Laboratory of Toxicology and Safety Science[1], Graduate School of Pharmaceutical Sciences, Osaka University; The Museum of Osaka University[2], Osaka; School of Pharmaceutical Sciences[3], Wakayama Medical University, Wakayama; Institute for Advanced Co-Creation Studies[4], Osaka University; Global Center for Medical Engineering and Informatics[5], Osaka University, Osaka, Japan

Progesterone receptor membrane component 2 expression leads to erlotinib resistance in lung adenocarcinoma cells

S. Hanamuro[1,#], Y. Lin[1,#], H. Konishi[1], K. Izusawa[1], L. Yang[1], Y. Haga[1], H. Tsujino[1,2], K. Nagano[1,3], K. Higashisaka[1,4,*], Y. Tsutsumi[1,5,*]

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**Corresponding authors: Kazuma Higashisaka, PhD, and Yasuo Tsutsumi, PhD, Laboratory of Toxicology and Safety Science, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6, Yamadaoka, Suita, Osaka 565-0871, Japan*
higashisaka@phs.osaka-u.ac.jp (Kazuma Higashisaka)
ytsutsumi@phs.osaka-u.ac.jp (Yasuo Tsutsumi)
#These authors contributed equally to the work.

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Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) provide a favorable treatment outcome in patients with EGFR mutation-positive non-small cell lung cancer. However, most of such patients become resistant to EGFR-TKIs within a year. Thus, clarifying the mechanism of acquired resistance to EGFR-TKIs has been a research focus. Here, we demonstrated that the expression of progesterone receptor membrane component 2 (PGRMC2) was upregulated in an erlotinib-resistant cell line, PC9/ER, compared with the parental PC9 lung cancer cells. Our previous study showed that PGRMC1 is responsible for acquired resistance to erlotinib; however, PGRMC2 has not been discussed yet. Thus, the aim of this study was to determine the role of PGRMC2 in acquired resistance to erlotinib. Transfection with PGRMC2 siRNA significantly enhanced the sensitivity to erlotinib in PC9/ER cells. Furthermore, knockdown of PGRMC2 reduced the expression of p21, which is known as cell-cycle inhibitor and antiproliferative effector. These results suggest that PGRMC2 partially contributes to erlotinib resistance in PC9/ER cells, and that investigation into the effect of PGRMC2 on apoptosis and the cell cycle are warranted.

1. Introduction

Non-small cell lung cancer (NSCLC), which accounts for more than 85% of patients with lung cancer world-wide, has a relatively gradual progression compared with small cell lung cancer (Torre et al. 2015). Approximately 50% of Asian patients with NSCLC have mutations in the epidermal growth factor receptor (*EGFR*) (Rosell et al. 2009; Takano et al. 2005; Taron et al. 2005), and EGFR tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib and erlotinib provide a favorable treatment outcome in these patients;[5] however, most of them acquire resistance to EGFR-TKIs within 12 months (Gainor and Shaw 2013; Wang et al. 2014). Thus, the mechanism of EGFR-TKI resistance has become a focus of clinical research. Genomic mutations in the EGFR tyrosine kinase domain, the activation of bypass signaling pathways, and phenotypic or histologic transformation have been identified as mechanisms of acquired resistance to first- and second-generation EGFR-TKIs (Gelatti et al. 2019). On the other hand, the mechanisms of acquired resistance remain unclear in up to 40% of lung cancer patients (Takeda et al. 2010). Our previous study showed that inhibition of progesterone receptor membrane component 1 (PGRMC1) increases erlotinib sensitivity in the erlotinib-resistant cell line PC9/ER, derived from PC9 lung cancer cells, suggesting that PGRMC1 is a potential target molecule for overcoming erlotinib resistance (Lin et al. 2020). PGRMC1 and the related protein, progesterone receptor membrane component 2 (PGRMC2), belong to the membrane-associated progesterone receptor family (Cahill 2007). PGRMC2 interacts with PGRMC1 in spontaneously immortalized rat granulosa cells (Peluso et al. 2014), and disrupting this interaction leads to inappropriate entry into the cell cycle, mitotic arrest, and cell death (Peluso et al. 2019).

Whereas PGRMC1 has been extensively characterized in *in vitro* studies, little is known about PGRMC2. Here, we attempted to determine the role of PGRMC2 in acquired resistance to EGFR-TKIs.

Abbreviations:
EGFR-TKIs, Epidermal growth factor receptor-tyrosine-kinase inhibitors; ER, Erlotinib resistance; NSCLC, Non-small cell lung cancer; PGRMC1, Progesterone receptor membrane component 1; PGRMC2, Progesterone receptor membrane component 2.

2. Investigations, results and discussion

Western blot analysis demonstrated that the protein level of PGRMC2 was upregulated in PC9/ER cells, an established erlotinib-resistant lung cancer cell line, compared with the parental PC9 cells (Fig. 1a). Furthermore, real-time RT-PCR analysis revealed that the mRNA expression of *PGRMC2* in PC9/ER cells was also significantly up-regulated compared with PC9 cells (Fig. 1b). To validate the clinical relevance of PGRMC2, according to the Kaplan-Meier plot and the results of the log-rank test (Fig. 1c), *PGRMC2* expression level was associated with overall survival in these patients: the high-PGRMC2 group had a lower overall survival rate than the low-PGRMC2 group (Fig. 1c). This result suggests that PGRMC2 might play a clinically relevant role in lung cancer. This is consistent with, but does not prove, that *PGRMC2*

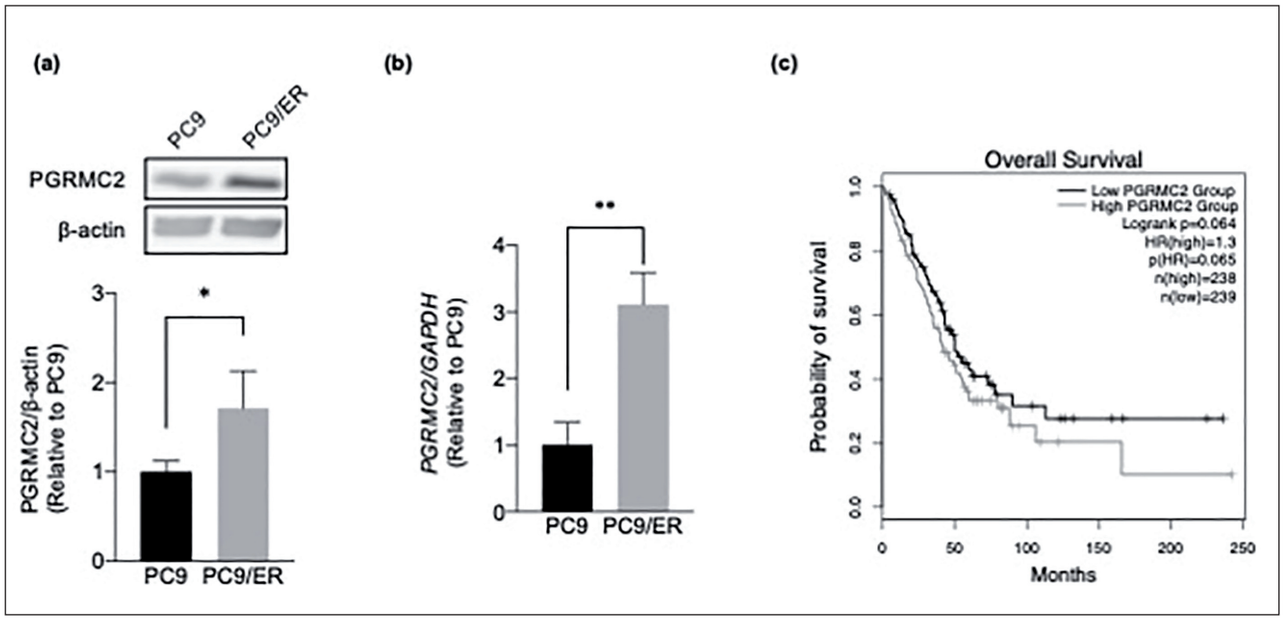

Fig. 1: Upregulation of PGRMC2 in PC9/ER cells. (a) Immunoblotting of PGRMC2 in PC9 and PC9/ER cells. The upper panel shows a representative blot. The lower panel shows means±S.D. (n = 3). *P < 0.05 vs. PC9 (Student's t-test). (b) Real-time RT-PCR analysis of *PGRMC2* mRNA levels in PC9 and PC9/ER cells. Data are means ± S.D. (n = 3). **P < 0.01 vs. PC9 (Student's t-test). (c) Kaplan–Meier plots of overall survival in lung adenocarcinoma patients. The plot was created, and statistical analyses were conducted using GEPIA2 (http://gepia2.cancer-pku.cn) from data in the TCGA/GTEx Dataset (see Experimental).

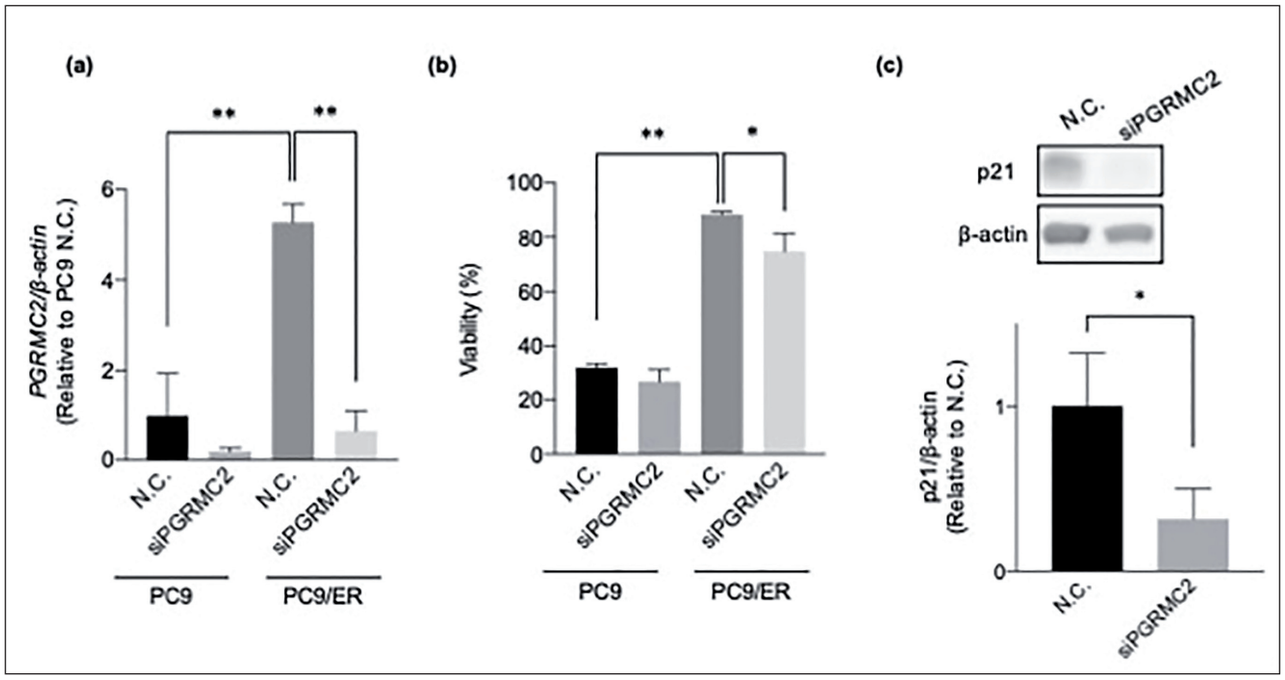

Fig. 2: Contribution of PGRMC2 to the resistance to erlotinib in lung adenocarcinoma cancer. (a) Real-time RT-PCR analysis of the efficiency of transfection of PC9 and PC9/ER cells with *PGRMC2* siRNA (siPGRMC2). N.C., negative control. (b) WST-8 assay of the effects of erlotinib (5 μM) on cell viability of *PGRMC2* siRNA–treated PC9 and PC9/ER cells. The viability to erlotinib (-) PC9 cells or PC9/ER cells were indicated. Data are means ± S.D. (n = 3). **P < 0.01, *P < 0.05 vs. PC9 by Tukey's multiple comparison test. N.S., not significant. (c) Immunoblotting analysis of the levels of p21 in PC9/ER cells treated with siPGRMC2. Data are means±S.D. (n = 3); **P < 0.01 vs. N.C. (Student's t-test).

expression contributes to acquired resistance in EGFR-TKI. To clarify the relation between *PGRMC2* expression and the resistance to EGFR-TKI in the clinic, it would be necessary to compare the expression levels in individual patients before and after they have acquired resistance.

To further evaluate the role of PGRMC2 in acquired resistance to erlotinib, we used the WST-8 assay to examine sensitivity to erlotinib in the presence or absence of siRNA for *PGRMC2* in the two cell lines; real-time RT-PCR analysis confirmed the efficient knockdown of *PGRMC2* by the siRNA (Fig. 2a). PC9/ER cells were more resistant to erlotinib than PC9 cells as we previously reported (Lin et al. 2020); the viability of PC9 cells transfected with *PGRMC2* siRNA was similar to that of those transfected with the siRNA negative control. On the other hand, PC9/ER cells transfected with *PGRMC2* siRNA displayed slightly but significantly lower viability than those transfected with the siRNA negative control (Fig. 2b). This result suggests that PGRMC2 partially contributes to erlotinib resistance in PC9/ER cells. Given that PGRMC1 interacts with PGRMC2 (Peluso et al. 2014), it is possible that the molecular relation between PGRMC1 and PGRMC2 affects the sensitivity to erlotinib in PC9/

ER cells. It is therefore essential to determine how PGRMC1 interacts with PGRMC2 in these cells, and to assess whether the two proteins have a synergistic or additive effect on acquired resistance to EGFR-TKIs.

PGRMC2 is required for delivery of heme to the nucleus and for the control of mitochondrial function (Galmozzi et al. 2019). Mitochondria play a central and multi-functional role in malignant tumor progression, cell cycle arrest, and apoptosis (Zhong et al. 2017). Thus, understanding the relation between the role of PGRMC2 and mitochondrial function in acquired resistance to EGFR-TKIs might reveal novel approaches for overcoming this resistance. In PC9/ER cells, knockdown of PGRMC2 significantly reduced the expression of p21 (Fig. 2c). Reports have implicated functional loss of p21 as cause of tamoxifen-resistance (Abukdeir and Park 2008), on the other hand, it is also suggested that p21 accumulation is related to prevent the induction of apoptosis in response to gefitinib (Guerard et al. 2018) and that p21 play an essential role in growth arrest after DNA damage (Gartel and Tyner 2002). Considering that PC9/ER cell viability was decreased by siPGRMC2, it could be presumed that cell death in PC9/ER cells was induced by inhibition of p21 expression. However, since the changes of cell viability due to siPGRMC2 was slight, it is necessary to investigate the effect of PGRMC2 via p21 on apoptosis and the cell cycle. Furthermore, disrupting the interaction between PGRMC1 and PGRMC2 through the use of siRNA against both proteins could clarify the effect of their interaction on downstream components of the PGRMC1 and PGRMC2 signal transduction pathways such as NF-κB signaling and Wnt/β-catenin signaling,[10] and progesterone-related signaling (Zhu et al. 2013).

Collectively, we showed that PGRMC2 knockdown reduced p21 expression and partially attenuated the resistance to erlotinib in PC9/ER cells. These findings suggest that PGRMC2 contributes to acquired EGFR-TKI resistance in NSCLC. Although further research is needed, our data help to clarify the mechanisms underlying acquired EGFR-TKI resistance.

3. Experimental

3.1. Cell lines and cell cultures

The NSCLC cell line PC9 was purchased from the RIKEN BioResource Center (Tsukuba, Japan). The erlotinib-resistant cell line PC9/ER was obtained previously (Shintani et al. 2018). The PC9 cell line was cultured in RPMI-1640 (Wako, Osaka, Japan) with 10% (v/v) fetal bovine serum (FBS; Biosera Inc., Manila, Philippines) and 1% (v/v) antibiotic–antimycotic solution (×100) (Wako) and maintained at 37°C at 5% CO_2 and >95% humidity. The PC9/ER cell line was cultured in the same conditions as the PC9 cell line but in the presence of 5 μM erlotinib (Selleckchem, Houston, TX, USA).

3.2. Survival analysis of PGRMC2 expression data in the TCGA/GTEx dataset

Survival analysis was performed using *PGRMC2* gene expression data and survival information of lung adenocarcinoma patients downloaded from the TCGA/GTEx dataset and the tools in GEPIA (Tang et al. 2018). The *PGRMC2* gene was entered into the GEPIA web interface (http://gepia2.cancer-pku.cn) as described (Tang et al. 2019) (last access time 03/08/2020) and a Kaplan–Meier survival plot, log-rank test *P*-value evaluating the difference between the two survival curves, and hazard ratio HR (High) of the high-expression group to the low-expression group were obtained. Survival analysis was performed with the following settings; Group Cutoff, expression threshold for splitting the high-expression and low-expression cohorts, was set as median (Cutoff-High is 50% and Cutoff-Low is 50%).

3.3. Transient transfection of small interfering RNA (siRNA)

Cells were transfected with 10 nM Stealth siRNA against *PGRMC2* (5′-CAUUUUGCCUAGAUAAAGAtt-3′ and 3′-UCUUUAUCUAGGCAAAAUGtg-5′), or the Stealth siRNA negative control with medium GC content (Invitrogen, Carlsbad, CA, USA). The transfection was executed with Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) for 72 h in RPMI-1640 containing 10% FBS and 1% (v/v) antibiotic–antimycotic solution (×100).

3.4. Cell viability analysis

PC9 and PC9/ER cells were seeded at 4000 cells/well in 96-well flat plates overnight without drugs; the cells were transfected with *PGRMC2* siRNAs or control siRNAs in complete medium containing 5 μM erlotinib for 72 h. Cell viability was evaluated by the WST-8 assay (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions.

3.5. Western blotting

Proteins were extracted by M-PER Mammalian Protein Extraction Reagent with Protease and Phosphatase Inhibitor Cocktail (both from Thermo Fisher Scientific, Waltham, MA, USA), and mixed with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) containing Dithiothreitol (Wako). Samples were boiled for 5 min and the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were then electro-transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) and blocked with 4% (w/v) Block Ace (KAC Co., Ltd., Kyoto, Japan) in phosphate buffered saline plus 0.01% (v/v) Triton X-100 (PBST). The membranes were incubated with primary antibody overnight at 4°C and then treated with secondary antibody for 1 h at room temperature. The following antibodies were used: anti-PGRMC2 monoclonal antibody (mAb) (sc-374624, 1:1000) and anti-mouse IgG-horseradish peroxidase (HRP)–conjugated secondary antibody (sc-516102, 1:1000) (Santa Cruz Biotechnology, Dallas, TX, USA); anti-β-actin mAb (A5316, 1:50,000) and anti-mouse IgG secondary antibody (A9044, 1:50,000) (Sigma-Aldrich, Darmstadt, DE, USA); anti-p21 Waf1/Cip1 mAb (#2947, 1:1000) and anti-rabbit IgG HRP-linked antibody (#7074, 1:2000) (Cell Signaling Technology, Danvers, MA, USA). All antibodies were diluted in 0.4% (w/v) Block Ace in PBST. The protein bands were detected by using ImmunoStar LD (Wako) and visualized with an ImageQuant LAS 4000 mini biomolecular imager (GE Healthcare Japan, Tokyo, Japan). The resultant images were analyzed by using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

3.6. Real-time RT-PCR analysis

Total RNA was extracted by using a FastGene RNA Basic Kit (Nippon Genetics, Tokyo, Japan) and reverse transcribed to cDNA by using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). A PCR mixture containing the above cDNA as the template and primers for *PGRMC2* (forward, 5′-AGGGGAAGAACCGTCAGAAT-3′; reverse 5′-AAGCCCCACCAGACATTACA-3′), *GAPDH* (forward, 5′-GAAGGTGAAGGTCGGAGTC-3′; reverse 5′-GAAGATGGTGATGGGATTTC-3′), and *Actin* (forward, 5'-GCCCTGAGGCACTCTTCCA-3′; reverse 5′-CGGATGTCCACGTCACACTTC-3′) (Eurofins Genomics, Tokyo, Japan) and GeneAce SYBR qPCR Mix α Low ROX (Nippon Gene) was prepared, and real-time RT-PCR was performed using a CFX-384 system (BioRad Laboratories).

3.7. Statistical analysis

All statistical analyses were conducted using Graph Pad Prism Mac version 7.0 (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). Data are expressed as means±S.D. For the results in Figs. 1a, 1b, and 2c, a two-sided Student's *t*-test was used to compare groups. For the results in Figures 2a and 2b, two-way ANOVA followed by Tukey's multiple comparison tests was used to compare groups.

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