online: 18 July 2012

Abstract
Purpose The prognosis of adult rhabdomyosarcoma (RMS) has been considered dismal. The question is raised that vincristine, *d*-actinomycin, and cyclophosphamide (VAC) chemotherapy may not be administered as per schedule for adult RMS; consequently, low dose intensity (DI) leads to poor prognosis. Herein, we examined whether the administration of VAC chemotherapy for adults and children with RMS is feasible with regard to the DIs of VAC.
Methods Chart review was retrospectively performed for all identified patients. The percentage of relative DI (RDI) was calculated according to the Children's Oncology Group D9803 protocol. Further, we examined the RDI in the first 6 cycles of VAC (induction phase) and the DI after the first 6 cycles of VAC (maintenance phase).
Results We identified a total of 27 adults and 18 children with RMS, respectively. The mean RDIs of vincristine in total phase were significantly lower in adults than that in children ($P = 0.04$). In induction phase, the mean RDIs of vincristine and cyclophosphamide were similar for both groups; however, they were dropped significantly in adults during maintenance phase ($P < 0.05$). Mean RDIs of vincristine in elderly patients tended to become low. Low RDI was mainly attributable to hematologic toxicity, infection, and peripheral neuropathy. The prognosis of low versus high RDI was similar.
Conclusions The RDIs of vincristine and cyclophosphamide in the maintenance phase were significantly lower than that in children. VAC chemotherapy for adults was not feasible; these patients require a different regimen.

Keywords Rhabdomyosarcoma · Adults · Children · Chemotherapy · Soft tissue sarcoma

Abbreviations

RMS	Rhabdomyosarcoma
VAC	Vincristine, *d*-actinomycin, and cyclophosphamide
DI	Dose intensity
RDI	Relative dose intensity
IRS	The International Rhabdomyosarcoma Study
PFS	Progression-free survival
OS	Overall survival
PCP	Pneumocystis jirovecci pneumonia

Introduction

Recent advances in the treatment for rhabdomyosarcoma have contributed to improve survival from 55 to 73 % [1–3]. Vincristine and *d*-actinomycin with or without cyclophosphamide (VAC) regimens for rhabdomyosarcoma are considered standard options according to the previous randomized trials [4–8]. However, these trials

Y. Kojima · K. Hashimoto (✉) · M. Ando · K. Yonemori · H. Yamamoto · M. Kodaira · M. Yunokawa · C. Shimizu · K. Tamura · Y. Fujiwara
Department of Medical Oncology and Breast Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
e-mail: gonmusao@k6.dion.ne.jp

Y. Kojima
Department of Hematology and Medical Oncology, Nagoya Medical Center, 4-1-1 Sannomaru, Naka-ku, Nagoya 460-0001, Japan

A. Hosono · A. Makimoto
Department of Pediatric Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

mainly focused on children or adolescents; therefore, the data of precious adult rhabdomyosarcoma patients were limited.

Sultan et al. [9] reported the prognosis of pediatric and adult rhabdomyosarcoma, and the data suggested that 5-year survival rate was significantly poor in adult rhabdomyosarcoma with 27 % compared to that of pediatrics with 61 %. Other facilities also reported small series of similar results [10–14]. Accordingly, the prognosis of adult rhabdomyosarcoma remained poor; however, the treatment strategy references along with childhood rhabdomyosarcoma.

There is no evidence available to uncover the reason for poor prognosis of adult rhabdomyosarcoma. The VAC chemotherapy was developed mainly for targeting pediatrics; therefore, adverse event may differ when we applied the regimen to adults. Accordingly, the VAC regimens may not be administered per schedule, which leads to poor prognosis due to low dose intensity. To our knowledge, there are no reports on dose intensity of VAC regimens in relation to prognosis. We launched this study to seek optimal treatment for adult rhabdomyosarcoma by comparing dose intensity to childhood or adolescent rhabdomyosarcoma.

Methods

Patients

All the patients included in this analysis meet the following criteria: histologically diagnosed with rhabdomyosarcoma, treated at the National Cancer Center Hospital in Tokyo between 1991 and 2010, and received VAC chemotherapy 6 cycles or over as a first-line treatment. The chart review was performed for all identified patients to obtain the following information: age, sex, primary tumor site, histopathology, tumor size, stage, group defined by the International Rhabdomyosarcoma Study (IRS) postsurgical grouping system [4], chemotherapy regimen, chemotherapy administration schedule, adverse event, date of radiotherapy, date of surgery, date of progression, and date of last follow-up.

Treatment

The details of VAC chemotherapy are shown as follows. VAC consisted of vincristine at a dose of 1.5 mg/m^2 (up to 2 mg/body), given intravenously on day 1, 8, 15, cyclophosphamide at a dose of 2.2 g/m^2, given intravenously on day 1, and *d*-actinomycin at a dose of 1.5 mg/m^2 (up to 2.5 mg/body), given intravenously on day 1. The treatment course was repeated every 3 weeks according to Children's Oncology Group Study D9803 [8]. *D*-actinomycin on day 1 and vincristine on day 8 and day 15 were omitted when radiotherapy was applicable.

Dose intensity calculation and definitions of terms

Dose intensity of vincristine and cyclophosphamide was calculated according to the following formula: Dose intensity (vincristine) = Total dose of vincristine (mg/m^2)/[(last date of injection − first date of injection) + 7]/7; Dose intensity (cyclophosphamide) = Total dose of cyclophosphamide (mg/m^2)/[(last date of injection − first date of injection) + 21]/7, respectively. The percentage of relative dose intensity (RDI) was calculated according to the Children's Oncology Group D9803 protocol [8]. We examined the RDI in induction phase and maintenance phase. Induction phase was defined as the first six cycles of VAC, and maintenance phase was defined as the latter cycles after induction phase. We defined cut-off points of RDI of vincristine and cyclophosphamide at 80 % and dichotomized into lower RDI (RDI $<$ 80 %) and higher RDI (RDI $\geq$ 80 %).

We defined adult as the patients aged greater than or equal to 21 years, and we used the term 'children' for the others. We attempted to retrospectively define the stage of disease according to the IRS staging system, which separates patients by site of the primary tumor, tumor size, and the presence or absence of tumor-involved regional lymph nodes and of distant metastases. Adverse events were retrospectively ranked by the National Cancer Institute Common Toxicity Criteria version 3.0. As a definition of febrile neutropenia, we used the guidelines established by the Infectious Disease Society of America [15]. Treatment delay was defined as more than a week of delay to start new cycle of VAC regimen.

Statistics

We used Student's *t* test to detect the difference of mean RDI. Progression-free survival (PFS) was defined as the time between the date of initial chemotherapy until the date of the recognition of local recurrence or distant metastases. Death was treated as an event, and the absence of disease progression was treated as a censored observation on the last day of follow-up. Overall survival (OS) was defined as the time from the date of initial chemotherapy until the date of death. Patients who were lost to follow up were treated as a censored observation on the last day of follow-up. We used the Kaplan–Meier method to draw survival curve, and significance was detected by the log-rank test. All statistical analyses were performed using SPSS ver. 19.0 (IBM Co. Inc, Okinawa, Japan).

Results

Patient characteristics

We identified 59 patients who received VAC regimen and of 45 patients met the eligibility criteria in the study period. Fourteen patients were excluded from this analysis by the following reasons: less than 6 cycles of VAC regimen in 12 patients, VAC chemotherapy of IRS II protocol in one, and clinical data unavailable in one. There were 27 adult patients and 18 were children.

The distribution of clinical and pathologic characteristics of these patients is listed in Table 1. The median age of adult was 28 years (range, 22–72) and that of children was 16 years (range, 2–19). Primary disease site, histopathology, and primary tumor size were similar among adult and children. Fourteen adults and 8 children had parameningeal primary site. Metastatic disease was somewhat high in adult.

Overall median follow-up period was 26 months (range, 6–114 months): 26 months in adults (range, 6–113 months) and 36 months in children (range, 6–114 months). We identified a total of 316 cycles of VAC chemotherapy in adults and a total of 209 cycles in children. The median number of chemotherapy cycles was 13 (range, 6–14) for adults and 12.5 (range, 6–14) for children.

Relative dose intensity of VAC chemotherapy

The mean RDIs of vincristine and cyclophosphamide in total phase for adults were 76.8 and 80.8 %, respectively, and those for children were 90.2 and 86.9 %, respectively. The mean RDI of vincristine in total phase was significantly lower in adults than that in children ($P < 0.05$) (Table 2).

In induction phase, the mean RDIs of vincristine and cyclophosphamide were similar for both groups; however, they were dropped significantly in adults during maintenance phase ($P < 0.05$) (Table 2). We examined the RDIs of vincristine and cyclophosphamide in relation to age. Mean RDIs of vincristine in elderly patients tended to become low (Fig. 1).

Chemotherapy efficacy by RDI

We analyzed the efficacy of RDI on PFS and OS. The PFS and OS were not statistically different in patients with high RDI and low RDI of vincristine and cyclophosphamide in total phase. For total phase of RDI for vincristine, median PFS was 28 months in patients with high RDI ($n = 15$) and 24 months in patients with low RDI ($n = 30$) ($P = 0.43$), and median OS was 60 months in patients with high RDI ($n = 15$) and 42 months in patients with low RDI ($n = 30$) ($P = 0.78$) (Fig. 2a). For total phase of RDI for cyclophosphamide, median PFS was 28 months in patients with high RDI ($n = 17$) and 24 months in patients with low RDI ($n = 28$) ($P = 0.78$), and median OS was 114 months in patients with high RDI ($n = 17$) and 60 months in patients with low RDI ($n = 28$) ($P = 0.67$) (Fig. 2b). We further analyzed induction phase and maintenance phase of RDIs for vincristine and cyclophosphamide or stratified them by adults and children on PFS and OS. None of the difference regarding RDI of both agents on PFS and OS was detected (Fig. 3).

Toxicity of VAC chemotherapy

Table 3 lists the worst degree of toxicity of VAC chemotherapy. Majority of patients experienced grade 3/4 neutropenia and anemia in both induction phase and maintenance phase. Grade 2–4 peripheral neuropathy was observed in 55.6 % of adult patients, while it was 33.3 % in children ($P = 0.37$). Approximately 80 % of patients needed granulocyte colony-stimulating factor, and 73.3 % of patients received trimethoprim-sulfamethoxazole for prevention of pneumocystis jirovecci pneumonia (PCP). Two patients who did not receive trimethoprim-sulfamethoxazole developed PCP; one experienced PCP in 4th cycle of VAC, and the other did in 14 the cycle of VAC.

Cause of dose reduction, skip, and delayed cycles

In induction phase, three of adults (11.1 %) and one of children (5.6 %) required dose reduction of vincristine due to peripheral neuropathy and myelosuppression. Eight of the patients (29.6 %) in adults and 4 of the patients (22.2 %) in children required dose reduction of cyclophosphamide, due to myelosuppression, febrile neutropenia, and liver dysfunction. In maintenance phase, four adults (16.0 %) and 2 children (11.8 %) required dose reduction of vincristine, due to peripheral neurotoxicity and myelosuppression. Nine adults (36.0 %) and 5 children (18.5 %) required dose reduction of cyclophosphamide, due to myelosuppression and febrile neutropenia.

Adult patients needed discontinuance of vincristine in total of 126 times out of 713 times (17.7 %) due to the following reasons: peripheral neurotoxicity in 75 times, myelosuppression in 35 times, and febrile neutropenia in 14 times. For these child patients, it was 55 times out of 483 times of vincristine administration (11.4 %) due to peripheral neurotoxicity in 16 times, myelosuppression in 16 times, and febrile neutropenia in 9 times.

In induction phase, 14 adult patients (51.9 %) and 9 child patients (50.0 %) experienced cycle delay. In maintenance phase, 17 adult patients (68.0 %) and 9 child patients (52.9 %) required cycle delay. The major causes of

Table 1 Patient characteristics

	Adults ($N = 27$)	Children ($N = 18$)
Age, median (range)	28 (22–72)	16 (2–19)
Gender		
Female	8 (29.6 %)	9 (50.0 %)
Male	19 (70.4 %)	9 (50.0 %)
Primary tumor site		
Head-neck	3 (11.1 %)	3 (16.7 %)
Parameningeal primary site	14 (51.9 %)	8 (44.4 %)
Unfavorable other	5 (18.5 %)	1 (5.6 %)
Extremity	3 (11.1 %)	5 (27.8 %)
Genitourinary primary site	2 (7.4 %)	1 (5.6 %)
Histopathology		
Alveolar	15 (55.6 %)	10 (55.6 %)
Embryonal	10 (37.0 %)	7 (38.9 %)
Others	2 (7.4 %)	1 (5.6 %)
Tumor size		
≥5 cm	10 (37.0 %)	8 (44.4 %)
<5 cm	15 (55.6 %)	9 (50.0 %)
Not available	2 (7.4 %)	1 (5.6 %)
IRS group		
I	3 (11.1 %)	2 (11.1 %)
II	3 (11.1 %)	1 (5.6 %)
III	12 (44.4 %)	12 (66.7 %)
IV	9 (33.3 %)	3 (16.7 %)
IRS pre-treatment stage		
Stage 1	4 (14.8 %)	4 (22.2 %)
Stage 2	4 (14.8 %)	1 (5.6 %)
Stage 3	10 (37.0 %)	10 (55.6 %)
Stage 4	9 (33.3 %)	3 (16.7 %)
Complete surgical resection		
Pre-chemotherapy	7 (25.9 %)	4 (22.2 %)
Post-chemotherapy	4 (14.8 %)	6 (33.3 %)
No surgery	16 (59.3 %)	8 (44.4 %)
Radiation therapy		
Pre-chemotherapy	2 (7.4 %)	2 (11.1 %)
Post-chemotherapy	20 (74.1 %)	14 (77.8 %)
No radiation therapy	5 (18.5 %)	2 (11.1 %)

IRS Intergroup Rhabdomyosarcoma Study

cycle delay were myelosuppression, febrile neutropenia, and patient preferences.

Discussion

In our analyses, 27 adults and 18 children of rhabdomyosarcoma who received VAC chemotherapy at a single institution were evaluated for RDIs of VAC chemotherapy. Adult patients had significantly lower RDIs of vincristine and cyclophosphamide in the maintenance phase compared

Table 2 Mean relative dose intensity in patients with adult and child rhabdomyosarcoma

	Adult (%)	Child (%)	*P* value
Vincristine			
Induction phase	77.7	86.4	0.109
Maintenance phase	71.9	100.1	0.042
Total phase	76.8	90.2	0.040
Cyclophosphamide			
Induction phase	87.1	88.2	0.820
Maintenance phase	69.7	86.4	0.011
Total phase	80.8	86.9	0.156

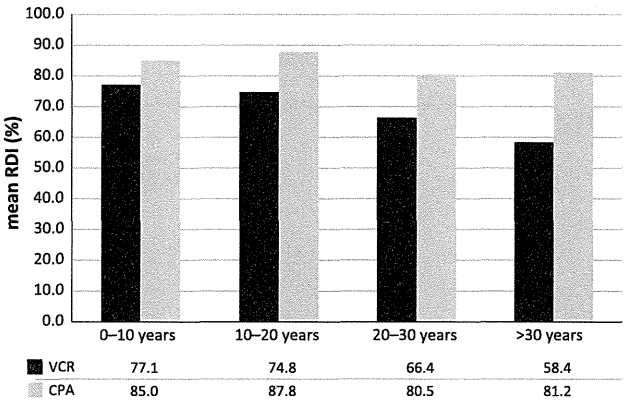

Fig. 1 Relative dose intensity of vincristine and cyclophosphamide in relation to age. *VCR* vincristine, *CPA* cyclophosphamide

to those of children. Further, the mean RDIs of vincristine tended to become low depending on patients' ages. The reasons for dose reduction, discontinuance, and treatment delay of vincristine and cyclophosphamide were generally due to myelosuppression, infection, and peripheral neurotoxicity. We revealed that the adult rhabdomyosarcoma patients significantly received lower RDIs of vincristine and cyclophosphamide than those of children; however, the PFS and the OS were not statistically different in patients with high RDI and low RDI in total phase and maintenance phase.

The prognosis of adult rhabdomyosarcoma remains poor, with approximately 30 % at 5-year survival [9–14, 16]. The reason for the poor outcome of adults remains unclear. Sultan et al. [9] reported the comparison of pediatric and adult rhabdomyosarcoma; they reported that the typical pediatric rhabdomyosarcoma variants (embryonal and alveolar subtypes) occurred less frequently in adult rhabdomyosarcoma and that the most common primary sites in adult were extremities and unfavorable sites and that radiation therapy was performed less frequently in adult rhabdomyosarcoma than in pediatric patients. In our study, adult rhabdomyosarcoma patients had lower RDIs in

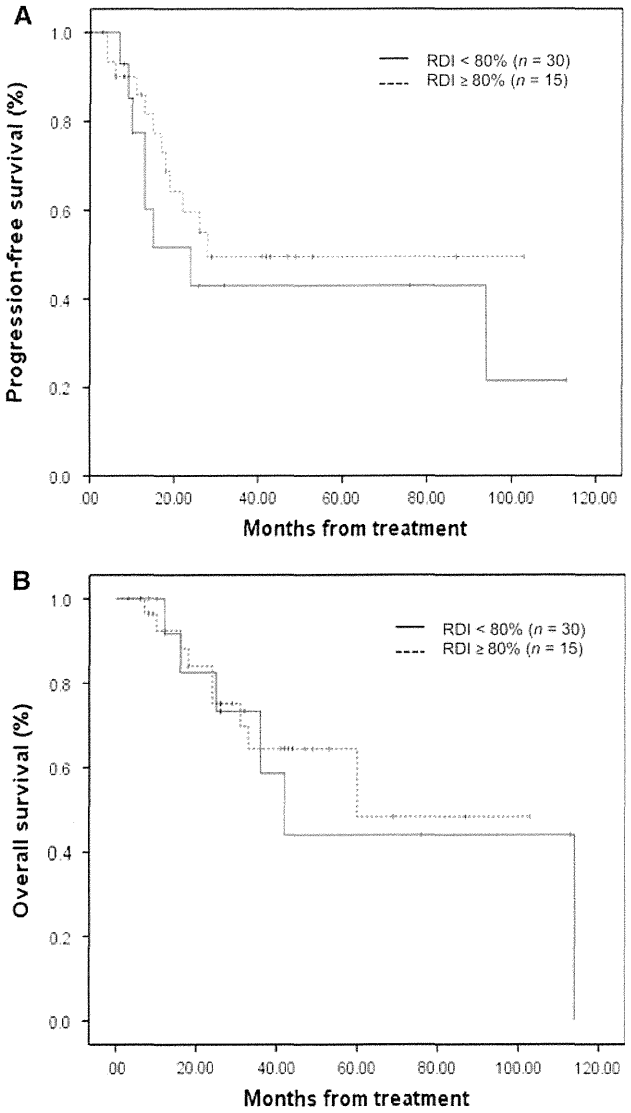

Fig. 2 **a** Kaplan–Meier curve of progression-free survival in patients with high relative dose intensity of vincristine (*dash line*) and low relative dose intensity of vincristine (*black line*). **b** Kaplan–Meier curve of overall survival in patients with high relative dose intensity of vincristine (*dash line*) and low relative dose intensity of vincristine (*black line*)

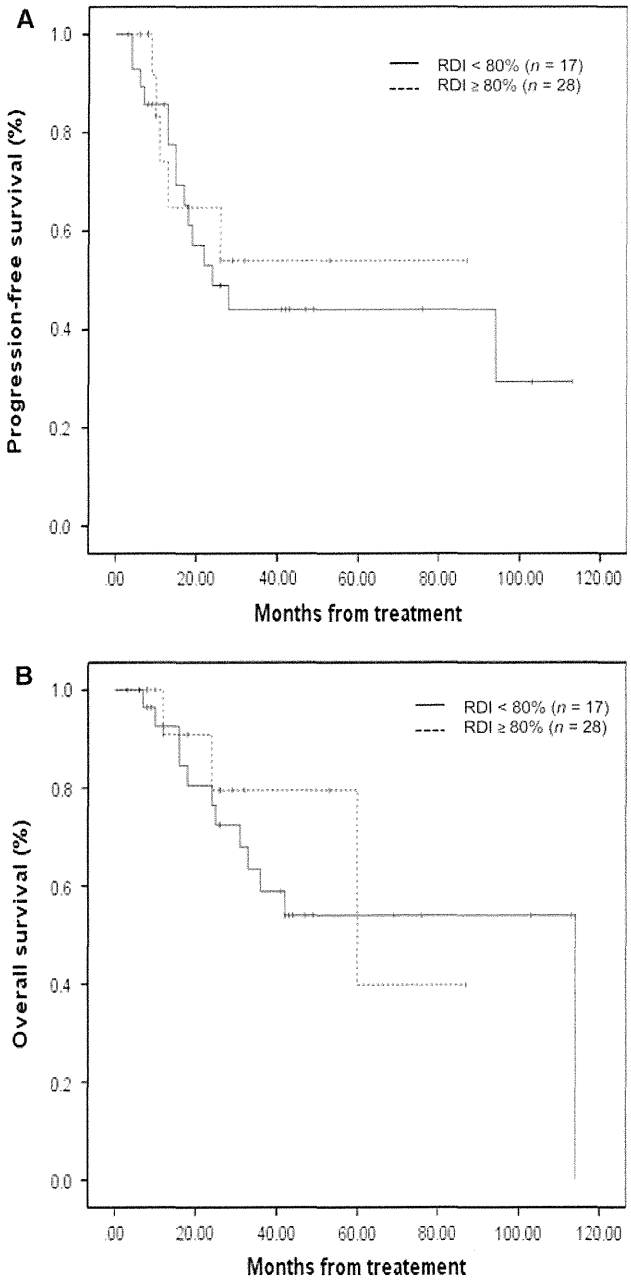

Fig. 3 **a** Kaplan–Meier curve of progression-free survival in patients with high relative dose intensity of cyclophosphamide (*dash line*) and low relative dose intensity of cyclophosphamide (*black line*). **b** Kaplan–Meier curve of overall survival in patients with high relative dose intensity of cyclophosphamide (*dash line*) and low relative dose intensity of cyclophosphamide (*black line*)

VAC chemotherapy, especially in maintenance phase. The reason for the low RDI was not apparent, but we demonstrated that VAC chemotherapy for adult rhabdomyosarcoma patient may not be feasible as VAC chemotherapy was developed mainly targeting pediatric patients. We also suggested that none of the differences regarding RDIs of both vincristine and cyclophosphamide on PFS and OS were detected. However, in our previous data focusing on childhood RMS and adult RMS survival suggested significant differences in survival in localized tumor [17]. Although we could not observe significant differences in survival in metastatic tumors for both childhood RMS and adult RMS, controversy remains as to the standard treatment for patients with adult rhabdomyosarcoma. Recently, several types of trials for these patients have demonstrated to show additional benefit of adding active new agents to standard VAC chemotherapy, which failed to show survival benefit [18–20].

In IRS-V study, the dosage of cyclophosphamide was designed 1.2 g/m^2/cycle. High-dose therapy with stem cell transplantation for high-risk metastatic RMS did not

Table 3 Adverse event in patients who received VAC regimen

	Adults			Children		
	Induction phase ($n = 27$)	Maintenance phase ($n = 25$)	Total phase	Induction phase ($n = 18$)	Maintenance phase ($n = 17$)	Total phase
Neutropenia Gr. 4	27 (100 %)	23 (92.0 %)	27 (100 %)	18 (100 %)	17 (100 %)	18 (100 %)
Anemia Gr. 3/4	24 (88.9 %)	21 (84.0 %)	27 (100 %)	16 (88.9 %)	14 (82.6 %)	16 (88.9 %)
Thrombocytopenia Gr. 4	12 (44.4 %)	14 (56.0 %)	19 (70.3 %)	13 (72.2 %)	10 (58.8 %)	13 (72.2 %)
FN Gr. 3/4	23 (85.2 %)	19 (76.0 %)	26 (96.3 %)	17 (94.4 %)	14 (82.6 %)	18 (100 %)
Peripheral neuropathy ≥Gr. 2	11 (40.7 %)	12 (48.0 %)	15 (55.6 %)	6 (33.3 %)	5 (29.4 %)	6 (33.3 %)

VAC vincristine, *d*-actinomycin, cyclophosphamide, *FN* febrile neutropenia, *Gr* grade

improve outcome significantly [21]. Accordingly, reduced dose of VAC chemotherapy for adults might not be inferior to standard VAC dose regarding survival.

This study has several limitations as of the nature of retrospective design and small sample size. More patients may be needed to detect the small differences regarding survival in low RDI patients and high RDI patients. Decision of administration of vincristine on day 8 and 15 may vary by physicians as evaluation of peripheral neuropathy by vincristine is not integrated. However, considering poor prognosis of adult RMS with highly toxic regimen of VAC, new strategy to treat this rare cohort must be developed urgently.

In summary, adult rhabdomyosarcoma is a rare malignancy with poor prognosis. The treatment strategy has been performed along with child rhabdomyosarcoma; however, the RDIs of vincristine and cyclophosphamide in the maintenance phase were significantly lower than that in children. Causes of low RDIs were generally myelosuppression, infection, and peripheral neurotoxicity in both groups. VAC chemotherapy for adults may not be feasible; thus, the prospective trial for adult rhabdomyosarcoma should be designed.

Acknowledgments We thank all the participants for supporting this study. Mrs. N. Nakamura supported for revising the manuscript.

Conflict of interest None declared.

References

1. Pappo AS, Shapiro DN, Crist WM (1997) Rhabdomyosarcoma. Biology and treatment. Pediatr Clin North Am 44(4): 953–972
2. Raney RB, Maurer HM, Anderson JR, Andrassy RJ, Donaldson SS, Qualman SJ, Wharam MD, Wiener ES, Crist WM (2001) The Intergroup Rhabdomyosarcoma Study Group (IRSG): major lessons from the IRS-I through IRS-IV studies as background for the current IRS-V treatment protocols. Sarcoma 5(1):9–15. doi: 10.1080/13577140120048890
3. Breitfeld PP, Meyer WH (2005) Rhabdomyosarcoma: new windows of opportunity. Oncologist 10(7):518–527. doi:10.1634/theoncologist.10-7-518
4. Maurer HM, Beltangady M, Gehan EA, Crist W, Hammond D, Hays DM, Heyn R, Lawrence W, Newton W, Ortega J et al (1988) The Intergroup Rhabdomyosarcoma Study-I. A final report. Cancer 61(2):209–220
5. Maurer HM, Gehan EA, Beltangady M, Crist W, Dickman PS, Donaldson SS, Fryer C, Hammond D, Hays DM, Herrmann J et al (1993) The Intergroup Rhabdomyosarcoma Study-II. Cancer 71(5):1904–1922
6. Crist W, Gehan EA, Ragab AH, Dickman PS, Donaldson SS, Fryer C, Hammond D, Hays DM, Herrmann J, Heyn R et al (1995) The Third Intergroup Rhabdomyosarcoma Study. J Clin Oncol 13(3):610–630
7. Crist WM, Anderson JR, Meza JL, Fryer C, Raney RB, Ruymann FB, Breneman J, Qualman SJ, Wiener E, Wharam M, Lobe T, Webber B, Maurer HM, Donaldson SS (2001) Intergroup Rhabdomyosarcoma Study-IV: results for patients with nonmetastatic disease. J Clin Oncol 19(12):3091–3102
8. Arndt CA, Stoner JA, Hawkins DS, Rodeberg DA, Hayes-Jordan AA, Paidas CN, Parham DM, Teot LA, Wharam MD, Breneman JC, Donaldson SS, Anderson JR, Meyer WH (2009) Vincristine, actinomycin, and cyclophosphamide compared with vincristine, actinomycin, and cyclophosphamide alternating with vincristine, topotecan, and cyclophosphamide for intermediate-risk rhabdomyosarcoma: Children's Oncology Group Study D9803. J Clin Oncol 27(31):5182–5188. doi:10.1200/JCO.2009.22.3768
9. Sultan I, Qaddoumi I, Yaser S, Rodriguez-Galindo C, Ferrari A (2009) Comparing adult and pediatric rhabdomyosarcoma in the surveillance, epidemiology and end results program, 1973 to 2005: an analysis of 2,600 patients. J Clin Oncol 27(20):3391–3397. doi: 10.1200/JCO.2008.19.7483
10. Ferrari A, Dileo P, Casanova M, Bertulli R, Meazza C, Gandola L, Navarria P, Collini P, Gronchi A, Olmi P, Fossati-Bellani F, Casali PG (2003) Rhabdomyosarcoma in adults. A retrospective analysis of 171 patients treated at a single institution. Cancer 98(3):571–580. doi:10.1002/cncr.11550
11. Little DJ, Ballo MT, Zagars GK, Pisters PW, Patel SR, El-Naggar AK, Garden AS, Benjamin RS (2002) Adult rhabdomyosarcoma: outcome following multimodality treatment. Cancer 95(2): 377–388. doi:10.1002/cncr.10669
12. Esnaola NF, Rubin BP, Baldini EH, Vasudevan N, Demetri GD, Fletcher CD, Singer S (2001) Response to chemotherapy and predictors of survival in adult rhabdomyosarcoma. Ann Surg 234(2):215–223
13. Hawkins WG, Hoos A, Antonescu CR, Urist MJ, Leung DH, Gold JS, Woodruff JM, Lewis JJ, Brennan MF (2001) Clinicopathologic analysis of patients with adult rhabdomyosarcoma.

Cancer 91(4):794–803. doi:10.1002/1097-0142(20010215)91:4<794:AID-CNCR1066>3.0.CO;2-Q
14. La Quaglia MP, Heller G, Ghavimi F, Casper ES, Vlamis V, Hajdu S, Brennan MF (1994) The effect of age at diagnosis on outcome in rhabdomyosarcoma. Cancer 73(1):109–117
15. Hughes WT, Armstrong D, Bodey GP, Bow EJ, Brown AE, Calandra T, Feld R, Pizzo PA, Rolston KV, Shenep JL, Young LS (2002) 2002 guidelines for the use of antimicrobial agents in neutropenic patients with cancer. Clin Infect Dis 34(6):730–751. doi:10.1086/339215
16. Lloyd RV, Hajdu SI, Knapper WH (1983) Embryonal rhabdomyosarcoma in adults. Cancer 51(3):557–565
17. Kojima Y, Hashimoto K, Ando M, Yonemori K, Hirakawa A, Kodaira M, Yunokawa M, Shimizu C, Tamura K, Katsumata N, Hosono A, Makimoto A, Fujiwara Y (2012) Clinical outcomes of adult and childhood rhabdomyosarcoma treated with vincristine, *d*-actinomycin, and cyclophosphamide chemotherapy. J Cancer Res Clin Oncol. doi:10.1007/s00432-012-1199-x
18. Klingebiel T, Boos J, Beske F, Hallmen E, Int-Veen C, Dantonello T, Treuner J, Gadner H, Marky I, Kazanowska B, Koscielniak E (2008) Treatment of children with metastatic soft tissue sarcoma with oral maintenance compared to high dose chemotherapy: report of the HD CWS-96 trial. Pediatr Blood Cancer 50(4):739–745. doi:10.1002/pbc.21494
19. Pappo AS, Lyden E, Breitfeld P, Donaldson SS, Wiener E, Parham D, Crews KR, Houghton P, Meyer WH (2007) Two consecutive phase II window trials of irinotecan alone or in combination with vincristine for the treatment of metastatic rhabdomyosarcoma: the Children's Oncology Group. J Clin Oncol 25(4):362–369. doi:10.1200/JCO.2006.07.1720
20. Lager JJ, Lyden ER, Anderson JR, Pappo AS, Meyer WH, Breitfeld PP (2006) Pooled analysis of phase II window studies in children with contemporary high-risk metastatic rhabdomyosarcoma: a report from the Soft Tissue Sarcoma Committee of the Children's Oncology Group. J Clin Oncol 24(21):3415–3422. doi:10.1200/JCO.2005.01.9497
21. Bisogno G, Ferrari A, Prete A, Messina C, Basso E, Cecchetto G, Indolfi P, Scarzello G, D'Angelo P, De Sio L, Di Cataldo A, Carli M (2009) Sequential high-dose chemotherapy for children with metastatic rhabdomyosarcoma. Eur J Cancer 45(17):3035–3041. doi:10.1016/j.ejca.2009.08.019

J Cancer Res Clin Oncol (2012) 138:1249–1257
DOI 10.1007/s00432-012-1199-x

ORIGINAL PAPER

Clinical outcomes of adult and childhood rhabdomyosarcoma treated with vincristine, *d*-actinomycin, and cyclophosphamide chemotherapy

Yuki Kojima · Kenji Hashimoto · Masashi Ando · Kan Yonemori · Akihiro Hirakawa · Makoto Kodaira · Mayu Yunokawa · Chikako Shimizu · Kenji Tamura · Noriyuki Katsumata · Ako Hosono · Atsushi Makimoto · Yasuhiro Fujiwara

Received: 30 January 2012 / Accepted: 6 March 2012 / Published online: 23 March 2012

Abstract
Background Outcomes in adult patients with rhabdomyosarcoma are poor, with a 5-year survival rate of approximately 30 %. The current study aimed to compare the clinical outcomes of adult and childhood rhabdomyosarcoma patients with local and metastatic disease and to examine the impact and timing of local therapy on metastasis.
Methods Clinicopathological features and patient outcomes were reviewed retrospectively for rhabdomyosarcoma patients receiving chemotherapy between 1981 and 2010 at our institution. Adults were defined as those aged 21 years or older.
Results Of the 98 patients identified, 36 were adults (median age, 29; range, 21–72) and 62 were children (median age, 11; range, 0.6–20). Median progression-free survival of localized and metastatic disease for children and adults was as follows: localized disease, 166.9 versus 22.4 months ($p = 0.005$), and metastatic disease, 13.3 versus 13.3 months ($p = 0.949$), respectively. Multivariate regression analysis revealed that older age (≥21 vs. <21) was a significant poor prognostic factor in localized disease. Conversely, age was not related to survival in metastatic disease. Receiving radiotherapy to the primary site was an independent factor indicating a better prognosis. An analysis of the optimal timing of local therapy was performed for 53 patients; however, its significance on survival could not be determined.
Conclusions Age was a negative prognostic factor in rhabdomyosarcoma patients with localized disease, but it did not affect the survival in metastatic disease. For metastatic disease, although local therapies may be effective for survival, the timing of such therapies should be determined individually.

Keywords Adult · Child · Rhabdomyosarcoma · Clinical outcome

This study was presented as part by ASCO 2011 in Chicago.

Y. Kojima · K. Hashimoto (✉) · M. Ando · K. Yonemori · M. Kodaira · M. Yunokawa · C. Shimizu · K. Tamura · N. Katsumata · Y. Fujiwara
Department of Medical Oncology and Breast Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
e-mail: gonmusao@k6.dion.ne.jp

Y. Kojima
Department of Hematology and Medical Oncology, Nagoya Medical Center, 4-1-1 Sannomaru, Naka-ku, Nagoya 460-0001, Japan

A. Hirakawa
Department of Management Science, Graduate School of Engineering, Tokyo University of Science, 3-3-2 Kasumigaseki Chiyoda-ku, Tokyo 100-0013, Japan

A. Hosono · A. Makimoto
Department of Pediatric Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children, but it is far less common in adults (Ferrari et al. 2003). Improvements in multimodal treatment of RMS have improved survival in children from 25 to approximately 70 % over the past 40 years (Pappo et al. 1997; Raney et al. 2001; Breitfeld and Meyer 2005; Leaphart and Rodeberg 2007). In contrast, the prognosis for adult RMS remains poor, with a 5-year survival rate of approximately 30 % (Sultan et al. 2009; Ferrari et al. 2003;

La Quaglia et al. 1994; Little et al. 2002; Esnaola et al. 2001; Hawkins et al. 2001).

Metastatic RMS affects approximately 15 % of all children with RMS (Breneman et al. 2003). According to the risk categories identified by the Intergroup Rhabdomyosarcoma Study (IRS), low- and intermediate-risk patients have improved outcomes, with 96–97 % of low-risk patients achieving 5-year survival (Meza et al. 2006) and 79 % of intermediate-risk patients attaining 4-year survival (Arndt et al. 2009). However, survival for high-risk patients remains poor, with 34 % achieving 3-year survival (Oberlin et al. 2008) and 24 % achieving 5-year survival (Carli et al. 2004). Consequently, standard treatment for high-risk patients remains controversial (Klingebiel et al. 2008; Pappo et al. 2007; Lager et al. 2006).

The treatment strategy for RMS requires multidisciplinary therapy, including chemotherapy, surgery, and radiation therapy. Vincristine and *d*-actinomycin, with or without cyclophosphamide (VAC) regimens, are considered the standard option for RMS (Maurer et al. 1988, 1993; Crist et al. 1995, 2001; Meza et al. 2006; Arndt et al. 2009), with wide surgical resection of tumor and postoperative radiation therapy required for local control of localized RMS (La et al. 2011; Rodeberg et al. 2011; Schuck et al. 2004). The timing of radiation therapy is critical: for localized RMS, radiation is appropriate to start during weeks 9–12, and for parameningeal RMS with intracranial extension, local radiation treatment should begin during the first 1–2 weeks of chemotherapy (Michalski et al. 2004; Raney et al. 2002). However, the impact and optimal timing of local therapy for metastatic disease is unknown. Therefore, the purpose of the current study was to compare the clinical outcomes of local or metastatic adult and childhood RMS and to examine the impact and timing of local therapies on metastatic disease.

Patients and methods

Patients

All patients included in this analysis met the following criteria: histologically diagnosed with RMS, treated at the National Cancer Center Hospital in Tokyo between 1981 and 2010, and received VAC or VAC-like chemotherapy. Medical records were then retrospectively reviewed to obtain the following information: date of birth, gender, date of diagnosis, primary tumor site, histopathology, initial tumor size, presence of central nervous system (CNS) invasion, clinical stage, group category as defined by the IRS (Breitfeld and Meyer 2005), date of treatment initiation, chemotherapy regimen, best response, chemotherapy administration schedule, date of radiotherapy, date of surgery, date of progression, date of last follow-up, and survival status.

Treatment

The VAC regimen after the year of 2000 consisted of vincristine at a dose of 1.5 mg/m^2 (up to 2 mg/body), given intravenously on days 1, 8, and 15; cyclophosphamide at a dose of 2.2 g/m^2, given intravenously on day 1; and *d*-actinomycin at a dose of 1.5 mg/m^2 (up to 2.5 mg/body), given intravenously on day 1. The treatment course was repeated according to IRS-IV or Children's Oncology Group (COG) Study (D9803) protocols (Arndt et al. 2009). Before the year of 2000, the VAC-like regimen includes the following regimens: vincristine, *d*-actinomycin, and either ifosfamide, etoposide, or doxorubicin. The treatment course was administered according to IRS-II or III protocols.

Local therapy includes surgery, radiation therapy, or both. Surgical resection defined in this manuscript includes only total gross resection. Microscopic complete resection (Group I) was confirmed microscopically later on. Radiation therapy was delivered to each primary tumor site and regional lymph nodes where applicable. The total dose ranged from 30 to 56.3 Gy, with a median dose of 45 Gy.

Definitions of terms

We defined adults as patients aged 21 years or more, and considered the remaining patients to be children. The induction phase was defined as the first six cycles of the VAC/VAC-like regimen, and the maintenance phase was defined as the later cycles following the induction phase. Response to chemotherapy was compared with baseline status. A complete response (CR) was defined as the disappearance of tumors with no evidence of disease. A partial response (PR) was a 50 % or greater decrease in the sum of tumor diameters. Stable disease (SD) was a less than 50 % decrease in the sum of tumor diameters. Progressive disease (PD) was defined as a 25 % or greater increase in the sum of tumor diameters and/or the appearance of new lesions.

Surgery or radiation therapy in this study means resection or radiation therapy to the primary site during primary treatment, respectively. Resection or radiation therapy to a relapsed site or metastatic site is not included in this category; likewise, biopsy only is not included in this definition.

Statistical analyses

Progression-free survival (PFS) was defined as the time from the date of initial chemotherapy to the date when

local recurrences or distant metastases were recognized. Overall survival (OS) was defined as the time from the date of initial chemotherapy to the date of death due to any cause. Patients who survived were treated as censored observations on the last day of follow-up. PFS and OS were estimated using the Kaplan–Meier method, and survival curves were compared using the log-rank test. Multivariate Cox regression analysis was used to estimate the hazard ratios and 95 % confidence intervals (CI). A two-sided $p < 0.05$ was considered statistically significant. All statistical analyses were performed using SAS (version 9.1.3; SAS Institute Inc., Cary, NC, USA).

Results

Patient characteristics

We identified 98 patients who met the eligibility criteria. The distributions of clinical and pathologic characteristics of patients are listed in Tables 1 and 2. There were 36 adults (age: median, 29; range 21–72) and 62 children (age: median, 11; range, 0.6–20). Seventy-three patients had localized disease (22 adults and 51 children) , while 25 patients had metastatic disease (14 adults and 11 children). The most common histology was embryonal in localized disease (56.2 %) and alveolar in metastatic disease (68.0 %). Botryoid was found in only one patient. Common primary sites in localized disease were parameningeal (38.4 %), head and neck (21.9 %), and extremity (15.1 %), while common primary sites in metastatic disease sites were parameningeal (32.0 %), extremity (28.0 %), and other (24.0 %) (Table 3). The most common metastatic

Table 1 Characteristics of patients with localized rhabdomyosarcoma ($n = 73$)

	Adults ($n = 22$)		Children ($n = 51$)	
	No.	%	No.	%
Gender				
Female	5	22.7	24	47.1
Male	17	77.3	27	52.9
Tumor size				
<5 cm	9	40.9	23	45.1
≥5 cm	13	59.1	28	51.9
Site				
Favorable	5	22.7	22	43.1
Unfavorable	17	77.3	29	56.9
Histology				
Alveolar	9	40.9	18	35.3
Embryonal	12	54.5	29	56.9
Other	1	4.5	4	7.8
Group				
1	3	13.6	9	17.6
2	3	13.6	5	9.8
3	16	72.7	37	72.5
Stage				
1	4	18.2	21	41.2
2	5	22.7	8	15.7
3	13	59.1	22	43.1
CNS invasion				
No	20	90.9	46	90.2
Yes	2	9.1	5	9.8

CNS central nervous system

Table 2 Characteristics of patients with metastatic rhabdomyosarcoma ($n = 25$)

	Adults ($n = 14$)		Children ($n = 11$)	
	No.	%	No.	%
Gender				
Female	6	42.9	6	54.5
Male	8	57.1	5	45.5
Tumor size				
<5 cm	2	14.3	4	36.4
≥5 cm	12	85.7	7	63.6
Site				
Favorable	1	7.1	1	9.1
Unfavorable	13	92.9	10	90.9
Histology				
Alveolar	11	78.6	6	54.5
Embryonal	2	14.3	5	45.5
Other	1	7.1	0	0
CNS invasion				
No	14	100	10	90.9
Yes	0	0	1	9.1

CNS central nervous system

Table 3 Primary site of rhabdomyosarcoma in localized and metastatic disease

	Localized disease No. (%)	Metastatic disease No. (%)
Extremity	11 (15.1)	7 (28.0)
Parameningeal site	28 (38.4)	8 (32.0)
Head and neck	16 (21.9)	1 (4.0)
Genitourinary primary site (non-bladder/prostate)	6 (8.2)	0 (0.0)
Orbit	5 (6.9)	1 (4.0)
Genitourinary primary site (bladder/prostate)	2 (2.7)	2 (8.0)
Unfavorable other	5 (6.9)	6 (24.0)

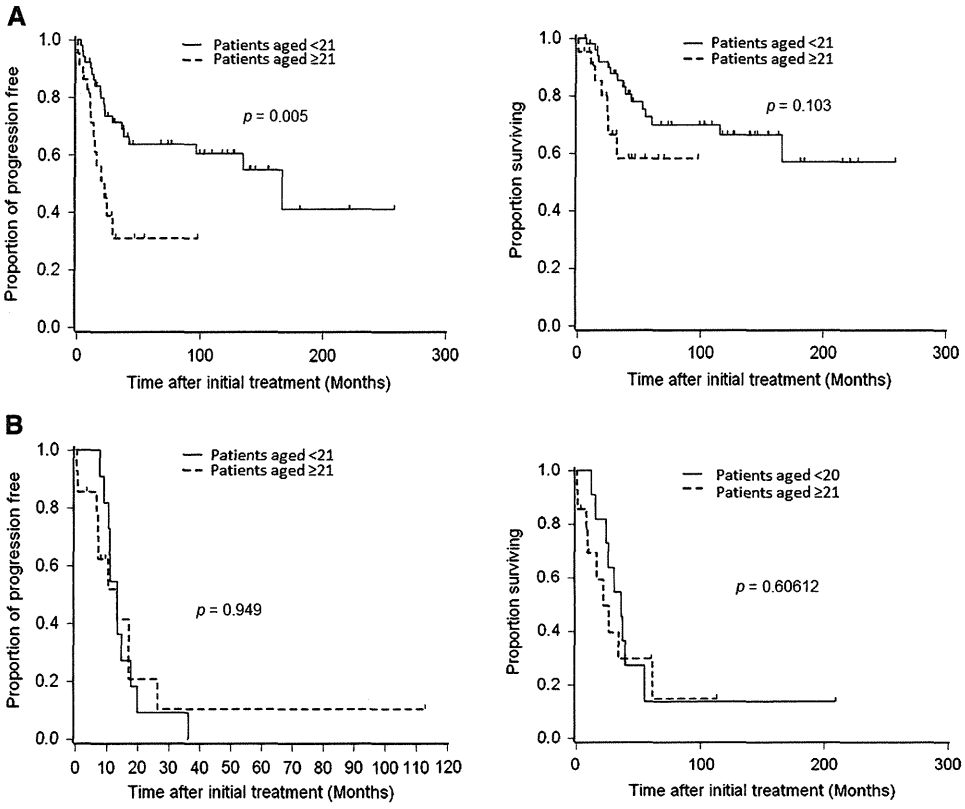

Fig. 1 **a** Kaplan–Meier curve of progression-free survival and overall survival in patients with localized disease of adults (*dashed line*) and children (*solid line*). **b** Kaplan–Meier curve of progression-free survival and overall survival in patients with metastatic disease of adults (*dashed line*) and children (*solid line*)

sites were bone ($n = 17$), bone marrow ($n = 7$), and lung ($n = 6$). Eleven patients had metastases to multiple sites.

Thirty-one patients (31.6 %) underwent surgical resection at RMS diagnosis. Ten patients were classified as IRSG Group I, 7 as Group II, and 14 as Groups III/IV. Surgery (primary tumor resection at diagnosis or second-look surgery) was performed in 43 patients (58.9 %) with localized disease and in 5 patients (20.0 %) with metastatic disease. We identified 59 patients (60.2 %) who had received VAC regimens and 39 patients (39.8 %) who had received VAC-like regimens. Radiation therapy was performed in 52 patients (71.2 %) with localized disease and in 18 patients (72.0 %) with metastatic disease during the course of treatment.

Patient outcomes in adults and children

The best responses to chemotherapy were as follows: among those with localized disease, 65 patients (89.0 %) achieved CR/PR, 5 patients (6.9 %) achieved SD/PD, and the data are not available for 3 patients (4.1 %); among patients with metastatic disease, 22 patients (88.0 %) achieved CR/PR and 3 patients (12.0 %) achieved SD/PD ($p = 1.000$). The overall median follow-up period was 37 months (range, 0–263 months); 37 months in metastatic disease (range, 0–213 months); and 43 months in localized disease (range, 0–263 months). At the time of analysis, 50 patients (51.0 %) experienced recurrence, and 41 of these patients (41.8 %) later died. Sites of first recurrence/progression were locoregional in 26 patients and distant metastases in 24 patients. Seven patients in whom the sites of first recurrence/progression were locoregional achieved CR and were still alive following second-line treatment.

Adult patients with localized disease had a significantly greater probability of poor outcome compared with children. The median PFS times for localized and metastatic disease for children and adults were as follows: localized disease, 166.9 versus 22.4 months ($p = 0.005$) (Fig. 1a), and metastatic disease, 13.3 versus 13.3 months ($p = 0.949$) (Fig. 1b), respectively. Median OS times were not statistically different in patients with metastatic disease for both adults and children.

Analyses of prognostic factors in localized and metastatic disease

To determine the independent predictors of survival, we used a multivariate Cox regression model. The results of multivariate analysis for PFS and OS in localized and metastatic disease are shown in Tables 4 and 5. According to Table 4, age (<21 vs. ≥21) was the only statistically significant negative predictor of PFS for patients with localized disease ($p = 0.018$). In contrast, for metastatic disease, age was not significantly different with respect to