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1 **Refractory lung metastasis from breast cancer treated with multidisciplinary**  
2 **therapy including an immunological approach**

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1 **ABSTRACT**

2 A suggestive case of metastatic disease from breast cancer is reported. The  
3 HER2-positive tumor was refractory to several agents, including anti-HER2 therapy,  
4 trastuzumab and lapatinib. After re-induction of trastuzumab in combination with  
5 activated natural killer cell injection therapy, tumor markers decreased, and finally a  
6 synergistic effect of taxane and capecitabine showed treatment response. This case  
7 suggests that multidisciplinary therapy including an immunological approach might be a  
8 breakthrough to refractory disease.

9

10 **KEYWORDS**

11 breast cancer, metastasis, natural killer cell, chemotherapy, trastuzumab

12

13

## 1 **Introduction**

2 Breast cancer can metastasize to lymph node, bone, lung, liver, and brain. Lung  
3 metastasis from breast cancer may not only decrease quality of life (QOL) but also  
4 threaten the patient's life. Therefore, it is important to control it.

5 Like endocrine therapy for estrogen-receptor (ER)-positive breast cancer,  
6 trastuzumab-based therapy is considered initially for tumors with overexpression of the  
7 HER-2/neu protein or amplification of the her-2/neu gene, which occur in 20-25% of  
8 metastatic breast cancers [1]. Treatment with trastuzumab, a humanized mAb directed  
9 against the extracellular domain of HER2, resulted in a response rate of 21% in patients  
10 pretreated with chemotherapy, as a single agent. Trastuzumab also increased the clinical  
11 benefit of chemotherapy in metastatic breast cancer with HER2 overexpression [2].  
12 Furthermore, adjuvant use of trastuzumab reduced recurrence remarkably in primary  
13 breast cancer [3-4].

14 The activity of trastuzumab was found to depend on the engagement of  
15 Fc-receptor-expressing lymphocytes, indicating antibody-dependent cellular  
16 cytotoxicity (ADCC) as the major mechanism of antibody action [5-7]. ADCC is  
17 triggered by interaction between antibody-coated target cells and Fc $\gamma$ RIII (CD16) on  
18 natural killer (NK) cells, which initiates a sequence of cellular events culminating in the

1 release of cytotoxic, granzyme-containing granules [8, 9]. Therefore, if we could  
2 increase the activity of NK cells, the efficacy of treatment with trastuzumab could be  
3 increased.

4 In this report, we present a case with refractory pulmonary metastasis, in which the  
5 tumor was successfully treated with trastuzumab, taxane, capecitabine, and activated  
6 NK cell injection therapy.

7

8

## 9 **Case Report**

10 A 55-year-old woman was treated in October 1999 for an invasive ductal carcinoma of  
11 the right breast by mastectomy (Auchincloss's procedure) at another hospital.  
12 Pathological examination showed pathological stage T2, positive lymph node for cancer  
13 (11/22), lymphatic invasion, and negative for ER and progesterone receptor (PgR). She  
14 was admitted to our hospital for adjuvant chemotherapy, which consisted of six courses  
15 of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF).

16 In August 2001, a chest CT scan performed as a follow-up study showed an abnormal  
17 shadow in the lung, which was diagnosed as a metastatic carcinoma from the breast  
18 cancer using transbronchial lung biopsy (TBLB). Because the tumor was positive for

1 human epidermal growth factor receptor type 2 (HER2/neu) (3+ by an  
2 immunohistochemical test), intravenous treatment with docetaxel and trastuzumab was  
3 started. In January 2003, doxifluridine (5'DFUR) was added by oral administration, and  
4 Response Evaluation Criteria in Solid Tumors (RECIST) indicated a partial tumor  
5 response. In April 2004, we changed the treatment to capecitabine with trastuzumab  
6 because of the tumor marker elevation. However, four months later, a chest CT scan  
7 revealed that lung metastasis had progressed. Although four courses of epirubicin  
8 monotherapy were started in September 2004, we could not stop disease progression. In  
9 January 2005, the patient joined a clinical trial of lapatinib. Eleven months later, a  
10 follow-up chest CT scan showed disease progression, and the patient subsequently  
11 withdrew from the trial.

12 After multidisciplinary consultation and with the written consent of the patient, we  
13 initiated therapy by injecting activated natural killer (NK) cells [9, 10]. Blood was  
14 collected, and peripheral blood mononuclear cells (PBMCs) were isolated from the  
15 blood by Ficoll–Hypaque gradient centrifugation (Amersham Biosciences, Uppsala,  
16 Sweden) and washed twice with RPMI 1640, and the number of cells was counted. To  
17 generate activated NK cells, PBMCs were cultured in an anti-CD16-coated flask with  
18 AIM-V (Invitrogen, Tokyo, Japan) medium supplemented with 5% auto-plasma, 700

1 U/ml IL-2 (Chiron, Amsterdam, Netherlands), and 1  $\mu$ l/ml OK432 (Chugai  
2 Pharmaceutical, Tokyo, Japan) for 24 h at 39°C. The cultured cells were then  
3 centrifuged at 1000 rpm for 10 min, and the supernatant was discarded. Next, the cells  
4 were again cultured in a flask (not coated with anti-CD16) in AIM-V medium  
5 supplemented with 5% auto-plasma and 700 U/ml IL-2 at 37°C for 2 to 3 weeks. During  
6 the culture periods, we added medium several times to expand and maintain the  
7 population of the activated NK cells; the purity of the NK cells was 92 to 94%. When  
8 the activated NK cells were injected intravenously by drip ( $2 \times 10^9$  cells/injection), blood  
9 was collected simultaneously. Then isolation and culture of NK cells were repeated at  
10 two-week intervals.

11 We also measured cytotoxic activity of NK cells by the following method. Freshly  
12 isolated PBMCs and activated NK cells were tested for cytotoxic activity at various  
13 effector-to-target (E/T) ratios in a Calcein-AM release assay using TERASCAN VP  
14 (Minerva Tech., Tokyo, Japan). We labeled the target cells, K562, with  
15 immunofluorescent-dye Calcein-AM solution (Do Jindo Lab., Kumamoto, Japan) and  
16 incubated for 30 min, washed the cells with PBS(-) and checked the fluorescence  
17 intensity of the cells. Target cells and effector cells were suspended with RPMI1640 and  
18 10% fetal bovine serum at various E/T ratios and added into a 96-well plate, incubated

1 for 2 hours and again checked for fluorescence intensity.

2 NK cell activity in the patient was extremely low before injection of activated NK

3 cells (Fig. 1); however, the activity increased after therapy began. Simultaneously, we

4 resumed intravenous treatment with trastuzumab. The concentrations of

5 carcinoembryonic antigen (CEA) and carbohydrate antigen 15-3 (CA15-3) had

6 decreased for six months, but began to increase following trastuzumab treatment. We

7 added capecitabine to trastuzumab; however, these tumor markers continued to increase.

8 At the same time, skin and bone metastases emerged. Therefore, weekly intravenous

9 injection of paclitaxel was added, and we also started hyperthermia for the skin

10 metastases. After that, NK cell activity increased again, and the tumor markers

11 decreased. A chest CT scan in November 2006 showed that the lung metastases had

12 reduced (Fig. 1). NK cell activity remained at a high level and the progression-free

13 survival was about 10 months.

14

15

## 16 **Discussion**

17 In the current report, lung metastasis which had been refractory to anti-HER2 therapy

18 responded to the combination therapy of trastuzumab, capecitabine, paclitaxel, and



1 activated NK cell injection therapy.

2 There are some reports that indicated a relation between the activity of NK cells and  
3 disease progression. When breast cancer cells were inoculated into NOD/SCID mice  
4 that possessed NK cell activity, only a small tumor grew at the inoculation site and no  
5 organ metastasis was shown. However, when NOD/SCID/ $\gamma_c^{\text{null}}$  (NOG) mice lacking  
6 T-cell, B-cell, and NK cell activity were inoculated with breast cancer cells, there was  
7 efficient formation of a relatively large tumor and spontaneous organ metastasis [10].

8 An epidemiological study demonstrated that the natural cytotoxic activity of PBMCs  
9 was significantly associated with reduced cancer risk [11]. We recently reported that NK  
10 cell activity in metastatic breast cancer patients was significantly reduced compared  
11 with that of healthy controls [12]. Furthermore, several exploratory studies have shown  
12 that NK cell therapy contributes to reduce the tumor burden in human cancers [13].

13 Antibody-dependent cellular cytotoxicity (ADCC) by NK cells is mediated by the  
14 binding of Fc $\gamma$ RIII (CD16) to the Fc portion of the antibody, which initiates a sequence  
15 of cellular events culminating in the release of cytotoxic, granzyme-containing granules  
16 [8]. Trastuzumab is known to mediate ADCC against a HER2/neu-positive breast cancer  
17 target [5-7]. In the present case, after induction of activated NK cell injection therapy  
18 and trastuzumab, the NK cell activity of the patient recovered and subsequently the

1 level of CEA decreased. This result indicated that activity of NK cells might influence  
2 the efficacy of trastuzumab.

3 Capecitabine is enzymatically converted to 5-fluorouracil in the tumor by thymidine  
4 phosphorylase (TP) [14]. We previously reported that docetaxel-containing regimens  
5 modulate TP in primary breast cancer tissues in a neoadjuvant setting [15]. Therefore, it  
6 was suggested that the combination of taxane and capecitabine was synergistically  
7 effective. Some experiments using human cancer xenografts have indicated this  
8 synergic effect and some clinical trials have also suggested that combination therapy of  
9 taxane and capecitabine provided favorable effects compared with capecitabine alone  
10 [16-17]. In the present case, the tumor responded to combination therapy of trastuzumab,  
11 docetaxel, and doxifluridine as a first-line therapy after recurrence. Furthermore, the  
12 combination therapy of paclitaxel and capecitabine was also effective in reducing  
13 re-growth of the tumor after induction of activated NK cell injection and trastuzumab.

14 Some reports have also shown that taxanes increased NK cell cytotoxicity. Paclitaxel  
15 induced mRNA and protein production of perforin and the activation of nuclear  
16 factor- $\kappa$ B (NF- $\kappa$ B) in NK cells in vitro [18]. In a taxane treatment group, NK cell  
17 cytotoxicity was 39% higher than that in a non-taxane treatment group [19].

18 Furthermore, several studies have indicated that hyperthermia therapy may increase NK

1 cell activity [20]. Although paclitaxel and hyperthermia might modulate the activity of  
2 NK cells, the relation between the modulation and the treatment response is still unclear.

3 In addition to downregulation of NK cell activity, various other mechanisms such as  
4 PTEN and phosphoinositol kinase abnormalities have been suggested to explain  
5 trastuzumab resistance [21, 22]. Therefore knowing the individual mechanism of  
6 resistance to anti-HER2 therapy such as trastuzumab or lapatinib is warranted to  
7 maximize the effect of anti-HER2 therapy.

8 Interestingly, although this patient was resistant to anti-HER2 therapy and showed an  
9 extremely low level of NK cell activity before NK cell injection therapy, NK cell  
10 activity increased and response was obtained after the patient received the combined  
11 treatment with highly purified and well activated NK cells and trastuzumab.  
12 Furthermore, adding TP-inducible chemotherapy and TP-targeting chemotherapy to the  
13 treatment yielded an objective response. Our case report indicates that multidisciplinary  
14 therapy, including an immunological approach, might be a breakthrough to refractory  
15 metastasis.

16

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2

3 **Conflicts of interest**

4 All authors declared they have no financial support that may pose a conflict of interest.

5

6 **Informed consent**

7 We obtained consent for publication in print and electronically from the patient's

8 husband.

9

10 **Figure legend:**

11 **Figure 1**

12 This figure shows changes of tumor markers and NK cell activity in the patient during

13 activated NK cell injection therapy. It also shows images from a chest CT scan above

14 the graph. NK cell activity in the patient was extremely low before injection of activated

15 NK cells. However, her NK cell activity increased after activated NK cell injection

16 therapy began. Because of disease progression, we also initiated trastuzumab,

17 capecitabine, and paclitaxel with activated NK cell injection therapy. Then tumor

18 markers became smaller, and the lung metastasis was reduced.