Table V. Concordance between the results for HER2 overexpression determined by IHC in surgically resected tumors and biopsy specimens.

Biopsy specimens	Surgically resected tumors			NPV (%)	PPV (%)
	Negative	Positive	Total		
Negative	144	13	157	91.7	-
Positive	10	33	43	-	76.7
Total	154	46	200		
Concordance rate (%)	93.5	71.7	88.5		

NPV, negative predictive value; PPV, positive predictive value.

of the biopsy specimens (Table V). A total 43 cases were evaluated as positive (21.5%) and 157 cases as negative (78.5%). The concordance rate for HER2 overexpression determined by IHC in the surgically resected tumors and the biopsy specimens was 88.5% (95% CI 87.1-89.9%). Of the 154 cases in which the surgically resected tumor was evaluated as negative for HER2 overexpression by IHC, the biopsy specimen was negative in 144 cases resulted in the concordance rate of 93.5% (95% CI 91-95%). Therefore, 144 of 157 negative cases with biopsy specimens could represent those in surgically resected specimen as HER2 negative (NPV: 91.7%). Of the 46 cases in which surgically resected tumor was evaluated as positive, the biopsy specimens was also positive in 33 cases with the concordance rate of 71.7% (95% CI 58.7-84.7%). Of the 43 cases in which the biopsy specimen was positive, 33 of 43 cases could represent those in surgically resected specimen as HER2 positive (PPV: 76.7%).

Concordance between the results of FISH in surgically resected tumors and biopsy specimens. We have evaluated all 54 biopsy specimens in which gene amplification was demonstrated in the surgically resected tumor: the FISH assay was technically successful in all 54 (100%) cases, despite the small specimens. HER2 was interpreted as being amplified in 33 of these 54 cases, which resulted in the concordance rate of 62.2% (95% CI 49.3-75.1%). The median number of signals per nucleus in the 54 cases was 3.5 (range 1.1-12.2), whereas the median number of signals per nucleus in the 33 amplified cases was 7.9.

Discussion

Gastric cancer is one of the leading causes of cancer death in the world. Despite improvements in survival as a result of early detection and curative surgery, approximately 50,000 patients died of gastric cancer in Japan in 2001 (20). Unresectable advanced cancer and recurrent gastric cancer, in particular, still have a poor prognosis. Randomized trials have demonstrated that fluorouracil (5FU)-based chemotherapy improves survival and quality of life compared with the best supportive care (21-23), however, no standard treatment regimen has been established yet. New and promising agents for gastric cancer are eagerly waited.

Although there is no relevant evidence Trastuzumab has been put forward as a potential candidate in gastric cancer therapy particularly in patients with HER2 expression. However, basic data is needed to decide whether it should be further developed in the treatment for gastric cancer. This is the first large study to evaluate concordance of HER2 status between protein expression and gene amplification in both surgical and endoscopic biopsy specimens of gastric cancer using two commercial kits, an IHC and a FISH.

In this study, HER2 protein overexpression was demonstrated in 23% formalin-fixed paraffin-embedded specimens of surgically resected advanced intestinal type gastric cancers, and *HER2* gene amplification was demonstrated in 27.1%. FISH indicated gene amplification in 86.7% of the cases in which HER2 protein overexpression was detected by IHC, and the concordance rate between the results obtained by IHC and FISH was 86.9%. Takehana *et al* performed a comparative study of IHC and FISH in gastric cancer (17). In their study, IHC revealed HER2 protein overexpression in 29 (8.2%) of 352 surgically resected gastric cancer not only for histologically intestinal type, and FISH showed gene amplification in 25 (86.2%) of the cases with HER2 overexpression. Our results are similar to their analysis and to those analyzed in breast cancer (5-7).

Ridolfi *et al* reported a low frequency of gene amplification in Hercep test 2+ breast cancer cases (24). They reported that FISH demonstrated gene amplification in only 36% of the 2+ cases. They claimed that 2+ IHC reactions are uncertain, that the majority of 2+ cases are a heterogeneous group, and concluded that FISH should be performed on all 2+ cases to confirm gene amplification. Although there have been a few reports of IHC studies of Hercep test 2+ cases, frequency of gene amplification in 2+ breast cancer cases varies widely and is generally lower than in 3+ cases (5-7). According to results of clinical trials of Trastuzumab in breast cancer, its antitumor activity in IHC 2+ cases was consistently lower than in 3+ patients (25,26), therefore Trastuzumab is considered an active agent for breast cancer evaluated as 3+ by Hercep test or as positive for gene amplification by FISH. FISH demonstrated gene amplification in 7 of 12 IHC 2+ cases (58.5%) in the present study, which is clearly lower than in the 3+ cases (88.2%). Taking these results into consideration in the target population for Trastuzumab, FISH should be assessed for patients with IHC 2+ cases even in gastric cancer.

The concordance between the IHC findings in surgically resected tumors and biopsy specimens is very important in gastric cancer clinically. Small specimens of tumors can

easily be obtained endoscopically. If a satisfactory concordance rate is obtained, HER2 status can be evaluated by IHC in unresectable cases as well as cases of recurrence after gastrectomy. In the present study, 21.5% of biopsy specimens were evaluated as positive and the concordance rate between the results in the surgically resected tumors and biopsy specimens was 93.5%. Furthermore, the NPV for the surgically resected tumors was 91.7%, and the PPV was 76.7%. Therefore, we considered it is appropriate to evaluate HER2 status using Hercep test in biopsy specimen for recruiting gastric cancer patients who become candidate for Trastuzumab.

Many investigators have examined that HER2 overexpression could be a predictor of survival outcome in gastric cancer (10-12,14,27-29). Brien *et al* tested 61 cases for gene amplification by performing FISH on sections of paraffin-embedded gastric cancer tissue, and 43% of the cases were positive (28). The multivariate analysis in their study showed that pathological stage and *HER2* gene amplification are independent prognostic factors of survival. Allgayer *et al* confirmed the importance of HER2 status as a prognostic factor in a prospective study of gastric cancer (29). They demonstrated a significant association between level of expression of HER2 and shorter disease-free and overall survival and concluded that HER2 is a promising target for anti-invasive therapy also in gastric cancer. In the present study, there are 36 stage IV patients (18%) who would be target population for systemic chemotherapy. Of the 36 surgically resected tumor specimens, 12 (33%) of the tumors were found to exhibit HER2 protein overexpression, 2+ in 4 (11%), and 3+ in 8 cases (22%), and *HER2* gene amplification were demonstrated in 15 cases (41%). These results might indicate higher tendency of HER2 overexpression and gene amplification in stage IV than in earlier stages, though this should be confirmed in large populations.

HER2 protein overexpression and *HER2* gene amplification can be assessed with commercial kits for breast cancer even in gastric cancer. Even small endoscopic biopsy specimens are suitable for evaluating HER2 overexpression in gastric cancer. Satisfactory concordance rates could be achieved between Hercep test and Pathvision. Development of Trastuzumab for gastric cancer will progress according to the result of this study therefore it could be possible to determine adaptation with Hercep test and PathVysion even in gastric cancer.

Acknowledgements

This study was supported by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan. We thank Mr. Tsutomu Yamamoto, Chugai Pharmaceutical Co., Ltd., for his assistance in the preparation of this study. We also thank Mrs. Mari Takahashi and Miss Yuki Yanagisawa for their technical assistance.

References

1. Akiyama T, Sudo C, Ogawara H, Toyoshima K and Yamamoto T: The product of the human c-erbB-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. Science 232: 1644-1646, 1986.
2. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A and McGuire L: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235: 177-182, 1987.
3. Press MF, Bernstein L, Thomas PA, Meisner LF, Zhou JY, Ma Y, Hung G, Robinson RA, Harris C, Naggar AE, Slamon DJ, Philips RN, Ross JS, Wolman SR and Folm KJ: Her-2/neu gene amplification characterized by fluorescence *in situ* hybridization: poor prognosis in node-negative breast carcinomas. J Clin Oncol 15: 2894-2904, 1997.
4. Slamon DJ, Jones BL, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J and Norton L: Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 344: 783-792, 2001.
5. Hoang MP, Salin AA, Ordonez NG and Sneige N: HER-2/neu gene amplification compared with Her-2/neu protein overexpression and interobserver reproducibility in invasive breast carcinoma. Am J Clin Pathol 113: 852-859, 2000.
6. Jacob TW, Gown AM, Yaziji H, Barnes MJ and Schnitt SJ: Comparison of fluorescence *in situ* hybridization and immunohistochemistry for the evaluation of HER-2/neu in breast cancer. J Clin Oncol 17: 1974-1982, 1999.
7. Lebeau A, Deimling D, Kaltz C, Sendelhofert A, Iff A, Luthardt B, Untch M and Lohrs U: HER-2/neu analysis in archival tissue samples of human breast cancer: comparison of immunohistochemistry and fluorescence *in situ* hybridization. J Clin Oncol 19: 354-363, 2001.
8. NCCN Practice Guidelines in Oncology. Version 1.2004, 04-19-04. National Comprehensive Cancer Network, Inc.
9. Falck VG and Gullick W: c-erbB-2 oncogene product staining in gastric adenocarcinoma. An immunohistochemical study. J Pathol 159: 107-111, 1989.
10. Tateishi M, Toda T, Minamisono Y and Nagasaki S: Clinicopathological significance of c-erbB-2 protein expression in human gastric cancer. J Surg Oncol 49: 209-212, 1992.
11. Uchino S, Tsuda H, Maruyama K, Kinoshita T, Sasako M, Saito T, Kobayashi M and Hirohashi S: Overexpression of c-erbB-2 protein in gastric cancer. Cancer 72: 3179-3184, 1993.
12. Motojima K, Furui J, Kohara N, Izawa K, Kanematsu T and Shiku H: *erb*B-2 expression in well-differentiated adenocarcinoma of the stomach predicts shorter survival after curative resection. Surgery 115: 349-354, 1994.
13. McCulloch PG, Ochiai A, O'Dowd GM, Nash JG, Sasako M and Hirohashi S: Comparison of the molecular genetics of c-erb-B2 and p53 expression in stomach cancer in Britain and Japan. Cancer 75: 920-925, 1995.
14. Nakajima M, Sawada H, Yamada Y, Watanabe A, Tatsumi M, Yamashita J, Matsuda M, Sakaguchi T, Hirao T and Nakano H: The prognostic significance of amplification and overexpression of c-met and c-erbB-2 in human gastric carcinomas. Cancer 85: 1894-1902, 1999.
15. Ishikawa T, Kobayashi M, Mai M, Suzuki T and Ooi A: Amplification of the c-erbB-2 (HER-2/neu) gene in gastric cancer cells. Am J Pathol 151: 761-768, 1997.
16. Sato T, Abe K, Kurose A, Uesugi N, Todoroki T and Sasaki K: Amplification of the c-erbB-2 gene detected by FISH in gastric cancers. Pathol Int 47: 179-182, 1997.
17. Takehana T, Kunitomo K, Kono K, Kitahara F, Iizuka H, Matsumoto Y, Fujino AF and Ooi A: Status of c-erbB-2 in gastric adenocarcinoma: a comparative study of immunohistochemistry, fluorescence *in situ* hybridization and enzyme-linked immunosorbent assay. Int J Cancer 98: 833-837, 2002.
18. Kaori F Ouchi, Sekiguchi F and Tanaka Y: Antitumor activity of trastuzumab (HERCEPTIN[R]) in human gastric cancer models. Eur J Cancer 1 (Suppl 5): S294, 2003.
19. Jacobs TW, Gown AM, Yaziji H, Barnes MJ and Schnitt SJ: Specificity of hercep test in determining HER-2/neu status of breast cancers using the United States food and drug administration-approved scoring system. J Clin Oncol 17: 1983-1987, 1999.
20. The Editorial Board of Cancer Statistics in Japan. Cancer Statistics in Japan, p36, 2003.
21. Murad AM, Santiago FF, Petroianu A, Rocha PRS, Rodrigues MAG and Rausch M: Modified therapy with 5-fluorourasil, doxorubicin, and methotrexate in advanced gastric cancer. Cancer 72: 37-41, 1993.
22. Glimelius B, Hotfmann K, Haglund U, Nyren O and Sjoden PO: Initial of delayed chemotherapy with best supportive care in advanced gastric cancer. Ann Oncol 5: 189-190, 1994.
23. Pyhonen S, Kuitumen T, Nyandoto P and Kouri M: Randomized comparison of fluorouracil, epidoxorubicin and methotrexate (FEMTX) plus best supportive care alone in patients with non-resectable gastric cancer. Br J Cancer 71: 587-591, 1995.

24. Ridolfi RL, Jamehdor MR and Arber JM: Her-2/neu testing in breast carcinoma: a combined immunohistochemical and fluorescence *in situ* hybridization approach. Mod Pathol 13: 866-873, 2000.
25. Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, Wolter JM, Paton V, Shak S, Leiberman G and Slamon DJ: Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 17: 2639-2648, 1999.
26. Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novothy WF, Burchmore M, Shak S, Stewart SJ and Press M: Efficacy and safety of Trastuzumab as a single agent in first line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol 20: 719-726, 2002.
27. Yonemura Y, Ninomiya I, Ohoyama S, Kimura H, Yamaguchi A, Fushida S, Kosaka T, Miwa K, Miyazaki I, Endou Y, Tanaka M and Sasaki T: Expression of c-erbB-2 oncoprotein in gastric carcinoma. Immunoreactivity for c-erbB-2 protein is an independent indicator of poor short-term prognosis in patients with gastric cancer. Cancer 67: 2914-2918, 1991.
28. Brien TP, Depowski PL, Sheehan CE, Ross JS and McKenna BJ: Prognostic factors in gastric cancer. Mod Pathol 11: 870-877, 1998.
29. Allgayer H, Babic R, Gruetzner KU, Tarabichi A, Schiidberg FW and Heiss MM: c-*erb*B-2 is of independent prognostic relevance in gastric cancer and is associated with the expression of tumor-associated protease systems. J Clin Oncol 18: 2201-2209, 2000.

Blockade of bulky lymphoma-associated CD55 expression by RNA interference overcomes resistance to complement-dependent cytotoxicity with rituximab

Yasuhito Terui,[1,2] Takuma Sakurai,[1,3] Yuko Mishima,[1] Yuji Mishima,[2] Natsuhiko Sugimura,[2] Chino Sasaoka,[2] Kiyotsugu Kojima,[2] Masahiro Yokoyama,[1] Nobuyuki Mizunuma,[1] Shunji Takahashi,[1] Yoshinori Ito[1] and Kiyohiko Hatake[1,2,4]

[1]Division of Clinical Chemotherapy, and [2]Olympus Bio-Imaging Laboratory, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo 135-8550, and [3]Material Research Laboratory, Morinaga Milk Industry, Zama, Kanagawa 228-8583, Japan

(Received August 29, 2005/Revised October 4, 2005/Accepted October 17, 2005/Online publication December 18, 2005)

Recently, anti-CD20 (rituximab) and anti-Her2/neu (trastuzumab) antibodies have been developed and applied to the treatment of malignant lymphoma and breast cancer, respectively. However, bulky lymphoma is known to be resistant to rituximab therapy, and this needs to be overcome. Fresh lymphoma cells were collected from 30 patients with non-Hodgkin's lymphoma, the expression of CD20 and CD55 was examined by flow cytometry, and complement-dependent cytotoxicity (CDC) assays were carried out. Susceptibility to CDC with rituximab was decreased in a tumor size-dependent manner ($r = -0.895$, $P < 0.0001$), but not in a CD20-dependent manner ($r = -0.076$, $P = 0.6807$) using clinical samples. One complement-inhibitory protein, CD55, contributed to bulky lymphoma-related resistance to CDC with rituximab. A decrease in susceptibility to CDC with rituximab was statistically dependent on CD55 expression ($r = -0.927$, $P < 0.0001$) and the relationship between tumor size and CD55 expression showed a significant positive correlation ($r = 0.921$, $P < 0.0001$) using clinical samples. To overcome the resistance to rituximab by high expression of CD55 in bulky lymphoma masses, small interfering RNA (siRNA) was designed from the DNA sequence corresponding to nucleic acids 1–380 of the CD55 cDNA. Introduction of this siRNA decreased CD55 expression in the breast cancer cell line SK-BR3 and in CD20-positive cells of patients with recurrent lymphoma; resistance to CDC was also inhibited. This observation gives us a novel strategy to suppress bulky disease-related resistance to monoclonal antibody treatment. (*Cancer Sci* 2006; 97: 72–79)

In recent years, monoclonal antibodies have been used increasingly to treat patients with malignancies such as lymphoma and breast cancer.[1–3] In particular, the anti-CD20 antibody, also called rituximab, is usually very effective for treatment of malignant lymphoma, and most patients can receive rituximab as monotherapy or combination chemotherapy.[4,5] However, in some cases with bulky mass and at stage IV, lymphoma cells become resistant to rituximab treatment.[6,7] Apart from the number of tumor cells being greater in these cases, how this resistance occurs has not yet been clarified.

Recently, some researchers have reported four mechanisms for the action of rituximab: (i) inhibition of proliferation; (ii) induction of apoptosis; (iii) complement-dependent cytotoxicity (CDC); and (iv) antibody-dependent cellular cytotoxicity (ADCC).[7,8] Because CDC could more rapidly and efficiently act on the target cells attacked by rituximab, CDC may be the most important of the mechanisms of rituximab.

The role of complementary regulatory proteins in the modulation of rituximab efficacy has been addressed, and several surface membrane proteins regulate the deposition of active complement proteins on cellular membranes to prevent cell lysis. Regulators of the complement system play an important role in CDC, and CD46, CD55 and CD59 are well known to inhibit the complement system.[9] Among these inhibitors, CD55 and CD59 seem to be the most important.[10] No differences in the expression of CD59 molecules have been reported between normal B cells and malignant B cells, whereas CD55 expression was shown to be different among individual patients with B-cell malignancy.[11] Nevertheless, *in vitro* susceptibility to rituximab-induced CDC could not be predicted by the level of these proteins in chronic lymphocytic leukemia (CLL) cells, and *in vivo* susceptibility could not be predicted in follicular lymphoma (FL) and CLL patients.[12,13] In contrast, some researchers have reported direct correlations among CDC, CD55 and CD59 using B-cell lines.[14]

CD55, also known as decay accelerating factor, is a major regulator of the alternative and classical pathways of complement activation and is expressed on all serum-exposed cells. CD55 is a 70-kDa glycoprotein, which is a glycosylphosphatidylinositol (GPI)-anchored protein.[15] CD55 can bind the complex of C3a and Bb, which is in the classical pathway, and it blocks the cascade of the complement system. A functional disorder of CD55 in blood cells causes paroxysmal nocturnal hemoglobinuria (PNH).[16] In these cases, the cascade of the complement system can not be controlled, and CDC activity is enhanced mainly against red blood cells. CD55 can enhance dissociation between C3 convertase and C4bC2/C3bBb complexes, and then inhibit the cascade of the

[4]To whom correspondence should be addressed. E-mail: khatake@jfcr.or.jp.

doi: 10.1111/j.1349-7006.2006.00139.x
© 2006 Japanese Cancer Association

complement system. While it is true that CD55 levels are low to absent in PNH, the disease is caused by phosphatidylinositol glycan-A (PIGA) gene mutations that lead to a failure to assemble GPI anchors. Hence, all GPI-anchored proteins are missing in this disease.

Previous researchers have shown that certain conditions for cancer cells, such as hypoxia, poor nutrition and bulky mass, make them chemoresistant.[17,18] When gastric cancer cells were exposed to hypoxia, hypoxia inducible factor (HIF)-1 was induced and the cells were resistant to Cis-platin (CDDP).[18] When lymphoid cells were able to resist doxorubicin (adriamycin), expression of nuclear factor (NF)-κB and its transcription activity were enhanced in doxorubicin (adriamycin)-resistant cells.[17]

Because CDC activity is especially important for rituximab therapy and CD55 may function as a mostly important inhibitor of CDC, it is possible that a decline in CDC activity by CD55 molecules may cause resistance to rituximab. CDC correlates directly with the expression of CD20 antigen in malignant B cells, and *in vitro* susceptibility to rituximab-mediated CDC depends primarily on CD20 protein expression. However, there have yet been no reports about the relationship between tumor size and sensitivity to CDC or between tumor size and CD55 expression.

More recently, small interfering RNA (siRNA) has been developed and applied to knock down target gene expression.[19] For example, the nuclear factor of activated T cells (NFAT) and NF-κB were shown to be constitutively active in large B-cell lymphoma cells, and downregulation of NFATc1 and NF-κB in malignant B-cell lymphoma with siRNA inhibited lymphoma cell growth.[20] Although many researchers tried siRNA for genes of membrane proteins such as growth factor receptors,[21] there have been no successful reports describing siRNA for complement inhibitors.

To clarify the resistance to rituximab and overcome the resistance, especially with regard to bulky mass unresponsiveness and efficacy for re-treatment, we examined the relationship between CDC activity and rituximab, and CD55 expression in our patients, using siRNA for CD55 to treat CDC with rituximab.

Materials and Methods

Cell lines

Human malignant B-cell lines as well as Daudi and Raji cells (ATCC) were cultured in RPMI-1640 (Gibco, Carlsbad, CA, USA) with 10% fetal calf serum (FCS) at 37°C. The cell lines were used as sensitive and resistant controls in CDC with anti-CD20 antibody. The human breast cancer cell lines MCF7 and SK-BR3 (ATCC) were cultured in Dulbecco's minimal essential medium (DMEM; Gibco) with 10% FCS.

Complement-mediated cytotoxicity assay

Cells were washed once with fresh complete medium, and anti-CD20 antibody (rituximab; Roche, Basel, Switzerland) or anti-Her2/neu antibody (trastuzumab; Roche) was added at a concentration of 20 μg/mL. Cells were incubated at 37°C for 1 h, and then human AB blood serum from healthy volunteers with informed consent was added at 20% (v/v). After incubation at 37°C for 1 h, propidium iodide (PI; Sigma, St Louis, MO, USA) was added and CDC assays were carried out by flow cytometry with FACscan (Becton Dickinson, San Jose, CA, USA). For CDC assays using a microplate reader, Daudi, Raji and SKBR3 cells were seeded at 1×10^5 cell/mL in each well, and then rituximab or trastuzumab (20 μg/mL) and normal AB serum (20% [v/v]) were added. The reaction was incubated at 37°C for 1 h, and the cells were washed with phosphate-buffered saline (PBS) at least three times. Ten microliters of Calcein-AM (2 μg/mL) (Dojindo, Kumamoto, Japan) was added to each well and mixed thoroughly. After incubation at room temperature, fluorescence intensity was measured at 485 nm/535 nm wavelengths with a microplate reader (Fluoroskan Ascent; Labsystems, Helsinki, Finland).

Surface markers

Cells were washed once with PBS, and were then were stained with phycoerythrin (PE)-conjugated anti-CD20, and fluoresceinisothiocyanate (FITC)-conjugated anti-CD55 (Becton Dickinson). Flow cytometry was then carried out using FACscan. The intensities of C20 and CD55 expression were normalized compared with a control. For confocal laser scanning microscopy, rituximab and trastuzumab were labeled with Alexa Fluor 594 (Molecular Probes; Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. In brief, 100 μg of antibody was labeled with Alexa Fluor 594 for 20 min after alkalization with carbonate. The mixture was put into a spin column and spun down at 1500*g*, and the flow-through was collected as Alexa Fluor 594-conjugated antibody.

Laser scanning confocal microscopy and phase-contrast microscopy

To see CDC activity on living cells, pictures were taken by a CDC camera with phase-contrast microscopy after the CDC assay with rituximab or trastuzumab. The cells were also stained with Alexa Fluor 594-labeled rituximab or Alexa Fluor 594-labeled trastuzumab and FITC-labeled anti-CD55 antibody, and serum was added to the culture medium. The stained cells were observed in real time under a confocal laser scanning microscopy system (Olympus, Tokyo, Japan).

Collection of clinical samples

Fresh lymphoma cells were collected from the lymph nodes of 30 patients with non-Hodgkin's lymphoma (11 cases of diffuse large B-cell type, 10 cases of marginal zone cell type, five cases of follicular cell type, two cases of small lymphocytic type, one case of B-cell immunoblastic type, and one case of diffuse small cell type) after receiving informed consent. In brief, the lymph nodes were resected surgically and specimens were broken into small pieces with scissors and ground between two glass slides. The cells were collected after centrifugation and washed with RPMI-1640 containing 10% FCS. Cell counting and viability were assessed by toluidine-blue exclusion dye test, and CD19-positive cells were isolated using a magnetic cell sorting (MACS) system. The isolated cells were stained with FITC-conjugated anti-CD19, PE-conjugated anti-CD20, and FITC-conjugated anti-CD55 antibodies and flow cytometry was then carried out.

© 2006 Japanese Cancer Association

Vector and siRNA for CD55

CD55 cDNA in Ultimate open reading frame (ORF) clones (clone *ID*: IOH3209) was purchased from Invitrogen, and amplified by polymerase chain reaction (PCR) (forward, 5′-CGCGGATCCGCGATGACCGTCGCGCGG-3′; and reverse, 5′-TCCCCCGGGGGACTAAGTCAGCAAGCC-3′). The PCR product was subcloned into the pEGFP-C1 vector (Clontech, Mountain View, CA, USA). To generate double-stranded RNA for CD55, three parts of the DNA sequence, corresponding to nucleic acids 1–380, 381–817 and 821–1146 in the CD55 cDNA, were amplified by PCR. These sequences were named CD55-N, CD55-M and CD55-C, respectively. RNA transcription was then performed with this DNA template to generate sense and antisense single-stranded RNA. After production of double-stranded RNA, a reaction with the Dicer enzyme was carried out using a BLOCK-iT Dicer RNAi kit (Invitrogen). For siRNA for CD55, the siRNA was transfected into Raji and SK-BR3 cells using Lipofectamine 2000 (Invitrogen). In brief, 0.75 ng of siRNA and 5 µL of Lipofectamine 2000 in Optimen medium were mixed and incubated at room temperature for 20 min. The mixture was added to culture medium with SK-BR3 cells and fresh lymphoma cells, and the cells were incubated at 37°C for 72 h and 24 h, respectively. To see downregulation of CD55 expression, the CD55-transfected cells were stained with FITC-conjugated anti-CD55 antibody, and then expression of CD55 was observed without fixation of the cells at the same intensity of emission and excision as under laser scanning confocal fluorescent microscopy.

Statistical analysis

Correlation of susceptibility to CDC with tumor size, CD20 expression and CD55 expression were tested using the Spearman rank correlation coefficient. Statistical comparisons were carried out using two-sided Student's *t*-tests. All statistical analyses were performed using StatView 5.0 software (SAS Institute, Cary, NC, USA).

Results

Negative correlation between tumor size and susceptibility to CDC with rituximab

Rituximab is known to be effective at the early stages of indolent and aggressive lymphomas, but the effect of rituximab declines in some patients with bulky disease and a large number of lymphoma cells. According to this fact, we investigated whether susceptibility to CDC is dependent on the size of the tumor. The diameter of extirpated lymph nodes, CDC assay and CD20 expression were examined in fresh samples from 30 patients with lymphoma, as described in 'Materials and Methods'. As shown in Fig. 1a, the relationship between susceptibility to CDC and size of extirpated lymph nodes showed a significant negative correlation ($R = -0.895$, $P < 0.001$). In contrast, the relationship between susceptibility to CDC and CD20 expression, and between size of extirpated lymph nodes and CD20 expression, did not reveal significant correlations, as shown in Fig. 1b,c ($R = -0.076$, $P = 0.6807$ and 0.072, $P = 0.6979$, respectively). This suggests that susceptibility to CDC is dependent on the size of the lymphoma tumor, and that expression of CD20 does not contribute to susceptibility to CDC with rituximab in non-Hodgkin's lymphoma.

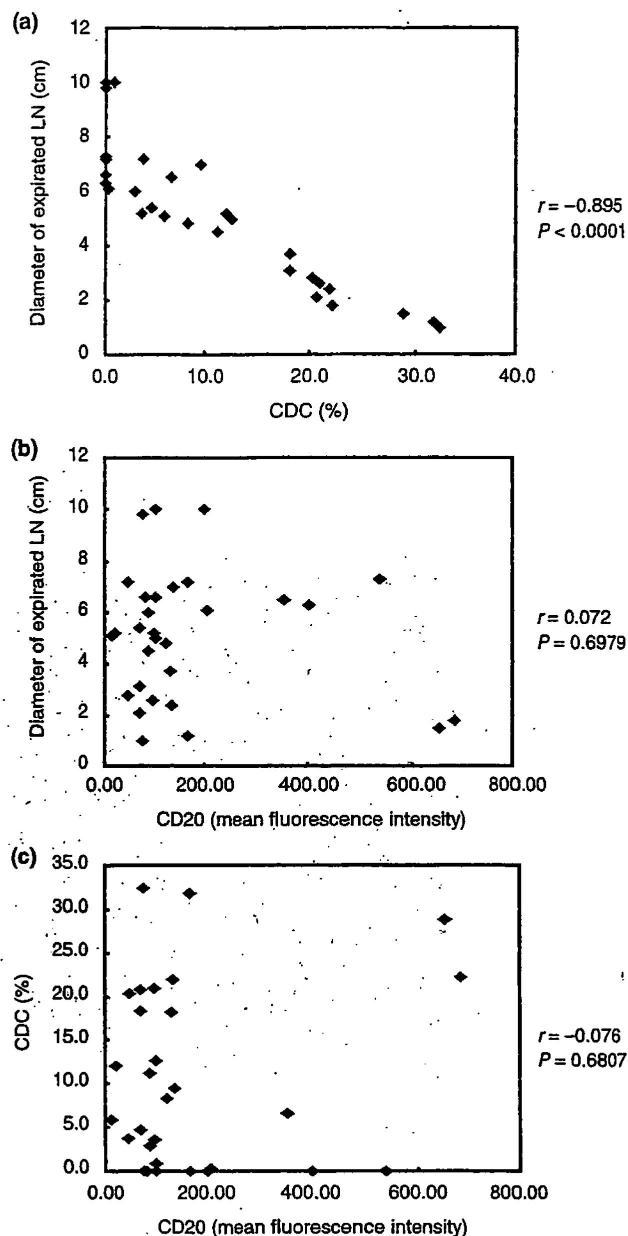

Fig. 1. Relationships between the size of extirpated tumors, susceptibility to complement-dependent cytotoxicity (CDC), and CD20 expression. The size of tumors from 30 patients with non-Hodgkin's lymphoma was measured and the cells were collected. After isolation of CD19-positive cells, FACScan analysis was carried out with anti-CD20 antibody, and CDC assay with rituximab was performed. Intensity of CD20 expression was normalized compared with a control. (a) Scatter plot and correlation analysis for size of extirpated tumor versus susceptibility to CDC. (b) Scatter plot and correlation analysis for size of extirpated tumor versus mean fluorescence intensity of CD20. (c) Scatter plot and correlation analysis for mean fluorescence intensity of CD20 versus susceptibility to CDC. All correlations were tested using the Spearman rank correlation coefficient.

doi: 10.1111/j.1349-7006.2006.00139.x
© 2006 Japanese Cancer Association

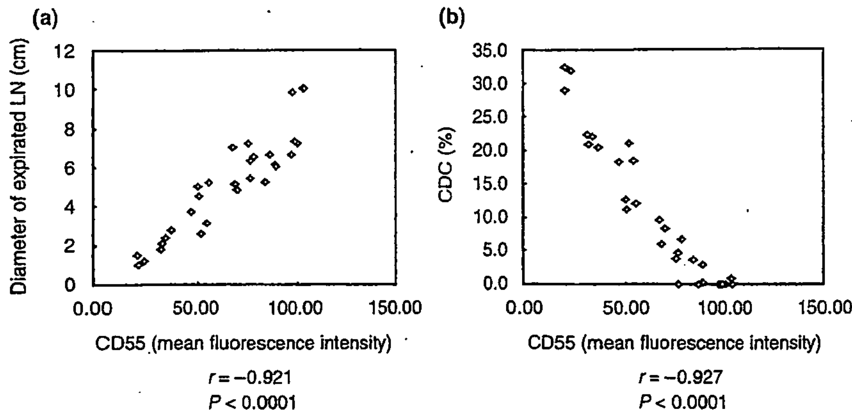

Fig. 2. Relationships between tumor size, CD55 expression and susceptibility to complement-dependent cytotoxicity (CDC). The size of tumors from 30 patients with lymphoma was measured and the cells were collected. After isolation of CD19+/CD20+ cells, FACscan analysis for CDC assay and CD55 expression were carried out. The intensity of CD55 expression was normalized compared with a control. (a) Scatter plot and correlation analysis for size of extirpated tumors versus CD55 expression. (b) Scatter plot and correlation analysis for CD55 expression versus susceptibility to CDC. All correlations were tested using the Spearman rank correlation coefficient.

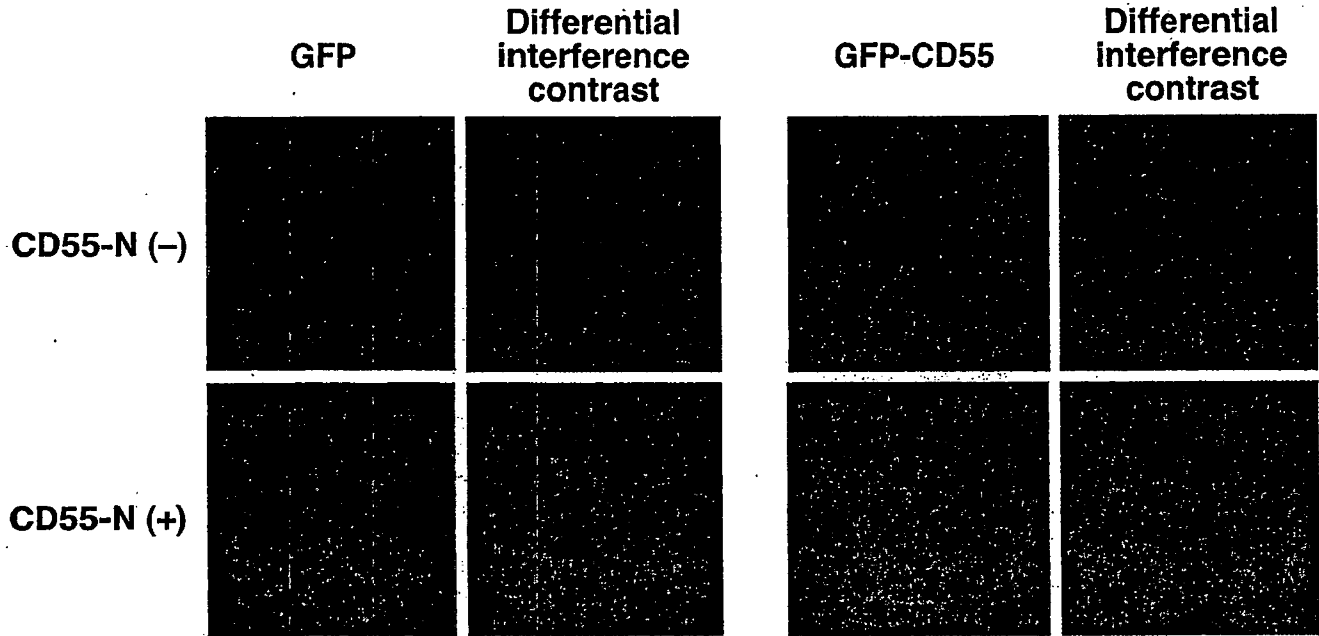

Fig. 3. Effect of small interfering RNA (siRNA) against the 5′-site of the *CD55* gene on expression of the exogenous *CD55* gene. MCF7 cells were transfected with pEGFP or pEGFP-CD55 in the presence or absence of siRNA. After 24 h, the cells were observed by laser scanning microscopy.

Size, CD55 expression and CDC in clinical samples

To investigate the relationship between the size of the extirpated tumor and CD55 expression in clinical samples, correlations between the size of extirpated tumor and fluorescence mean intensity of CD55, and between susceptibility to CDC with rituximab and fluorescence mean intensity of CD55, were analyzed statistically (Fig. 2). As shown in Fig. 2a, the level of CD55 expression on lymphoma cells was statistically correlated with the size of the lymph node ($r = 0.921$, $P < 0.001$). In contrast, the relationship between susceptibility to CDC with rituximab and fluorescence mean intensity of CD55 statistically revealed a negative correlation ($r = -0.927$, $P < 0.001$) (Fig. 2b). This suggests that increasing size of tumor contributes to higher or enhanced CD55 expression and resistance to CDC with rituximab.

Effect of siRNA for CD55 on CD55-transfected MCF7 cells

To overcome the resistance to CDC with rituximab on bulky mass, siRNA against a part of CD55 (CD55-N for 1–380 nucleotides) was designed and cotransfected with the pEGFP or pEGFP-CD55 plasmid into MCF7 cells (Fig. 3). When the cells were cotransfected with both pEGFP and siRNA for CD55, the expression of green fluorescent protein (GFP) did not change compared with transfection with only pEGFP vector (Fig. 3, upper panels). On the other hand, when the cells were cotransfected with both pEGFP-CD55 and siRNA for CD55, the expression of GFP-CD55 disappeared compared with transfection with only the pEGFP-CD55 vector (Fig. 3, lower panels). This suggests that CD55-N, siRNA against 1–380 nucleotides in the CD55 gene, is effective for blocking the expression of CD55.

Decrease in CD55 expression by siRNA overcomes resistance to CDC in breast cancer cell line SK-BR3

We investigated the use of a monoclonal antibody against the Her2/neu molecule for breast cancer, named trastuzumab.

© 2006 Japanese Cancer Association

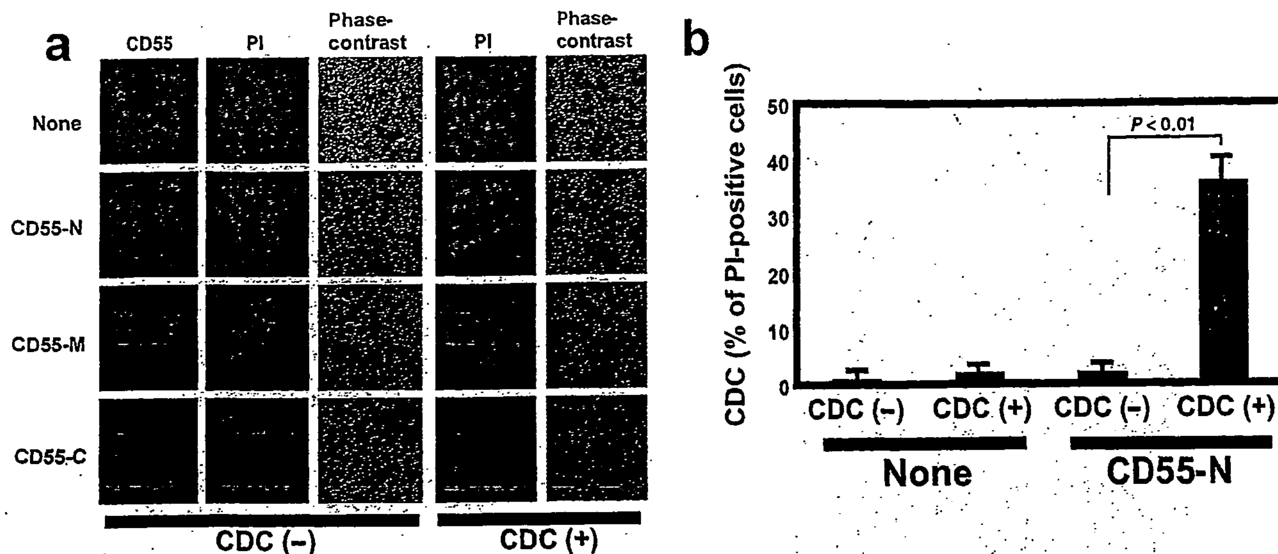

Fig. 4. Blockade of endogenous CD55 on breast cancer cells by small interfering RNA (siRNA). (a,b) SK-BR3 cells were transfected with siRNA against three parts of CD55, namely CD55-N, CD55-M and CD55-C, for 72 h. After transfection, the cells were stained with the anti-CD55 antibody and DAPI, and then the complement-dependent cytotoxicity (CDC) assay with trastuzumab was carried out with or without adding fresh human AB serum (a, left and right panels). (b) The percentage of propidium iodide-positive cells was calculated by counting 100 cells. Data are the mean ± SD (error bars) from experiments with triplicate samples. All statistical tests were two-sided Student's t-tests.

Because the breast cancer cell line SK-BR3 expresses Her2/neu and CD55 on its cell surface, siRNAs against three parts of CD55 (CD55-N for 1–380 nucleotides; CD55-M for 381–817 nucleotides; and CD55-C for 821–1146 nucleotides) were designed and introduced into SK-BR3 cells (Fig. 4). To detect dying cells, PI staining was used for the CDC assay with trastuzumab, and then the percentage of PI-positive cells was evaluated under laser scanning confocal microscopy. Most SK-BR3 cells expressed CD55 molecules without transfection of siRNA against CD55 (Fig. 4a, left). In contrast, expression of CD55 on SK-BR3 cells transfected with CD55-N disappeared 72 h after transfection, or became much weaker than without transfection of siRNA against CD55 (Fig. 4a, right). SK-BR3 cells transfected with CD55-M or CD55-C did not reveal knock down of CD55 expression to the level seen with CD55-N (Fig. 4a). Only 3.0 ± 1.0% of SK-BR3 cells without transfection of siRNA (mock transfection) against CD55 became PI-positive by CDC with trastuzumab, whereas 36.0 ± 6.0% of cells were PI-positive by CDC with trastuzumab after the transfection of siRNA (Fig. 4b). This suggested that siRNA against nucleotides 1–380 of CD55 (i.e. CD55-N) was effective for decreasing CD55 expression and sensitivity to CDC on adherent cells such as SK-BR3.

Blockade of CD55 expression by siRNA overcomes resistance to CDC in fresh lymphoma cells

To investigate the effect of siRNA against CD55 on fresh lymphoma cells, lymphoma cells were isolated from the lymph nodes of five patients with recurrent lymphomas and transfected with siRNA against CD55 (Fig. 5). As shown in Fig. 5a, lymphoma cells from all five cases with recurrent lymphoma strongly expressed CD55 molecules under laser scanning confocal microscopy. When fresh lymphoma cells were transfected with CD55-N for 24 h, but not CD55-M and CD55-C, CD55 expression on fresh lymphoma cells was significantly knocked down under laser scanning confocal microscopy, compared with the control (Fig. 5a, left columns). The percentage of PI-positive cells showed no significant differences among transfections with and without CD55-N, CD55-M and CD55-C before the CDC assay (Fig. 5b). The percentage of PI-positive cells in the transfection with CD55-N significantly increased from 7.1 ± 2.8% to 67.9 ± 8.1%. This indicates that the siRNA against CD55 (CD55-N) could efficiently knock down the expression of CD55 on SK-BR3 and freshly isolated lymphoma cells from recurrent lymphomas, and that it could induce cell death in SK-BR3 and freshly isolated lymphoma cells from recurrent lymphomas by CDC. This suggests that the degree of CD55 expression can determine resistance to CDC with antibody therapy, and that the therapies, which target CD55 molecules such as siRNA and its monoclonal antibody, would be helpful in antibody therapy for bulky disease.

Discussion

Treatment of malignancies has been largely based on chemotherapy and radiotherapy. Although improvement in response rates and survival has been obtained with these therapies over the years, a significant proportion of patients do not respond to treatment, or they relapse. Moreover, conventional cytotoxic therapy is often associated with significant morbidity. Recently, molecular targeting therapy has been developed[22] and monoclonal antibodies against CD20 and HER2/neu have been used for molecular targeting therapy.[1–3] Also, in recent therapies for malignancies, monoclonal antibodies have emerged as important therapeutic agents.

In the preset study, we have shown a negative correlation between the size of extirpated lymph nodes and susceptibility to CDC with rituximab, but the level of CD20 expression did

doi: 10.1111/j.1349-7006.2006.00139.x
© 2006 Japanese Cancer Association

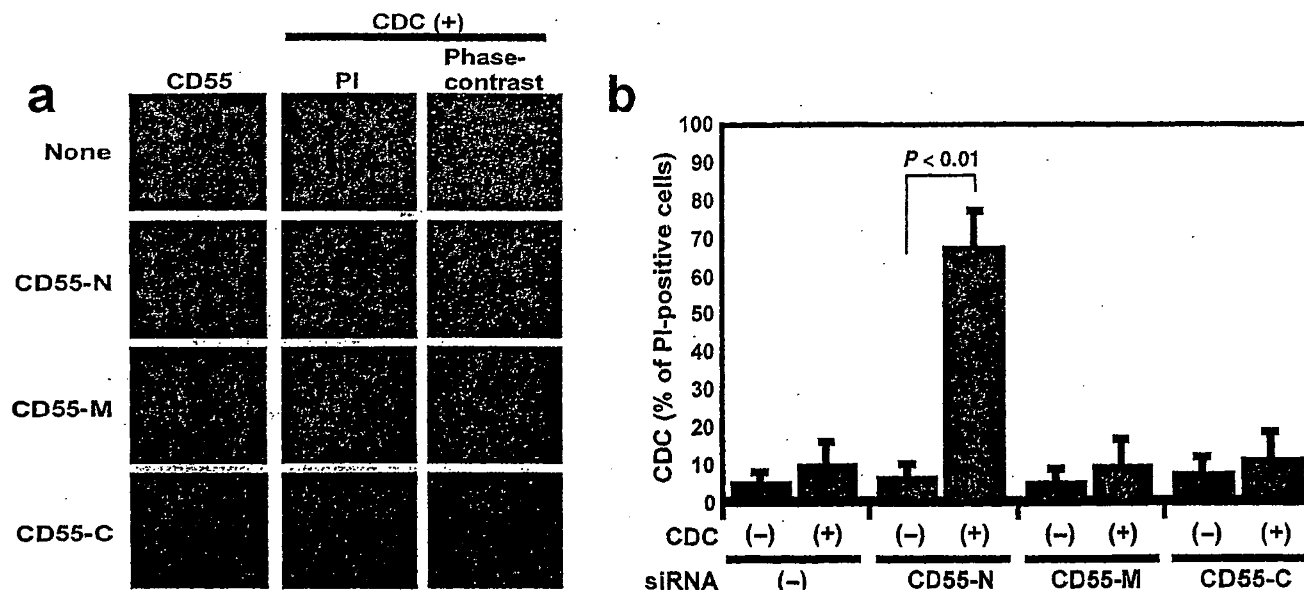

Fig. 5. Blockade of CD55 on primary lymphoma cells by small interfering RNA (siRNA). (a,b) Lymphoma cells from the lymph nodes of five patients with chemotherapy refractory and resistant lymphoma were transfected with siRNA against three parts of CD55, namely CD55-N, CD55-M and CD55-C, for 24 h. (a) After transfection, the cells were stained with anti-CD55 antibody and propidium iodide (PI), and then the complement-dependent cytotoxicity (CDC) assay with rituximab was carried out with or without adding fresh human AB serum. (b) The percentage of PI-positive cells was calculated by counting 100 cells. Data are the mean ± SD (error bars) from experiments with triplicate samples. All statistical tests were two-sided Student's *t*-tests.

not correlate with the size of the lymph node or susceptibility to CDC with rituximab. To date, no other studies have analyzed the relationship between size of lymph node and susceptibility to CDC with rituximab. It has been shown previously that CDC is directly correlated with CD20 expression.[11,23] In contrast, Manches *et al.*[24] have reported in detail that there is no direct correlation between lysis and expression of CD20 in global lymphoma such as FL, mantle cell lymphoma (MCL), small lymphocytic lymphoma (SLL), diffuse large B cell lymphoma (DLCL), and non-tumor B cells, as we showed in the current study. They also suggested that other regulators such as C-reactive protein (CRP) might play important roles in this complement system.

Although antibody therapy is a good tool, resistance sometimes occurs due to unknown mechanisms.[8,25] Patients with bulky mass, especially more than 7 cm of lymphoma mass, often show resistance to rituximab and are not curable.[26] We have demonstrated that CDC activity negatively correlates with the size of extirpated lymph nodes, and that the formula's intercept is 7.447 cm. This suggests that CDC is ineffective to tumors greater than 7.447 cm in size, and that our observation is consistent with the report of Coiffier *et al.*[26] Additionally, CD55 expression significantly correlates with the size of extirpated lymph nodes, suggesting that CD55 expression may play an important role in CDC resistance with antibody therapy. High densities of Daudi and Raji cells, associated with bulky mass, also became resistant to CDC with rituximab, and expression of CD55 increased during cell culture (Terui *et al.*, unpublished data). The relationship between cell density and size of tumors, resistance to CDC and CD55 expression are the same in not only extirpated lymph nodes from patients but also in experimental cell lines. Although previous reports have discussed whether CD55 can be an indicator of prognosis, no one has reported the relationship between cell density and tumor size, resistance to CDC and CD55 expression. Low or high CD55 expression has been reported in CLL cells.[11] However, some researchers have reported that *in vitro* susceptibility to rituximab-induced CDC could not be predicted by the levels of CD55 protein in CLL cells, nor *in vivo* in FL and CLL patients.[12,13] On the other hand, Golay *et al.*[27] have reported that relative levels of CD55 and CD59 may become useful markers to predict clinical responses. Overexpression of CD55 on some tumor cell lines and in colorectal carcinomas has been shown to be an indicator of poor prognosis. This result is consistent with the present study, as we found that CD55 expression in bulky disease may be a useful indicator of this prognosis. Recently, Madjd *et al.*[28] reported that loss of CD55 is related to poor prognosis in breast cancer. High expression of CD55 was significantly associated with low-grade lymph node negativity and with good prognosis. Survival analysis showed that CD55 overexpression was associated with a more favorable outcome. On the other hand, loss of CD55 is associated with poor survival. They established a novel anti-CD55 antibody for use in immunohistochemistry. Although they classified weak to strong intensity of CD55, it is possible that the antibody recognized the non-glycosylated SCR3 domain of CD55 molecule, but not the glycosylated CD55 molecule. The authors pointed out that loss of CD55 is associated with poor prognosis, but not with monoclonal antibody resistance. In the present study, we demonstrated that blockage of CD55 overcomes resistance to antibody therapy and that CDC plays an important role in tumor attack in antibody therapy. As the mechanism that we refer to is different from their study, it may depend on the type of cancer investigated.

© 2006 Japanese Cancer Association

Malignant progression has been reported to be associated with tumor hypoxia, and the inside of the bulky mass showed low oxygen partial pressure (PO_2) (<10 mmHg).[29] Because hypoxia induces COX-2 expression and prostaglandin E_2 (PGE_2) production in not only human vascular endothelial cells[30] but also tumor cells,[31,32] PGE_2 may be produced more in bulky tumors with hypoxia. Recently, it has been reported that PGE_2 upregulates expression of the complement inhibitor CD55 in colorectal cancer.[33] This suggests that bulky mass of lymphoma and other cancers may express CD55 to high levels via PGE_2 production.

It has been reported that the protective activity of rituximab or the 1F5 antibody is completely abolished in syngeneic knockout animals lacking C1q, the first component of the classical complement pathway C (C1qa$^{-/-}$).[34] This indicates that complement activation is fundamental for rituximab therapeutic activity *in vivo*. As CDC is more rapidly and efficiently triggered by monoclonal antibodies in cells with higher expression of their target molecules, we focused on how sensitivity to CDC can be recovered in the resistance to monoclonal antibody therapy. In antibody therapy, blockage of CD55 may be useful for recovery of sensitivity to CDC. It has been reported that anti-CD55 and anti-CD59 antibodies can enhance CDC sensitivity with rituximab, and that CD55 and CD59 may become useful markers to predict the clinical response.[24] Although they did not mention the therapy against resistance to antibody therapy using anti-CD55 and anti-CD59 antibodies,[24] there are three ways to block the function of CD55: (i) blocking the antibody against CD55; (ii) siRNA[35] for CD55; and (iii) small molecules as CD55 inhibitors. We have demonstrated that siRNA for CD55 successfully inhibited functional CD55 protein, and that CDC activity was enhanced in the CD55-knock down breast cancer cell line SK-BR3 and in clinical samples from lymphoma patients. In particular, siRNA is a better tool for blocking CD55, as siRNA can inhibit not only expression of CD55 but also the function of CD55. Nagajothi *et al.* also showed genetic and biochemical methods to decrease CD55 expression and other GPI-anchored proteins.[36] This suggests that a decline in CD55 levels could be enough to make the tumor sensitive to CDC with rituximab and trastuzumab.

In conclusion, we have shown that CD55 blockade by siRNA enhances rituximab-mediated cytotoxicity. This observation gives us a novel strategy to suppress bulky disease-related resistance to monoclonal antibody treatment.

Acknowledgments

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan; a Research on Advanced Medical Technology grant from the Ministry of Health, Welfare and Labor; and a grant for International Health Cooperation Research from the Ministry of Health, Welfare and Labor of Japan. We appreciate the assistance of Dr Dovie Wylie for English editing and correction, and thank Ms Tomomi Sagawa and Noriko Yamamichi for technical assistance.

References

1 Coiffier B. Immunochemotherapy: the new standard in aggressive non-Hodgkin's lymphoma in the elderly. *Semin Oncol* 2003; **30** (1 Suppl. 2): 21–7.
2 Tan AR, Swain SM. Ongoing adjuvant trials with trastuzumab in breast cancer. *Semin Oncol* 2003; **30** (5 Suppl. 16): 54–64.
3 Hiddemann W, Dreyling M, Unterhalt M. Rituximab plus chemotherapy in follicular and mantle cell lymphomas. *Semin Oncol* 2003; **30** (1 Suppl. 2): 16–20.
4 Dillman RO. Treatment of low-grade B-cell lymphoma with the monoclonal antibody rituximab. *Semin Oncol* 2003; **30**: 434–47.
5 Blum KA, Bartlett NL. Antibodies for the treatment of diffuse large cell lymphoma. *Semin Oncol* 2003; **30**: 448–56.
6 Grillo-Lopez AJ. Rituximab: an insider's historical perspective. *Semin Oncol* 2000; **27** (6 Suppl. 12): 9–16.
7 Maloney DG, Smith B, Rose A. Rituximab: mechanism of action and resistance. *Semin Oncol* 2002; **29** (1 Suppl. 2): 2–9.
8 Villamor N, Montserrat E, Colomer D. Mechanism of action and resistance to monoclonal antibody therapy. *Semin Oncol* 2003; **30**: 424–33.
9 Wojnicz D, Bar J, Jankowski S. [The role of membrane glycoproteins CD46, CD55 and CD59 in protection of tumor cells against complement lysis]. *Postepy Hig Med Dosw* 2002; **56** (5): 603–16. (In Polish.)
10 Cerny T, Borisch B, Introna M, Johnson P, Rose AL. Mechanism of action of rituximab. *Anticancer Drugs* 2002; 13 (Suppl. 2): S3–10.
11 Bellosillo B, Villamor N, Lopez-Guillermo A *et al.* Complement-mediated cell death induced by rituximab in B-cell lymphoproliferative disorders is mediated *in vitro* by a caspase-independent mechanism involving the generation of reactive oxygen species. *Blood* 2001; **98**: 2771–7.
12 Bannerji R, Kitada S, Flinn IW *et al.* Apoptotic-regulatory and complement-protecting protein expression in chronic lymphocytic leukemia: relationship to *in vivo* rituximab resistance. *J Clin Oncol* 2003; **21**: 1466–71.
13 Weng WK, Levy R. Expression of complement inhibitors CD46, CD55, and CD59 on tumor cells does not predict clinical outcome after rituximab treatment in follicular non-Hodgkin lymphoma. *Blood* 2001; **98**: 1352–7.
14 Cardarelli PM, Quinn M, Buckman D *et al.* Binding to CD20 by anti-B1 antibody or F (ab') (2) is sufficient for induction of apoptosis in B-cell lines. *Cancer Immunol Immunother* 2002; **51**: 15–24.
15 Hourcade D, Liszewski MK, Krych-Goldberg M, Atkinson JP. Functional domains, structural variations and pathogen interactions of MCP, DAF and CR1. *Immunopharmacology* 2000; **49**: 103–16.
16 Jarva H, Meri S. Paroxysmal nocturnal haemoglobinuria: the disease and a hypothesis for a new treatment. *Scand J Immunol* 1999; **49**: 119–25.
17 Jeremias I, Kupatt C, Baumann B, Herr I, Wirth T, Debatin KM. Inhibition of nuclear factor κB activation attenuates apoptosis resistance in lymphoid cells. *Blood* 1998; **9**: 4624–31.
18 Unruh A, Ressel A, Mohamed HG *et al.* The hypoxia-inducible factor-1α is a negative factor for tumor therapy. *Oncogene* 2003; **22**: 3213–20.
19 Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001; **411**: 494–8.
20 Pham LV, Tamayo AT, Yoshimura LC, Lin-Lee YC, Ford RJ. Constitutive NF-κB and NFAT activation in aggressive B cell lymphomas synergistically activates the CD154 gene and maintains lymphoma cell survival. *Blood* 2005; **106**: 3940–7.
21 Surmacz E. Growth factor receptors as therapeutic targets: strategies to inhibit the insulin-like growth factor I receptor. *Oncogene* 2003; **22**: 6589–97.
22 Gale DM. Molecular targets in cancer therapy. *Semin Oncol Nurs* 2003; **19**: 193–205.
23 Golay J, Lazzari M, Facchinetti V *et al.* CD20 levels determine the *in vitro* susceptibility to rituximab and complement of B-cell chronic lymphocytic leukemia: further regulation by CD55 and CD59. *Blood* 2001; **98**: 3383–9.
24 Manches O, Lui G, Chaperot L *et al. In vitro* mechanisms of action of rituximab on primary non-Hodgkin lymphomas. *Blood* 2003; **101**: 949–54.
25 Smith MR. Rituximab (monoclonal anti-CD20 antibody): mechanisms of action and resistance. *Oncogene* 2003; **22**: 7359–68.
26 Coiffier B, Haioun C, Ketterer N *et al.* Rituximab (anti-CD20 monoclonal antibody) for the treatment of patients with relapsing or

doi: 10.1111/j.1349-7006.2006.00139.x
© 2006 Japanese Cancer Association

refractory aggressive lymphoma: a multicenter phase II study. *Blood* 1998; 92: 1927–32.

27 Golay J, Zaffaroni L, Vaccari T *et al.* Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab *in vitro*: CD55 and CD59 regulate complement-mediated cell lysis. *Blood* 2000; 95: 3900–8.

28 Madjd Z, Durrant LG, Bradley R, Spendlove I, Ellis IO, Pinder SE. Loss of CD55 is associated with aggressive breast tumors. *Clin Cancer Res* 2004; 10: 2797–803.

29 Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res* 1996; 56: 4509–15.

30 Schmedtje JF Jr, Ji YS, Liu WL, DuBois RN, Runge MS. Hypoxia induces cyclooxygenase-2 via the NF-κB p65 transcription factor in human vascular endothelial cells. *J Biol Chem* 1997; 272: 601–8.

31 Liu XH, Kirschenbaum A, Yu K, Yao S, Levine AC. Cyclooxygenase-2 suppresses hypoxia-induced apoptosis via a combination of direct and indirect inhibition of p53 activity in a human prostate cancer cell line. *J Biol Chem* 2005; 280: 3817–23.

32 Liu XH, Kirschenbaum A, Yao S *et al.* Upregulation of vascular endothelial growth factor by cobalt chloride-simulated hypoxia is mediated by persistent induction of cyclooxygenase-2 in a metastatic human prostate cancer cell line. *Clin Exp Metastasis* 1999; 17: 687–94.

33 Holla VR, Wang D, Brown JR, Mann JR, Katkuri S, DuBois RN. Prostaglandin E2 regulates the complement inhibitor CD55/decay-accelerating factor in colorectal cancer. *J Biol Chem* 2005; 280: 476–83.

34 Di Gaetano N, Cittera E, Nota R *et al.* Complement activation determines the therapeutic activity of rituximab *in vivo*. *J Immunol* 2003; 171: 1581–7.

35 Elbashir SM, Harborth J, Lendecke W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001; 411: 494–8.

36 Nagajothi N, Matsui WH, Mukhina GL, Brodsky RA. Enhanced cytotoxicity of rituximab following genetic and biochemical disruption of glycosylphosphatidylinositol anchored proteins. *Leuk Lymphoma* 2004; 45: 795–9.

© 2006 Japanese Cancer Association

Breast Cancer
Vol. 13 No. 1 January 2006

Review Article

Gene Therapy for Breast Cancer. – Review of Clinical Gene Therapy Trials for Breast Cancer and *MDR1* Gene Therapy Trial in Cancer Institute Hospital

Shunji Takahashi*[1], Yoshinori Ito*[1], Kiyohiko Hatake*[1], and Yoshikazu Sugimoto*[2,3]

*[1]*Department of Medical Oncology, Cancer Institute Hospital and* *[2]*Department of Gene Therapy, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research,* *[3]*Department of Chemotherapy, Kyoritsu University of Pharmacy, Japan.*

Gene therapy for advanced breast cancer is anticipated to be a useful therapeutic approach. Strategies in ongoing clinical protocols can be divided into four groups: (1) suppression of oncogenes or transfer of tumor-suppressor genes; (2) enhancement of immunological response; (3) transfer of suicide genes; (4) protection of bone marrow using drug resistance genes. We have started a clinical study of multidrug resistance (*MDR1*) gene therapy. Advanced breast cancer patients received high dose chemotherapy and autologous peripheral blood stem cell transplantation (PBSCT) with *MDR1*-transduced hematopoietic cells, and then were treated with docetaxel. Two patients have been treated so far, and *in vivo* enrichment of *MDR1*-transduced cells with docetaxel treatment has been seen. Both patients are in complete remission and had no apparent adverse effects from the *MDR1* gene transfer.

Breast Cancer 13:8-15, 2006.

Key words: Breast cancer, Gene therapy, *MDR1*, Adenoviral vector, Retroviral vector

The cure rate of advanced or recurring breast cancer is under 5%, so the usual goal of treatment is prolongation of survival or improvement of quality of life (QOL), not cure[1]. Endocrine therapy for hormone-receptor-positive patients, chemotherapy, radiation therapy, bisphosphonates for bone diseases, and trastuzumab for HER2-overexpressed patients, have all been shown to be effective for advanced breast cancer, but none has been shown to increase the cure rate.

Gene therapy for advanced breast cancer is expected to be a useful therapeutic approach. Strategies in ongoing clinical protocols can be divided into four groups: (1) suppression of oncogenes or transfer of tumor-suppressor genes; (2) enhancement of immunological response; (3) transfer of suicide genes; (4) protection of bone marrow using drug resistance genes (Table 1)[2,3]. There are three major methods for gene transfer: (1) transduction of naked DNA such as lipofection (transient expression); (2) transduction of adenoviral vector or vaccinia virus vector (transient expression); (3) transduction of retroviral vector (stable expression). In this paper, ongoing clinical trials of gene therapy for breast cancer are reviewed, and a clinical trial of multiple drug resistance 1 (*MDR1*) gene therapy at our institution is described.

Reprint requests to Shunji Takahashi, Department of Medical Oncology, Cancer Chemotherapy Center and Cancer Institute Hospital, Japanese Foundation for Cancer Research, 3-10-6 Ariake, Koto-ku, Tokyo, 135-8500, Japan.
E-mail: stakahas@jfcr.or.jp

Present Status of Clinical Trials of Gene Therapy for Breast Cancer

Suppression of Oncogene Expression or Transfer of Tumor-Suppressor Gene

The carcinogenic process requires an accumulation of multiple gene mutations or abnormalities of gene expression. Common gene abnormalities in breast cancer include p53 gene mutation, ErbB2/HER2 gene amplification, c-myc gene amplification, and cyclin D1 gene amplification[4]. Several clinical trials aim to improve those gene abnormalities by local or systemic gene transfer.

A) Transfer of the normal p53 gene: Mutations of the p53 gene are the most frequently found gene abnormalities among various malignancies, including breast cancer[5]. Tumor cells with mutated p53 genes show defects of cell-cycle regulation,

Table 1. Clinical Studies of Gene Therapy for Breast Cancer

Strategy	Gene	vector	Investigator
1 suppression of oncogene or transfer of tumor suppressor gene	p53	adenovirus	von Mehren
			Cristofanilli
			Baynes
	E1A	lipofection	Hortobagyi
	antisense (c-fos, c-myc)	retrovirus	Holt
	MDA-7	adenovirus	Bucholz
2-A transfer of cytokine gene	IL-2	lipofection	Lyerly
		adenovirus	Stewart
	IL-12	retrovirus	Park
	GM-CSF	adenovirus	Suzuki
	TNF + NeoR	retrovirus	Rosenberg
2-B transfer of costimulatory molecule gene	B7.1 (CD80)	lipofection	Urba
		adenovirus	Schuchter
2-C transfer of antigen gene	MUC1	vaccinia virus	Kufe
	HER-2	naked DNA	Patel
	MUC1 + CD80	vaccinia virus	Eder
	MUC1 + IL-2	vaccinia virus	Velu
3 transfer of suicide gene	HSV-TK	retrovirus	Favrot
	Cytosine deaminase	lipofection	Lemoine
	CYP 2B6	retrovirus	Harris
4 transfer of drug resistance gene	MDR1	retrovirus	Stewart
			Cowan
			Deisseroth
			Hesdorffer
			O'Shaughnessy
			Takahashi

according to http://www.wiley.co.uk/genetherapy/clinical

and transfer of normal p53 genes causes cell-cycle arrest or apoptosis. Clinical studies of p53 gene therapy using adenoviral vectors (Advexin, Introgen *et al.*) for various tumor types, including breast cancer, are ongoing. Von Mehren and Cristofanilli have begun clinical studies of a combination of local injection of p53-adenoviral vector into skin metastatic lesions or locally advanced breast cancer and systemic chemotherapy. Baynes has initiated a clinical study of high dose chemotherapy associated with transplantation of autologous peripheral blood stem cells (PBSC) that have been purged *ex vivo* by p53-adrnovirus infection. Baynes's group has shown that p53 gene transfer has no effect on normal PBSC.

B) Suppression of the ErbB2/HER2 gene: The ErbB2/HER2 gene encodes an 185 kD protein and is a member of the epidermal growth-factor receptor family. This gene is amplified in 20-30% of breast cancer patients, and correlates with a poor prognosis and resistance to hormone therapy[4]. Monoclonal humanized murine antibody to ErbB2/HER2 protein (trastuzumab/Herceptin™) is effective in advanced, ErbB2/HER2-overexpressing breast cancer patients[6]. The adenovirus type 2 or type 5 E1A gene inhibits expression of the ErbB2/HER2 gene, and E1A gene transfer into ErbB2/HER2-overexpressed tumors causes tumor reduction and enhances sensitivity to chemotherapy *in vitro* and *in vivo*[7]. At MD Anderson Cancer Center, patients with breast cancer or ovarian cancer overexpressing ErbB2/HER2 were treated with gene therapy using a local injection of E1A gene-liposome into skin lesions or pleural/peritoneal effusion[8]. There was no serious adverse effect other than fever or pain at the injection sites. In

six cases in which tumor cells in body fluids could be analyzed, reduction of ErbB2/HER2 expression and a decrease in tumor cells were shown. E1A gene transfer also reduced tumor growth of non-HER2-overexpressing cells, and E1A gene transfer to tumor tissues of breast cancer or head and neck cancer by lipofection showed minor response in HER2-negative tumors[9].

C) Suppression of c-myc and c-fos gene: Arteaga and Holt made a retroviral vector which overexpresses antisense mRNA to c-myc and c-fos genes under the control of mammary tumor virus (MMTV) promoter. Transfer of this vector into a breast cancer cell line suppressed tumor formation in animal models[10]. They have started a clinical trial of gene therapy for malignant effusion or meningitis in breast cancer patients who have failed standard therapy. Effusions will be drained and replaced with a solution of the vector, then periodically drained to follow the disease and assess gene transfer[11].

D) Transfer of melanoma differentiation associated protein 7 (MDA-7): MDA-7 is a novel tumor suppressor gene, and its transfer into tumor cells causes growth suppression and apoptosis. However, MDA-7 gene transfer into normal cell lines does not[12]. A clinical trial of gene therapy that injects MDA-7- adenoviral vector (Ad-mda7, ISGN 241) into tumor cells has started (Buchholz). There was no serious adverse effect in a phase I study, and a combination phase I/II study with irradiation has begun.

Augmentation of Immunological Response to Cancer Cells

Breast cancer cells have long been supposed to have low antigenecity and to be resistant to immune therapy. So far, reports of nonspecific immune therapies such as BCG have shown that those therapies are not effective for breast cancer[13]. But since the 1990s, many breast cancer-associated antigens have been reported, and various clinical studies of specific immune therapy for breast cancer, such as vaccination therapy targeted to ErbB2/HER2, are ongoing[14,15]. Immune therapy by gene transfer includes: 1) transfer of cytokine genes that enhance immune response, 2) transfer of co-stimulatory molecule genes, and 3) transfer of antigen molecule genes.

A) Transfer of cytokine genes

i) Interleukin-2 (IL-2): Injection of IL-2 gene-adenoviral vector into tumor tissues[16], or subcutaneous injection of inactivated tumor cells that were transduced *ex vivo* by IL-2 gene lipofection (Lyerly) may cause a systemic immune reaction in tumor cells. In a phase I/II study, Stewart *et al.*[17] treated 23 cases with breast cancer or malignant melanoma by injection of 10^7-10^{10}pfu adenovirus-IL-2 into subcutaneous tumors. There was no side effect other than local inflammation of injection sites, and reduction in diameter of subcutaneous tumors was reported in 24% of patients, but there was no PR.

ii) Interleukin-12 (IL-12): Retroviral transfer of IL-12 gene into skin fibroblasts of patients *ex vivo*, then injection of the fibroblasts into tumor tissues may activate a tumor-specific immune response. In a phase I study, nine cases with advanced neoplasm including breast cancer were treated by Kang *et al.* Reduction of tumor at injection sites was shown in four cases, and reduction of tumor at remote sites was shown in one melanoma case. There was no side effect other than slight pain at the injection sites[18].

iii) Granulocyte-macrophage colony stimulating factor (GM-CSF): Retroviral transfer of GM-CSF gene into tumor cells and injection of those cells into subcutaneous tissue may activate systemic immune reaction to tumor cells (Suzuki). The same gene therapy for renal cell cancer has been done in Japan.

iv) Tumor necrosis factor (TNF): Retroviral transfer of TNF gene and Neo gene into tumor cells *ex vivo* and subcutaneous injection of tumor cells may activate systemic immune response to tumor cells[19].

B) Transfer of co-stimulatory molecule gene: Transfer of T cell co-stimulatory molecule CD80 (B7.1) gene into tumor cells by lipofection and injection of those tumor cells into subcutaneous tissue (Urba), or direct injection of CD80-adenoviral vector into tumor tissue (Schuchter) may activate T cell growth and immune response.

C) Transfer of antigen gene: Clinical studies of MUC1(CA15-3) gene transfer by vaccinia virus into tumor cells and injection of tumor cells into subcutaneous tissue (Kufe), simultaneous transfer of MUC1 and CD80 gene (Eder), or HER2 gene transfer (Patel), have been ongoing. Scholl *et al.* repeatedly administered vaccinia virus containing MUC1 and IL-2 genes (TG1031) intramuscularly to patients with metastatic breast cancer. In 31 patients, two patients (6%) had PR and 15 patients had SD[20].

Suicide Gene Therapy

Transfer of drug-activating enzyme gene into tumor cells and treatment with a prodrug form of chemotherapeutic agents causes a high concentration of the activated drug in the tumor tissue and apoptosis of tumor cells. Not only transduced cells, but also circumferential cells are reported to die with this gene therapy (bystander effect).

A clinical trial of retroviral herpes simplex virus thymidine kinase (HSV-TK) gene transfer into breast cancer tumor tissues and treatment with gancylovir is ongoing (Favrot).

A phase I study of injection of HER2 promoter-driven cytosine deaminase (CD) gene plasmid into metastatic skin lesions of breast cancer and treatment with prodrug (fluorocytosine) has been reported. Fluorocytosine is transformed into 5FU by the CD gene. Expression of the CD gene in HER2-positive tumor cells has been shown in 9/11 cases at day 2 and 3/10 cases at day 7. Tumor reduction was shown in 4 of 12 cases[21].

Retroviral P450 2B6 (CYP2B6) gene transfer into metastatic cutaneous tissues and oral cyclophosphamide therapy causes efficient conversion of prodrug cyclophosphamide into active metabolite phosphoramide mustard in the tumor tissues. In a phase I study, nine breast cancer and three melanoma patients were treated with CYP2B6 vector (MetXia-P450). One breast cancer patient had a PR and four (33%) had stable diseases (SD) ≧ 3 months[22].

Bone Marrow Protection by Drug-Resistance Gene

Breast cancer is sensitive to chemotherapy. Response rates of advanced breast cancer for most combination chemotherapy are between 40% and 70% (complete response (CR) rate 10-30%), but duration of response is 7-10 months for PR, and 9-18 months for CR. High dose chemotherapy with autologous blood stem cell transplantation for advanced breast cancer has shown high complete response rates (up to 50%), and 10-15% patients have enjoyed durable remission[23,24]. However, most patients will relapse after transplantation. Randomized studies comparing high dose chemotherapy and conventional chemotherapy showed that median survival times appear to be no better than those achieved with conventional chemotherapy, so far[25]. Probably high dose chemotherapy cannot completely eradicate residual disease, and insufficient bone marrow function after the reconstitution is a major problem in post-transplantation chemotherapy. One approach to overcome the current situation would be the transplantation of the drug-resistant gene-transduced hematopoietic stem cells so that normal bone-marrow cells will be protected from the toxic effect of anticancer drugs.

A multidrug resistance 1 (*MDR1*) gene was cloned from cancer cell lines resistant to various anticancer drugs[26]. The *MDR1* gene product (P-glycoprotein, P-gp) is a 170 kD glycoprotein consisting of two trans-membranous domains and two ATP-binding domains. P-gp ATP-dependently excretes various drugs such as doxorubicin, vinka-alkaloids, or taxanes from cytoplasm to extra-cellular fluid. *Ex vivo* transfer of *MDR1* genes into hematopoietic stem cells and transplantation might make post-transplant chemotherapy feasible. Chemotherapeutic drugs such as docetaxel and paclitaxel, which have good clinical activity in the treatment of breast cancer and are efficiently effluxed by P-gp, might be the best choice for this strategy. Using a retroviral vector, Sorrentino *et al.*[27] transplanted *MDR1*-transduced bone marrow into irradiated mice and then treated them with paclitaxel. Paclitaxel treatment increased *MDR1*-transduced leukocytes in peripheral blood (*in vivo* amplification), and *MDR1*-transduced mice showed reduced bone marrow suppression by paclitaxel (bone marrow protection). Then, several groups have undertaken clinical studies of *MDR1* gene therapy for advanced breast cancer or other neoplasms[28-30].

A group at MD Anderson Cancer Center first reported the results of clinical trials[28]. They performed retroviral gene transfer without using cytokines, and in suspension or with autologous stromal cells. *In vitro* transduction efficiency was 2.8% with the solution method and 5.6% with the stromal method, detected by *in situ* PCR. But three to four weeks after transplantation, direct PCR assay of peripheral blood leukocytes in patients showed positive results in 0/10 with the solution method, and 5/8 with the stromal method. These data show insufficient transduction efficiency without using cytokines. NCI also reported the results of a clinical trial of retroviral *MDR1* gene therapy[30]. They transferred *MDR1* genes into bone marrow mononuclear cells or peripheral blood stem cells stimulated by IL-3, IL-6, and SCF. *Ex vivo* transduction efficiency was 0.2-0.5%. They treated transplanted patients with paclitaxel, but

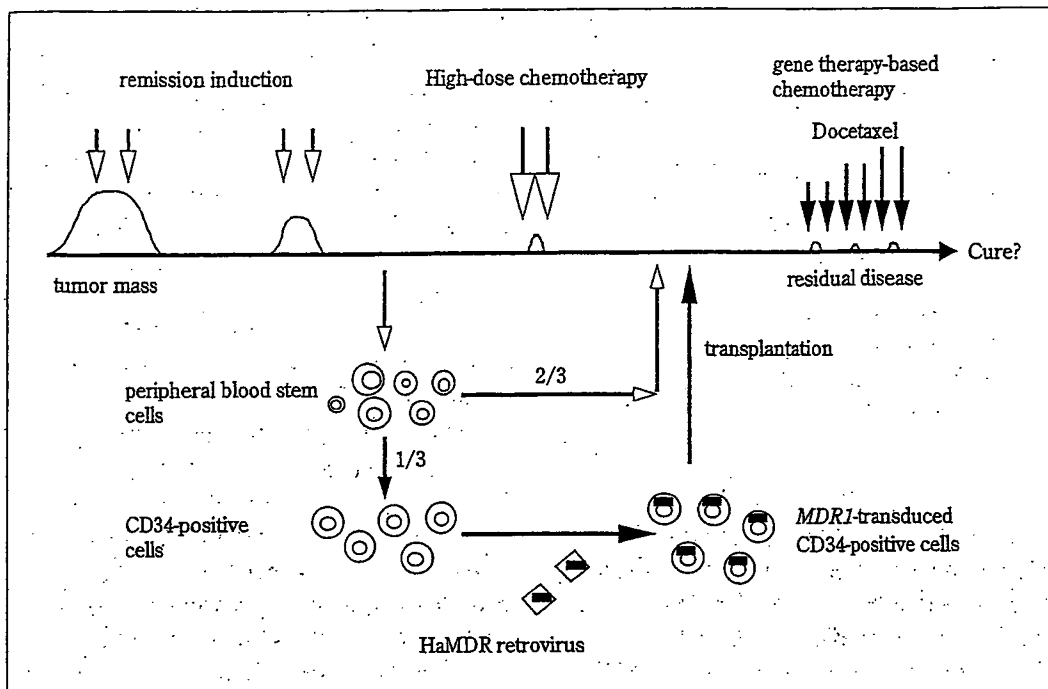

Fig 1. Schema of *MDR1* gene therapy for advanced breast cancer patients in Cancer Institute Hospital.

they could not show any enrichment of *MDR1*-transduced white blood cells by PCR. A group at Columbia University also transferred *MDR1* genes into bone marrow mononuclear cells or peripheral blood stem cells stimulated by IL-3, IL-6, and stem cell factor (SCF). They showed that 20-70% of BFU-E or CFU-GM colonies from transferred CD34-positive cells were positive for *MDR1* by PCR. BM from patients 3-12 weeks after transplantation showed *MDR1*-positivity by PCR in 2/5 patients. They also analyzed P-gp expression in bone marrow cells using flow cytometry, but they could not show any expression. Clinical studies of *MDR1* gene therapy are now ongoing at several institutions (Stewart, Cowan, Disseroth, Hesdorffer, O'Shaughnessy).

MDR1 Gene Therapy in Cancer Institute Hospital

Our group also started *MDR1* gene therapy for breast cancer. This study was approved by the Ministry of Health and the Ministry of Education and Science on February 24, 2000. The outline of the protocol is shown in Fig 1. We selected histologically confirmed, metastatic breast cancer patients who achieved good PR or CR to a precedent conventional dose chemotherapy regimen (using anthracycline and/or taxane). We used a HaMDR vector in which wild type *MDR1* cDNA (Kyoto University) had been inserted into pHa vector (NCI) derived from Harvey mice sarcoma virus (HaMSV). Peripheral blood stem cells (PBSC) were harvested by cyclophosphamide and G-CSF. CD34-positive cells were selected from about one third of PBSC, and HaMDR was transferred into those cells stimulated by SCF, thrombopoietin, IL-6, Flt-3 ligand, and soluble IL-6 receptor. Transduced PBSC were checked for safety (presence of replication-competent retrovirus, etc.) and then frozen. Patients were treated with high-dose cyclophosphamide, thiotepa, and carboplatin. Then unprocessed and *MDR1* gene-transduced PBSC were transplanted together. After bone marrow was reconstituted and patient status was normalized, patients were treated with 50% of standard dose docetaxel, then with increased doses up to 100% if grade 4 neutropenia was not recorded. Gene transfer efficiency and P-gp expression were checked with PCR and flowcytometry analysis, using peripheral leukocytes and bone marrow cells.

So far, two patients have finished high-dose chemotherapy, PBSC transplantation with *MDR1* gene transfer, and then docetaxel chemotherapy (Table 2). Peripheral blood P-gp-positive leuko-

Table 2. Case 1 of *MDR1* Gene Therapy in Cancer Insitute Hospital

October-00	Informed consent, approval by Insitutinal Review Board
November-00	PBSC harvest and *MDR1* gene transfer #1
February-01	PBSC harvest and *MDR1* gene transfer #2
April-01	High dose chemotherapy and transplantation of *MDR1*-transuced PBSC
June-01	Start of docetaxel chemotherapy
October-01	CR after 5 cycles of docetaxel
February-02	Final docetaxel therapy (#10)
March-05	No sign of relapse/leukemia

cytes increased to 5% after transplantation but decreased gradually. During docetaxel chemotherapy after transplantation, *in vivo* expansion of the *MDR1*-transduced cells (up to 10%) was observed. Comparison of two patients suggests the presence of a bone-marrow protection effect by *MDR1* expression during docetaxel chemotherapy, but this is not clear. No serious side effect was observed, and the patients have been in complete remission for 3 years.

Retroviral gene therapy causes random insertion of exogenous genes into genome DNA of target cells, so it may cause carcinogenesis by activation of oncogene or inactivation of tumor suppressor gene. At the end of 2002, occurrence of T cell leukemia in two patients after gene therapy for X-linked severe combined immune deficiency (X-SCID) was reported. A genetic defect in the γC gene, which is a common domain of multiple interleukin receptors (IL-2R, IL-4R, IL-7R, *et al.*), causes severe defects of T cell and natural killer cells as well as severe immune deficiency in X-SCID patients. Retroviral γC gene transfer using autologous CD34-positive hematopoietic cells in X-SCID patients restored immune system in 9 of 11 patients[31]. But T cell leukemia occurred in three patients (one more patient in January 2005) of those 9. In the leukemic cells, retroviral vector was inserted in the LMO2 gene, which causes T cell leukemia[32]. Then the FDA recommended suspension of all clinical trials of retroviral gene therapy for hematopoietic stem cells. We also suspended *MDR1* gene therapy for the third patient in January 2003. After thorough investigation of retroviral gene therapy trials for hematopoietic stem cells all over the world, no leukemia event has been found in clinical gene therapy trials, other than the French X-SCID trial (American Society for Gene Therapy Annual Meeting, 2003). Screening of the Mouse Retroviral Cancer Gene database showed that retroviral insertion into γC and LMO2 gene was found in two cases each, and insertion into both genes were found in one case. This fact suggests that both genes are oncogenes, and that the two genes can collaborate[33]. In X-SCID gene therapy, a double hit with retroviral activation of LMO2 gene and exogenous activated γC gene might be necessary for leukemogenesis. If so, retroviral gene therapy with non-oncogenic genes might have a low risk of cancer[34].

Thereafter, gene therapy using retroviral vector resumed, and retroviral gene transfer into hematopoietic cells of adenosine deaminase deficiency patients was begun in Japan at the end of 2003. We also resumed our *MDR1* gene therapy after changing the protocol (informed consent with regard to the adverse effects and more thorough investigation of patients' peripheral blood), and started high-dose chemotherapy and transplantation of PBSC with *MDR1* gene transfer to the third patient in July 2004.

We also started investigation of insertion sites of HaMDR vector in the first two patients. A clonality study of leukocytes from case 1 showed eight long-lived clones of *MDR1*-tranduced hematopoietic stem cells. No sign of expansion of any clones has been observed.

To summarize the data of our own and other institutions' clinical studies of retroviral *MDR1* gene therapy, first, there has been no serious side effect, including secondary neoplasm, but thorough investigations including retroviral insertion sites are necessary. Second, maintenance of *MDR1*-transduced hematopoietic cells for more than one year was confirmed. Third, the *MDR1*-transduced cells were selectively enriched *in vivo* by chemotherapy. Whether *MDR1* gene therapy can protect bone marrow from chemotherapy is not yet certain. We have almost finished proof-of-concept stage for the gene therapy, and we should be able to show clinical benefits compared with conventional therapy.

The techniques and knowledge of gene therapy are still limited, so we must proceed with caution, and we must inform patients of both the risks and benefits of the therapy.

Acknowledgements

This work was supported by the grants from the Min-

istry of Health, Labor and Welfare, and the Ministry of Education, Culture, Sports, Science and Technology and the Nippon Foundation, Japan.

References

1) Hortobagyi GN: Treatment of breast cancer. *N Engl J Med* 339:974-984, 1998.
2) http://www4.od.nih.gov/oba/rac/clinicaltrial.htm.
3) http://www.wiley.co.uk/genetherapy/clinical/.
4) Osborne C, Wilson P, Tripathy D: Oncogenes and tumor suppressor genes in breast cancer: potential diagnostic and therapeutic applications. *Oncologist* 9:361-377, 2004.
5) Coles C, Condie A, Chetty U, Steel CM, Evans HJ, Prosser J: p53 mutations in breast cancer. *Cancer Res* 52:5291-5298, 1992.
6) Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, *et al*: Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 344:783-792, 2001.
7) Ueno NT, Bartholomeusz C, Herrmann JL, Estrov Z, Shao R, Andreeff M, Price J, Paul RW, Anklesaria P, Yu D, *et al*: E1A-mediated paclitaxel sensitization in HER-2/neu-overexpressing ovarian cancer SKOV3.ip1 through apoptosis involving the caspase-3 pathway. *Clin Cancer Res* 6:250-259, 2000.
8) Hortobagyi GN, Ueno NT, Xia W, Zhang S, Wolf JK, Putnam JB, Weiden PL, Willey JS, Carey M, Branham DL, *et al*: Cationic liposome-mediated E1A gene transfer to human breast and ovarian cancer cells and its biologic effects: a phase I clinical trial. *J Clin Oncol* 19:3422-3433, 2001.
9) Yoo GH, Hung MC, Lopez-Berestein G, LaFollette S, Ensley JF, Carey M, Batson E, Reynolds TC, Murray JL: Phase I trial of intratumoral liposome E1A gene therapy in patients with recurrent breast and head and neck cancer. *Clin Cancer Res* 7:1237-1245, 2001.
10) Arteaga CL, Holt JT: Tissue-targeted antisense c-fos retroviral vector inhibits established breast cancer xenografts in nude mice. *Cancer Res* 56:1098-1103, 1996.
11) Holt JT, Arteaga CB, Robertson D, Moses HL: Gene therapy for the treatment of metastatic breast cancer by in vivo transduction with breast-targeted retroviral vector expressing antisense c-fos RNA. *Hum Gene Ther* 7:1367-1380, 1996.
12) Mhashilkar AM, Schrock RD, Hindi M, Liao J, Sieger K, Kourouma F, Zou-Yang XH, Onishi E, Takh O, Vedvick TS, *et al*: Melanoma differentiation associated gene-7 (mda-7): a novel anti-tumor gene for cancer gene therapy. *Mol Med* 7:271-282, 2001.
13) Fisher B, Brown A, Wolmark N, Fisher ER, Redmond C, Wickerham DL, Margolese R, Dimitrov N, Pilch Y, Glass A, *et al*: Evaluation of the worth of corynebacterium parvum in conjunction with chemotherapy as adjuvant treatment for primary breast cancer. Eight-year results from the National Surgical Adjuvant Breast and Bowel Project B-10. *Cancer* 66:220-227, 1990.
14) Foy TM, Fanger GR, Hand S, Gerard C, Bruck C, Cheever MA: Designing HER2 vaccines. *Semin Oncol* 29:53-61, 2002.
15) Sivanandham M, Kim E, Wallack M Immunology, serum markers, and immunotherapy of mammary tumors. In: W. Donegan and J. Spratt (eds.), Cancer of the Breast, 5th edition. St Louis: Sanders, 2002.
16) Stewart AK, Lassam NJ, Graham FL, Gauldie J, Addison CL, Bailey DJ, Dessureault S, Dube ID, Gallenger S, Krajden M, *et al*: A phase I study of adenovirus mediated gene transfer of interleukin 2 cDNA into metastatic breast cancer or melanoma. *Hum Gene Ther* 8:1403-1414, 1997.
17) Stewart AK, Lassam NJ, Quirt IC, Bailey DJ, Rotstein LE, Krajden M, Dessureault S, Gallinger S, Cappe D, Wan Y, *et al*: Adenovector-mediated gene delivery of interleukin-2 in metastatic breast cancer and melanoma: results of a phase 1 clinical trial. *Gene Ther* 6:350-363, 1999.
18) Kang WK, Park C, Yoon HL, Kim WS, Yoon SS, Lee MH, Park K, Kim K, Jeong HS, Kim JA, *et al*: Interleukin 12 gene therapy of cancer by peritumoral injection of transduced autologous fibroblasts: outcome of a phase I study. *Hum Gene Ther* 12:671-684, 2001.
19) Immunization of cancer patients using autologous cancer cells modified by insertion of the gene for tumor necrosis factor. *Hum Gene Ther* 3:57-73, 1992.
20) Scholl S, Squiban P, Bizouarne N, Baudin M, Acres B, Von Mensdorff-Pouilly S, Shearer M, Beuzeboc P, Van Belle S, Uzielly B, *et al*: Metastatic Breast Tumour Regression Following Treatment by a Gene-Modified Vaccinia Virus Expressing MUC1 and IL-2. *J Biomed Biotechnol* 2003:194-201, 2003.
21) Pandha HS, Martin LA, Rigg A, Hurst HC, Stamp GW, Sikora K, Lemoine NR: Genetic prodrug activation therapy for breast cancer: A phase I clinical trial of erbB-2-directed suicide gene expression. *J Clin Oncol* 17:2180-2189, 1999.
22) Braybrooke JP, Slade A, Deplanque G, Harrop R, Madhusudan S, Forster MD, Gibson R, Makris A, Talbot DC, Steiner J, *et al*: Phase I study of MetXia-P450 gene therapy and oral cyclophosphamide for patients with advanced breast cancer or melanoma. *Clin Cancer Res* 11:1512-1520, 2005.
23) Dunphy FR, Spitzer G, Fornoff JE, Yau JC, Huan SD, Dicke KA, Buzdar AU, Hortobagyi GN: Factors predicting long-term survival for metastatic breast cancer patients treated with high-dose chemotherapy and bone marrow support. *Cancer* 73:2157-2167, 1994.
24) Peters WP, Dansey RD, Klein JL, Baynes RD: High-dose chemotherapy and peripheral blood progenitor cell transplantation in the treatment of breast cancer. *Oncologist* 5:1-13, 2000.
25) Berry DA, Broadwater G, Klein JP, Antman K, Aisner J, Bitran J, Costanza M, Freytes CO, Stadtmauer E, Gale RP, *et al*: High-dose versus standard chemotherapy in metastatic breast cancer: comparison of Cancer and Leukemia Group B trials with data from the Autologous Blood and Marrow Transplant Registry. *J Clin Oncol* 20:743-750, 2002.
26) Sugimoto Y, Tsuruo T: DNA-mediated transfer and cloning of a human multidrug-resistant gene of adriamycin-resistant myelogenous leukemia K562. *Cancer Res* 47:2620-2625, 1987.
27) Sorrentino BP, Brandt SJ, Bodine D, Gottesman M, Pastan I, Cline A, Nienhuis AW: Selection of drug-resistant bone marrow cells in vivo after retroviral transfer of human MDR1. *Science* 257:99-103, 1992.
28) Hanania EG, Giles RE, Kavanagh J, Fu SQ, Ellerson

D, Zu Z, Wang T, Su Y, Kudelka A, Rahman Z, *et al*: Results of MDR1 vector modification trial indicate that granulocyte/macrophage colony-forming unit cells do not contribute to posttransplant hematopoietic recovery following intensive systemic therapy. *Proc Natl Acad Sci USA* 93:15346-15351, 1996.

29) Hesdorffer C, Ayello J, Ward M, Kaubisch A, Vahdat L, Balmaceda C, Garrett T, Fetell M, Reiss R, Bank A, *et al*: Phase I trial of retroviral-mediated transfer of the human MDR1 gene as marrow chemoprotection in patients undergoing high-dose chemotherapy and autologous stem-cell transplantation. *J Clin Oncol* 16:165-172, 1998.

30) Cowan KH, Moscow JA, Huang H, Zujewski JA, O'Shaughnessy J, Sorrentino B, Hines K, Carter C, Schneider E, Cusack G, *et al*: Paclitaxel chemotherapy after autologous stem-cell transplantation and engraftment of hematopoietic cells transduced with a retrovirus containing the multidrug resistance complementary DNA (MDR1) in metastatic breast cancer patients. *Clin Cancer Res* 5:1619-1628, 1999.

31) Hacein-Bey-Abina S, Le Deist F, Carlier F, Bouneaud C, Hue C, De Villartay JP, Thrasher AJ, Wulffraat N, Sorensen R, Dupuis-Girod S, *et al*: Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N Engl J Med* 346:1185-1193, 2002.

32) McCormack MP, Rabbitts TH: Activation of the T-cell oncogene LMO2 after gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* 350:913-922, 2004.

33) Dave UP, Jenkins NA, Copeland NG: Gene therapy insertional mutagenesis insights. *Science* 303:333, 2004.

34) Berns A: Good news for gene therapy. *N Engl J Med* 350:1679-1680, 2004.

Cancer Chemother Pharmacol (2006) 57: 114–119
DOI 10.1007/s00280-005-0021-8

CLINICAL TRIAL REPORT

Baoli Qin · Ken Kato · Kenji Mitsugi
Minoru Nakamura · Risa Tanaka · Eishi Baba
Hiroshi Ariyama · Toshiro Kuroiwa · Mine Harada
Shuji Nakano

Feasibility study of ambulatory continuous infusion of 5-fluorouracil followed by cisplatin through hepatic artery for metastatic colorectal cancer

Received: 27 December 2004 / Accepted: 25 February 2005 / Published online: 5 July 2005
© Springer-Verlag 2005

Abstract *Purpose*: A great synergy has been reported in a number of preclinical studies when 5-fluorouracil (5-FU) precedes cisplatin (CDDP). The objective of this study was to determine the feasibility of ambulatory continuous infusion of 5-FU followed by CDDP through hepatic artery for metastatic colorectal cancer. *Patients and methods*: Seventeen patients with unresectable liver metastases, who underwent primary tumor resection, were treated with 5-FU (450 mg/m^2/day) for seven consecutive days followed by CDDP (100 mg/body/week) for seven consecutive days, each administered continuously by using a balloon pump via Infuse-A-Port catheter inserted into common hepatic artery. The doses of drugs were reduced 20% in patients older than 70 years. The treatment was repeated every 4–6 weeks until disease progression. *Results*: Of 17 assessable patients, nine patients showed PR (53%; 95% CI, 29.3–76.7%) and eight patients had SD (47%; 95% CI, 23.3–70.7%), with disease control rate of 100%. The median overall survival was 26 months (95% CI: 17.5–41 months) and TTP 14 months (95% CI: 11–20.3 months). Two patients (11.8%), who showed progression due to collateral feeding arteries, responded to HAI again after occlusion. Grade 3 toxicity included leukopenia (12%) and anemia (24%). Grade 4 toxicity was absent. Four patients (23.5%) progressed at extrahepatic sites. *Conclusions*: This sequential combination of 5-FU followed by CDDP through hepatic artery is active and safe in an outpatient setting, and warrants further multi-institutional study, although prevention of micrometastasis would be mandatory to further prolong overall survival.

Keywords 5-Fluorouracil · Cisplatin · Hepatic arterial infusion · Colorectal cancer · Liver metastasis

B. Qin · K. Kato · K. Mitsugi · M. Nakamura
R. Tanaka · E. Baba · H. Ariyama · M. Harada · S. Nakano (✉)
First Department of Internal Medicine and Department of Biosystemic Science of Medicine, Graduate School of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-Ku, Fukuoka, 812-8582 Japan
E-mail: sn@intmed1.med.kyushu-u.ac.jp
Tel.: +81-92-6425226
Fax: +81-92-6425247

T. Kuroiwa
Department of Radiology, Graduate School of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-Ku, Fukuoka, 812-8582 Japan

Introduction

The incidence of colorectal cancer (CRC) is increasing worldwide. Approximately 20% of patients have metastatic liver disease when the primary tumor is diagnosed [10]. Furthermore, an additional 35–45% of patients will develop hepatic metastases during the course of their disease [1]. Complete resection of hepatic metastases yields 3- and 5-year average survival rates of 23–65% and 25–45%, respectively [13]. Approximately, 75% of the patients who undergo resection of liver metastases will have a recurrence, 50% in the liver [11]. Therapeutic options are limited for patients who are not resectable, and such patients with liver metastases have an median survival of approximately 9 months, with three year survival less than 3% [3, 17, 28]. Conventional systemic chemotherapy is associated with low response rate and overall survival remains poor. Therefore it is of extreme importance to define ideal regional remedies for maximizing benefits but minimizing mortality and adverse effects for those patients.

Metastatic liver cancers derive approximately 80% of their blood supply from the hepatic artery [4]. This unique blood supply of the liver allows hepatic arterial infusion active and feasible for patients with liver metastasis, not only because when injected into the hepatic artery, the regional drug concentration is