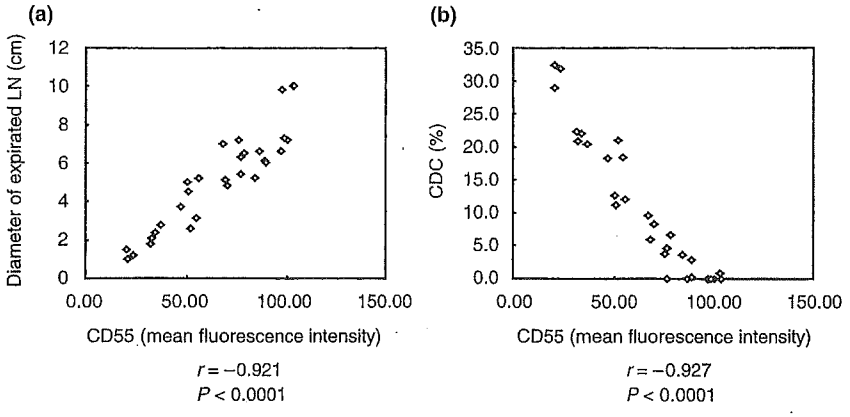

Fig. 2. Relationships between tumor size, CD55 expression and susceptibility to complement-dependent cytotoxicity (CDC). The size of tumors from 30 patients with lymphoma was measured and the cells were collected. After isolation of $CD19^+/CD20^+$ cells, FACscan analysis for CDC assay and CD55 expression 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.

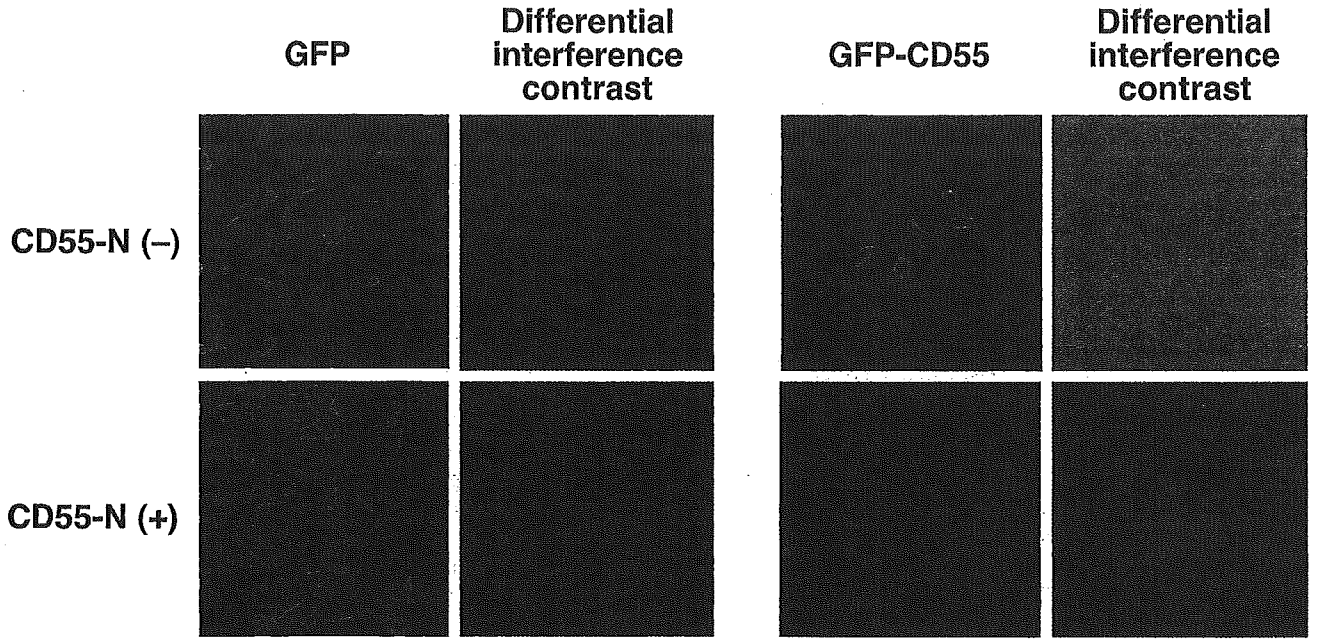

Fig. 3. Effect of small interfering RNA (siRNA) against the 5′-site of the *CD55* gene on expression of the exogenous *CD55* gene. MCF7 cells were transfected with pEGFP or pEGFP-CD55 in the presence or absence of siRNA. After 24 h, the cells were observed by laser scanning microscopy.

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.

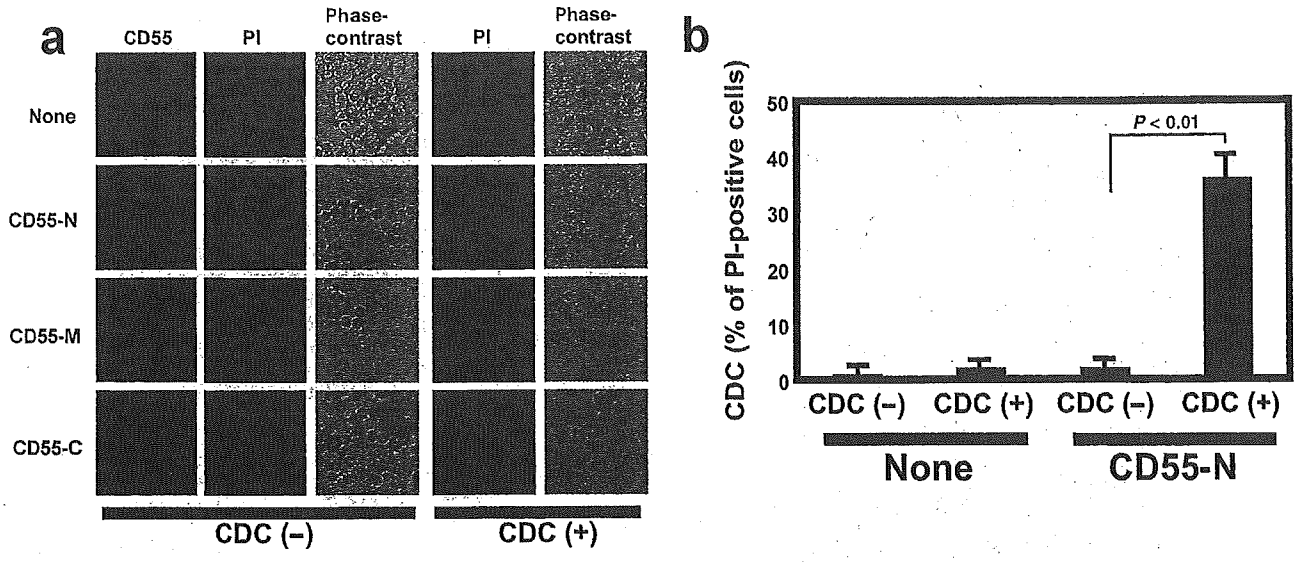

Fig. 4. Blockade of endogenous CD55 on breast cancer cells by small interfering RNA (siRNA). (a,b) SK-BR3 cells were transfected with siRNA against three parts of CD55, namely CD55-N, CD55-M and CD55-C, for 72 h. After transfection, the cells were stained with the anti-CD55 antibody and DAPI, and then 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

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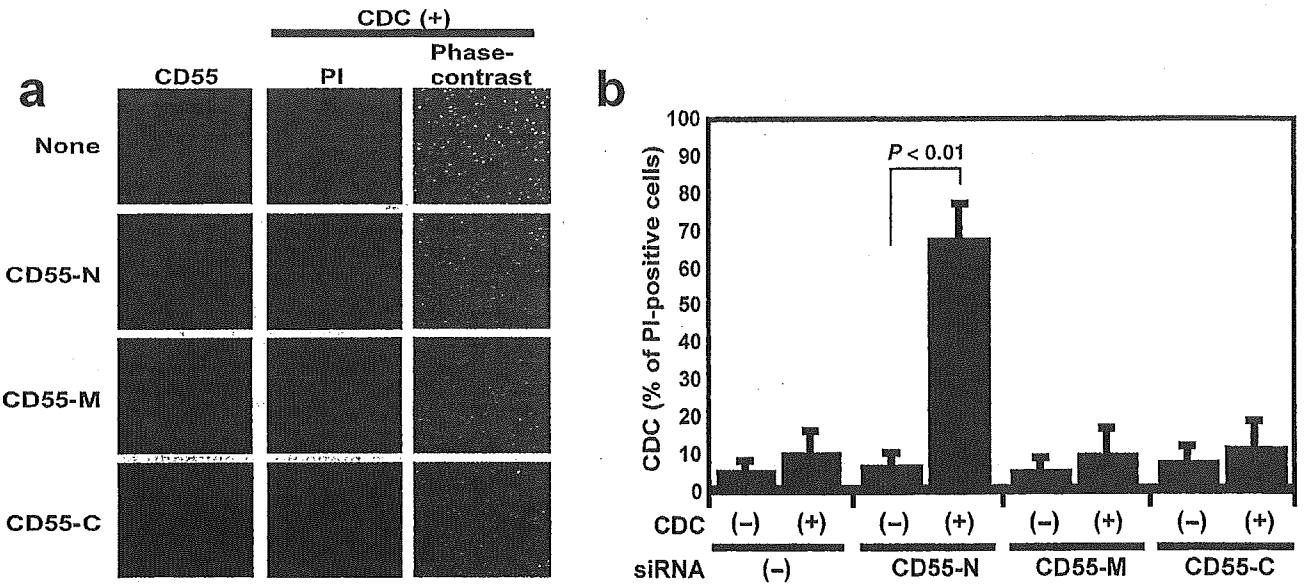

Fig. 5. Blockade of CD55 on primary lymphoma cells by small interfering RNA (siRNA). (a,b) Lymphoma cells from the lymph nodes of five patients with chemotherapy refractory and resistant lymphoma were transfected with siRNA against three parts of CD55, namely CD55-N, CD55-M and CD55-C, for 24 h. (a) 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.

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

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Breast Cancer
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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

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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.

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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 adenovirus	Lyerly Stewart
	IL-12	retrovirus	Park
	GM-CSF	adenovirus	Suzuki
	TNF + NeoR	retrovirus	Rosenberg
2-B transfer of costimulatory molecule gene	B7.1 (CD80)	lipofection adenovirus	Urba 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

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) $\geqq$ 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

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].

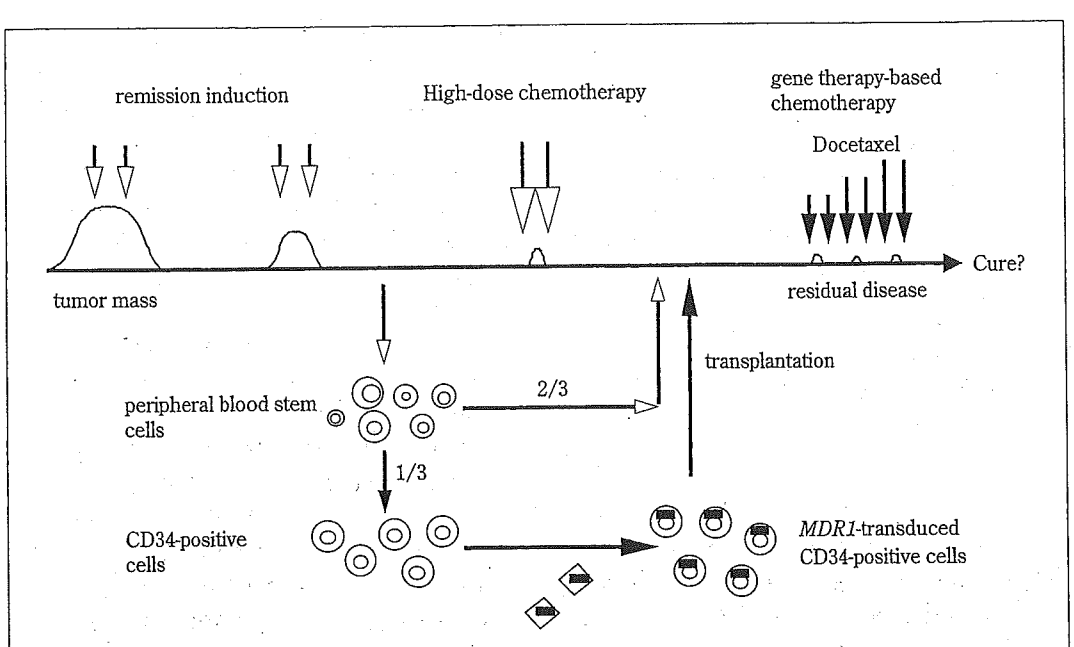

Fig 1. Schema of *MDR1* gene therapy for advanced breast cancer patients in Cancer Institute Hospital.

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 obser ved. 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.

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CLINICAL TRIAL REPORT

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

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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

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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

significantly higher than systemic concentration, but also because the drugs are largely extracted by the liver during the first pass, resulting in minimal systemic toxicity [18]. In a number of randomized studies using fluoropyrimidine derivatives, response rates were significantly higher for hepatic arterial infusion (HAI), as compared with intravenous administration. However, the prolongation of survival in patients treated with HAI still remains controversial in the United States and Europe where floxuridine (FUDR) alone has been used for hepatic arterial infusion [2, 5, 16, 19, 20, 22, 26]. FUDR is almost exclusively extracted by the liver, therefore it seems to be difficult to control the micrometastases in the extrahepatic region. Unlike FUDR, a certain level of 5-fluorouracil (5-FU) remains in the systemic when injected into the hepatic artery [6, 23].

The combination of 5-FU and cisplatin (CDDP) exhibits sequence-dependent synergy both in vitro and in tumor-bearing animals [25, 29, 30]. Several in vivo studies including the investigations of human tumor xenografts in nude mice demonstrated that the sequence of 5-FU followed by CDDP was more active than the reverse sequence or either drug alone [21, 25, 32, 33]. The clinical efficacy of 5-FU and CDDP combination has been confirmed. However, such a sequence-dependent antitumor activity has yet to be clinically determined in the treatment of metastatic colorectal cancer confined to the liver. Therefore, the current study was designed to assess the efficacy and tolerability of ambulatory continuous HAI of 5-FU followed by CDDP for such patients who underwent complete resection of primary tumor. The primary objectives of the research were to observe objective response rate, survival, time to progression (TTP) and toxicities in outpatient setting.

Patients and methods

Eligibility criteria

We included patients with histologically confirmed colorectal cancer, who had multiple and/or massive metastases confined to the liver that were not amenable to surgery replacement (the presence of more than 60% liver by unresectable metastasis) after complete resection of primary tumor. Inclusion criteria were as follows: a history of primary colorectal cancer excision; no evidence of extrahepatic metastasis on computed tomography, bone scintigraphy, and magnetic resonance imaging (MRI) if needed; age 20–80 years; Eastern Cooperative Oncology Group performance status ≤ 2; measurable liver lesions; leukocyte count $\geq 3{,}500/mm^3$; neutrophil count $\geq 1{,}500/mm^3$; platelet count $\geq 100{,}000/mm^3$; serum creatinine ≤ 1.5 mg/dl; serum bilirubin ≤ 2.0 mg/dl; AST ≤ 100 IU/l, ALT ≤ 100 IU/l; a life expectancy ≥ 3 months; and adequate cardiac function. Previous fluorouracil-based treatments were eligible if treatment had been completed more than 4 weeks before study entry. Exclusion criteria were: extrahepatic metastases; active uncontrolled infection; unresolved bowel obstruction; known contraindications to fluorouracil (angina pectoris, myocardial infarction in the past 6 months); and portal vein occlusion. This study was approved by the local ethics committee, and patients were informed of the investigational nature of the study and provided their written informed consent before registration in the study.

Treatment plan

Hepatic arterial infusion was given by percutaneous catheterization of the femoral artery. Procedures for pump placement are as follows. A catheter was inserted into common hepatic artery from right femoral artery. Under celiac angiography, the right gastric artery, and gastroduodenal artery were occluded by a steel coil, to avoid inflow of anticancer drugs to other organs. The collateral arteries which feed the liver, if any, were also occluded. After confirming the presence of the tip of a heparin-coated catheter in the common hepatic artery, a reservoir connected to the catheter was implanted in a subcutaneous pocket in the right subinguinal portion and the catheter was secured in the artery. An intraoperative injection of contrast material was used to check the flow immediately after placement.

Patients were treated with 5-FU (450 mg/m^2/day) on days 1–7, which was followed by CDDP (100 mg/body/week) on days 8–14. Each of them was administered continuously using a LV 1.5 Baxter balloon pump (275 ml) at a flow rate of 1.5 ml/h via Infuse-A-Port catheter inserted into common hepatic artery. Dexamethasone 8 mg and heparin 35,000 unit were mixed in balloon pump and concurrently infused. The doses of 5-FU and CDDP were reduced 20% in patients above 70 years. To prevent nausea and vomiting, 5-hydroxytryptamine-3 antagonists were intravenously administered before chemotherapy. G-CSF was used when neutropenia less than $500/mm^2$ or febrile neutropenia less than $1{,}000/mm^3$ were present. Treatment was continued until evidence of progression, unacceptable toxicity, or patient refusal. Treatment was delayed if, on the planned day of treatment, there was leucopenia less than $3{,}000/mm^3$, thrombocytopenia less than $100{,}000/mm^3$, infectious fever, persistent diarrhea, or nonhematological toxicities greater than grade 3, except for nausea and vomiting. If toxicities greater than grade 3 were observed, the doses of both 5-FU and cisplatin were reduced by 20% on the next cycle. This treatment was repeated every 4–6 weeks.

Assessment of treatment and response

Pretreatment evaluation included a complete history, physical examination, performance status assessment and laboratory examinations including hepatic and renal

functions, urinalysis, complete blood count with differential leukocyte profile, serum alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA) and CA19-9. Computed tomography (CT) scans of the chest, abdomen and pelvis were performed before commencement of chemotherapy. During treatment, a physical examination, a complete blood count and urinalysis were performed once a week. Hepatic and renal functions were examined once a month. CEA and CA19-9 were checked every 2 months. Liver metastatic lesions were reevaluated every 8 weeks. A chest radiograph and abdominal ultrasonography or CT scan were repeated at least every 2 month to exclude lung or other abdominal metastases. For evaluating the response to the treatment, the tumors were measured bidimensionally by computed tomography (CT) both before and after chemotherapy. Responses were evaluated every 8 weeks according to World Health Organization Criteria. Toxicities were monitored weekly and scored according to standard NCI-CTC.

Statistical analysis

The data were statistically analyzed using JMP software (SAS Institute Inc, Cary, NC, USA). Survival estimates were calculated using Kaplan-Meier curves and confidence intervals were calculated using Greenwood variance formula. Survival was calculated until death as a result of any cause, and progression-free survival was calculated from start of chemotherapy until progression of disease or death as a result of any cause.

Results

From May 1997 to September 2003, we randomly enrolled 17 patients with extensive and/or massive metastases confined to the liver from colorectal cancer. Patient characteristics were listed in Table 1. There were 4 women and 13 men. The median age was 68 years, with a range from 45-year-old to 80-year-old. Among 17 patients recruited in the group, 15 were in ECOG performance status 0 and the other two in performance status 1 and 2, respectively. Of the five patients who received prior chemotherapy, three were administered CPT-11-based systemic regimen, one 5-FU-based systemic treatment, and one HAI treatment. Patients were given the HAI treatment after we confirmed that they had normal vasculature and could have a catheter inserted to perfuse the liver completely. Infusion via collateral arteries was done in two patients. Total 176 cycles (median 10, range 3–20 per patient) were done so far.

All patients enrolled were assessable for responses. Nine out of 17 patients (53%) showed PR (95% CI, 29.3–76.7%), one of them received a resection of liver metastases after the treatment. Eight patients (47%; 95% CI, 23.3–70.7%) experienced NC. Therefore, disease control rate was 100%. Two patients (11.8%), who showed progression due to collateral feeding arteries, responded to HAI again after occlusion of these vasculature. As shown in Fig. 1, the median overall survival was 26 months (95% CI, 17.5–41 months). The

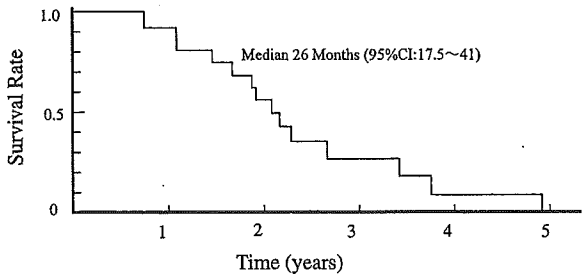

Fig. 1 Kaplan-Meier survival curve of overall survival

Table 1 Patient characteristics and treatment results (As of October 30th, 2004)

Pts	Age/Sex	ECOG PS	Primary site	Histology	Liver metastases	No. of cycles	Response	Survival (Months)
1	56/M	0	Rectum	Well-mod	Multiple	17	PR	27.5
2	70/F	0	Sigmoid	Well-mod	Multiple	18	PR	32
3	77/M	1	Rectum	Well-mod	Multiple	11	NC	13
4	73/M	0	Rectum	Mod	Multiple	9	PR	23
5	73/M	0	Descending	Well	Multiple	10	PR	59
6	80/F	0	Rectum	Well	Multiple	19	PR	45
7	55/M	0	Sigmoid	Mod	Multiple	20	PR	41
8	72/M	0	Sigmoid	Well	Multiple	11	NC	20
9	76/M	2	Sigmoid	Well-mod	Multiple	7	NC	13
10	59/M	0	Rectum	Well-mod	Multiple	6	PR	17.5
11	63/F	0	Ascending	Well-mod	Multiple	6	NC	9
12	70/M	0	Rectum	Mod	Multiple	3	NC	22.5
13	56/F	0	Rectum	Well-mod	Multiple	14	NC	29+
14	67/M	0	Descending	Well-mod	Multiple	3	PR	26
15	45/M	0	Rectum	Well	Massive	12	PR	25+
16	59/M	0	Sigmoid	Mod	Multiple	7	NC	13+
17	68/M	0	Sigmoid	Well	Multiple	10	NC	25

ECOG Eastern Cooperative Oncology Group, *PS* performance status, *Well* well-differentiated adenocarcinoma, *Mod* moderately-differentiated adenocarcinoma, *Well-mod* well to moderately differentiated adenocarcinoma. + alive, *Pts* patients

1-, 2-, and 3-year overall survival rates were 94.1, 57, and 27.1%, respectively. Three patients (17.6%) are alive at present. Median time to progression (TTP) was 14 months (95% CI, 11–20.3 months) (Fig. 2). Four patients (23.5%) progressed at extrahepatic sites, mostly lung (three patients), bone (one patient), brain (one patient).

All the patients were assessable for toxicities and catheter-related complications. There were no treatment-related deaths during the entire courses of study. Five patients experienced the replacement of catheter due to the obstruction. There was no evidence of chemical hepatitis, biliary sclerosis, catheter-induced thrombosis, and duodenal ulceration and hemorrhage that have been associated with HAI administration of chemotherapy. No patient developed severe abdominal pain suggestive of gastroduodenal ulcer or gastroduodenitis. Elevated liver enzymes in documented disease progression were not considered treatment-related toxicities. Non-hematological toxicity was rare with one patient (6%) showing grade 1 vomiting. Hematological and renal toxicities were summarized in Table 2. Grade 3 toxic effects were leukocytopenia (12%) and anemia (24%). No grade 4 toxicities were observed. Cardiac and neurological adverse effects were not encountered in any of the patients. As a result, all the patients received the doses as scheduled.

Discussion

The unique blood supply of the liver allows hepatic arterial infusion active and feasible for patients with liver metastasis [18]. In a number of randomized studies using fluoropyrimidine derivatives, response rates were significantly higher for hepatic arterial infusion (22–62%), as compared with intravenous administration (9–19.6%). However median survivals for HAI treatment groups (12.6–17 month) were not always significantly longer than those for systemic treatment groups, which ranged from 7.5 months to 16 months, indicating that the prolongation of survival remains controversial [2, 5, 16, 19, 20, 22, 26]. Moreover in a randomized study to compare an intrahepatic arterial 5-FU plus leucovorin regimen with the standard intravenous de Gramont fluorouracil plus leucovorin regimen for patients with metastatic colorectal cancer confined to the liver, the objective response rate was 22% and the stable disease rate 32%, median overall survival and TTP were 14.7 and 7.7 months, respectively, 57% of patients were alive at 1 year, and 22% at 2 years in the HAI treatment group. Moreover, there was no evidence of advantage in TTP and overall survival in HAI treatment group, as compared to systemic treatment group [20]. In the present study, sequential hepatic arterial infusion of 5-FU followed by CDDP demonstrated PR and SD in 53 and 47% of treated patients respectively, with disease control rate (PR + SD) of 100%. The median overall survival was 26 months (95% CI: 17.5–41 months) and median TTP 14 months (95% CI: 11–20.3 months). Therefore, sequential hepatic arterial infusion of 5-FU followed by CDDP appears superior to previous HAI treatment employing fluoropyrimidine derivatives. In addition, 3-year overall survival rate was 27.1%, being significantly higher than patients with unresectable liver metastases, who showed a median survival of approximately 9 months and 3 year survival of less than 3% [3, 17, 28].

Recent progresses have been achieved in the treatment of colorectal cancer by introducing CPT-11 or oxaliplatin. Several phase III trials investigating combination regimens with FU/LV plus CPT-11 or FU/LV plus oxaliplatin as a first-line therapy have achieved overall survival of 14.8–21.5 months [14]. The use of all three active drugs in advanced colorectal cancer produced the longest overall survival [15]. Indeed, triple-combination protocols using FU-LV plus irinotecan plus oxaliplatin have consistently resulted in high response rates of 57–78% in patients with previously untreated advanced CRC and have produced the longest overall survival of 22.5 months in one trial [12, 24, 27, 31]. In comparison with these systemic treatments, we obtained a survival benefit of at least 3.5 months with sequential HAI treatment despite patients having liver metastasis, an extremely poor prognostic factor.

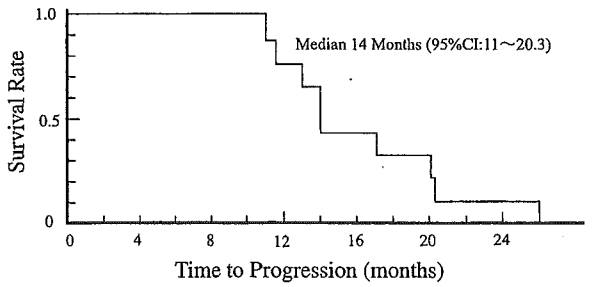

Fig. 2 Kaplan-Meier survival curve of time to progression

Table 2 Hematological and renal toxicities

Data are indicated as the maximum number of patients with the most severe grade of toxicity

NCI-CTC Grade	Number of patients (%)			
	Leucocytopenia	Thrombocytopenia	Anemia	Renal dysfunction
1	1 (6%)	7 (41%)	2 (12%)	1 (6%)
2	1 (6%)	–	4 (24%)	2 (12%)
3	2 (12%)	–	4 (24%)	–
4	–	–	–	–

Therefore, it seems likely that this sequential treatment would be a much better option for patients with metastatic CRC confined to the liver.

The rationale of HAI is based on increased local drug concentrations and hepatic clearance of the drug before entering systemic flow. Continuous hepatic infusion of 5-FU and CDDP has been shown to yield fivefold to tenfold and fourfold to sevenfold higher local concentration than systemic administration, respectively [7]. In addition, protracted infusion may expose a relatively larger proportion of cycling tumor cells to 5-FU, thereby increasing the efficacy of 5-FU. Moreover, the combination of 5-FU and CDDP has been shown to exhibit a sequence-dependent synergy in vitro and in vivo, with sequence of 5-FU followed by CDDP being the most active schedule. This sequence-dependent synergy can be explained by the mechanism of DNA damage repair and detoxification processes; i.e., pretreatment of 5-FU increased CDDP cytotoxicity and even circumvents CDDP resistance by inhibiting repair of platinum-DNA interstrand cross-links as well as by reducing the cellular GSH levels [8, 9].

Although our treatment improved response rate and prolonged the survival of the CRC patients with liver metastases, further follow-up of these patients and accrual of more numbers of patients are needed. Moreover, four patients (23.5%) experienced extrahepatic metastases which led to the patients' death. Therefore, prevention of extrahepatic micrometastases with systemic chemotherapy appears to be mandatory to further improve overall survival. In conclusion, this sequential combination of 5-FU followed by CDDP through hepatic artery is active and safe in an outpatient setting for patients with colorectal cancer metastasized only to the liver, and warrants further multi-institutional studies.

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Gefitinib, a Selective EGFR Tyrosine Kinase Inhibitor, Induces Apoptosis Through Activation of Bax in Human Gallbladder Adenocarcinoma Cells

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Abstract Although gefitinib, a selective inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase, has been clinically demonstrated to be effective for certain cancer cell types, the molecular mechanisms of the anti-tumor activity have not been fully elucidated. In this study, we investigated the mechanism of gefitinib-induced growth inhibition and apoptosis in HAG-1 human gallbladder adenocarcinoma cells. Treatment of gefitinib at a dose of 1 μM resulted in a significant growth inhibition, and the cell number irreversibly declined after 72-h incubation, with a progressive expansion of apoptotic cell population over 120-h. Following 2-h treatment, gefitinib significantly inhibited EGFR autophosphorylation and subsequent downstream signaling pathway through Erk and Akt, and induced accumulation of cells in the G0/G1 phase of the cell cycle at 24-h, accompanied by a concomitant increase in p21 transcript and increased expression of p27. Gefitinib did not affect the amount of total and phosphorylated p53 at serine 15, but upregulated the expression of total Bax, with subsequent increase in p18 Bax, an active form of Bax. The expression of Bcl-2 and Bad was unchanged. An increase in gefitinib-induced expression of total Bax might be due to the decreased degradation of Bax, because the level of Bax mRNA has not been altered by gefitinib treatment. Gefitinib promoted the cleavage of full-length p21 Bax into p18 Bax in mitochondrial-enriched fraction, a characteristic feature of Bax activation toward apoptosis. Moreover, blockade of Bax by using anti-Bax small interfering double stranded RNA (siRNA) significantly reduced gefitinib-induced apoptosis. Taken together, these data suggest a critical role of p18 Bax in gefitinib-induced apoptosis. J. Cell. Biochem. 97: 724–734, 2006.

Key words: gefitinib; EGFR; Bax; apoptosis; Akt; Erk

The EGFR, a receptor tyrosine kinase, is overexpressed in a wide variety of epithelial malignancies including non-small cell lung, head, neck, colon, and breast cancers [Salomon et al., 1995; Shirai et al., 1995; Grandis et al., 1998; Brabender et al., 2001] and enhanced expression of epidermal growth factor receptor (EGFR) is associated with more aggressive disease and a poor patient prognosis [Fox et al., 1994; Rusch et al., 1997]. Upon ligand binding, EGFR is activated through autophosphorylation by forming homodimerization or heterodimerization with other members of the HER family tyrosine kinases [Olayioye et al., 1998; Muthuswamy et al., 1999], and transduces a variety of signals to downstream signal transduction cascades that lead to cellular proliferation and survival [Alroy and Yarden, 1997; Schlessinger, 2000].

Gefitinib, a quinazoline derivative that inhibits EGFR tyrosine kinase activity, has been shown to be effective in preclinical studies and in late stages of clinical trials for non-small cell lung cancer [Fukuoka et al., 2003; Sirotnak, 2003], although the activity is associated with

Abbreviations used: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; RTK, receptor tyrosine kinase; MAPK, mitogen activated protein kinase; Erk, extracellular signal-regulated kinase; PI-3K, phosphatidylinositol 3′-kinase.

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