Cetuximab inhibits the growth of mucinous ovarian carcinoma tumor cells lacking *KRAS* gene mutations

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Received November 15, 2011; Accepted December 29, 2011

DOI: 10.3892/or.2012.1626

Abstract. The purpose of this study was to explore the possibility of targeted molecular therapy with anti-epidermal growth factor receptor (anti-EGFR) antibody (cetuximab) for the treatment of mucinous ovarian carcinoma. We analyzed EGFR protein expression and *KRAS* gene mutations in 5 mucinous ovarian carcinoma cell lines RMUG-L, RMUG-S, MN-1, OMC-1 and MCAS and evaluated the *in vitro* and *in vivo* effects of cetuximab on each. EGFR expression was observed in all cell lines except for MN-1 cells, and a *KRAS* gene mutation at codon 12 was detected only in the MCAS cell line. Cetuximab inhibited RMUG-L and OMC-1 cell growth *in vitro* and completely blocked RMUG-L tumor growth *in vivo*. On the other hand, cetuximab did not affect MCAS cell growth *in vitro* and only partially reduced the MCAS tumor growth *in vivo*. These results suggest the possibility of targeted molecular therapy with cetuximab for mucinous ovarian carcinoma cells lacking a *KRAS* gene mutation.

Introduction

Ovarian cancer is the fifth leading cause of cancer-related-death in the United States. Ovarian cancer was reported in ~22,000 women in 2010, ~14,000 of whom ultimately died of this disease (1). Since most patients with early-stage ovarian cancer seldom have symptoms, by the time they are diagnosed, >75% are already in the advanced stage (2). The standard treatment for ovarian cancer is cytoreductive surgery with platinum/taxane combination chemotherapy. Although ovarian cancer is generally sensitive to chemotherapy (3,4), there are cases that exhibit both natively drug-resistant tumors as well as tumors that eventually acquire drug tolerance. The 5-year survival rate is only 40% and has not improved in the last decade (1). Therefore, new strategies, especially targeted molecular therapy, require more attention in order to improve the prognosis of ovarian cancer.

Mucinous ovarian adenocarcinoma (MAC) accounts for 10-14% of all types of epithelial ovarian cancers (EOC) (5,6). Compared to serous adenocarcinoma (SAC), which is the most common histopathologic subgroup of EOC, MAC is relatively resistant to the conventional platinum or taxane-based chemotherapy, thereby leading to a poor prognosis (7-10). It has been reported that MAC differs from SAC pathologically and cytogenetically, and more closely resembles colorectal cancer (11). These results suggest that therapeutic agents that are effective in treating colorectal cancer may also be effective for treating MAC.

Epidermal growth factor (EGF) and its receptor (EGFR) are reportedly involved in the growth and extension of malignant tumors (12). In particular, EGFR overexpression has been observed in various malignant tumors (13). Further, EGFR overexpression has been reported to be a poor prognostic factor for various malignant tumors (14,15). It has been reported that EGFR is expressed in 48% of MAC tumors, and its expression is correlated with the histologic grade, stage and death rate (16).

Cetuximab, an anti-EGFR monoclonal antibody, is a molecular-targeted therapeutic agent that was produced as a human-mouse chimeric antibody; it has a higher binding affinity for EGFR than natural ligands and inhibits tyrosine kinase phosphorylation (17,18). In addition, cetuximab reportedly induces EGFR internalization and degradation (19). Recently, it has been widely used in the medical treatment of colorectal cancer (20).

The purpose of this study was to explore the possibility of molecular-targeted therapy using anti-EGFR antibody (cetuximab) for MAC as a potential new treatment for this disease.

Materials and methods

Cell culture. The 5 MAC cell lines used in this study (RMUG-L, RMUG-S, MN-1, OMC-1 and MCAS) (21-25) were obtained as follows: the RMUG-L and RMUG-S lines were obtained from Dr Daisuke Aoki (Keio University, Tokyo, Japan); the MN-1

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Key words: ovarian cancer, mucinous ovarian carcinoma, cetuximab, epidermal growth factor receptor, KRAS

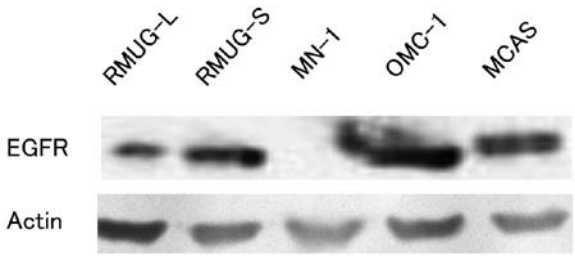

Figure 1. Western blotting using an anti-EGFR polyclonal antibody. EGFR expression was detected at the position corresponding to a molecular weight of 170 kDa in all cell lines except for MN-1 cells.

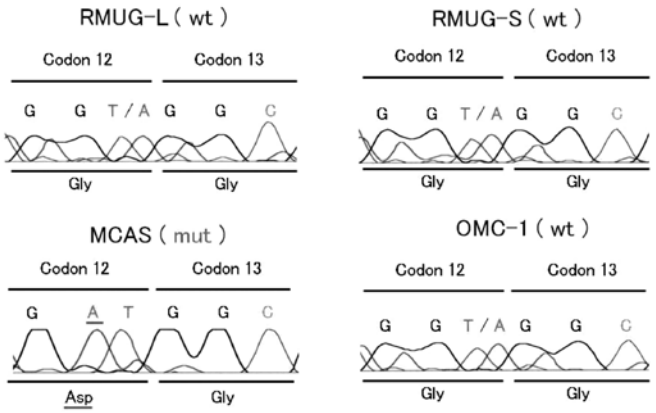

Figure 2. DNA sequence analysis of the *KRAS* gene at codon 12 in exon 2 in 4 MAC cell lines. No mutations were detected in RMUG-L, RMUG-S and OMC-1 cell lines. A point mutation [GGT (Gly) to GAT (Asp)] was observed only in the MCAS cell line.

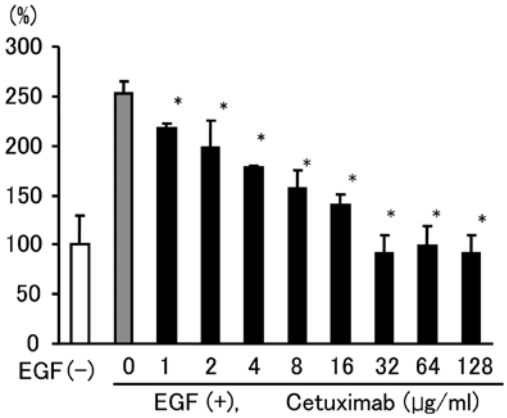

Figure 3. Analysis of the inhibitory effect of cetuximab on *in vitro* cell growth of RMUG-L cells. Cetuximab inhibited the *in vitro* cell growth in a concentration-dependent manner. *P<0.01. Results are expressed as mean ± SD.

line, from Dr Yasuhiko Kiyozuka (Kansai Medical University, Osaka, Japan); the OMC-1 line, from Dr Tsuyoshi Saito (School of Medicine, Sapporo Medical University, Sapporo, Japan); and the MCAS cell line, purchased from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). These cell lines were maintained in D-MEM/Ham's F-12 medium (DMEM/F12, Gibco, Grand Island, NY) containing 10% inactivated fetal calf serum (Sigma, St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) at 37˚C in a 5% CO_2 atmosphere for no longer than 8 weeks after recovery from frozen stocks.

Antibodies. Both the anti-EGFR polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and the anti-human actin polyclonal antibody (Sigma) were used according to the manufacturer's protocols.

Anti-EGFR monoclonal antibody (cetuximab). Cetuximab was purchased from Bristol-Myers Squibb (Tokyo, Japan) and used undiluted at a concentration of 2 mg/ml in animal experiments.

Western blotting. Protein extracted (10 μg) from a homogenate of cultured cells was mixed with 2X SDS-PAGE sample buffer [120 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.004% bromophenol blue, and 10% 2-mercaptoethanol]. The mixture was heated at 95˚C for 2 min, electrophoresed on a 0.1-5% SDS-polyacrylamide gel, and then the proteins were blotted onto a polyfluorovinylidene membrane. The membranes were blocked with Non-Protein Blocking Agent (ATTO Corporation, Tokyo, Japan) at room temperature for 1 h, and incubated with anti-EGFR polyclonal antibody (1:1,000) and anti-human actin polyclonal antibody (1:200) for 1 h at room temperature. Each membrane was washed with phosphate-buffered saline (PBS)-Tween-20 three times, and then incubated with a horseradish peroxidase-conjugated secondary anti-rabbit antibody (Thermo, Rockford, IL). Signals were detected by chemiluminescence (ECL kit, Amersham Biosciences, Piscataway, NJ) on X-ray film.

KRAS gene mutations. Each of the 5 MAC cell lines were analyzed for KRAS gene mutations. Genomic DNA was extracted from cells by using a QIAamp DNA Mini kit (Qiagen, Valencia, CA). The hot-spots (exon 2) of KRAS gene mutations were amplified by PCR with EX Taq (Takara, Tokyo, Japan) and primers as described previously (26) and sequenced to confirm the presence or absence of mutations by using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Applied Biosystems Division, Darmstadt, Germany) with the ABI PRISM 310 genetic analyzer (Perkin Elmer).

Effects of cetuximab in vitro. RMUG-L, OMC-1 and MCAS were cultured in DMEM/F12 medium supplemented with 100 pg/ml EGF (R&D Systems, Minneapolis, MN) without fetal calf serum and exposed to cetuximab at concentrations of 0-128 μg/ml. To examine the inhibitory effect of cetuximab on cell growth, 5,000 cells/well were dispensed into 96-well plates. After 48 h, the viable cell count was determined by a colorimetric assay with the Cell Proliferation kit II (XTT) (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) and calculated as the percent of control cells (cultured without cetuximab).

Experimental animals. Four- to six-week-old female BALB/c nude mice (Japan Clea Laboratories, Tokyo, Japan) were used. All animal experiments were conducted according to the institutional and national guidelines for animal experiments.

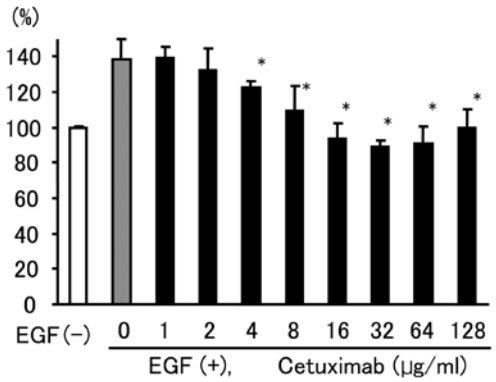

Figure 4. Analysis of the inhibitory effect of cetuximab on *in vitro* cell growth of OMC-1 cells. Cetuximab inhibited *in vitro* cell growth in a concentration-dependent manner. *P<0.01. The results are expressed as mean ± SD.

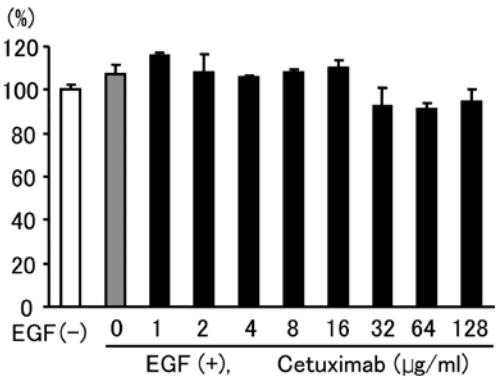

Figure 5. Analysis of the inhibitory effect of cetuximab on *in vitro* cell growth of MCAS cells. Cetuximab did not influence the *in vitro* cell growth of MCAS cells. The results are expressed as mean ± SD.

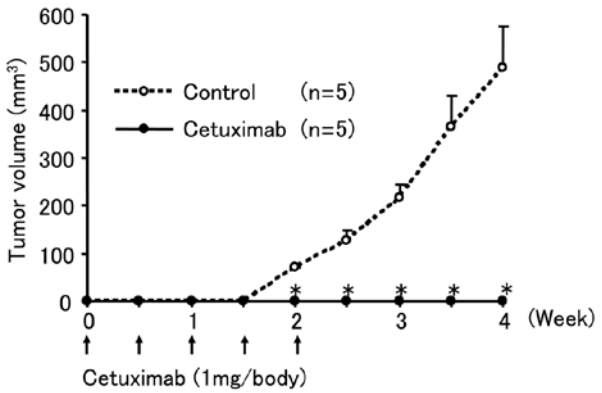

Figure 6. Analysis of the inhibitory effect of cetuximab on *in vivo* tumor growth of RMUG-L cells. The tumor growth was completely abolished at 4 weeks after inoculation in the cetuximab-administered group in comparison with the control group. *P<0.05. The results are expressed as mean ± SD.

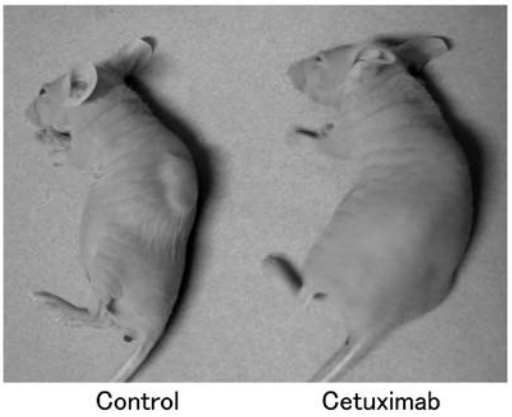

Figure 7. Subcutaneous tumors of RMUG-L cells at 4 weeks after inoculation. A clearly identifiable tumor was formed on the back of the control mouse, while in contrast, no tumor was found on the back of the cetuximab-treated mouse.

Effects of cetuximab in vivo. The RMUG-L or MCAS lines of concentrations of $5x10^6$ cells were inoculated subcutaneously into the back of each mouse to induce tumor growth. On the day after inoculation with the tumor cells, cetuximab was intraperitoneally administered 2 times per week at a dose of 1 mg/body until 2 weeks after inoculation. PBS was administered to the control group. The tumor volume [(long diameter) x (short diameter)^2 x 1/2] was measured twice a week to obtain a tumor growth curve.

Statistical analysis. The test of significance between the 2 groups was performed using Student's t-test. A P-value <0.05 was considered significant.

Results

EGFR expression. As shown in Fig. 1, EGFR expression was detected by western blotting at the position corresponding to a molecular weight of 170 kDa. EGFR was detected in all tested cell lines except for the MN-1 line.

KRAS gene mutations. No mutations at codon 12 in exon 2 of the *KRAS* gene were detected in the RMUG-L, RMUG-S, or OMC-1 cell lines. A single point mutation, GGT (Gly) to GAT (Asp), was observed only in the MCAS line at codon 12 in exon 2 of the *KRAS* gene (Fig. 2). The sequence of the MN-1 line was not confirmed because the PCR did not work.

Effects of cetuximab on in vitro cell growth. As shown in Figs. 3 and 4, cetuximab inhibited the *in vitro* cell growth of both RMUG-L and OMC-1 cells in a concentration-dependent manner. On the other hand, cetuximab did not influence *in vitro* cell growth of MCAS cells (Fig. 5). Therefore, cetuximab inhibited growth of those MAC cells that lacked a KRAS gene mutation, while cetuximab did not affect growth of MAC cells with a *KRAS* gene mutation.

Effects of cetuximab in vivo tumor growth. As shown in Figs. 6 and 7, RMUG-L tumor growth was completely abolished at 4 weeks after inoculation in the cetuximab-treated group in comparison with the control group. On the other hand, MCAS tumor growth was only partially reduced in the cetuximab-treated group in comparison with the control group (Fig. 8). Therefore, cetuximab completely inhibited MAC tumor growth in those cell lines that lacked a KRAS gene mutation and only partially reduced the MAC tumor growth in those lines with a mutation in the *KRAS* gene.

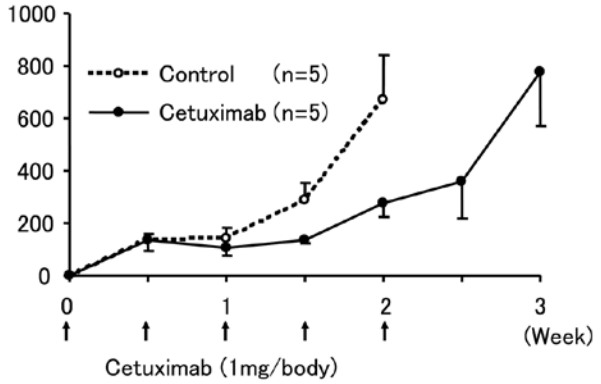

Figure 8. Analysis of the inhibitory effect of cetuximab on *in vivo* tumor growth of MCAS. The tumor growth of MCAS was partially reduced in the cetuximab-treated group in comparison with the control group.

Discussion

In this basic study, we explored the possibility of targeted molecular therapy using cetuximab for MAC in order to develop a new treatment for this disease. First, we investigated the expression of EGFR in 5 MAC cell lines and observed that all of them, except the MN-1 line, expressed EGFR. Next, we screened each cell line for *KRAS* gene mutations and found that only the MCAS line carried a point mutation at codon 12. Finally, we examined the effect of cetuximab on MAC. We observed that cetuximab inhibited the growth of those MAC cell lines that lacked a *KRAS* gene mutation, and by contrast, we showed that cetuximab could not inhibit the growth of MAC cells that carried a mutation in the *KRAS* gene.

MAC is the third most common type of EOC, comprising 10-14% of EOC (5,6). MAC appears to have a distinctly different clinical progression from that of other types of EOC (5,6). Several studies show that MAC is often diagnosed at an early stage, and therefore, it presents a relatively good prognosis (5,6). However, advanced MAC has a poorer prognosis than other histopathologic subgroups (6). MAC's low response (26-42%) to conventional platinum-based chemotherapy is associated with a poor prognosis because chemosensitivity is one of the main prognostic factors for patients with advanced EOC (7-10). Although MAC is known to be resistant to platinum/taxane combination chemotherapy (10), patients with MAC are usually treated with this first-line chemotherapy regimen. A novel treatment strategy for advanced MAC is urgently needed. The histopathology of MAC is similar to that of colorectal cancer. Further, it has been reported that the serum tumor marker and molecular marker expression pattern of MAC differs from those of SAC, and is more similar to colorectal cancer (11). These results suggest that therapeutic agents effective in treating colorectal cancer may also be effective in treating MAC.

Cetuximab is an anti-EGFR monoclonal antibody that binds to EGFR to inhibit its activity, and it is used as a targeted molecular therapeutic agent against specific molecules involved in tumor growth. It is a human-mouse chimeric antibody of the IgG1 subclass and is clinically administered as an intravenous infusion. Recently, cetuximab has been widely used in the medical treatment of colorectal cancer (26). It has been reported that EGFR is expressed in 35-70% of ovarian cancer (13). While there are few studies that have examined MAC, Alshenawy reported that EGFR is expressed in 10 of 21 cases of MAC (16). In the present study, we examined the effect of cetuximab on MAC and found that cetuximab inhibited MAC cell growth *in vitro* and MAC tumor growth *in vivo*. These results suggest the possibility of targeted molecular therapy using cetuximab for MAC.

KRAS, a small G-protein downstream of EGFR and an essential component of the EGFR signaling cascade, can acquire activating mutations in exon 2, thus isolating the pathway from the effect of EGFR (27) and rendering EGFR inhibitors ineffective (28-30). Karapetis *et al* reported that the mutation status of the KRAS gene is associated with overall survival among patients with advanced colorectal cancer who were treated with cetuximab after previous chemotherapy had failed. Compared to only supportive care, treatment with cetuximab was associated with almost a doubling of the median overall and progression-free survival rates among patients with wild-type KRAS tumors. However, there was no significant survival benefit from cetuximab among patients with tumors that had KRAS mutations (26). Mutations in exon 2 of the KRAS gene are observed in ~50% of MAC (31,32). In the present study, cetuximab was unable to inhibit growth of a cell line that had a mutation in exon 2 of the *KRAS* gene. Although this was observed in just 1 cell line, these results suggest that patients with MAC bearing mutated KRAS would not benefit from cetuximab, similar to patients with colorectal cancer. Additionally, in the present study, we observed that cetuximab partially inhibited *in vivo* tumor growth of the cell line that carried the mutation. It has been reported that cetuximab has the potential to inhibit angiogenesis in malignant tumors (33,34) and the potential to enhance a host's tumor immunity (35). Our results described above might reflect these actions.

Thus far, there have been no published studies of targeted molecular therapy for MAC. Targeted molecular therapy for MAC with cetuximab, which we propose here, could be very advantageous for patients with advanced MAC, which is resistant to conventional chemotherapy.

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