Trastuzumab Activates Allogeneic or Autologous Antibody-Dependent Cellular Cytotoxicity against Malignant Rhabdoid Tumor Cells and Interleukin-2 Augments the Cytotoxicity

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Abstract

**Purpose:** Malignant rhabdoid tumor (MRT) is an early childhood cancer with poor prognosis. Trastuzumab, a humanized monoclonal antibody against human epidermal growth factor receptor-2 (HER-2), has been shown to be effective against breast cancer and other cancers. We investigated the effect of trastuzumab on MRT cell lines.

**Experimental Design:** We examined expression of HER-2 on four MRT cell lines and two tumor tissues by indirect immunofluorescence, flow cytometry, and immunohistochemistry. The effect of trastuzumab against MRT cells was examined by cell growth assay. To observe the antibody-dependent cellular cytotoxicity of effector cells, we examined the cytotoxicity of trastuzumab in combination with allogeneic or autologous human peripheral blood mononuclear cells with and without IL-2 using the chromium release assay.

**Results:** All four MRT cell lines and both MRT tissues expressed HER-2 protein. Trastuzumab alone did not reduce the viability of the MRT cell lines. On the other hand, the cytotoxicity of trastuzumab against each of the MRT cell lines was significantly increased by the presence of allogeneic and autologous peripheral blood mononuclear cells ($P < 0.01$). There was a strong correlation coefficient ($r = 0.825$) between HER-2 expression and the cytotoxicity enhanced by trastuzumab. Moreover, trastuzumab in combination with peripheral blood mononuclear cells augmented by interleukin-2 (IL-2) was significantly more cytotoxic than trastuzumab alone or IL-2 alone ($P < 0.01$).

**Conclusions:** Our results indicate that (1) trastuzumab can exert antitumor effects on MRT cells by using the antibody-dependent cellular cytotoxicity of effector cells and (2) IL-2 can enhance the cytotoxicity of trastuzumab against MRT cells.

Malignant rhabdoid tumor (MRT) is a highly aggressive malignancy of early childhood. The most common locations are in the central nervous system and kidney, although such tumors may arise in almost any site (1). In the past several years, there has been significant progress in the genetic studies, which indicated that the majority of MRT harbor biallelic inactivation in the integrase interactor 1 (INI1) tumor suppressor gene, located in chromosome 22q11.2 (2). However, the overall survival rate of patients with MRT of the kidney is not more than 20% to 25%. For example, only 8.8% of infants that were diagnosed before the age of 6 months were living 4 years after diagnosis (3). Therefore, the search for effective treatments against MRT is needed.

Human epidermal growth factor receptor type 2 (HER-2) belongs to the epidermal growth factor receptor (EGFR) family. HER-2 can mediate signal transduction from all EGFR family and is thus involved in a variety of cell functions, including cell proliferation, differentiation, and apoptosis. In addition, HER-2 also plays a pivotal role in oncogenic transformation and tumorigenesis (4–8). Overexpression and amplification of HER-2 has been identified in a variety of cancer types, including non-small cell lung cancer and carcinomas of the breast, bladder, pancreas, and ovary (9, 10). The humanized anti-HER-2 monoclonal antibody, trastuzumab (Herceptin), has already shown significant activity clinically in women with metastatic breast cancer overexpressing HER-2 (11). There are several reports on HER-2 expression and the effectiveness of trastuzumab against not only breast cancer, but also the other cancers, such as colon cancer, ovarian cancer (12), gastric cancer (13), and esophageal cancer (14) in *in vitro* and *in vivo* experiments. The effects of trastuzumab against HER-2 include direct downregulation of HER-2 and activating antibody-dependent cellular cytotoxicity (ADCC) in the presence of human
were discussed. Against this aggressive and extremely poor prognostic tumor assay. In addition, possible clinical applications of trastuzumab were established, to MRT by the this study, we confirmed the diagnosis of four cell lines, which we established, to MRT by the INI1 gene and INI1 protein. Next, we confirmed the expression of HER-2 on the four MRT cell lines and two tumor tissues, from which the cell lines were established, by indirect immunofluorescence, flow cytometry analysis, and immunohistochemistry. Furthermore, the effect of trastuzumab against MRT cells in vitro was examined by cell growth assay, and a cytotoxicity effect of trastuzumab with or without IL-2 was examined by chromium ($^{31}$Cr) release assay. In addition, possible clinical applications of trastuzumab against this aggressive and extremely poor prognostic tumor were discussed.

### Materials and Methods

**Tumor tissues, cell lines, and cell culture.** MRT tissues were confirmed in the four patients by clinical and histologic examinations. MRT tissues were obtained from patients NS and YM in 1991 and 2000, respectively. Ten percent formalin-fixed tumor tissues embedded in paraffin were used for the HER-2 expressions in MRT tumor tissues. We used three of our previously established MRT cell lines, KP-MRT-NS (22), MP-MRT-AN (25), KP-MRT-YM (24), and KP-MRT-RY, which was our newly established cell line. Clinical data on the four MRT patients are given in Table 1.

Cell lines were cultured in RPMI1640 medium containing 10% fetal bovine serum and were subcultured as previously described (22). The SKBR3 breast cancer cell line and the HL60 promyelocytic leukemia cell line were used as positive and negative controls of HER-2 expression, respectively.

**Reagents.** Trastuzumab was purchased from Chugai Pharmaceutical Company (Tokyo, Japan). Human recombinant IL-2 was kindly provided by Shionogi Pharmaceutical Co. (Osaka, Japan). Stock solutions of each compound were prepared in saline at the concentrations as previously described (26). The lysates were suspended according to an article that we previously published (26). Two pairs of primers were designed to amplify the INI1-1 cDNA: INI1CD1, forward, 5'-CTG ATC AGG ACC TTC TCC GGC CAG-3' and INI1CD1 reverse, 5'-GAT TGC TGG CAT AAA CGT CAG-3' and INI1CD2.forward, 5'-AGA TCG ATG GGC AGA AGC TGC-3' and INI1CD2.reverse, 5'-TGC AAT GTG TAC CGG GAA GGC-3'. The primers of β-actin were as follows: forward, 5'-GAT GGC GCC CCC ACC CAC CA-3' and reverse, 5'-CTC CTT AAT GTC ACG CAC GAC TAT TGC-3' (22). The annealing temperature of each PCR was at 66°C and 58°C, respectively.

**Direct sequencing analysis.** The cDNA sequence including exon 2 of the INI1 gene of the KP-MRT-RY cell line was amplified with primers: forward, 5'-GAC GAC GCC GAG TAC TAC AT-3' and reverse 5'-CAT GGC AGC GCA TCT AAG T-3'. The amplicon was directly sequenced with a Dynamic ET Terminator Cycle Sequencing Kit (Amersham, St. Louis, MO) and analyzed with the RISA-384 Sequence Detection System (Shimadzu, Kyoto, Japan). The sequence was compared with the NCBI database (Gene bank accession No. NM_003073 and NM_001007468).

**Western blotting.** Cell lysates were purified and adjusted to equal concentrations as previously described (26). The lysates were suspended in 2 × SDS sample buffer, boiled for 5 minutes, separated by SDS-PAGE, transferred to an Immobilon-P membrane, and immunoblotted using anti-IN1 polyclonal antibody (1:200, Santa Cruz Biotechnology, CA) and β-actin antibody (1:2500, Sigma Chemical, St. Louis, MO). Antibody binding was detected with an enhanced chemiluminescence detection system (Amersham, St. Louis, MO) and analyzed with the RISA-384 Sequence Detection System (Shimadzu, Kyoto, Japan). The sequence was compared with the NCBI database (Gene bank accession No. NM_003073 and NM_001007468).

**Immunofluorescence.** Immunofluorescence staining of MRT cells was performed as previously described (22). Anti-c-erbB-2/HER-2/neu oncoprotein Ab-2 (Clone 9G6.10; 1:10; NeoMarker, Union City, CA) was used as the first antibody, and fluorescein isothiocyanate-conjugated antimonique Ig (1:80, Cappel Products, Aurora, OH) was used as the second antibody. Cell nuclei were stained with 4-diamidino-2-phenyindole (1:2000, Kirkegaard & Perry, Gaithersburg, MD). All cells were observed with a Keyence (Osaka) Japan) BZ-8000 immunofluorescent microscope using the same settings.

**Histochemistry and immunohistochemistry.** Histochemistry and immunofluorescence staining of MRT tissues was performed as previously described (26). In brief, sections were blocked for 10 minutes with BlockAce at a dilution of 1:4 (Dai-Nippon Pharmaceutical, Osaka, Japan), stained with anti-c-erbB-2/HER-2/neu oncoprotein Ab-15 (Clone 3B3) (1:200; NeoMarker) overnight at 4°C, and exposed to the secondary antibody (1:400; Dako, Glostrup, Denmark) for 30 minutes.

**Flow cytometry analysis.** Adherent MRT cells were harvested by scraper, suspended in PBS with 2% fetal bovine serum, blocked with rabbit serum, incubated for 30 minutes at 37°C, washed with PBS, incubated with trastuzumab (10 μg/mL) for 30 minutes at 37°C, washed with PBS, and incubated with fluorescein isothiocyanate-conjugated rabbit antihuman IgG (Dako, 40 μg/mL) for 30 minutes at 37°C. Fluorescein isothiocyanate-positive cells were detected with a FACSCaliber cytometer (Becton Dickinson, San Jose, CA). Expression levels of HER-2 in MRT and control cell lines were determined by mean fluorescence index (MFI).

| Table 1. Clinical histories of MRT patients from which MRT cell lines were established |
|------------------|------------------|------------------|------------------|------------------|
| Characteristic   | KP-MRT-NS         | KP-MRT-RY         | MP-MRT-AN         | KP-MRT-YM         |
| Age at diagnosis | 2 mo             | 1 mo             | 3 mo             | 5 mo             |
| Gender           | Female            | Male             | Female            | Male             |
| Site of primary tumor | Left kidney     | Left kidney      | Liver             | Chest wall        |
| Source of cell line | Ascitic fluid   | Left kidney      | Peripheral blood  | Chest wall        |
| Outcome          | Death             | Death            | Death             | Alive             |

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Trastuzumab activates ADCC against MRT cells

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Cell viability assay. Cells were seeded in normal growth medium into 96-well cell plates (3,000 per well). After 24 hours, trastuzumab or human IgG was added. Cells were cultured and treated in triplicate. Cell viability was determined every 24 hours for 4 days by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphonyl)-2H-tetrazolium (WST-8) assay using a Cell Counting Kit-8 (Dojin East, Tokyo, Japan) as described previously (28). In brief, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well. After incubation for another 2 hours at 37°C, we determined cell viability colorimetrically by the optical density (OD) at 450 nm with a microplate reader (Multiscan IX, Dainippon Pharmaceutical, Osaka, Japan). Each OD 450 was calculated according to the following formula: OD 450 = experimental well-blank well.

Chromium release assay. Adherent MRT cells were harvested by trypsinization and suspended in growth medium. For using target cells, MRT cells (1 × 10⁶ cells) were labeled with 740 kBq of ⁵¹Cr for 120 minutes. PBMC were used as human effector cells. They were obtained from a healthy donor or from patient YM from which the KP-MRT-YM cell line had been established. Identical HLA phenotype and genotype showed that the KP-MRT-YM cells and the PBMC, taken from patient YM, had the same origins. The PBMC were separated on a Ficoll-Hypaque density gradient. Then, we coincubated target cells (1 × 10⁶/well) and effector cells at various effector/target (E:T) ratios in 150-μL medium in a 96-well plate in triplicate for 4 hours at 37°C with trastuzumab or a control antibody, human IgG. After 4 hours of incubation, the radioactivity of the supernatant (100 μL) was measured with a γ counter. The percentage of specific lysis was calculated according to the following formula: % cytotoxicity = 100 × (experimental cpm - spontaneous cpm)/(maximum cpm - spontaneous cpm). Enhancement of the cytotoxicity by trastuzumab was analyzed by Pearson’s correlation coefficient test.

Statistical analysis. Values are expressed as the mean ± SE. We used the two-sided Student t test to statistically evaluate the differences in cytotoxicity. P < 0.05 was considered to indicate a statistically significant difference. The relation between HER-2 expression and the cytotoxicity enhanced by trastuzumab was analyzed by Pearson’s correlation coefficient test.

Results

Confirmation of cell lines as MRT cells. In RT-PCR, INI1CD1 was not detected in the KP-MRT-AN, KP-MRT-YM, or KP-MRT-NS cell lines. INI1CD2 was not detected in the KP-MRT-AN or KP-MRT-YM cell lines, and a shortened form was detected in the KP-MRT-NS cell line (Fig. 1A). These results were consistent with the previous reports (24–26). However, both INI1CD1 and INI1CD2 were detected in our newly established KP-MRT-RY cell line, as well as in the HL60 cell line, which was used as a positive control. Therefore, we directly sequenced the amplicon corresponding to INI1 exon 2. The mutation in exon 2 (C157T) was not detected in any of the four MRT cell lines by Western blotting (Fig. 1C).

Expression of HER-2 in MRT cell lines and clinical tissues. In immunofluorescence, all four of the MRT cell lines and the SKBR3 cell line expressed HER-2 protein, whereas the HL60 cell line did not (Fig. 2A through F). Both MRT tissues had round or polygonal cells with eosinophilic hyaline material in the cytoplasm in hematoxylin and eosin staining, consistent with MRT tissue (Fig. 2G and H). They were also immunopositive for HER-2 expression (Fig. 2I and J).

In the flow cytometry analysis, the KP-MRT-NS cell line had the highest HER-2 expression (MFI 50.6 ± 8.65) of the four MRT cell lines. HER-2 levels of the KP-MRT-RY (MFI 35.9 ± 4.74), MP-MRT-AN (23.4 ± 2.99), and KP-MRT-YM (13.8 ± 0.47) cell lines were higher than that of HL60 (10.7 ± 0.92), which was used as a negative control, but lower than that of SKBR3 (856 ± 98.8), which was used as a positive control (Fig. 2K).

From these results, the MRT cell lines and the clinical tissues were also confirmed to express HER-2 protein.
Trastuzumab alone does not inhibit viability of MRT cells. Because all four of the MRT cell lines expressed HER-2 in immunofluorescence and flow cytometry, we examined whether trastuzumab directly inhibited the viability of MRT cells by WST-8 assay. Treatment with either 1 to 100 μg/mL trastuzumab had no effect on viability of any of the cells (Fig. 3A through D).

Trastuzumab enhances cytotoxicity against MRT cell lines using allogeneic or autologous human peripheral blood mononuclear cells. We examined the cytotoxic ability of trastuzumab through allogeneic human PBMC indirectly, which was known as the ADCC effect, by $^{51}$Cr release assay in vitro. MRT cells as target (T) cells and allogeneic human PBMC as effector (E) cells were mixed at different E:T ratios. Cytotoxicity tended to be higher at higher E:T ratios for all of the MRT cell lines. In each MRT cell line, the treatment with trastuzumab was significantly increased cytotoxicity, as compared with that of human IgG, used as control ($P < 0.01$; Fig. 3E through H). Trastuzumab showed the highest cytotoxicity for the KP-MRT-NS cell line, which has the highest HER-2 expression of the four cell lines, compared with human IgG (cytotoxicity ratio with E:T 20:...
Effect of trastuzumab on four MRT cell lines. A-D, trastuzumab alone does not inhibit the viability of MRT cells. MRT cells were seeded, allowed to attach for 24 h, and added trastuzumab or human IgG in triplicate cultures. Cell viability was determined every 24 h for 72 h by WST-8 assay. Values are the mean of results from three wells. Bars, ± SE.

E-J, trastuzumab enhances cytotoxicity against MRT cell lines using allogeneic (E through H) or autologous (J) human peripheral blood mononuclear cells. MRT cells were labeled with chromium for 120 min. MRT cells (target cells) and human peripheral blood mononuclear cells (effector cells) were coincubated at various E:T ratios for 4 h with trastuzumab or human IgG in triplicate cultures. Each cytotoxicity was measured with 51Cr counter. The HL60 cell line was used as a negative control cell of HER-2 (I). Values are the mean of results from three wells. Bars, ± SE. * P < 0.01 relative to human IgG alone.

Fig. 3. Effect of trastuzumab on four MRT cell lines. A-D, trastuzumab alone does not inhibit the viability of MRT cells. MRT cells were seeded, allowed to attach for 24 h, and added trastuzumab or human IgG in triplicate cultures. Cell viability was determined every 24 h for 72 h by WST-8 assay. Values are the mean of results from three wells. Bars, ± SE. E-J, trastuzumab enhances cytotoxicity against MRT cell lines using allogeneic (E through H) or autologous (J) human peripheral blood mononuclear cells. MRT cells were labeled with chromium for 120 min. MRT cells (target cells) and human peripheral blood mononuclear cells (effector cells) were coincubated at various E:T ratios for 4 h with trastuzumab or human IgG in triplicate cultures. Each cytotoxicity was measured with 51Cr counter. The HL60 cell line was used as a negative control cell of HER-2 (I). Values are the mean of results from three wells. Bars, ± SE. * P < 0.01 relative to human IgG alone.
MRT is still a disease with a poor prognosis and refractory to other chemotherapeutic drugs. Novel effective treatments for patients with MRT are needed. The objective of our study was to determine the effect of trastuzumab, an anti-HER-2 humanized monoclonal antibody, and/or IL-2 against MRT cells and to evaluate the possibility of using trastuzumab and IL-2 as novel therapeutic agents for MRT patients. This is the first report on the expression of HER-2 in MRT cell lines, in clinical MRT tissues, and on the evaluation of the effect of trastuzumab and IL-2 against MRT cells.

Deletion or mutation of the INI1 gene was previously shown in the KP-MRT-NS, MP-MRT-AN, and KP-MRT-YM cell lines (24, 26), but not in the KP-MRT-RY cell line. Only the KP-MRT-RY cell line expressed both INI1CD1 and CD2, in contrast to the other cell lines, by RT-PCR (Fig. 1A). The KP-MRT-RY cells have a nonsense mutation in exon 2 of the INI1 gene (C157T in codon 53; Fig. 1B). The same mutation was found in genomic DNA extracted from the KP-MRT-RY cell line (data not shown). This mutation, C157T in codon 53, is a novel mutation of the INI1 gene in MRT, although the other mutation of exon 2 was frequently found in MRT of the kidney (1, 29). From these results and deletion of INI1 protein (Fig. 1C), our four cell lines were indicated as MRT cells.

HER-2 expression was detected in the four MRT cell lines by immunofluorescence staining (Fig. 2A through F) and flow cytometry (Fig. 2K), as well as in two MRT clinical tissues by immunohistochemistry (Fig. 2I and J). The HER-2 levels of all of the MRT cell lines were significantly higher than that of HL60 cell line, although the levels were lower than that in the SKBR3 cell line, which was known for cell overexpressed HER-2 (30) in flow cytometry analysis, in which trastuzumab was used for the first antibody because the effects of trastuzumab against MRT cells could be directly examined in our study. These results indicated that the four MRT cells expressed HER-2 and did not overexpress it.

A patient with cancer of HER-2 overexpression, such as metastatic breast cancer, should be treated with trastuzumab. However, in basic research, trastuzumab is also effective against cancer cells which do not express HER-2 as highly as breast cancer cells (31, 32). These results prompted us to evaluate the possibility of targeting HER-2 for treatment of MRT.

We can think of two reasons why trastuzumab directly did not inhibit cell viability of MRT by WST-8 assay (Fig. 3A through D). First, HER-2 gene amplification does not occur in MRT cell lines. Most cancer cells that overexpress HER-2 protein and have viability inhibited by trastuzumab, such as SKBR3...
cells, possess HER-2 gene amplification. In contrast, none of the MRT cell lines amplify the HER-2 gene as shown by real-time PCR (refs. 33–35; Supple. 1). Second, the pathways downstream of HER-2 may not be associated with cell growth in MRT cells. In our experiments, HER-2 expression was significantly decreased with trastuzumab alone in the KP-MRT-NS cell line by Western blot analysis, using another HER-2 monoclonal antibody having a different epitope from trastuzumab (Supple. 2). This result is consistent with a report that trastuzumab enhanced ubiquitination of HER-2 (36). The above results suggest that the pathways downstream of HER-2, such as mitogen-activated protein kinase pathway and phosphatidylinositol 3’ kinase pathway, are not important for the growth of MRT cells (37–39). Moreover, trastuzumab may induce upregulation of tyrosine kinases other than HER-2, as well as downregulation of HER-2 in MRT cells. This upregulation may compensate for change in the pathways downstream of HER-2. These results suggest that the dependency on HER-2 of MRT cells is less than that of SKBR3 cells. Proliferation of MRT cells may depend on EGFR rather than HER-2. In fact, the growth of the KP-MRT-NS and the MP-MRT-AN cells, both of which express EGFR, was inhibited by gefitinib, a selective EGFR-tyrosine kinase inhibitor (26).

Our results (Fig. 3E through I), in 51Cr release assay, suggest that the degree of cytotoxicity of trastuzumab against MRT cells depends on their level of HER-2 expression. The ADCC effect of trastuzumab was also highly correlated with HER-2 expression in other cancer cells (9, 32, 40). Moreover, using autologous human PBMC of the patient from which the cell line had been established, the cytotoxicity was also significantly enhanced by trastuzumab against the KP-MRT-NS cell line (Fig. 3I). These results indicate that trastuzumab enhances the cytotoxicity of autologous as well as allogeneic human PBMC against MRT cells. In a previous article, trastuzumab did not inhibit the growth of pancreatic cancer cell lines expressing HER-2 in vitro, but it did induce an antibody-dependent antitumor effect both in vitro and in vivo (32). The main mechanism tumor growth arrest with trastuzumab was the ADCC effect (16). Therefore, our results suggest that trastuzumab might induce antibody-dependent antitumor effect in vivo as well as in vitro.

ADCC is a well-recognized immune effector mechanism in which antigen-specific antibodies direct immune effector cells to the killing of the antigen-expressing cancer cells (41). The main effector cells of ADCC are natural killer (NK) cells. Also in our experiment, the most enhanced ADCC with trastuzumab against the KP-MRT-NS cells was induced by CD56-positive NK cells, which were isolated from human PBMC by magnetic beads (Miltenyi-Biotech, Bergisch Gladbach, Germany; Supple. 3). Consequently, we noticed that NK cells enhance the ADCC with trastuzumab also against MRT cells. In general, however, the activity of NK cells is lower in children than in adults. Therefore, we suggested that IL-2 might enhance ADCC activity of PBMC with trastuzumab against MRT cells. The additive-enhanced cytotoxicity against the KP-MRT-NS cell line was obtained by the allogeneic PBMC, cocultivated with IL-2 and following trastuzumab (Fig. 4A). Similar results could be shown in the autologous PBMC and the KP-MRT-YM cells (Fig. 4B). From these results, it is indicated that IL-2 can enhance the cytotoxicity of trastuzumab against MRT cells, in autologous PBMC as well as in allogeneic PBMC. Additive enhanced ADCC with IL-2 and trastuzumab against MRT cells was first shown in autologous PBMC as well as in allogeneic PBMC in vitro.

Trastuzumab is presently used only to treat patients with metastatic breast cancer, whose tumors overexpress the HER-2 protein. However, combinations of trastuzumab and anticancer drugs have also been used in many clinical trials for patients with breast cancer. Moreover, a phase 1 study of erlotinib, trastuzumab, and paclitaxel has begun for patients with advanced solid tumors that express HER-2 and EGFR and that are refractory to standard treatments (42). MRT cells express EGFR as previously described (26), and MRT is refractory to chemotherapy and radiotherapy and frequently leads to metastatic solid tumors.

Conclusion

Our results and this clinical trial indicate that trastuzumab and gefitinib may effectively contribute to the therapy of patients with MRT. Because the number of MRT patients is small, additional studies are needed to determine whether these individuals expressing HER-2 tend to have a poor prognosis and to determine the efficacy of trastuzumab with or without IL-2 in clinical trials.

In conclusion, our results indicate that trastuzumab was able to have antitumor effects on MRT cells by ADCC, and IL-2 was able to further enhance the effect of trastuzumab on MRT cells.

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Trastuzumab activates ADCC against MRT cells


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