Table 2. Relationship between gemcitabine therapy and clinicopathological factors

	Gemcitabine therapy		
	Treated (n = 23)	Not treated (n = 28)	*P*-value
Age (<65 : ⩾ 65 years)	13 : 10	12 : 16	0.3314
Sex (male:female)	11 : 12	14 : 14	0.8772
Histopathological type (well or mod:poor)	21 : 2	22 : 6	0.2134
Tumour size (<27 : ⩾27 mm)	12 : 11	12 : 16	0.5071
Tumour location (head:body or tail)	18 : 5	22 : 6	0.9786
Pathological depth of invasion pT (T1 or T2:T3)	2 : 21	1 : 27	0.4390
Pathological lymph node metastasis pN (negative:positive)	5 : 18	11 : 17	0.1790
Pathological stage (IA or IB or IIA:IIB or IV)	5 : 18	11 : 17	0.1790
SMARCC1 expression (−:+)	11 : 12	15 : 13	0.6830

Abbreviations: mod = moderately differentiated; poor = poorly differentiated; well = well differentiated.

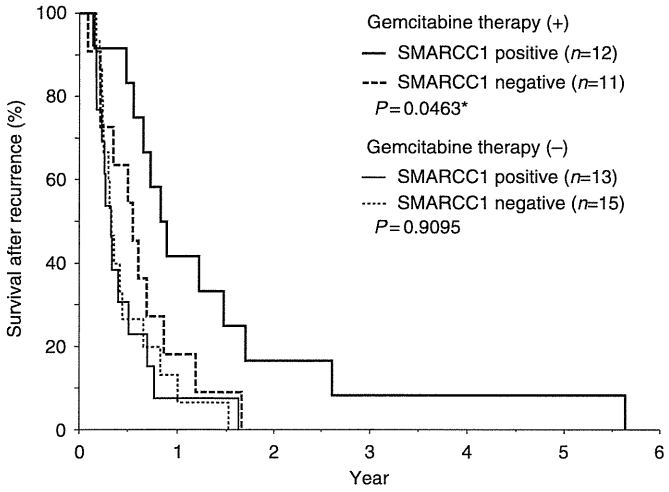


Figure 5. Relationship between SMARCC1 expression and survival after recurrence. Survival after recurrence curves showed a significantly better survival rate for SMARCC1-positive patients than for SMARCC1-negative patients treated with gemcitabine therapy (*$P = 0.0463$), but survival was not significantly different in patients treated without gemcitabine therapy ($P = 0.9095$).

miRNAs in gemcitabine-resistant pancreatic cancer cells compared with their parental cells, and we showed that miR-320c induced the resistance to gemcitabine. Among the putative targets of miR-320c by TargetScan, we focussed on SMARCC1, a switch/sucrose nonfermentable (SWI/SNF)-related matrix-associated actin-dependent regulator of chromatin subfamily C member 1, also known as BAF155 (BRG1-associated factor 155), as a candidate molecule responsible for miR-320-mediated drug resistance because of recent studies suggesting a role of chromatin remodeling in some cancers. Drug resistance is the major cause of treatment failure in cancer, yet the multifactorial mechanisms responsible for resistance remain largely unknown. Recently, several studies reported the contribution of chromatin remodeling in drug resistance in various types of cancer, such as the DEK oncogene in melanoma (Khodadoust *et al*, 2009), remodeling and spacing factor 1 in ovarian cancer (Choi *et al*, 2009), enhancer of zeste homolog 2 in pancreatic cancer (Ougolkov *et al*, 2008), and chromatin remodeling at the topoisomerase II-beta promoter in neuroblastoma (Das *et al*, 2010). Unlike DNA mutations, which are essentially irreversible in cancer, chromatin alterations, including both histone modifications and nucleosome remodeling, are potentially reversible and thus might constitute attractive therapeutic targets (Wilson and Roberts, 2011). The SWI/SNF chromatin remodeling complex is a 2-Mda multisubunit complex first identified in yeast and highly conserved among eukaryotes (Peterson, 1996). Transcriptional activation and efficient transcription of genes require dynamic structural changes in chromatin, and the ATP-dependent SWI/SNF complex is involved in chromatin restructuring (Percipalle and Farrants, 2006).

The SWI/SNF chromatin remodeling complex consists of a catalytic ATPase subunit, core subunits, and variant subunits. SMARCC1 is contained in the core subunits. The SWI/SNF complexes have a widespread role in tumour suppression (Wilson and Roberts, 2011). Inactivating deletion and mutations in SWI/SNF subunits have been reported at high frequency in various cancers, such as SNF5 in rhabdoid tumours (Versteege *et al*, 1998), BAF180 and ARID1A in renal cell carcinoma (Varela *et al*, 2011), ARID1A (Jones *et al*, 2010; Wiegand *et al*, 2010) and BAF155 (DelBove *et al*, 2011) in ovarian carcinoma, and BRG1, BRM, ARID1A, ARID1B, and BAF180 in pancreatic cancer (Shain *et al*, 2012). In addition, some SWI/SNF subunit deficiencies correlate with malignant potential, including drug resistance and shorter survival in melanoma (Lin *et al*, 2009) and in ovarian carcinoma (Katagiri *et al*, 2012) and glucocorticoid resistance in acute lymphoblastic leukaemia (Pottier *et al*, 2008), and as a severe risk factor for histologically malignant gastric cancer (Yamamichi *et al*, 2007). Some studies have reported that SMARCC1 deficiency prevents DNA damage–induced cell death (Ahn *et al*, 2011) and predicts short-term survival of colorectal cancer (Andersen *et al*, 2009). In addition, knockdown of SMARCC1 promotes self-renewal gene expression in embryonic stem cells (Schaniel *et al*, 2009). In the present study, we used MiaPaCa2-P and MiaPaCa2-RG1 and showed that knockdown of SMARCC1 induced gemcitabine resistance, and both gain-of-function and loss-of-function of miR-320c inversely altered the expression level of SMARCC1 protein. Although SMARCC1 may be only one of the responsible molecules, the molecule is shown to be involved, at least partly, in the miR-320c-related drug-resistance.

Evaluating the expression of miR-320c in clinical specimens may be crucial in predicting the drug-resistance, yet SMARCC1 may be practically easier and more useful than miR-320c. Thus, in the present study, we evaluated the clinical importance of SMARCC1 rather than miR-320c. We have previously reported RRM1 expression as the beneficial predictor of the clinical response to gemcitabine in pancreatic cancer patients after a complete resection (Akita *et al*, 2009). The present study revealed a significant association between SMARCC1 expression and the clinical response to gemcitabine therapy in completely resected pancreatic cancer patients. Therefore, RRM1, the key enzyme involved in DNA synthesis, and SMARCC1, the core subunit of the SWI/SNF chromatin remodeling complex, appear to make a contribution to drug-resistance mechanisms in separate processes and not to depend on each other. SMARCC1 expression could be a newly independent predictor of the clinical response to gemcitabine in pancreatic cancer patients.

In conclusion, we demonstrated in the present study that miR-320c inhibited the anti-cancer effect of gemcitabine in pancreatic cells and that SMARCC1 mediated this effect. The response to gemcitabine in MiaPaCa2 cells was controlled by genetic manipulation of miR-320c and SMARCC1. In addition, clinical examination revealed that only patients who were SMARCC1 positive benefited from gemcitabine therapy with regard to survival after recurrence. Considered together, the results suggest that miR-320c/SMARCC1-mediated gemcitabine resistance is a potential legitimate target for the treatment of pancreatic cancer.

ACKNOWLEDGEMENTS

We thank Nariaki Matsuura (Department of Molecular Pathology, Graduate School of Medicine, Osaka University, Osaka, Japan) for advice on immunohistochemical analysis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Ahn J, Ko M, Lee C, Kim J, Yoon H, Seong RH (2011) Srg3, a mouse homolog of BAF155, is a novel p53 target and acts as a tumor suppressor by modulating p21(WAF1/CIP1) expression. *Oncogene* **30**(4): 445–456.

Akita H, Zheng Z, Takeda Y, Kim C, Kittaka N, Kobayashi S, Marubashi S, Takemasa I, Nagano H, Dono K, Nakamori S, Monden M, Mori M, Doki Y, Bepler G (2009) Significance of RRM1 and ERCC1 expression in resectable pancreatic adenocarcinoma. *Oncogene* **28**(32): 2903–2909.

Ali S, Ahmad A, Banerjee S, Padhye S, Dominiak K, Schaffert JM, Wang Z, Philip PA, Sarkar FH (2010) Gemcitabine sensitivity can be induced in pancreatic cancer cells through modulation of miR-200 and miR-21 expression by curcumin or its analogue CDF. *Cancer Res* **70**(9): 3606–3617.

Andersen CL, Christensen LL, Thorsen K, Schepeler T, Sorensen FB, Verspaget HW, Simon R, Kruhoffer M, Aaltonen LA, Laurberg S, Orntoft TF (2009) Dysregulation of the transcription factors SOX4, CBFB and SMARCC1 correlates with outcome of colorectal cancer. *Br J Cancer* **100**(3): 511–523.

Bronisz A, Godlewski J, Wallace JA, Merchant AS, Nowicki MO, Mathsyaraja H, Srinivasan R, Trimboli AJ, Martin CK, Li F, Yu L, Fernandez SA, Pecot T, Rosol TJ, Cory S, Hallett M, Park M, Piper MG, Marsh CB, Yee LD, Jimenez RE, Nuovo G, Lawler SE, Chiocca EA, Leone G, Ostrowski MC (2012) Reprogramming of the tumour microenvironment by stromal PTEN-regulated miR-320. *Nat Cell Biol* **14**(2): 159–167.

Chen L, Yan HX, Yang W, Hu L, Yu LX, Liu Q, Li L, Huang DD, Ding J, Shen F, Zhou WP, Wu MC, Wang HY (2009) The role of microRNA expression pattern in human intrahepatic cholangiocarcinoma. *J Hepatol* **50**(2): 358–369.

Choi JH, Sheu JJ, Guan B, Jinawath N, Markowski P, Wang TL, Shih IeM (2009) Functional analysis of 11q13.5 amplicon identifies Rsf-1 (HBXAP) as a gene involved in paclitaxel resistance in ovarian cancer. *Cancer Res* **69**(4): 1407–1415.

Das CM, Zage PE, Taylor P, Aguilera D, Wolff JE, Lee D, Gopalakrishnan V (2010) Chromatin remodelling at the topoisomerase II-beta promoter is associated with enhanced sensitivity to etoposide in human neuroblastoma cell lines. *Eur J Cancer* **46**(15): 2771–2780.

Davidson JD, Ma L, Flagella M, Geeganage S, Gelbert LM, Slapak CA (2004) An increase in the expression of ribonucleotide reductase large subunit 1 is associated with gemcitabine resistance in non-small cell lung cancer cell lines. *Cancer Res* **64**(11): 3761–3766.

DelBove J, Rosson G, Strobeck M, Chen J, Archer TK, Wang W, Knudsen ES, Weissman BE (2011) Identification of a core member of the SWI/SNF complex, BAF155/SMARCC1, as a human tumor suppressor gene. *Epigenetics* **6**(12): 1444–1453.

Duan H, Jiang Y, Zhang H, Wu Y (2010) MiR-320 and miR-494 affect cell cycles of primary murine bronchial epithelial cells exposed to benzo[a]pyrene. *Toxicol In Vitro* **24**(3): 928–935.

Eguchi H, Nagano H, Yamamoto H, Miyamoto A, Kondo M, Dono K, Nakamori S, Umeshita K, Sakon M, Monden M (2000) Augmentation of antitumor activity of 5-fluorouracil by interferon alpha is associated with up-regulation of p27Kip1 in human hepatocellular carcinoma cells. *Clin Cancer Res* **6**(7): 2881–2890.

Gao W, Shen H, Liu L, Xu J, Shu Y (2011) MiR-21 overexpression in human primary squamous cell lung carcinoma is associated with poor patient prognosis. *J Cancer Res Clin Oncol* **137**(4): 557–566.

Giovannetti E, Funel N, Peters GJ, Del Chiaro M, Erozenci LA, Vasile E, Leon LG, Pollina LE, Groen A, Falcone A, Danesi R, Campani D, Verheul HM, Boggi U (2010) MicroRNA-21 in pancreatic cancer: correlation with clinical outcome and pharmacologic aspects underlying its role in the modulation of gemcitabine activity. *Cancer Res* **70**(11): 4528–4538.

Goan YG, Zhou B, Hu E, Mi S, Yen Y (1999) Overexpression of ribonucleotide reductase as a mechanism of resistance to 2,2-difluorodeoxycytidine in the human KB cancer cell line. *Cancer Res* **59**(17): 4204–4207.

Hwang JH, Voortman J, Giovannetti E, Steinberg SM, Leon LG, Kim YT, Funel N, Park JK, Kim MA, Kang GH, Kim SW, Del Chiaro M, Peters GJ, Giaccone G (2010) Identification of microRNA-21 as a biomarker for chemoresistance and clinical outcome following adjuvant therapy in resectable pancreatic cancer. *PloS One* **5**(5): e10630.

Ji Q, Hao X, Zhang M, Tang W, Yang M, Li L, Xiang D, Desano JT, Bommer GT, Fan D, Fearon ER, Lawrence TS, Xu L (2009) MicroRNA miR-34 inhibits human pancreatic cancer tumor-initiating cells. *PloS One* **4**(8): e6816.

Jones S, Wang TL, Shih IeM, Mao TL, Nakayama K, Roden R, Glas R, Slamon D, Diaz Jr. LA, Vogelstein B, Kinzler KW, Velculescu VE, Papadopoulos N (2010) Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. *Science* **330**(6001): 228–231.

Katagiri A, Nakayama K, Rahman MT, Rahman M, Katagiri H, Nakayama N, Ishikawa M, Ishibashi T, Iida K, Kobayashi H, Otsuki Y, Nakayama S, Miyazaki K (2012) Loss of ARID1A expression is related to shorter progression-free survival and chemoresistance in ovarian clear cell carcinoma. *Mod Pathol* **25**(2): 282–288.

Khodadoust MS, Verhaegen M, Kappes F, Riveiro-Falkenbach E, Cigudosa JC, Kim DS, Chinnaiyan AM, Markovitz DM, Soengas MS (2009) Melanoma proliferation and chemoresistance controlled by the DEK oncogene. *Cancer Res* **69**(16): 6405–6413.

Kondo M, Yamamoto H, Nagano H, Okami J, Ito Y, Shimizu J, Eguchi H, Miyamoto A, Dono K, Umeshita K, Matsuura N, Wakasa K, Nakamori S, Sakon M, Monden M (1999) Increased expression of COX-2 in nontumor liver tissue is associated with shorter disease-free survival in patients with hepatocellular carcinoma. *Clin Cancer Res* **5**(12): 4005–4012.

Li D, Xie K, Wolff R, Abbruzzese JL (2004) Pancreatic cancer. *Lancet* **363**(9414): 1049–1057.

Li Y, VandenBoom 2nd TG, Kong D, Wang Z, Ali S, Philip PA, Sarkar FH (2009) Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. *Cancer Res* **69**(16): 6704–6712.

Lin H, Wong RP, Martinka M, Li G (2009) Loss of SNF5 expression correlates with poor patient survival in melanoma. *Clin Cancer Res* **15**(20): 6404–6411.

Nakahira S, Nakamori S, Tsujie M, Takahashi Y, Okami J, Yoshioka S, Yamasaki M, Marubashi S, Takemasa I, Miyamoto A, Takeda Y, Nagano H, Dono K, Umeshita K, Sakon M, Monden M (2007) Involvement of ribonucleotide reductase M1 subunit overexpression in gemcitabine resistance of human pancreatic cancer. *Int J Cancer* **120**(6): 1355–1363.

Oettle H, Post S, Neuhaus P, Gellert K, Langrehr J, Ridwelski K, Schramm H, Fahlke J, Zuelke C, Burkart C, Gutberlet K, Kettner E, Schmalenberg H, Weigang-Koehler K, Bechstein WO, Niedergethmann M, Schmidt-Wolf I, Roll L, Doerken B, Riess H (2007) Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. *JAMA* **297**(3): 267–277.

Ougolkov AV, Bilim VN, Billadeau DD (2008) Regulation of pancreatic tumor cell proliferation and chemoresistance by the histone methyltransferase enhancer of zeste homologue 2. *Clin Cancer Res* **14**(21): 6790–6796.

Park JK, Lee EJ, Esau C, Schmittgen TD (2009) Antisense inhibition of microRNA-21 or -221 arrests cell cycle, induces apoptosis, and sensitizes

the effects of gemcitabine in pancreatic adenocarcinoma. *Pancreas* **38**(7): e190–e199.
Percipalle P, Farrants AK (2006) Chromatin remodelling and transcription: be-WICHed by nuclear myosin 1. *Curr Opin Cell Biol* **18**(3): 267–274.
Peterson CL (1996) Multiple SWItches to turn on chromatin? *Curr Opin Genet Dev* **6**(2): 171–175.
Pottier N, Yang W, Assem M, Panetta JC, Pei D, Paugh SW, Cheng C, Den Boer ML, Relling MV, Pieters R, Evans WE, Cheok MH (2008) The SWI/SNF chromatin-remodeling complex and glucocorticoid resistance in acute lymphoblastic leukemia. *J Natl Cancer Inst* **100**(24): 1792–1803.
Schaar DG, Medina DJ, Moore DF, Strair RK, Ting Y (2009) miR-320 targets transferrin receptor 1 (CD71) and inhibits cell proliferation. *Exp Hematol* **37**(2): 245–255.
Schaniel C, Ang YS, Ratnakumar K, Cormier C, James T, Bernstein E, Lemischka IR, Paddison PJ (2009) Smarcc1/Baf155 couples self-renewal gene repression with changes in chromatin structure in mouse embryonic stem cells. *Stem Cells* **27**(12): 2979–2991.
Schepeler T, Reinert JT, Ostenfeld MS, Christensen LL, Silahtaroglu AN, Dyrskjot L, Wiuf C, Sorensen FJ, Kruhoffer M, Laurberg S, Kauppinen S, Orntoft TF, Andersen CL (2008) Diagnostic and prognostic microRNAs in stage II colon cancer. *Cancer Res* **68**(15): 6416–6424.
Schmittgen TD, Jiang J, Liu Q, Yang L (2004) A high-throughput method to monitor the expression of microRNA precursors. *Nucleic Acids Res* **32**(4): e43.
Shain AH, Giacomini CP, Matsukuma K, Karikari CA, Bashyam MD, Hidalgo M, Maitra A, Pollack JR (2012) Convergent structural alterations define SWItch/Sucrose NonFermentable (SWI/SNF) chromatin remodeler as a central tumor suppressive complex in pancreatic cancer. *Proc Natl Acad Sci USA* **109**(5): E252–E259.
Shen YL, Jiang YG, Greenlee AR, Zhou LL, Liu LH (2009) MicroRNA expression profiles and miR-10a target in anti-benzo[a] pyrene-7, 8-diol-9, 10-epoxide-transformed human 16HBE cells. *Biomed Environ Sci* **22**(1): 14–21.
Tomimaru Y, Eguchi H, Nagano H, Wada H, Tomokuni A, Kobayashi S, Marubashi S, Takeda Y, Tanemura M, Umeshita K, Doki Y, Mori M (2010) MicroRNA-21 induces resistance to the anti-tumour effect of interferon-alpha/5-fluorouracil in hepatocellular carcinoma cells. *Br J Cancer* **103**(10): 1617–1626.
Tomokuni A, Eguchi H, Tomimaru Y, Wada H, Kawamoto K, Kobayashi S, Marubashi S, Tanemura M, Nagano H, Mori M, Doki Y (2011) miR-146a suppresses the sensitivity to interferon-alpha in hepatocellular carcinoma cells. *Biochem Biophys Res Commun* **414**(4): 675–680.
Varela I, Tarpey P, Raine K, Huang D, Ong CK, Stephens P, Davies H, Jones D, Lin ML, Teague J, Bignell G, Butler A, Cho J, Dalgliesh GL, Galappaththige D, Greenman C, Hardy C, Jia M, Latimer C, Lau KW, Marshall J, McLaren S, Menzies A, Mudie L, Stebbings L, Largaespada DA, Wessels LF, Richard S, Kahnoski RJ, Anema J, Tuveson DA, Perez-Mancera PA, Mustonen V, Fischer A, Adams DJ, Rust A, Chan-on W, Subimerb C, Dykema K, Furge K, Campbell PJ, Teh BT, Stratton MR, Futreal PA (2011) Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. *Nature* **469**(7331): 539–542.
Versteege I, Sevenet N, Lange J, Rousseau-Merck MF, Ambros P, Handgretinger R, Aurias A, Delattre O (1998) Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. *Nature* **394**(6689): 203–206.
Wiegand KC, Shah SP, Al-Agha OM, Zhao Y, Tse K, Zeng T, Senz J, McConechy MK, Anglesio MS, Kalloger SE, Yang W, Heravi-Moussavi A, Giuliany R, Chow C, Fee J, Zayed A, Prentice L, Melnyk N, Turashvili G, Delaney AD, Madore J, Yip S, McPherson AW, Ha G, Bell L, Fereday S, Tam A, Galletta L, Tonin PN, Provencher D, Miller D, Jones SJ, Moore RA, Morin GB, Oloumi A, Boyd N, Aparicio SA, Shih IeM, Mes-Masson AM, Bowtell DD, Hirst M, Gilks B, Marra MA, Huntsman DG (2010) ARID1A mutations in endometriosis-associated ovarian carcinomas. *N Engl J Med* **363**(16): 1532–1543.
Wilson BG, Roberts CW (2011) SWI/SNF nucleosome remodellers and cancer. *Nat Rev Cancer* **11**(7): 481–492.
Yamamichi N, Inada K, Ichinose M, Yamamichi-Nishina M, Mizutani T, Watanabe H, Shiogama K, Fujishiro M, Okazaki T, Yahagi N, Haraguchi T, Fujita S, Tsutsumi Y, Omata M, Iba H (2007) Frequent loss of Brm expression in gastric cancer correlates with histologic features and differentiation state. *Cancer Res* **67**(22): 10727–10735.
Yamamoto H, Kondo M, Nakamori S, Nagano H, Wakasa K, Sugita Y, Chang-De J, Kobayashi S, Damdinsuren B, Dono K, Umeshita K, Sekimoto M, Sakon M, Matsuura N, Monden M (2003) JTE-522, a cyclooxygenase-2 inhibitor, is an effective chemopreventive agent against rat experimental liver fibrosis1. *Gastroenterology* **125**(2): 556–571.
Yamamoto T, Nagano H, Sakon M, Wada H, Eguchi H, Kondo M, Damdinsuren B, Ota H, Nakamura M, Marubashi S, Miyamoto A, Dono K, Umeshita K, Nakamori S, Yagita H, Monden M (2004) Partial contribution of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor pathway to antitumor effects of interferon-alpha/5-fluorouracil against hepatocellular carcinoma. *Clin Cancer Res* **10**(23): 7884–7895.
Yan LX, Huang XF, Shao Q, Huang MY, Deng L, Wu QL, Zeng YX, Shao JY (2008) MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. *RNA* **14**(11): 2348–2360.
Yang MH, Chen CL, Chau GY, Chiou SH, Su CW, Chou TY, Peng WL, Wu JC (2009) Comprehensive analysis of the independent effect of twist and snail in promoting metastasis of hepatocellular carcinoma. *Hepatology* **50**(5): 1464–1474.
Zhang XJ, Ye H, Zeng CW, He B, Zhang H, Chen YQ (2010) Dysregulation of miR-15a and miR-214 in human pancreatic cancer. *J Hematol Oncol* **3**: 46.
Zhao JJ, Yang J, Lin J, Yao N, Zhu Y, Zheng J, Xu J, Cheng JQ, Lin JY, Ma X (2009) Identification of miRNAs associated with tumorigenesis of retinoblastoma by miRNA microarray analysis. *Childs Nerv Syst* **25**(1): 13–20.

Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)

IJC
International Journal of Cancer

Induction of CD8 T-cell responses restricted to multiple HLA class I alleles in a cancer patient by immunization with a 20-mer NY-ESO-1f (NY-ESO-1 91-110) peptide

Shingo Eikawa[1,2], Kazuhiro Kakimi[3], Midori Isobe[2], Kiyotaka Kuzushima[4], Immanuel Luescher[5], Yoshihiro Ohue[2], Kazuhiro Ikeuchi[1], Akiko Uenaka[6], Hiroyoshi Nishikawa[7], Heiichiro Udono[1], Mikio Oka[2] and Eiichi Nakayama[6]

[1] Department of Immunology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan
[2] Department of Respiratory Medicine, Kawasaki Medical School, Kurashiki, Japan
[3] Department of Immunotherapeutics, University of Tokyo Hospital, Tokyo, Japan
[4] Department of Immunology, Aichi Cancer Center, Nagoya, Japan
[5] Ludwig Institute for Cancer Research, University of Lausanne, Epalinges, Switzerland
[6] Faculty of Health and Welfare, Kawasaki University of Medical Welfare, Kurashiki, Japan
[7] Department of Experimental Immunology, Immunology Frontier Research Center, Osaka University, Osaka, Japan

Immunogenicity of a long 20-mer NY-ESO-1f peptide vaccine was evaluated in a lung cancer patient TK-f01, immunized with the peptide with Picibanil OK-432 and Montanide ISA-51. We showed that internalization of the peptide was necessary to present CD8 T-cell epitopes on APC, contrasting with the direct presentation of the short epitope. CD8 T-cell responses restricted to all five HLA class I alleles were induced in the patient after the peptide vaccination. Clonal analysis showed that B*35:01 and B*52:01-restricted CD8 T-cell responses were the two dominant responses. The minimal epitopes recognized by A*24:02, B*35:01, B*52:01 and C*12:02-restricted CD8 T-cell clones were defined and peptide/HLA tetramers were produced. NY-ESO-1 91-101 on A*24:02, NY-ESO-1 92-102 on B*35:01, NY-ESO-1 96-104 on B*52:01 and NY-ESO-1 96-104 on C*12:02 were new epitopes first defined in this study. Identification of the A*24:02 epitope is highly relevant for studying the Japanese population because of its high expression frequency (60%). High affinity CD8 T-cells recognizing tumor cells naturally expressing the epitopes and matched HLA were induced at a significant level. The findings suggest the usefulness of a long 20-mer NY-ESO-1f peptide harboring multiple CD8 T-cell epitopes as an NY-ESO-1 vaccine. Characterization of CD8 T-cell responses in immunomonitoring using peptide/HLA tetramers revealed that multiple CD8 T-cell responses comprised the dominant response.

The NY-ESO-1 antigen was originally identified in esophageal cancer by serological expression cloning (SEREX) using autologous patient serum.[1,2] NY-ESO-1 expression is observed in a wide range of human malignancies, but the expression is restricted to germ cells in the testes in normal adult tissues.[1–4] Therefore, NY-ESO-1 has emerged as a prototype of a class of cancer/testis (CT) antigens.[5] The efficacy of the NY-ESO-1 antigen as a cancer vaccine has been studied extensively using various preparations, *e.g.*, peptide, protein or DNA, etc. of the antigen with various adjuvants.[6–14] These studies established the safety of the NY-ESO-1 vaccine and demonstrated its immunogenicity.

In a phase I clinical trial, we immunized cancer patients with a complex of cholesterol-bearing hydrophobized pullulan and NY-ESO-1 whole protein (CHP-NY-ESO-1) and showed that the vaccine had potent capacity to induce the NY-ESO-1 antibody in vaccinated patients.[13,14] The most dominant serological antigenic epitope was NY-ESO-1 91-108. The CHP-NY-ESO-1 vaccine also elicited CD4 and CD8 T-cell responses in immunized patients.[14] Analysis of T cell responses against overlapping peptides (OLPs) spanning the NY-ESO-1 molecule revealed that two dominant NY-ESO-1 regions, regions II (73-114) and III (121-144), were recognized by either CD4 or CD8 T-cells in most patients irrespective of their HLA type. Essentially similar findings were obtained by studies using other preparations of NY-ESO-1 protein vaccine.[11,12,15]

Key words: cancer vaccine, NY-ESO-1, long peptide, CD8 T-cell response
Additional Supporting Information may be found in the online version of this article.
This article was published online on 22 June 2012. An error was subsequently identified. This notice is included in the online and print versions to indicate that both have been corrected 21 November 2012.
Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant-in-Aid for Scientific Research (B)), New Energy and Industrial Technology Development Organization (NEDO), Japan
DOI: 10.1002/ijc.27682
History: Received 11 Jan 2012; Accepted 1 Jun 2012; Online 22 Jun 2012
Correspondence to: Eiichi Nakayama, M.D., Faculty of Health and Welfare, Kawasaki University of Medical Welfare, 288 Matsushima, Kurashiki, Okayama 701-0193, Japan, Tel.: +81-86-462-1111 ext. 54954, Fax: +81-86-464-1109, E-mail: nakayama@mw.kawasaki-m.ac.jp

What's new?

An antigen called NY-ESO-1 is expressed by a wide range of human cancers, and has shown promise as a cancer vaccine. In this study, the authors studied a peptide derived from that antigen, and analyzed the cellular and molecular mechanisms that allow the peptide to provoke an immune response. They found that the peptide must be internalized by antigen-presenting cells (APCs) in order to yield T-cells that can attack tumours via the NY-ESO-1 antigen. These data increase our understanding of the requirements for an effective therapeutic cancer vaccine. (This section added after initial online publication.)

CD8 T-cells induced by immunization with NY-ESO-1 class I short epitope peptides have been shown to be of low affinity and do not recognize naturally processed NY-ESO-1 on tumor cells.[16] However, the advantage of synthetic long peptides over short peptides for use as vaccines has been reported.[17] Long peptides do not bind to MHC class I molecules directly, and require antigen processing by dendritic cells to be presented. Therefore, the use of long peptides prevents the antigen peptides from direct binding to MHC class I molecules on nonprofessional antigen-presenting cells (APC), which may cause transient activation and subsequent anergy of CTLs in the absence of appropriate costimulatory signals.[17–19] Based on these findings, we recently used a long peptide spanning a peptide region NY-ESO-1 91-110 (NY-ESO-1f peptide) which included the dominant serological antigenic epitope and overlapped one of the two dominant regions recognized by CD4 and CD8 T-cells for a vaccine in a clinical trial.[20] Ten patients received the NY-ESO-1f peptide vaccine. The NY-ESO-1f peptide vaccine was well tolerated and elicited humoral, CD4 and CD8 T-cell responses in immunized patients.

In this study, we demonstrated that internalization of the peptide was necessary to present CD8 T-cell epitopes on APC treated with the long 20-mer NY-ESO-1f peptide. Analysis of the CD8 T-cell response in an NY-ESO-1f peptide-immunized patient revealed occurrence of responses restricted to all five HLA class I alleles defined in the patient. The frequency of A*24:02, B*35:01, B*52:01, C*03:03 and C*12:02-restricted CD8 T-cells in PBMCs was defined by clonal analysis revealing B*35:01- and B*52:01-restricted CD8 T-cell responses as dominant. By establishing clones from those HLA-restricted CD8 T-cells, new epitopes on A*24:02, B*35:01, B*52:01 and C*12:02 were defined and peptide/HLA tetramers were prepared. Clonal analysis showed that CD8 T-cells that recognize natural epitopes on tumor cells were induced in a significant proportion by immunization with the NY-ESO-1f peptide. Immunomonitoring using the tetramers revealed that multiple CD8 T-cell responses comprised the dominant response.

Material and Methods

Clinical trial

A phase I clinical trial of the NY-ESO-1f peptide vaccine was conducted to evaluate the safety, immune response and tumor response.[20] Patients with advanced cancers that were refractory to standard therapy and expressed NY-ESO-1 as assessed by immunohistochemistry (IHC) were eligible. The protocol was approved by the Ethics Committee of Tokyo, Osaka and Okayama Universities in light of the Declaration of Helsinki. Written informed consent was obtained from each patient before enrolling in the study. The study was performed in compliance with Good Clinical Practice. The study was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (Unique trial number: UMIN000001260) on July 24, 2008 (UMIN-CTRURL: http://www.umin.ac.jp/ctr/ index.htm).

Blood samples

Patient TK-f01 was a lung cancer patient who received a right middle lobectomy in October, 2004.[20] As the tumor continued to grow despite chemotherapy, he was enrolled in the study in June, 2008. The patient received 12 vaccinations once every 3 weeks. Peripheral blood was drawn from patient TK-f01 with informed consent for immunological monitoring. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density gradient centrifugation using a Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). CD4-, CD8- and CD19-positive cells were purified by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). The residual cells were kept for use as APC. The cells were stored in liquid N_2 until use. HLA tying was done with PBMCs by a sequence-specific oligonucleotide probe and sequence-specific priming of genomic cDNA using a standard procedure.

Cell lines

LC99A and OU-LC-OK are lung cancer cell lines. SK-OV3 is an ovarian cancer cell line and SK-MEL37 is a melanoma cell line. These cell lines were kept by serial passage in tissue culture. EBV-B cells were generated from CD19-positive peripheral blood B cells using a culture supernatant from EBV-producing B95-8 cells. The medium used to maintain these cell lines was RPMI1640 supplemented with 10% FCS (JRM, Bioscience, Lenexa, KA), 2 mmol/l Glutamax, antibitotics, and 10 mmol/l HEPES (Invitrogen, Carlsbad, CA).

Antibodies

Anti-human CD4, anti-human CD8, anti-HLA class I and anti-HLA class II mAbs were purchased from BD Bioscience (San Jose, CA).

Peptides

The following series of 28 18-mer OLPs and a C-terminal 30-mer peptide spanning the entire NY-ESO-1 protein were used: 18.1 (1-18), 18.2 (7-24), 18.3 (13-30), 18.4 (19-36), 18.5 (25-42), 18.6 (31- 48), 18.7 (37-54), 18.8 (43-60), 18.9 (49-

66), 18.10 (55-72), 18.11 (61-78), 18.12 (67-84), 18.13 (73-90), 18.14 (79-96), 18.15 (85-102), 18.16 (91-108), 18.17 (97-114), 18.18 (103-120), 18.19 (109-126), 18.20 (115-132), 18.21 (121-138), 18.22 (127-144), 18.23 (133-150), 18.24 (139-156), 18.25 (145-162), 18.26 (149-166), 18.27 (153-170), 18.28 (156-173) and 30.9 (151-180). Various N- and C-termini truncated peptides in the NY-ESO-1f peptide were also used. These peptides were synthesized using standard solid-phase methods based on *N*-(9-fluorenyl)-methoxycarbonyl (Fmoc) chemistry on an ABIMED Multiple Peptide Synthesizer (AMS422, ABIMED, Langenfeld, Germany) at Okayama University (Okayama, Japan). The carboxyfluorescein (FAM)-conjugated NY-ESO-1f peptide (5(6)-FAM-YLAMPFATPMEAELARRSLA) was synthesized by Operon (Tokyo, Japan).

Recombinant NY-ESO-1 protein

Recombinant NY-ESO-1 protein was prepared as described earlier.[13] NY-ESO-1 cDNA was cloned into the *Sph*I/*Sal*I and *Bam*HI/*Sph*I sites of the pQE-30 vector. N-His tagged protein was purified by nickel-ion affinity chromatography under denaturing conditions.

Preparation of immature dendritic cells

Monocytes were isolated from PBMCs using anti-CD14 mAb-coated magnetic beads (Miltenyi Biotec) and cultured in AIM-V medium supplemented with 5% heat-inactivated pooled human serum, 10 ng/ml rhGM-CSF (Kyowa Hakko Kirin, Tokyo, Japan) and 10 ng/ml rhIL-4 (PeproTech) for 7 days at 37°C in a 5% CO_2 atmosphere.

In vitro stimulation of bulk CD4 and CD8 T-cells

CD4 and CD8 T-cells (2×10^6/well) were cultured with a mixture of 29 NY-ESO-1 OLPs (10^{-6} M) in the presence of an equal number of irradiated (40 Gy), autologous CD4- and CD8-depleted cells as APC in a 24-well culture plate (BD Bioscience) for 12 days at 37°C in a 5% CO_2 atmosphere. The medium was AIM-V (Invitrogen) supplemented with 5% heat-inactivated pooled human serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 units/ml recombinant human (rh) IL-2 (Takeda Chemical Industries, Osaka, Japan) and 10 ng/ml rhIL-7 (Peprotech, London, UK).

Cloning of CD8 T-cells

CD8 T-cells were cloned by limiting dilution after *in vitro* stimulation in round-bottomed 96-well plates in the presence of irradiated (40 Gy) allogeneic PBMCs freshly prepared from the healthy donor as feeder cells. The medium used was AIM-V (Invitrogen) supplemented with 5% heat-inactivated pooled human serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 units/ml rhIL-2 (Takeda Chemical Industries), 10 ng/ml rhIL-7 (Peprotech) and 1 μg/ml phytohemagglutinin (PHA)-L (Sigma-Aldrich).

Clonal expansion

Cloned CD8 T-cells (1×10^3) obtained by limiting dilution were expanded in a round-bottomed 96 well plate in the presence of irradiated (40 Gy) PBMCs (5×10^4) freshly prepared from allogeneic healthy donors as feeder cells. Fresh medium was added every 3 days. After culture for 14 days, the cells were harvested and kept frozen at 2×10^6/tube. The medium used was AIM-V supplemented with 5% heat-inactivated pooled human serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 units/ml rhIL-2, 10 ng/ml rhIL-7 and 1 μg/ml phytohemagglutinin (PHA)-L.

IFNγ capture assay

Bulk CD4 or CD8 T-cells (1×10^5) from the *in vitro* stimulation culture were cultured with autologous or allogeneic EBV-B cells (1×10^5) pulsed with OLPs for 4 hr. The cells were then treated with a bi-specific CD45 and IFNγ antibody (IFNγ catch reagent) (2 μl) for 5 min on ice. The cells were diluted in AIM-V medium (3 ml) and placed on a slow rotating device (Miltenyi Biotec) to allow IFNγ secretion at 37°C in a 5% CO_2 atmosphere. After incubation for 45 min, the cells were washed with cold buffer and treated with PE-conjugated anti-IFNγ (detection reagent), and FITC-conjugated anti-CD4 or anti-CD8 mAb. After incubation for 10 min at 4°C, the cells were washed and analyzed by a FACS Canto II (BD Bioscience).

Tetramer construction and staining

Peptide/HLA tetramers were produced as described earlier.[21,22] NY-ESO-1 91-101/A*24:02, NY-ESO-1 92-100/B*35:01, NY-ESO-1 92-102/B*35:01, NY-ESO-1 94-104/B*35:01 and NY-ESO-1 96-106/C*12:02 tetramers were used. The HIV Env/A*24:02 tetramer was used as a control. For staining, cells were incubated with tetramer at a concentration of 20 μg/ml for 15 min at 37°C, followed by incubation with an FITC-conjugated anti-CD8 mAb (Miltenyi Biotec) on ice for 15 min and analyzed by a FACS Canto II (Becton Dickinson).

Cytotoxicity assay

Cytotoxicity was assayed by a luminescent method using the aCella-Tox kit (Cell Technology, Mountain View, CA). Effector cells were incubated with 5,000 target cells at various ratios in 96-well round bottomed culture plates for 12 hr at 37°C in a 5% CO_2 atmosphere. The plate was read by a luminometer (multi-detection microplate reader, DS Pharma, Osaka, Japan).

IFNγ ELISA

CD8 T-cell clones (5×10^3) were cultured with autologous or allogeneic EBV-B cells (5×10^3) pulsed with the peptides in a 96-well round bottomed culture plate for 24 hr at 37°C in a 5% CO_2 atmosphere. Culture supernatants were then collected and the amount of IFNγ was measured by sandwich ELISA. For antibody blocking experiments, each mAb (5 μg/ml) was added to the assay culture. To inhibit internalization of the antigens to DCs, cytochalasin B (10 μM) was added to the culture.

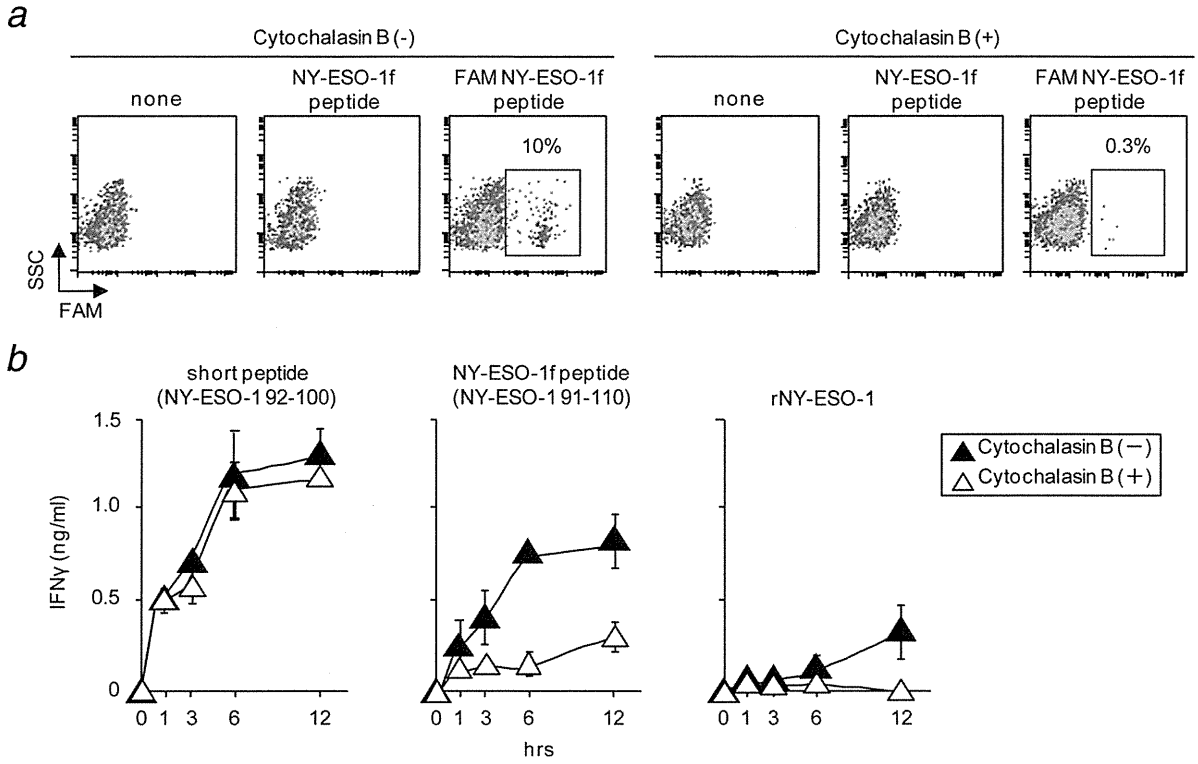


Figure 1. Internalization and processing of the long 20-mer NY-ESO-1f-peptide by APC in CD8 T-cell recognition. In (*a*), immature dendritic cells (iDCs) from a healthy donor (HD) NO PBMCs (A*02:07/*26:02, B*35:01/*46:01, C*08:03/*14:03) were cultured with an NY-ESO-1f peptide (10 μM) or a FAM NY-ESO-1f peptide (10 μM) for 12 hr in the presence or absence of cytochalasin B (10 μM). After culture, the internalization of the FAM NY-ESO-1f peptide was analyzed by FACS Canto II. In (*b*), the CD8 T-cell clone 2H10 (5×10^3) was stimulated with iDCs (5×10^3) pulsed with the short peptide (NY-ESO-1 92-100: LAMPFATPM, 9-mer; 1 μM), the NY-ESO-1f peptide (NY-ESO-1 91-110: YLAMPFATPMEAELARRSLA, 20-mer; 1 μM) or recombinant NY-ESO-1 protein (1 μM) for the indicated time in the presence or absence of cytochalasin B (10 μM). The amount of IFNγ in the culture supernatant was determined by ELISA. One representative of three independent experiments is shown.

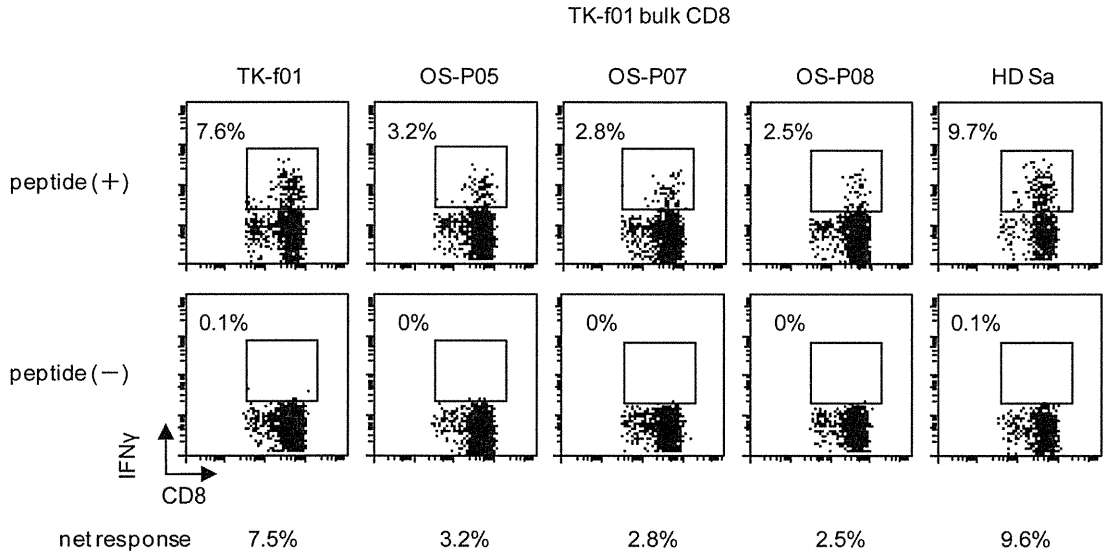
Results

Internalization of 20-mer NY-ESO-1f peptide to present CD8 T-cell epitopes on antigen presenting cells

We investigated the need for internalization of the 20-mer NY-ESO-1f peptide (NY-ESO-1 91-110: YLAMPFATPMEAELARRSLA) to present CD8 T-cell epitopes on APC. Immature dendritic cells (iDCs) were prepared from purified CD14-positive cells from a B*35:01 healthy donor by treating them with IL-4 and GM-CSF for 7 days and used as APC. As shown in Figure 1*a*, internalization of the FAM-conjugated NY-ESO-1f peptide was observed in approximately 10% of DCs after culture with the peptide for 12 hr. Treatment of DCs with the peptide in the presence of cytochalasin B diminished internalization. Recognition by a B*35:01-restricted CD8 T-cell clone 2H10 of DCs treated with the peptides was investigated. As shown in Figure 1*b*, clone 2H10 recognized DCs pulsed with the short peptide NY-ESO-1 92-100 in the presence of cytochalasin B. On the other hand, recognition of DCs pulsed with NY-ESO-1f peptide was inhibited in the presence of cytochalasin B. Only marginal recognition was observed with DCs pulsed with recombinant NY-ESO-1 protein.

Multiple HLA class I-restricted CD8 T-cell responses in PBMCs from patient TK-f01 immunized with the 20-mer NY-ESO-1f peptide

TK-f01 was a lung adenocarcinoma patient immunized with the NY-ESO-1f peptide (600 μg) with Picibanil OK-432 and Montanide ISA-51 12 times once every 3 weeks.[20] We investigated multiple HLA class I-restricted CD8 T-cell responses in PBMCs from patient TK-f01. The patient's HLA class I was A*24:02, B*35:01, B*52:01, C*03:03 and C*12:02. Purified CD8 T-cells from the patient's PBMCs obtained at day 64 after the third vaccination were stimulated once with a mixture of 29 18-mer series of NY-ESO-1 OLPs spanning the entire protein for 12 days using CD4 and CD8-depleted PBMCs as APC. The cells were harvested and stimulated with NY-ESO-1f peptide for 4 hrs using autologous and allogeneic EBV-B cells as APC and the response was assayed by IFNγ capture assay. As shown in Figure 2, TK-f01 CD8 T-cells responded strongly to NY-ESO-1f peptide-pulsed autologous EBV-B cells. Use of various allogeneic EBV-B cells as APC showed TK-f01 CD8 T-cell responses against NY-ESO-1f peptide presented on multiple HLA class I molecules shared with patient TK-f01.



HLA class I

EBV-B	A	B	C
TK-f01	***24:02**	***35:01**, ***52:01**	***03:03**, ***12:02**
OS-P05	***24:02**, *24:20	*51:01, ***52:01**	***12:02**, *14:02
OS-P07	*02:06, ***24:02**	*46:01, *51:01	*01:02, *14:02
OS-P08	***24:02**	*40:02, *59:01	*01:02, ***03:03**
HD Sa	***24:02**, *33:03	***35:01**, *44:03	***03:03**

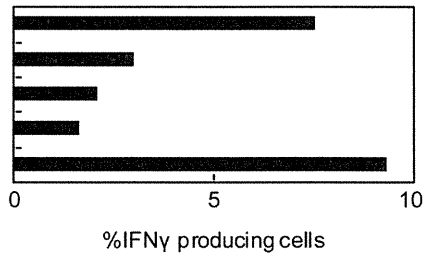


Figure 2. Multiple HLA class I-restricted CD8 T-cell responses in patient TK-f01 immunized with the 20-mer NY-ESO-1f peptide. CD8 T-cells (2×10^6) were stimulated with a mixture of NY-ESO-1 OLPs (10^{-6} M) in the presence of APC for 12 days. The cells were harvested and stimulated with NY-ESO-1f peptide for 4 hr using autologous and allogeneic EBV-B cells as APC and the response was assayed by an IFNγ capture assay.

Clonal analysis of the multiple HLA class I-restricted TK-f01 CD8 T-cell responses against the NY-ESO-1f peptide

The multiple HLA class I-restricted TK-f01 CD8 T-cell responses against the NY-ESO-1f peptide were investigated by clonal analysis. Purified CD8 T-cells from the patient's PBMCs obtained at day 64 after the third vaccination were stimulated once with a mixture of NY-ESO-1 OLPs for 12 days using CD4 and CD8-depleted PBMCs as APC as above. The cells were then collected and seeded at 3 cells per well in 96-well culture plates to expand clones. After culture for 14 days, 300 clones were obtained. Each of the 300 clones were split into 7 and the responses against NY-ESO-1f peptide on autologous, OS-P06, OS-P08, HD NM, HD NO, OY-P04 and OY-P02 EBV-B cells shown in Figure 3*b* were investigated by IFNγ ELISA for the culture supernatant to determine restriction molecules. As shown in Table 1, the frequencies of B*35:01-, B*52:01-, A*24:02-, C*12:02- and C*03:03-restricted CD8 T-cell clones were 11.0, 9.3, 2.3, 1.3 and 0.7%, respectively. The frequencies of CD8 T-cell clones determined after clonal expansion were comparable to the results shown in Figure 2.

The frequency of NY-ESO-1f peptide-reactive CD8 T-cells in PBMCs restricted to each allele was calculated assuming the doubling time of the cells to be 24 hr during the culture period for 12 days as listed in Table 1.

Determination of minimal epitopes derived from the 20-mer NY-ESO-1f peptide recognized by TK-f01 CD8 T-cell clones in restriction to A*24:02, B*35:01, B*52:01 and C*12:02

We established stably proliferating TK-f01 CD8 T-cell clones: 4E10, 2H10, 8D5, 10-10U and 7B recognizing the 20-mer NY-ESO-1f peptide in restriction to A*24:02, B*35:01, B*35:01, B*52:01 and C*12:02, respectively, from the culture shown in Table 1 and determined the minimal epitopes. Figure 3 shows the confirmation of the restriction molecule and determination of a minimal epitope in recognition of the NY-ESO-1f peptide by the CD8 T-cell clone 4E10. As shown in Figure 3*a*, the response was blocked by anti-CD3 mAb, anti-CD8 mAb and anti-HLA class I mAb, but not anti-CD4 mAb or anti-class II mAb, confirming that the response was HLA class I restricted. As shown in Figure 3*b*, use of a panel

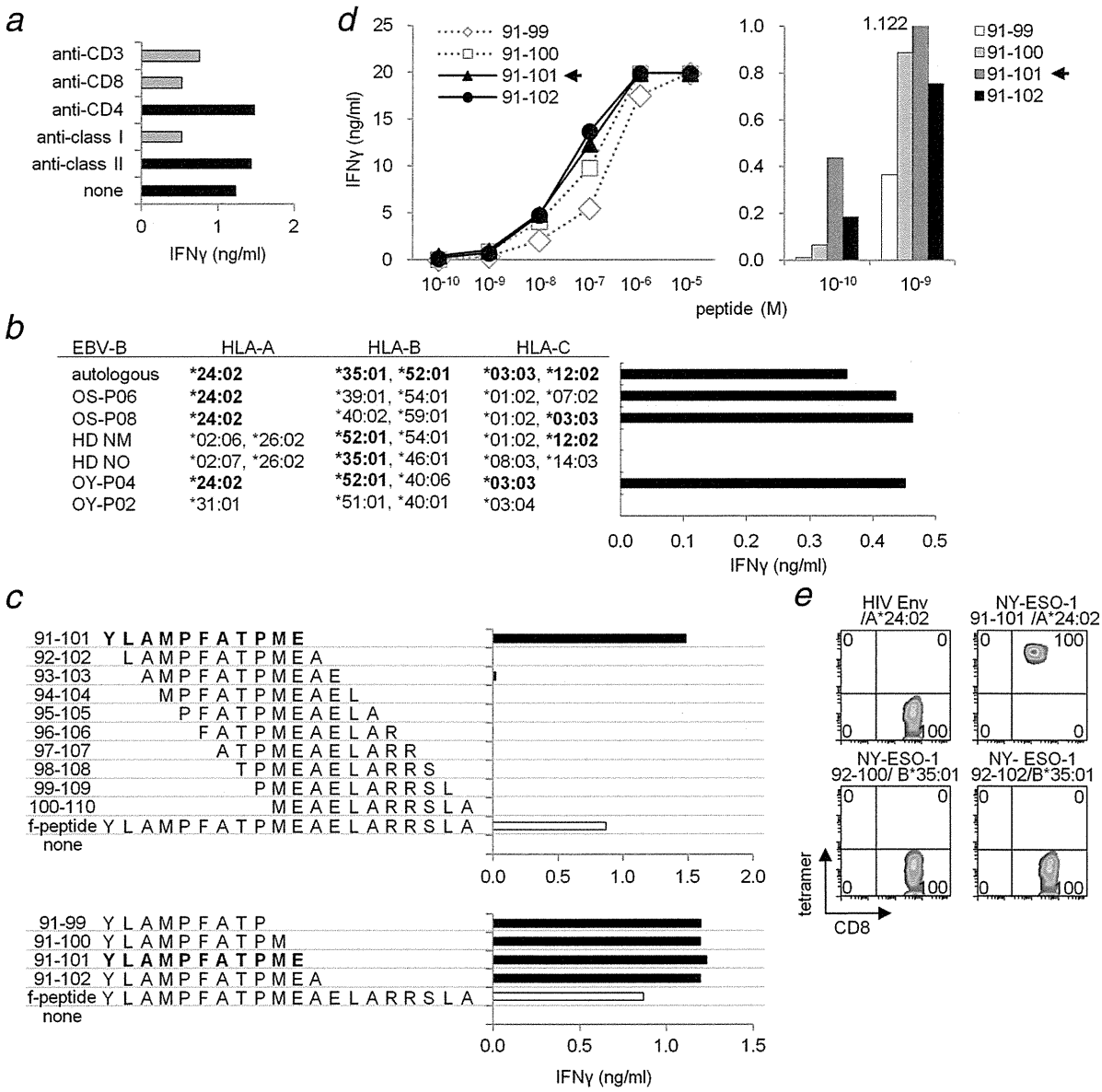


Figure 3. The restriction molecule and the minimal epitope in recognition of NY-ESO-1f peptide by the CD8 T-cell clone 4E10. A CD8 T-cell clone 4E10 (5×10^3) obtained from the culture shown in Table 1 was cultured with autologous EBV-B cells pulsed with the NY-ESO-1f-peptide (1 μM). The restriction molecule was analyzed by antibody blocking (5 μg/ml) (*a*) and using various EBV-B cells as APC (*b*). The minimal epitope was determined by using various N- and C-termini truncated peptides (1 μM) (*c*) and by titration of the peptide concentration (*d*). In (*e*), the binding of the NY-ESO-1 91-101/A*24:02 tetramer to the clone 4E10 is shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 1. Clonal analysis of multiple HLA class I-restricted CD8 T cell responses against the NY-ESO-1f peptide

HLA	Positives/total number of clones	Calculated frequency of CD8 T-cells in PBMCs
A*24:02	7/300 (2.3%)	5.7×10^{-6}
B*35:01	33/300 (11.0%)	2.7×10^{-5}
B*52:01	28/300 (9.3%)	2.3×10^{-5}
C*03:03	2/300 (0.7%)	1.6×10^{-6}
C*12:02	4/300 (1.3%)	3.3×10^{-6}

The other 226 clones (75.4%) showed no reaction against NY-ESO-1f peptide on seven EBV-B cells (see Text).

of allogeneic EBV-B cells as APC to present the NY-ESO-1f peptide confirmed the response of clone 4E10 was A*24:02-restricted. As shown in Figures 3*c* and 3*d*, use of various N- and C-termini truncated peptides and titration of the peptide concentration revealed that the minimal epitope was NY-ESO-1 91-101 (11-mer). Figure 3*e* shows the binding of the tetramer constructed using the epitope peptide to the clone 4E10.

Similarly, as shown in Supporting Information Figures S1–S4, we confirmed B*35:01-, B*35:01-, B*52:01- and C*12:02-restricted recognition of the NY-ESO-1f peptide by clones 2H10, 8D5, 10-10U and 7B, respectively, determined minimal epitopes and successfully produced tetramers.

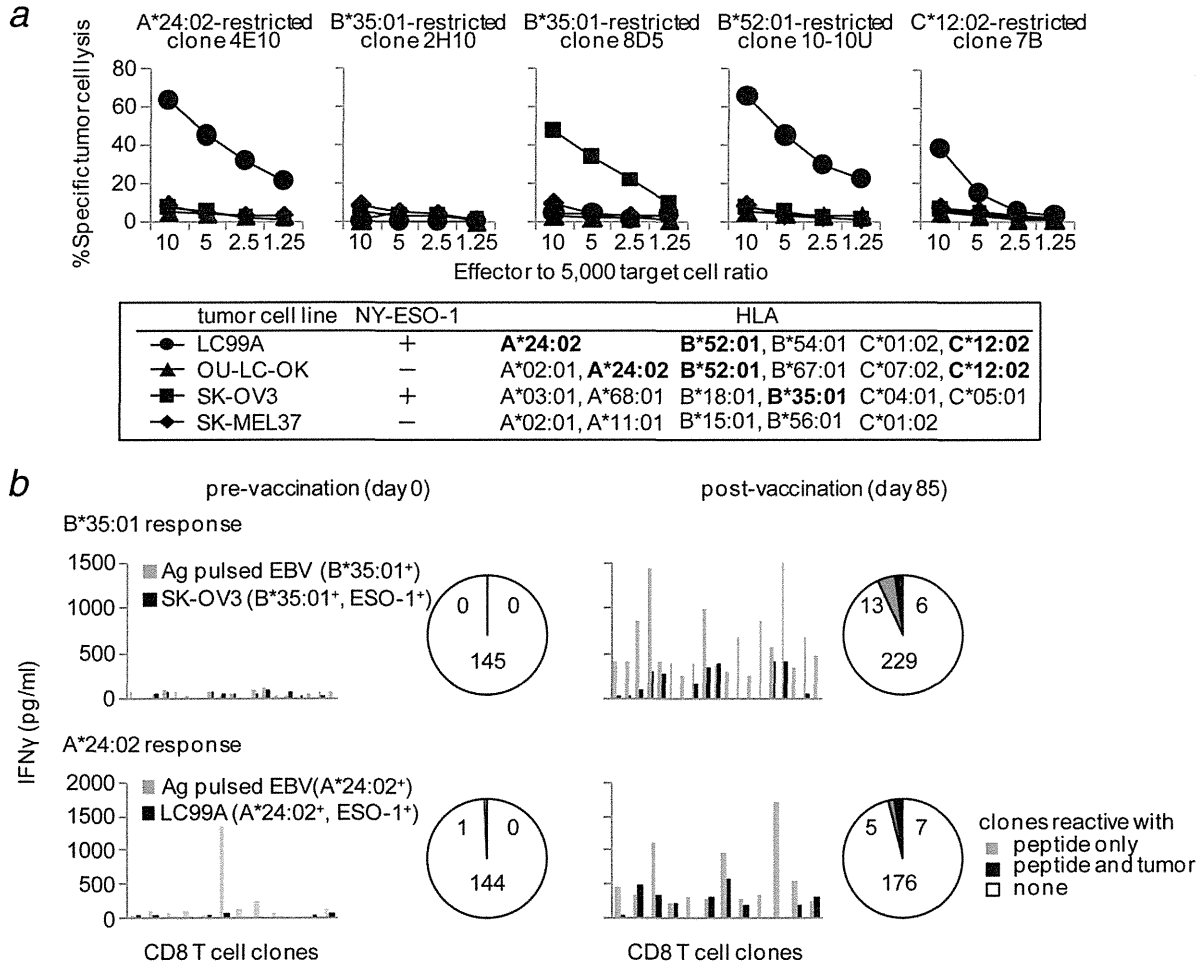


Figure 4. Clonal analysis of CD8 T-cells recognizing tumor cells induced by NY-ESO-1f peptide vaccination. In (*a*), cytotoxicity of the CD8 T-cell clones shown in Figure 3, and Supporting Information Figures S1–S4 was assayed by a luminescent method using the aCella-Tox kit. Shared HLA class I alleles in target tumors are shown in bold. In (*b*), purified CD8 T-cells (2×10^6) from TK-f01 PBMCs obtained at day 0 and at day 85 (after the fourth vaccination) were stimulated once with a mixture of NY-ESO-1 OLPs for 12 days in the presence of APC for 12 days. The cells were collected and seeded at 3 cells per well in 96-well culture plates to expand clones. After culture for 14 days, 290 clones were obtained from a culture at day 0 and 436 clones were obtained from a culture at day 85. Those clones were split into two groups and cultured with the NY-ESO-1f peptide (1 μM) pulsed-HD NO EBV-B cells or NY-ESO-1 positive SK-OV3 both sharing only B*35:01 with the patient to determine the B*35:01-restricted response and with NY-ESO-1f (1 μM) peptide pulsed-OS-P06 EBV-B cells or NY-ESO-1-positive LC99A both sharing A*24:02 with the patient to determine the A*24:02-restricted response. The amount of IFNγ in the culture supernatant was determined by ELISA.

We investigated the cytotoxicity of CD8 T-cell clones using an aCella-Tox system to examine G3PDH release from target tumor cell lines. As shown in Figure 4*a*, cytotoxicity against tumor cell lines expressing NY-ESO-1 and an appropriate HLA class I allele was observed with the A*24:02-restricted clone 4E10, B*35:01-restricted clone 8D5, B*52:01-restricted clone 10-10U and C*12:02-restricted clone 7B, but not with the B*35:01-restricted clone 2H10. With those clones, no cytotoxicity was observed against tumor cell lines which lacked either NY-ESO-1 or a matched HLA class I allele.

Frequency of CD8 T-cells recognizing tumor cells

We then investigated the frequency of CD8 T-cells recognizing tumor cells by clonal analysis. Purified CD8 T-cells from TK-f01 PBMCs obtained at day 0 and at day 85 (after the fourth vaccination) were stimulated once with a mixture of NY-ESO-1 OLPs for 12 days using CD4 and CD8-depleted PBMCs as APC as above. The cells were collected and seeded at 3 cells per well in 96-well culture plates to expand clones. After culture for 14 days, 290 clones were obtained from a culture of CD8 T-cells at day 0 and 436 clones were obtained from a culture of CD8 T-cells at day 85. Those clones were split into two groups and the B*35:01- and A*24:02-restricted responses were investigated. As shown in Figure 4*b*, in CD8 T-cells obtained at day 0, no B*35:01-restricted clone reactive against NY-ESO-1f peptide was obtained. There was only one A*24:02-restricted clone reactive against the NY-ESO-1f peptide (1/145). On the other hand, in CD8 T-cells obtained at

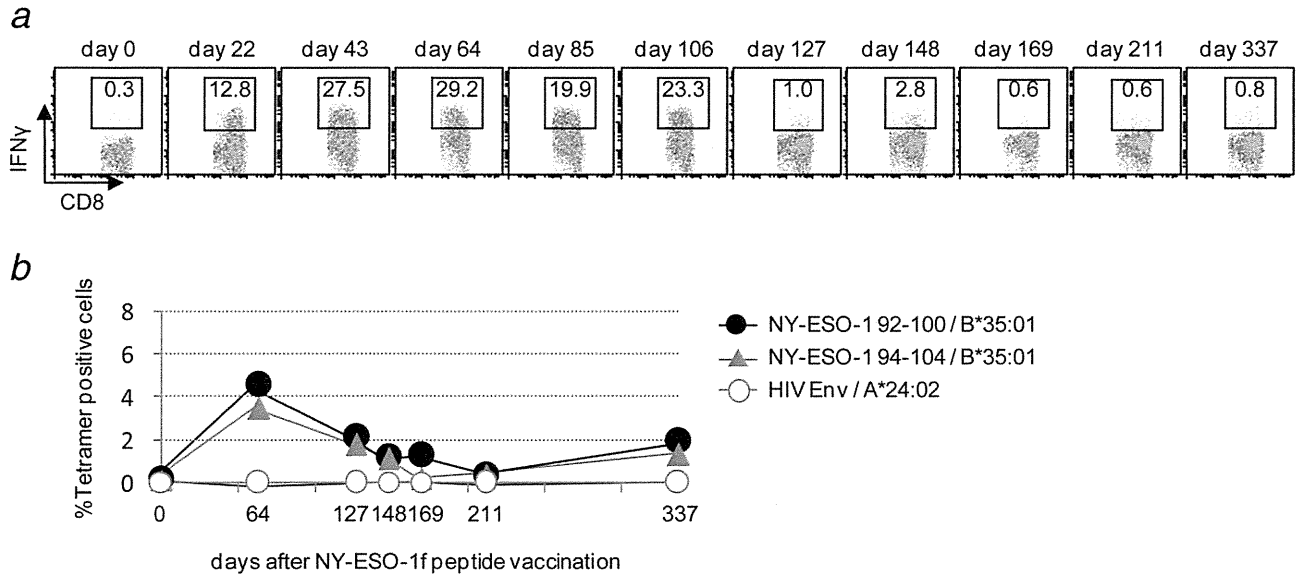


Figure 5. Immunomonitoring of the CD8 T-cell response by IFNγ capture assay and by peptide/HLA class I tetramers in PBMCs from TK-f01 during vaccination. CD8 T-cells (2×10^6) were cultured with a mixture of NY-ESO-1 OLPs (1 μM) in the presence of APC for 12 days. In (*a*), the response was assayed by an IFNγ capture assay. In (*b*), staining by PE-labeled NY-ESO-1 92-100/B*35:01 and NY-ESO-1 94-104/B*35:01 tetramers is shown. An HIV Env/A*24:02 tetramer was used as control.

day 85, the number of B*35:01-restricted clones reactive against the NY-ESO-1f peptide was 19 (7.7%), and 6 of 19 (31.6%) clones were also reactive against the NY-ESO-1-positive and B*35:01-positive tumor SK-OV3. The number of A*24:02-restricted clones reactive against the NY-ESO-1f peptide was 12 (6.4%), and 7 of 12 (58.3%) clones were also reactive against the NY-ESO-1-positive and A*24:02-positive tumor LC99A. Recognition of tumor cells was also confirmed by an aCella-Tox system (data not shown).

Immunomonitoring of CD8 T-cell responses by IFNγ capture assay and by peptide/HLA class I tetramers in PBMCs from TK-f01 during vaccination

After initiating the vaccine, the tumor remained stable for 6 months and was classed as SD at the end of the sixth vaccination in this patient.[20] However, the tumor started to grow after the eighth vaccination, consistent with an accelerated elevation in the serum CEA level.

Figure 5*a* shows the results of the IFNγ capture assay demonstrating the CD8 T-cell response against NY-ESO-1f peptide after stimulation with a mixture of NY-ESO-1 OLPs for 12 days. A vigorous CD8 T-cell response was observed even after the first vaccination (day 22), continuing until after the fifth vaccination (day 106). Thereafter, the response decreased suddenly. As shown in Figure 5*b*, the number of CD8 T-cells in the same culture positively stained with NY-ESO-1 92-100 and NY-ESO-1 94-104/B*35:01 tetramers was parallel with that of CD8 T-cells in an IFNγ capture assay. The findings indicated that CD8 T-cell responses against the peptides on B*35:01 comprised the dominant response, consistent with the clonal analysis shown in Table 1.

Discussion

In this study, we showed that internalization of the peptide was necessary for presenting CD8 T-cell epitopes derived from the long 20-mer NY-ESO-1f peptide on APC. Labeled NY-ESO-1f peptide was detected in approximately 10% of DCs after incubation with the peptide for 12 hr. No CD8 T-cell recognition of NY-ESO-1f peptide-pulsed DCs was observed after blocking internalization with cytochalasin B. Thus, the CD8 T-cell epitope was presented following internalization with a long 20-mer NY-ESO-1f peptide, contrasting with the direct presentation of the short epitope. Recognition by CD8 T-cells of NY-ESO-1f peptide-pulsed DCs, but not the recombinant NY-ESO-1 protein-pulsed DCs, indicated the occurrence of efficient cross presentation on MHC class I molecules after internalization of the 20-mer NY-ESO-1f peptide. Cross presentation of the long peptides on MHC class I molecules after internalization was previously shown with a peptide derived from HPV.[23] Processing of the peptide in APC may facilitate the presentation of natural epitopes. This study showed a high frequency of CD8 T-cells recognizing natural epitopes on tumor cells (see below). It has been shown previously that immunization with the short peptide induced CD8 T-cells with low affinity incapable of recognizing natural epitopes on tumor cells.[16] Use of the long peptide for immunization is beneficial to induce CD8 T-cells that recognize tumor cells.

We showed multiple HLA class I-restricted CD8 T-cell responses against a long 20-mer NY-ESO-1f peptide in the lung cancer patient TK-f01, who was immunized with the peptide with Picibanil OK-432 and Montanide ISA-51. Clonal analysis showed that the frequencies of A*24:02, B*35:01,

B*52:01, C*03:03 and C*12:02-restricted CD8 T-cells were 2.3, 11.0, 9.3, 0.7 and 1.3%, respectively, in the bulk CD8 T-cell population stimulated with NY-ESO-1 OLPs, indicating that B*35:01 and B*52:01-restricted CD8 T-cell responses were the two dominant CD8 T-cell responses. The minimal epitopes recognized by A*24:02, B*35:01, B*52:01 and C*12:02, but not C*03:03-restricted CD8 T-cell clones, were defined and the peptide/HLA tetramers were produced using those epitopes. NY-ESO-1 91-101 (11-mer) on A*24:02, NY-ESO-1 92-102 (11-mer) on B*35:01, NY-ESO-1 96-104 (9-mer) on B*52:01 and NY-ESO-1 96-106 (9-mer) on C*12:02 were new epitopes first defined in this study. Particularly, identification of this A*24:02 binding epitope and successful production of a tetramer with the epitope will be highly relevant when studying Japanese populations because of its high expression frequency (60%). Interestingly, within 25 HLA class I epitopes in the NY-ESO-1 molecule defined to date,[24–26] 15 epitopes including the epitopes shown in this study clustered in the peptide region NY-ESO-1 89-104. These findings suggested the usefulness of a long 20-mer NY-ESO-1f peptide harboring multiple CD8 T-cell epitopes for an NY-ESO-1 vaccine and this study indeed showed that vaccination with a long 20-mer NY-ESO-1f peptide elicited multiple HLA class I-restricted CD8 T-cell responses against the respective epitopes.

We also estimated the frequency of B*35:01- and A*24:02-restricted CD8 T-cells recognizing only the peptide-pulsed DCs, or both the peptide-pulsed DCs and the tumor cells naturally expressing the epitope and the matched HLA class I. At day 0, no B*35:01-restricted CD8 T-cell clones were detected from 145 clones obtained from 960 wells in total of the bulk CD8 T-cells stimulated with NY-ESO-1 OLPs for 12 days. Only a single A*24:02-restricted CD8 T-cell clone reactive against the peptide, but not the tumor, was obtained from the same number of clones. On the other hand, at day 85 after the fourth vaccination, 19 B*35:01-restricted and 12 A*24:02-restricted CD8 T-cell clones reactive against the peptide were detected from 248 and 188 clones, respectively, obtained from 960 wells for each of the bulk CD8 T-cells stimulated as above. 31.6% (6 of 19) of B*35:01-restricted and 58.3% (7 of 12) of A*24:02-restricted CD8 T-cell clones were also reactive against the tumors naturally expressing the epitope and the matched HLA class I. Thus, a significant frequency of CD8 T-cells recognizing tumor cells naturally expressing the epitopes and matched HLA was induced. This was likely to be related to the internalization and processing of the peptide in APC.

It has been reported that vaccination with NY-ESO-1 protein with CpG and Montanide elicited detectable CD8 T-cell responses in half of the immunized patients (9/18) and that vaccine-induced CD8 T-cells mostly recognized NY-ESO-1 81-110 in restriction to either HLA-B35 or HLA-Cw3.[27] B35 binding peptide epitopes 94-102 and 94-104 and Cw3 binding peptide epitopes 92-100 and 96-104 have been described.[25,28] In our previous study, we showed that an NY-ESO-1f peptide (NY-ESO-1 91-110) vaccine elicited a response to peptide 16 (NY-ESO-1 91-108) in all six patients analyzed with or without B35 and/or Cw3. The fact that the NY-ESO-1f peptide vaccine elicited CD8 T cell responses in patients with various HLA types suggests the advantage of a long peptide over the whole protein for vaccination.

Patient TK-f01 showed stable disease until the eighth vaccination and the disease exacerbated thereafter. In immunomonitoring CD8 T-cell responses following 20-mer NY-ESO-1f peptide vaccination, we observed vigorous CD8 T-cell responses, even after the first vaccination (day 22), lasting until the fifth vaccination (day 106) by IFNγ capture assay with a decrease thereafter, consistent with the clinical course. Characterization of CD8 T-cell responses using the various peptide/HLA tetramers showed that vigorous CD8 T-cell responses against the peptides restricted to B*35:01 were observed at day 64 after the third vaccination. These findings suggested that the multiple B*35:01-restricted CD8 T-cell responses comprised the dominant response.

Because Th cells help CTL induction, introduction of a Th epitope into the vaccine or physical linking of Th and CTL epitope peptides facilitated an increase in the immunogenicity of CTL vaccines.[29,30] The synthetic long peptide containing overlapping CD4 and CD8 T cell epitope sequences in the antigens is expected to generate both CD4 and CD8 T cell responses as a vaccine. In our study, the NY-ESO-1f peptide was shown to contain multiple HLA class II epitopes, as well as class I epitopes,[20] and is considered to be beneficial to elicit CD8 T-cell responses efficiently.

Acknowledgements

We thank Ms. Junko Mizuuchi for preparation of the manuscript. This article is dedicated to the memory of Lloyd J. Old, M.D.

References

1. Chen YT, Scanlan MJ, Sahin U, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci U S A* 1997;94:1914–8.
2. Gnjatic S, Nishikawa H, Jungbluth AA, et al. NY-ESO-1: review of an immunogenic tumor antigen. *Adv Cancer Res* 2006;95:1–30.
3. Jungbluth AA, Chen YT, Stockert E, et al. Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues. *Int J Cancer* 2001;92:856–60.
4. Scanlan MJ, Gure AO, Jungbluth AA, et al. Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. *Immunol Rev* 2002;188:22–32.
5. Simpson AJ, Caballero OL, Jungbluth A, et al. Cancer/testis antigens, gametogenesis and cancer. *Nat Rev Cancer* 2005;5:615–25.
6. Jager E, Gnjatic S, Nagata Y, et al. Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers. *Proc Natl Acad Sci U S A* 2000;97:12198–203.
7. Gnjatic S, Jager E, Chen W, et al. CD8(+) T cell responses against a dominant cryptic HLA-A2 epitope after NY-ESO-1 peptide immunization of cancer patients. *Proc Natl Acad Sci U S A* 2002;99:11813–8.
8. Valmori D, Dutoit V, Ayyoub M, et al. Simultaneous CD8+ T cell responses to multiple tumor antigen epitopes in a multipeptide melanoma vaccine. *Cancer Immun* 2003;3:15.

9. Shackleton M, Davis ID, Hopkins W, et al. The impact of imiquimod, a Toll-like receptor-7 ligand (TLR7L), on the immunogenicity of melanoma peptide vaccination with adjuvant Flt3 ligand. *Cancer Immun* 2004;4:9.
10. Davis ID, Chen W, Jackson H, et al. Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4(+) and CD8(+) T cell responses in humans. *Proc Natl Acad Sci U S A* 2004;101:10697–702.
11. Valmori D, Souleimanian NE, Tosello V, et al. Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T cells through cross-priming. *Proc Natl Acad Sci U S A* 2007;104: 8947–52.
12. Jager E, Karbach J, Gnjatic S, et al. Recombinant vaccinia/fowlpox NY-ESO-1 vaccines induce both humoral and cellular NY-ESO-1-specific immune responses in cancer patients. *Proc Natl Acad Sci U S A* 2006;103:14453–8.
13. Kawabata R, Wada H, Isobe M, et al. Antibody response against NY-ESO-1 in CHP-NY-ESO-1 vaccinated patients. *Int J Cancer* 2007;120: 2178–84.
14. Uenaka A, Wada H, Isobe M, et al. 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* 2007;7:9.
15. Chen Q, Jackson H, Parente P, et al. Immunodominant CD4+ responses identified in a patient vaccinated with full-length NY-ESO-1 formulated with ISCOMATRIX adjuvant. *Proc Natl Acad Sci U S A* 2004;101: 9363–8.
16. Le Gal FA, Ayyoub M, Dutoit V, et al. Distinct structural TCR repertoires in naturally occurring versus vaccine-induced CD8+ T-cell responses to the tumor-specific antigen NY-ESO-1. *J Immunother* 2005;28:252–7.
17. Melief CJ, van der Burg SH. Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. *Nat Rev Cancer* 2008;8: 351–60.
18. Bijker MS, van den Eeden SJ, Franken KL, et al. CD8+ CTL priming by exact peptide epitopes in incomplete Freund's adjuvant induces a vanishing CTL response, whereas long peptides induce sustained CTL reactivity. *J Immunol* 2007;179: 5033–40.
19. van der Burg SH, Bijker MS, Welters MJ, et al. Improved peptide vaccine strategies, creating synthetic artificial infections to maximize immune efficacy. *Adv Drug Deliv Rev* 2006;58: 916–30.
20. Kakimi K, Isobe M, Uenaka A, et al. 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* 2011;129:2836–46.
21. Altman JD, Moss PA, Goulder PJ, et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 1996;274:94–6.
22. Morishima S, Akatsuka Y, Nawa A, et al. Identification of an HLA-A24-restricted cytotoxic T lymphocyte epitope from human papillomavirus type-16 E6: the combined effects of bortezomib and interferon-gamma on the presentation of a cryptic epitope. *Int J Cancer* 2007;120:594–604.
23. Zwaveling S, Ferreira Mota SC, Nouta J, et al. Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. *J Immunol* 2002;169:350–8.
24. Jager E, Chen YT, Drijfhout JW, et al. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. *J Exp Med* 1998;187:265–70.
25. Gnjatic S, Nagata Y, Jager E, et al. Strategy for monitoring T cell responses to NY-ESO-1 in patients with any HLA class I allele. *Proc Natl Acad Sci U S A* 2000;97:10917–22.
26. Jager E, Karbach J, Gnjatic S, et al. Identification of a naturally processed NY-ESO-1 peptide recognized by CD8+ T cells in the context of HLA-B51. *Cancer Immun* 2002; 2:12.
27. Bioley G, Guillaume P, Luescher I, et al. HLA class I – associated immunodominance affects CTL responsiveness to an ESO recombinant protein tumor antigen vaccine. *Clin Cancer Res* 2009;15:299–306.
28. Jackson H, Dimopoulos N, Mifsud NA, et al. Striking immunodominance hierarchy of naturally occurring CD8+ and CD4+ T cell responses to tumor antigen NY-ESO-1. *J Immunol* 2006;176:5908–17.
29. Ossendorp F, Mengede E, Camps M, et al. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J Exp Med* 1998;187: 693–702.
30. Shirai M, Pendleton CD, Ahlers J, et al. Helper-cytotoxic T lymphocyte (CTL) determinant linkage required for priming of anti-HIV CD8+ CTL in vivo with peptide vaccine constructs. *J Immunol* 1994;152:549–56.