

# Neutralization of complement regulatory proteins CD55 and CD59 augments therapeutic effect of herceptin against lung carcinoma cells

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**Abstract.** Human monoclonal anti-Her2/neu antibody (herceptin, also named trastuzumab) failed in the treatment of lung cancer when in combination with two chemotherapy agents gemcitabine and cisplatin, despite of its clinical benefit in women with Her2 positive breast cancer. The capacity of herceptin to activate human complement and complement-dependent cytotoxicity against tumor cells was investigated in a study of tumor immunotherapy. We found that the expression of membrane complement regulatory proteins (mCRPs), CD55 and CD59 on non-small cell lung cancer (NSCLC) cells was closely correlated with histological types, prognosis and preoperational adjuvant chemotherapy of the disease. Herceptin-mediated complement cytotoxicity to two human lung carcinoma cell lines exerted stronger killing effect on tumor cells after the neutralization of mCRPs via their antibodies. Furthermore, treatment of herceptin combined with chemo-agents had advantages over chemotherapy alone, while CD55 and CD59 expression levels both declined remarkably in A549 and H157 cell lines after incubation with IC<sub>50</sub> cisplatin for 72 h. Our data indicated that overexpression of mCRPs on tumor cells contributes to herceptin's acquisition of resistance to NSCLC, and its anticancer efficacy was enhanced when mCRPs were neutralized or cisplatin could be used to down-regulate their expression.

## Introduction

Her2, an oncogene encoding a 185-kDa transmembrane glycoprotein tyrosine kinase, is proved to be tumorigenic and

plays a certain role in metastasis as well as chemoresistance (1). With the development of monoclonal antibody technology, a humanized anti-Her2 monoclonal antibody (herceptin, also called trastuzumab) was developed which can bind the Her2 receptor and exert anticancer effect (2). Herceptin has been proven to be an effective treatment in breast cancer with Her2 overexpression and approved by the Food and Drug Administration (FDA) of USA for use in Her2-overexpressed metastatic breast cancer. Her2 receptor overexpression also occurs in 11-32% of non-small cell lung carcinoma (NSCLC) (3), and herceptin can inhibit the proliferation of Her2 overexpressing NSCLC cells (4). Studies on Her2 positive NSCLC cell lines showed that herceptin had an even greater synergy with gemcitabine and cisplatin than in breast cancer cells, therefore, it might also benefit lung cancer patients. However, the clinical therapeutic effect of herceptin was as low as 15% to NSCLC. We wondered why herceptin was of limited use in Her2 overexpressing NSCLC cells, but was very effective for breast cancer. We assumed that the anticancer effect of herceptin might be suppressed when the antibody was used to treat NSCLC. In addition, some *in vitro* evidence showed that herceptin had a synergistic effect with cisplatin, vinorelbine, gemcitabine and paclitaxel in Her2-overexpressing NSCLC cell lines (5). Thus, chemotherapeutic agents might eliminate or inhibit the mechanism responsible for the anticancer effect when herceptin was used to treat NSCLC.

Herceptin is thought to act *in vivo* mostly through activation of antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (6), although diminishing growth receptor signaling or induction of apoptosis may also play a great role (7). The complement system as an essential component of innate immunity, is actively involved in host defense against infectious agents and in the removal of immune complexes and apoptotic cells. It has a definite advantage over cytotoxic cells as a defense system because it is made up of soluble molecules that can easily reach the tumor site and diffuse inside the tumor mass. Moreover, complement components are readily available as a first line of defense because they are synthesized locally by many cell types, including macrophages (8), fibroblasts (9) and endothelial cells (10). However, the efficiency of complement-mediated tumor cell lysis is hampered by various protective mechanisms employed by the tumor cells (11).

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The intrinsic protection is provided by expression of membrane complement regulatory proteins (mCRPs), such as CD55 (decay-accelerating factor, DAF) and CD59 (membrane inhibitor of reactive lysis factor, MIRL). CD55 controls the complement cascade at the level of C3, supervising the generation of the membrane attack complex (MAC) (12). CD55 inhibits formation and dissociates preformed C3/C5 convertases, independently of other proteins, and CD59 prevents cytolysis by binding to C8 and C9 and inhibiting the C9-polymerization process (13). These mCRPs restrict the susceptibility of tumor cells to CDC and provide a mechanism of escape for tumor cells to counteract complement attack. The sensitivity to CDC induced by rituximab and that complement resistance have been reported dependent on the expression level of CD55 and CD59 among lymphomas (14). Neutralization of mCRPs would enhance the therapeutic effect of the monoclonal antibody.

This study investigated the possible roles of CD55 and CD59 in the putative complement activation by herceptin in NSCLC. We used immunochemical assay to analyze the CD55 and CD59 expression in lung carcinoma tissues of NSCLC and explored the relationship between the expression and histo-logical types of tumor and chemotherapy. We presumed that blocking of mCRPs may enhance the killing activity of Her2 monoclonal antibody against carcinoma cells. We proposed that mCRPs might be down-regulated by chemo-agents, thereby improving CDC, and the anticancer effect of the monoclonal antibody.

## Materials and methods

**Lung carcinoma tissues.** Thirty-six non-small cell lung cancer (NSCLC) tissue samples and 8 adjacent tissue samples were obtained from patients who had received surgery in the Xinqiao Hospital (Chongqing, China) between January 1999 and September 2006 with informed consent and approval of the Ethics Committee of our hospital. They were identified as 21 cases of adenocarcinoma, 15 cases of squamous cell carcinoma, 25 cases were well differentiated and 11 cases poorly differentiated. The chemotherapeutic regimen was mainly TP (paclitaxel+cisplatin) and NP (vinorelbine+cisplatin). Only 15 of them received preoperative chemotherapy. According to UICC Proposal 2006, the age, sex, histologic types, lymphatic invasion and pTNM (pathologic tumor-node-metastasis) stage were evaluated by reviewing medical charts and pathological records.

**Immunohistochemical staining for CD55 and CD59.** The avidin-biotin-peroxidase complex technique was used with diaminobenzidine tetrahydrochloride as the chromagen. Briefly, after the formalin fixing and paraffin embedding, 4- $\mu$ m-thick sections were deparaffinized with xylene, and rehydrated through graded concentration of ethanol. Tissue sections were treated in a microwave oven at low power for 10 min in 10 mmol/l sodium citrate buffer (pH 6.0). After being washed with distilled water, endogenous peroxidase was inactivated using hydrogen peroxide in methanol for 20 min. Subsequently, the sections were incubated in 1% sheep serum in Tris-buffered saline, pH 6.0, for 3 min, followed by incubation with mouse monoclonal antibodies against CD55

(provided by Immunology Research Institute of Third Military Medical University, China) (1:50 in PBS containing 2% normal sheep serum) overnight at 4°C. The same procedure was performed to stain CD59 with mouse monoclonal antibodies against CD59 (SeroTec, UK). Normal pulmonary alveolus present in the tumor slides was used as an internal negative control. Negative control samples were created by replacing the primary antibody with PBS.

**Cell lines.** Lung carcinoma cell lines with different expression level of Her2, as estimated by an immunohistochemical scale, were used (15). Human lung adenocarcinoma cell line A549 was restored in the cell bank of our oncology institute. Human lung squamous carcinoma cell line H157 was provided by the Cell Center of Preclinical Medicine Institute of Chinese Academy of Sciences (Beijing, China). Human small cell lung carcinoma cell line H146, negative to Her2 expression, was kindly gifted by the Institute of Respiratory Diseases of PLA, Xinqiao Hospital. All these cell lines were cultured in RPMI-1640 medium (Sigma) supplemented with 10% FBS.

**Antibodies and reagents.** Herceptin was kindly provided by Shanghai CP Guojian Pharmaceutical Co. (China). The anti-CD55 blocking antibody was from Immunology Research Institute of Third Military Medical University, China. The anti-CD59 blocking antibody was purchased from SeroTec (YTH53.1). Normal human serum (NHS), used as a source for complements, was prepared from flesh collected human blood and frozen at -70°C.

FITC-labeled anti-CD55 antibody, FITC-labeled isotype control antibody IgG1 and PE-labeled isotype control antibody IgG2a were purchased from Jingmei Biotech (Shenzhen, China). PE-labeled anti-CD59 was obtained from BioLegend (San Diego, USA).

**Cell surface phenotyping by FACS analysis.** Cultured lung carcinoma cells were washed and resuspended at  $2 \times 10^5$  cells in 200  $\mu$ l of cold PBS containing 5% FBS. Subsequent staining with labeled (FITC-anti-CD55 or PE-anti-CD59) antibodies or appropriate isotypic controls was performed for 30 min on ice. Cells were then washed and resuspended in 200  $\mu$ l of cold PBS containing 5% FBS and 2% paraformaldehyde (Electron Microscopy Sciences). Stained cells were analyzed for a single or double color immunofluorescence with a FACS Calibur (BD Biosciences, San Jose, USA).

**Cytotoxicity assays.** The procedure of CDC described previously (16) was performed to evaluate the effect of the neutralizing antibody on the complement susceptibility of A549 and H157 cells with some modifications. Briefly,  $1 \times 10^6/50$   $\mu$ l cells were incubated with 1.5 mg/ml herceptin ( $IC_{20}$ ) in the presence or absence of blocking antibodies (10  $\mu$ g/ml), and the mixture was adjusted to a final volume of 100  $\mu$ l for 10 min at room temperature prior to the addition of NHS (20%).

Cell viability was assessed using the Dojindo Cell Counting kit-8 (Dojindo Laboratories, Gaithersburg, MD), according to the supplier recommendations. Toxicity data were expressed as the percentage of remaining viable cells relative to untreated

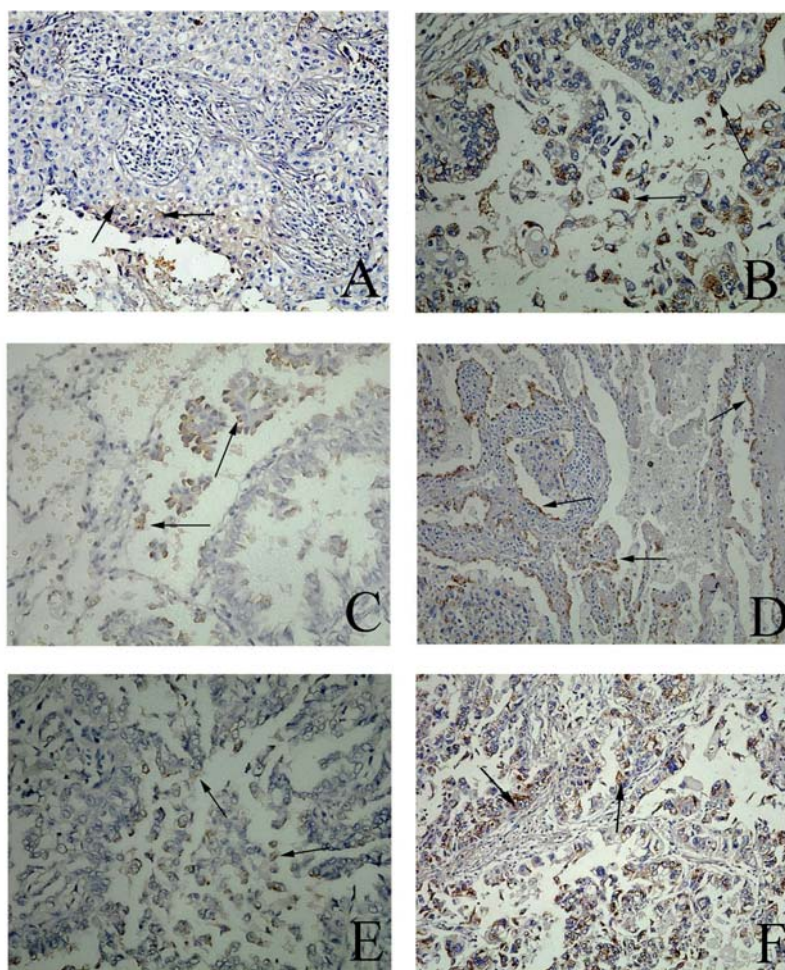


Figure 1. Expression of CD55 and CD59 in NSCLC tissue (SP x200). (A) CD55 antigen positive in lung squamous carcinoma; (B) CD59 antigen in lung squamous carcinoma; (C) CD55 antigen positive in lung adenocarcinoma; (D) CD59 antigen in lung adenocarcinoma; (E and F) CD55 and CD59 antigen double positive in lung adenocarcinoma (the arrows indicate positive expression).

controls, calculated using the absorbance ratio of the formazan dye product at 450 nm generated from the Dojindo reagent. All experiments were performed in triplicate on three separate occasions (17).

**Western blot analysis.** After the treatment of 0.8  $\mu\text{g/ml}$  cisplatin ( $\text{IC}_{50}$ ) for 24, 48 or 72 h, the A549 cells were washed in cold-PBS and then scraped and lysed in ice-cold lysis buffer (50 mmol/l Tris, pH 7.5, 150 mmol/l NaCl, 1% Triton X-100, 1% sodium deoxychlorate, 1 mmol/l phenylmethyl sulfonylfluoride, 50 mmol/l sodium fluoride, 1 mmol/l sodium ortho anadate, 50 g/ml aprotinin, and 50 g/ml leupeptin). Lysate from  $1 \times 10^6/\text{ml}$  cells was microcentrifuged for 15 min at  $4^\circ\text{C}$ , and the supernatant was collected as total protein. Samples (25  $\mu\text{g}$ ) were denatured at  $99^\circ\text{C}$  for 5 min and separated by 12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Proteins from polyacrylamide gel were electrotransferred onto PVDF (polyvinylidene difluoride) film at 40 mA for 1 h at  $4^\circ\text{C}$ , then blocked with 5% non-fat milk in TBST (20 mmol/l Tris, 150 mmol/l NaCl, and 0.1% Triton 20, pH 7.4) for 2 h at  $4^\circ\text{C}$  and then incubated overnight at  $4^\circ\text{C}$  with a 1:400 dilution of mouse monoclonal antibodies against CD55, CD59 and  $\beta$ -actin. After being washed extensively, they were incubated with

biotinylated secondary antibody (1:1000) (Dako, Carpinteria, CA). Blots were developed using ECL Plus (Perbio Science, Northumberland, UK) and images captured on autoradiographic film (Kodak, Jingumae, Japan). The results for the expressions of CD55 and CD59 were presented relative to the expression of the control gene ( $\beta$ -actin).

**Statistical analysis.** The results were expressed as mean  $\pm$  SD. The significance between different groups was evaluated by Fisher's exact test 2x2 contingency table analyses.

## Results

**Expression of CD55/CD59 antigen in lung cancer tissues and their clinical significance.** Of the assessable tumor samples, 21 (58%) were positive to CD55 staining, including 11 cases of lung squamous carcinoma and 10 cases of lung adenocarcinoma samples. No significant correlations with age, gender, histologic type, histologic differentiation, pathologic stage or pathologic N factor were observed. Fig. 1 shows CD55/CD59 staining in different lung carcinomas. Patients without preoperative chemotherapy tended to have a higher positive percentage of CD55, than those with preoperative treatment ( $P < 0.05$ ), positive percentage of CD59 between the



Table I. Correlation of clinicopathological features and expression of CD55 and CD59 in patients with non-small cell lung carcinoma.

Characteristic	CD55 expression			CD59 expression		
	Negative	Positive	P-value	Negative	Positive	P-value
Age						
≤65	7	15	0.175	8	14	0.171
>65	8	6		9	5	
Gender						
Male	11	12	0.484	3	10	0.177
Female	4	9		4	9	
Histologic type						
Squamous carcinoma	10	11	0.501	12	9	0.192
Adenocarcinoma	5	10		5	10	
Differentiation						
Well	13	12	0.077	10	15	0.281
Poorly	2	9		7	4	
Pathologic stage						
I	11	13	0.721	12	12	0.732
II+III	4	8		5	7	
Pathologic N factor						
N0+N1	9	11	0.741	12	8	0.106
N2+N3	6	10		5	11	
Preoperative chemotherapy						
With preoperative chemotherapy	3	12	0.041 <sup>a</sup>	5	10	0.192
Without preoperative chemotherapy	12	9		12	9	

<sup>a</sup>Fisher's exact probability test showed that the ratio of expression of CD55 with preoperative chemotherapy is lower than in the group not receiving preoperative chemotherapy (P<0.05).

preoperative chemotherapy and without preoperative chemotherapy was not statistically significant, as shown in Table I.

**CD55/CD59 expressions in lung cancer cells.** Expression of CD55/CD59 on lung carcinoma cell lines was detected by flow cytometry. Data were compared to isotype control. The results showed that in A549 cells, mean fluorescence of CD55 was 5.40 times higher than that of isotype control; and that of CD59 was 10.53 times higher. In H157 cell line mean fluorescence of CD55 was 3.52 times higher than that of isotype control; and that of CD59 was 6.11 times higher than its isotype control. The data indicated that CD55/CD59 were overexpressed in A549 and H157 cells (Fig. 2).

**Anti-CD55 and anti-CD59 bind to lung carcinoma cell lines and promote herceptin-mediated CDC.** To overcome the restricted complement action of herceptin on the lung tumor cells, the mCRPs CD55 and CD59 were inhibited with non-complement fixing antibodies. Assessing the neutralizing activity of anti-CD55 and anti-CD59 antibody, we evaluated

the effect on the susceptibility of A549 and H157 cells to complement-mediated damage stimulated by herceptin. The number of A549 cells sensitized by herceptin and killed by complement was about 22%, but 86% when blocking CD55 antibody was pre-added to the test system. Blocking CD59 antibody also enhanced the complement-dependent killing of the cells to lysis percentage 62%. The data of H157 cells was similar to that of A549 cells (Figs. 3 and 4). Herceptin had no effect on cell line H146 (the data not showed).

**CD55 and CD59 expression in A549 cells after cisplatin treatment.** Western blotting showed that in the A549 cells with IC<sub>50</sub> treatment of cisplatin, the expression of CD55 was decreased at 24 and 48 h, and then recovered to the level without cisplatin treatment in 72 h, while the expression of CD59 tended to decline with the elapse of time (Fig. 5).

## Discussion

Monoclonal antibodies have been used, with a limited success, in therapy of a number of tumors, including lymphomas (18),

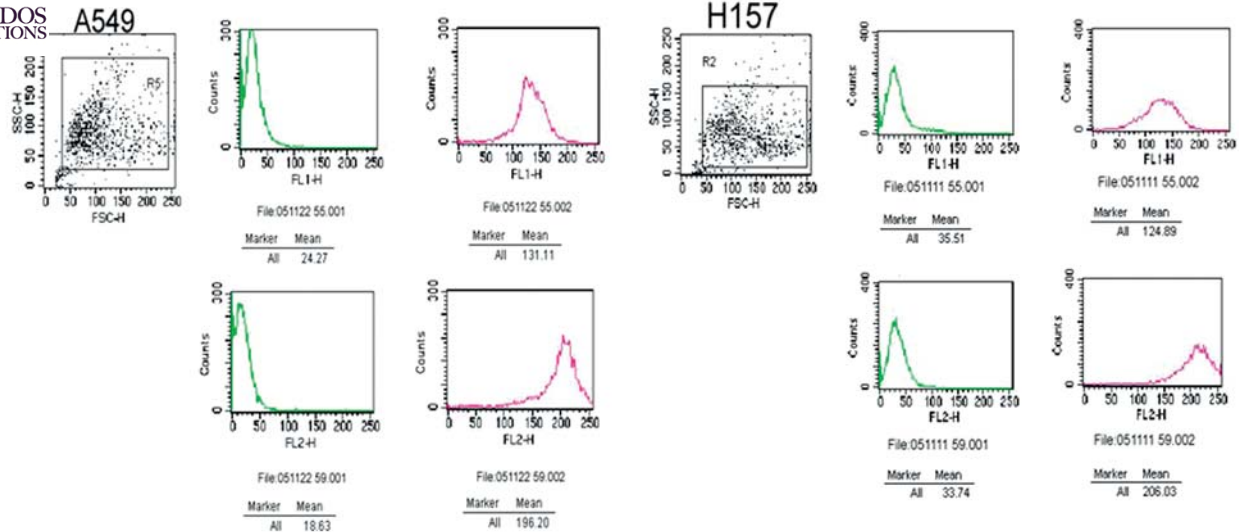
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Figure 2. Mean fluorescence of CD55 and CD59 in A549 and H157 cells with their isotype control, respectively.

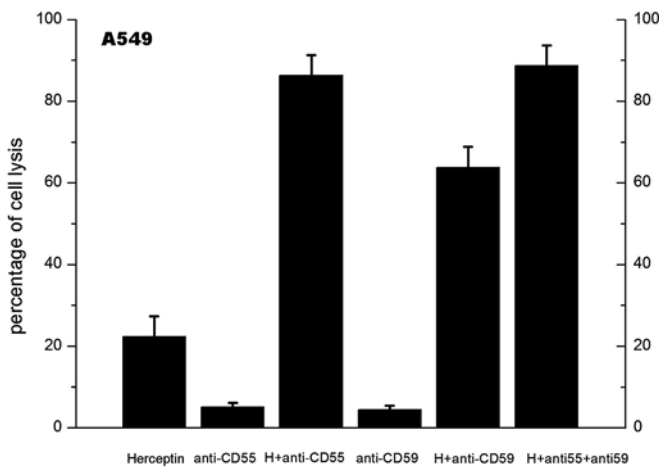


Figure 3. Effect of anti-CD55 and anti-CD59 on complement-mediated lysis of A549. The cells ( $1 \times 10^6$ ) were incubated with herceptin (1.5 mg/ml) and with each Ab (anti-CD55, anti-CD59, both anti-CD55 and anti-CD59) (10 lg/ml) for 10 min followed by NHS (20%). Residual viable cells were measured after 1 h at 37°C and the number of lysed cells was calculated. Herceptin had no effect on cell line H146.

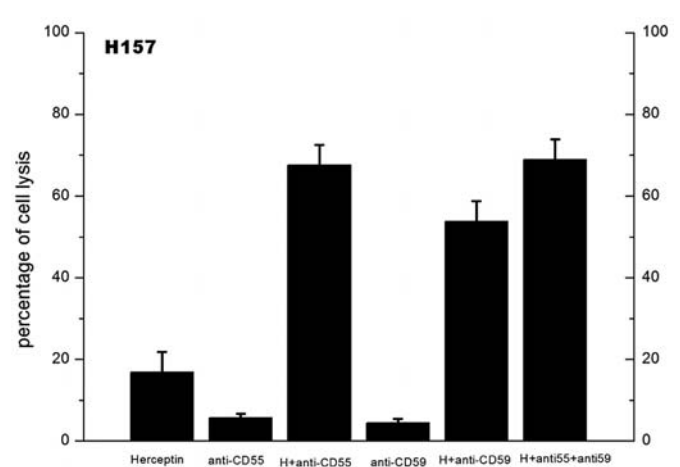


Figure 4. Effect of anti-CD55 and anti-CD59 on complement-mediated lysis of H157. The cells ( $1 \times 10^6$ ) were incubated with herceptin (1.5 mg/ml) and with each Ab (anti-CD55, anti-CD59, both anti-CD55 and anti-CD59) (10 lg/ml) for 10 min followed by NHS (20%). Residual viable cells were measured after 1 h at 37°C and the number of lysed cells was calculated.

breast carcinomas (19) and other solid carcinomas. To our knowledge, the therapeutic efficacy usually varies because of poor penetration of mAb into solid tumor tissue and patient's immunological incompetence due to tumor-induced immunosuppression or therapeutical interventions and/or by inefficient recruitment of host effector mechanism (20). Targeting complement to tumor cells is a critical step in the initiation of complement-mediated cell damage. CDC effect by the mAb may play a great role in immunotherapeutic mechanism (Fig. 6). Our data showed that the positive rate of CD55 had a higher expression in those patients who had not received preoperative chemotherapy than those who had. Western blot analyses also indicated that cisplatin down-regulated the expressions of CD55 and CD59 in A549 cells. Thus, we assumed that high expression of CD55 might be one of the reasons for tumor cells to evade the killing effect of monoclonal antibodies, such as herceptin. However, the up-

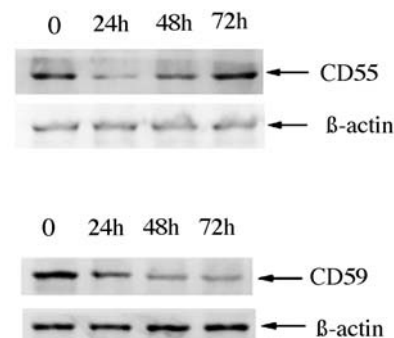


Figure 5. Western blot analysis was performed on lysates from A549 that were either untreated (control) or treated with IC<sub>50</sub> cisplatin 24, 48 and 72 h.

regulated expression of CD55 might be associated with our limited cases of NSCLC, or it was chemotherapeutic agents

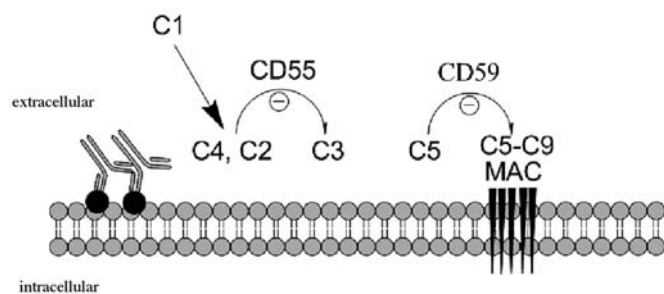


Figure 6. Membrane complement regulatory proteins control the complement activation mechanism.

that reduced the expression of CD55 and thus enhanced the complement activities. This hypothesis calls for further studies.

The complement system is one of the targeted cell lysis systems called into action by therapeutic mAb, but the overexpression of mCRPs in tumor cells restricts the destructive effect of the complement (21). Moreover, complement activation partially determines targeted cell lysis. Thus, the overexpression of mCRP plays a certain role in the evasion of the lung carcinoma cells against complement-mediated damage. Overexpression of mCRPs has been described in many tumors as well as in tumor cell lines from different origins, such as, renal cell carcinoma (22), melanoma (23), tumors of the B lymphoma (24) and ovarian carcinoma (25). Despite the apparent suitability of mAb for adjuvant immunotherapy of lung cancer, it is likely that the expression of intrinsic complement-regulators on tumor cells interfere with the potential cytotoxic effect of the antibody. It is reported that blocking CD55 can enhance rituximab-mediated CDC in B lymphoma cells, especially when monoclonal antibody DG3 is used. Previous studies indicated that expression of CD55 and CD59 may counteract the therapeutic potential of tumor-directed antibodies (26). To explore the inhibitory activity of mCRP and to potentiate complement-mediated killing of carcinoma cells, we have found that CD55 and CD59 were overexpressed in adenocarcinoma cell line A549 and squamous carcinoma cell line H157. In addition, after neutralization of the mCRP, the cells became more sensitive to complement attack.

Herceptin, used in combination with chemotherapeutic agent, such as cisplatin, showed major clinical benefit in women with Her2-positive breast cancer and those with NSCLC, including a significant increase in survival. Zinner *et al* (27) reported that cisplatin 75 mg/m<sup>2</sup> + herceptin 4 mg/kg every day are well tolerated to NSCLC patients, with an encouraging 1-year survival rate of 62%, indicating a synergic effect of herceptin and cisplatin. In our study, cisplatin at IC<sub>50</sub> down-regulated the protein expressions of mCRPs CD55 and CD59 in A549 cells, so we supposed that cisplatin enhanced the antitumor effect of herceptin by decreasing the expressions of CD55 and CD59, and thus improving the activity of complement cascade. The mechanism by which cisplatin down-regulates the mCRP expression may be due to the reduced activity of mitogen-activated protein kinase (MAPK) in the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway (ERK) (28).

In summary, we showed that increased CD55 and CD59 expressions are not only related to herceptin-induced CDC, but further result in suppressed antitumor effectiveness of herceptin, suggesting that CD55 and CD59 may be useful markers for predicting the clinical response to herceptin therapy. They play an important role in protecting the tumor cells from complement attack. Neutralizing these mCRPs improves the complement-depending killing of lung carcinoma cell lines induced by herceptin.

As we studied only cell lines, it remains unclear how herceptin therapy against NSCLC in the clinical setting might benefit. Further studies using primary samples are needed to clarify these issues, but we believe our findings will contribute toward improving treatment results in patients with NSCLC by prevention of, or relief, from the acquisition of herceptin resistance.

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