

The role of PRMT1 in EGFR methylation and signaling in MDA-MB-468 triple-negative breast cancer cells

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Abstract

Background Epidermal growth factor receptor (EGFR) is often overexpressed in triple-negative breast cancer (TNBC). However, clinical studies have shown that therapies against EGFR are not effective in patients with TNBC. Recently, it has been reported that arginine 198/200 in EGFR extracellular domain is methylated by PRMT1 and that the methylation confers resistance to EGFR monoclonal antibody cetuximab in colorectal cancer cells. To explore a potential mechanism underlying intrinsic resistance to anti-EGFR therapy in TNBC, we investigated the role of PRMT1 in EGFR methylation and signaling in MDA-MB-468 (468) TNBC cells.

Methods We knocked down PRMT1 in 468 cells by shRNA, and subjected the cell lysates to Western blot analysis to examine EGFR activation and its downstream molecules. We also evaluated cell proliferation and sphere formation of PRMT1-knockdown cells. Finally, we examined the effects of pan-PRMT inhibitor, AMI-1, on cetuximab by colony formation and soft agar assays.

Results EGFR methylation and activity was significantly reduced in PRMT1-knockdown cells compared to the parental cells. Knockdown of PRMT1 also reduced cell

proliferation and sphere formation. Moreover, AMI-1 sensitized 468 cells to cetuximab.

Conclusion The results indicate that PRMT1 is critical for EGFR activity in 468 cells. Our data also suggest that inhibition of PRMT1 sensitizes TNBC cells to cetuximab. Thus, inhibition of PRMT1 may be an effective therapeutic strategy to overcome intrinsic resistance to cetuximab in TNBC.

Keywords PRMT1 · EGFR · Triple-negative breast cancer · Arginine methylation

Introduction

Triple-negative breast cancer (TNBC), which does not express estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), is the most aggressive subset of breast cancer [1]. Because ER or HER2-targeted therapies cannot be used for the treatment for TNBC, conventional chemotherapy is still the primary option for systemic treatment for TNBC patients [2]. Although a large number of TNBC patients initially respond to the chemotherapy, the disease eventually relapses and becomes resistant [2]. Therefore, the effective therapeutic approaches for TNBC are urgently necessary.

The receptor tyrosine kinase epidermal growth factor receptor (EGFR) is frequently mutated or overexpressed in various cancer types [3], and many therapies targeting EGFR, including EGFR tyrosine kinase inhibitors (TKI), e.g., gefitinib and erlotinib, and monoclonal antibodies (mAb), e.g., cetuximab and panitumumab, have been developed and are used in the clinic [4]. EGFR is expressed more frequently in TNBC than other subtypes of breast cancer [5]. High EGFR expression is also correlated with

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poor prognosis in TNBC. Thus, several clinical trials evaluating anti-EGFR therapies in TNBC patients have been conducted, but none of these drugs has yet to be approved for TNBC [6]. Although a small subpopulation of TNBC patients seems to respond to cetuximab therapy [7, 8], the results are still far from satisfactory.

Protein arginine methylation is an important post-translational modification that is involved in various cellular processes, including RNA processing, DNA damage repair, gene transcription, signal transduction, and protein translocation [9]. Arginine methylation includes monomethylarginines, asymmetric di-methylarginines, and symmetric di-methylarginines in mammalian cells. The protein arginine *N*-methyltransferases (PRMTs) are responsible for arginine methylation. PRMTs have been shown to be overexpressed in multiple cancer types, including breast cancer [9], and some of them are shown to be critical for breast cancer cells proliferation and migration [10, 11]. Therefore, PRMTs are potential targets for cancer therapy. Among the PRMT family members (PRMT1–9), PRMT1 is a major asymmetric arginine *N*-methyltransferase in mammalian cells [9]. PRMT1 catalyzes histone H4 arginine 3 asymmetric dimethylation (H4R3me_{2a}), which is a critical modification for transcriptional activation [12]. In addition, PRMT1 regulates various cellular functions via arginine methylation of important proteins, such as FOXO1, ER α , MRE11, and 53BP1 [13–16].

PRMT5-mediated arginine methylation of EGFR at R1175 was previously reported to attenuate EGFR activity [17]. More recently, Liao et al. reported EGFR methylation at R198 and R200, which locate in its extracellular domain, by PRMT1 [18]. Notably, EGFR methylation at R198/200 enhances EGFR receptor dimerization, activation, and cetuximab resistance [18]. Moreover, higher levels of R198/200-methylated EGFR expression in tumors from patients with colorectal cancer correlates with higher recurrence rate after cetuximab treatment, and methylated EGFR level positively correlates with PRMT1 expression [18]. Although EGFR methylation at R198/200 seems to play a role in cetuximab sensitivity in colorectal cancer, EGFR methylation in breast cancer has not yet been examined. Thus, in the present study, we investigated the EGFR methylation at 198/200, as well as the role of PRMT1 in MDA-MB-468 (468) TNBC cells.

Materials and methods

Plasmids, reagents, and antibodies

Lentivirus expression plasmids of shRNA for PRMT1 were described previously [18]. EGF and tubulin antibodies

were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against EGFR, p-EGFR (pY1068), ERK, p-ERK (pT202/pY204), AKT, p-AKT (p-Ser473), STAT3, p-STAT3 (p-Y705), PRMT1 were obtained from Cell Signaling Technology (Danvers, MA, USA). R198/200-methylated EGFR antibody was developed in Mien-Chie Hung lab as described previously [18]. AMI-1 was purchased from Cayman Chemical (Ann Arbor, MI, USA). Cetuximab was purchased from the pharmacy at the MD Anderson Cancer Center.

Cell lines

TNBC cell lines (HCC38, HCC1937, MDA-MB-468, BT-549, Hs-578T, MDA-MB-436, MDA-MB-231, MDA-MB-453), luminal cell lines (MCF-7, T47D), and human embryonic kidney cell line (293T) were obtained from ATCC. Cells were maintained in Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Before EGF stimulation, 80% confluent cells were serum-starved for 24 h and then stimulated with 50 ng/ml EGF for 30 min.

shRNA lentivirus production

For lentivirus production, PLKO.1 PRMT1 shRNA vector and packaging plasmids were co-transfected into 293T cells using a standard calcium phosphate transfection method. After 48-h transfection, breast cancer cells were infected with viral particles. Stable knockdown clones were selected by culturing cells in medium with 2 μ g/ml puromycin.

Western blot assay

Whole cell extracts were subjected to 8–12% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane using transfer cassettes according to the manufacturer's protocols (Bio-Rad). After incubation with 3% skimmed milk in TBS-T (Tris-buffered saline, 0.1% Tween 20) for 60 min, the membranes were incubated with various antibodies at 4 °C overnight. The membranes were then washed with TBS-T 3 times for 10 min and incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h. After washing the membrane with TBS-T 3 times, signals were detected with an enhanced chemiluminescence (ECL) reagent (Bio-Rad; Hercules, CA).

Immunohistochemical staining

Tissue microarray (TMA) with TNBC tissues were obtained from MD Anderson Cancer Center ($n = 81$), and

IHC staining was performed as described previously [18]. In brief, following preincubation in 10% normal serum for 1 h, TMA was incubated with the primary antibody (anti-methyl EGFR) at 4 degree overnight. The TMA was then incubated with a biotin-conjugated secondary antibody, followed by the incubation with an avidin–biotin–peroxidase complex. The signals were visualized with the 3-amino-9-ethylcarbazole solution, while Mayer's hematoxylin was used for the counterstaining. The stained TMA was scanned on the Automated Cellular Image System III (ACIS III) for quantification by digital image analysis. According to histologic scoring, the intensity of staining was ranked into 1 of 4 groups: high (score 3), medium (score 2), low (score 1), and negative (score 0).

Co-immunoprecipitation assay

Cells were lysed in NP-40 lysis buffer (150 mM NaCl, 10 mM Tris–HCl pH 7.5, 1% NP-40, protease inhibitors and phosphatase inhibitors cocktails), and the lysates containing 1 mg total proteins were incubated overnight at 4 °C with 1 µg of anti-EGFR antibody (Ab-13, ThermoFisher Scientific; Waltham, MA, USA), anti-RPMT1 or control IgG, followed by additional 4 h incubation with 15 µl of protein G or A-agarose beads (Santa Cruz Biotechnology; Santa Cruz, CA, USA). After washing three times with NP40 lysis buffer, the beads were boiled in 2× SDS sample buffer for 5 min for extracting proteins. The signals were then detected by Western blot. 50 µg of the total lysates used for the immunoprecipitation was also applied to Western blot to verify the protein expression.

Cell proliferation assay

Cells were seeded in triplicate at 5000 cells per chamber of 12-well culture plates, and fresh medium was added every day. Cells were then trypsinized, and cell numbers were counted on a daily basis. The experiments were performed in triplicate.

Cancer sphere formation assay

Cells were suspended in complete MammoCult™ medium (Stem Cell Technology; Vancouver, BC, Canada) in 12-well plates (1.0 ml per well) pre-coated with 0.5% agar incubated at 37 °C, 5% CO₂. After 14 days, spheres were photographed. The experiments were performed in triplicate.

Colony formation assay

MDA-MB-468 cells were seeded in 12-well plates (2000 cells/well) and treated with or without cetuximab 200 µg/

ml, and/or AIM-1 10 µM for 10 days. Medium containing drugs was replaced every 4 days. Cells were then fixed with 3.7% of formaldehyde and stained with crystal violet solution. After taking images of the plates, 0.5% of SDS solution was added to each well and the plates were incubated for 2 h at room temperature. The relative densities of cells were then determined by measuring the absorbance of the solution at 570 nm using microplate reader. The experiments were performed in triplicate.

Soft agar assay

MDA-MB-468 cells were suspended in DMEM/F12 medium containing 0.35% agarose and seeded on top of 0.5% base agar in 12-well plates (2000 cells/well). Each well was covered with complete medium with or without cetuximab 200 µg/ml, AIM-1 10 µmol/l or the combination, and medium was replaced every 4 days. After 14 days, viable cells were stained with MTT solution and colonies larger than 100 µm were counted under microscope. The experiments were performed in triplicate and repeated three times.

Results

EGFR is methylated in 468 cells and TNBC patient tissues

To determine whether EGFR is also methylated in TNBC, we first examined the status of EGFR methylation in various TNBC cell lines (HCC38, HCC1937, 468, BT-549, Hs-578T, MDA-MB-436, MDA-MB-231, and MDA-MB-453) by Western blot analysis using the specific antibody against methylated EGFR at R198/200 (Fig. 1a) [18]. We also examined the expression of total EGFR and PRMT1 (Fig. 1a). These lines were also subdivided into 4 subgroups (Basal-Like: HCC38, HCC1937, and 468; Mesenchymal: BT-549; Mesenchymal Stem Like: Hs-578, MDA-MB-436, and MDA-MB-231; and Luminal AR: MDA-MB-453) according to previous studies [19]. As controls, we also included ER-positive luminal cell lines, MCF-7 and T47D. EGFR expression was detected in all TNBC cells except MDA-MB-453. Consistent with the previous report, EGFR was overexpressed in 468 cells [20]. Whereas expression of PRMT1 was found in all breast cancer cell lines and appeared to be independent of the breast cancer subtype, methylated EGFR was detected only in 468 cells. To further validate the EGFR R198/200 methylation in TNBC, we next studied EGFR methylations status in TNBC patient tissues by immunohistochemical (IHC) staining of a TNBC tissue microarray (Fig. 1b). 19.8 and 30.9% of patient tissues showed high and median

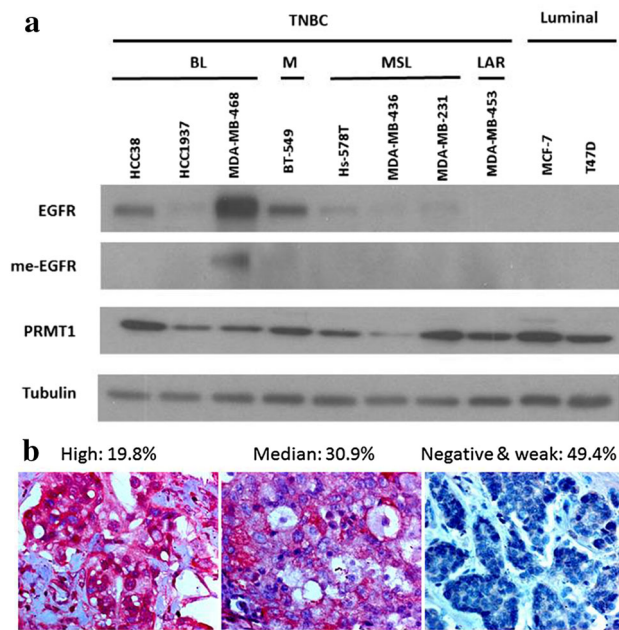


Fig. 1 EGFR methylation in different breast cancer cell lines and TNBC patient tissues. **a** The indicated cells were grown under normal culture conditions, and the cell lysates were subjected to western blot analysis with the antibodies against EGFR, R198/200-methylated EGFR (me-EGFR), PRMT1, and tubulin. *BL* Basal-Like, *M* Mesenchymal, *Hs-578*, *MSL* Mesenchymal Stem Like, *LAR* Luminal AR. **b** The tissue microarray with TNBC tissues ($n = 81$) were subjected to immunohistochemical staining with the antibody specific to EGFR R198/200 methylation. Representative images are shown. 16 cases (19.8%), 25 cases (30.9%), and 40 cases (49.4%) show strong, median, and negative or weak signals, respectively

signals, respectively. These results suggest that similar to colorectal cancer, EGFR is methylated at R198/200 in TNBC.

PRMT1 regulates EGFR methylation and EGFR downstream signaling

PRMT1 is responsible for EGFR methylation in colon cancer cells [18]. To determine whether PRMT1 also regulates EGFR methylation in 468 cells, we first performed co-immunoprecipitation assay, which showed that EGFR interacted with PRMT1 in 468 cells (Fig. 2a). Next, to validate that PRMT1 is responsible for EGFR methylation, we knocked down PRMT1 in 468 cells and examined EGFR methylation by Western blot with the specific antibody against methylated EGFR at R198/200. As shown in Fig. 2b, EGFR methylation was significantly reduced in the PRMT1-knockdown cells. Moreover, we validated the effect of PRMT1 knockdown on EGFR activation. Serum-starved 468 PRMT1 knockdown and control cells were treated with EGF, and the phosphorylation of EGFR and its downstream molecules including AKT, ERK, and STAT3 were examined (Fig. 2c). The results showed that EGF-

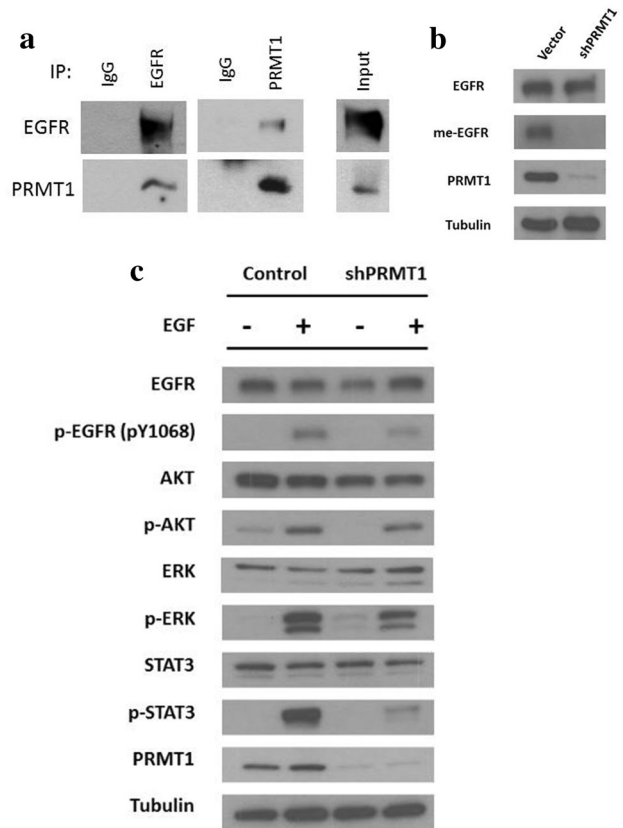


Fig. 2 PRMT1 interacts with EGFR and regulates EGFR methylation and signaling in 468 cells. **a** 468 cells were grown under normal culture conditions, and the cell lysates were subjected to immunoprecipitation with either the anti-PRMT1 or anti-EGFR antibody, followed by Western blot analysis with the indicated antibodies. The total cell lysate was also subjected to Western blot to verify the protein expression (Input). **b** The PRMT1-knockdown and vector control 468 cells were subjected to western blot analysis with the antibodies against EGFR, R198/200-methylated EGFR (me-EGFR), PRMT1, and tubulin. **c** The PRMT1-knockdown and vector control 468 cells were grown under serum-deprived conditions overnight, and then stimulated with 10 ng/ml of EGF for 30 min. Then, the activation of EGFR downstream signaling was evaluated by western blot analysis with the indicated antibodies

induced phosphorylation of AKT, ERK, and STAT3 was reduced in the PRMT1-knockdown cells compared to the control cells. In particular, EGF-induced STAT3 phosphorylation was strongly downregulated in the PRMT1 knockdown cells. Together, these results suggested that PRMT1 regulates EGFR activity through EGFR methylation.

PRMT1 promotes cell proliferation and sphere formation of 468 cells

Our results above suggested that PRMT1 might regulate EGFR activity in 468 cells through methylation of EGFR. Given that EGFR signaling pathway is critical for cell growth

and proliferation, we compared cell proliferation of the 468 PRMT1-knockdown and control cells by counting cell numbers under the normal culture condition. Consistent with the downregulation of EGFR signaling in the PRMT1 knockdown cells, cell proliferation was significantly reduced in the PRMT1-knockdown cells compared to the control cells (Fig. 3a). Moreover, we also investigated sphere formation activity of these cells, which represents cancer stem cell activity, as EGFR signaling has been shown to be critical for cancer stem cell property [21]. As shown in Fig. 3b, knock-down of PRMT1 strongly reduced sphere formation of 468 cells. These results indicated that PRMT1 is important for cell proliferation and the maintenance of a stem cell property, and the effects may be attributed to enhanced EGFR signaling via PRMT1-mediated EGFR methylation.

PRMT inhibitor AMI-1 sensitizes 468 cells to cetuximab

Because the previous study demonstrated that EGFR methylation at R198/200 confers resistance to cetuximab in colon cancer cells [20], we next studied the effects of the inhibition of PRMT1 on cetuximab sensitivity in 468 cells. To this end, we used a pan-PRMT inhibitor, AMI-1, as

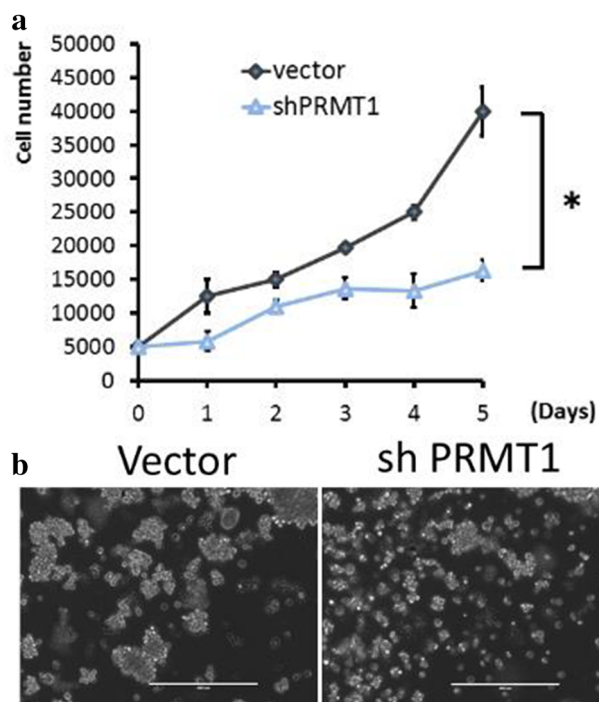


Fig. 3 PRMT1 is involved in cell proliferation and the regulation of a stem cell property in 468 cells. **a** The PRMT1-knockdown and vector control 468 cells were grown under the normal culture condition, and cell numbers were counted daily. Error bars mean \pm SD, $*P < 0.01$. **b** The PRMT1-knockdown and vector control 468 cells were grown in mammosphere culture media for 14 days and the cell images were captured under the microscope

currently no PRMT1-specific inhibitors are commercially available. We then evaluated the effects of AMI-1, cetuximab, and their combination in standard colony formation and soft agar assays. Both drugs alone showed some inhibitory effects on cell proliferation in two- and three-dimensional culture conditions (Fig. 4a, b). However, the effects were significantly enhanced when AMI-1 and cetuximab were combined (Fig. 4a, b). Thus, similar to colorectal cancer cells, inhibition of PRMT1 may sensitize TNBC cells to cetuximab.

Discussion

Arginine methylation of EGFR extracellular domain enhances ligand-dependent receptor dimerization and EGFR activity, and contributes to cetuximab resistance in

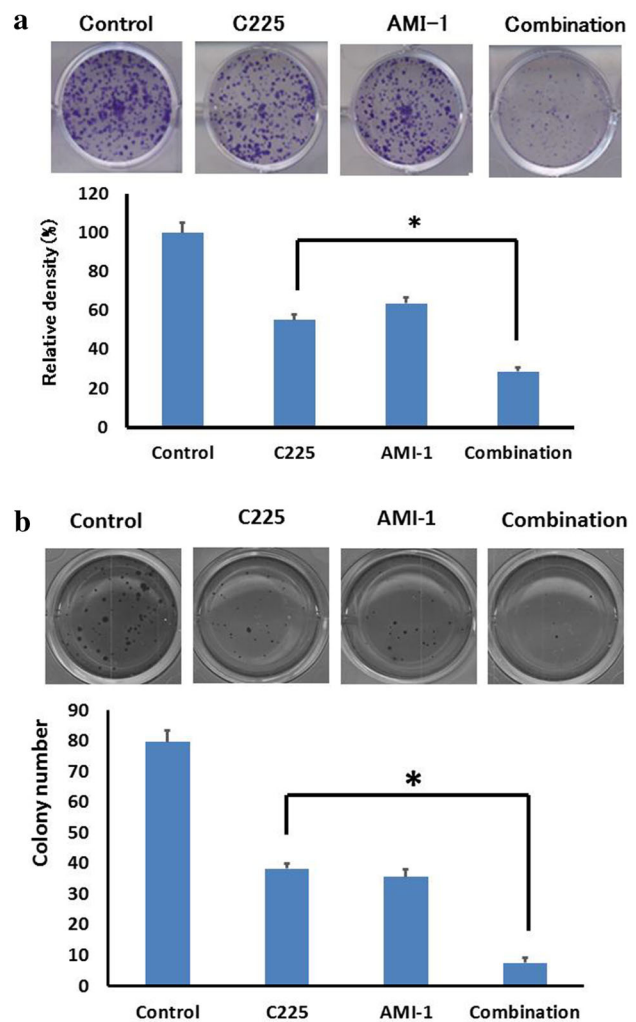


Fig. 4 A pan-PRMT inhibitor AMI-1 sensitizes 468 cells to cetuximab. The effect of cetuximab (C225), AMI-1, and the combination of cetuximab and AMI-1 were determined by colony formation (a) and soft agar assays (b) ($*P < 0.01$)

colorectal cancer cells [18], suggesting that EGFR arginine methylation in colorectal cancer could serve as a predictive marker for cetuximab treatment. There is a clinical trial (NCT02022995) examining the EGFR arginine methylation in response of the cetuximab in colorectal cancer patients. In the present study, we showed that arginine methylation of the extracellular domain of EGFR also contributed to EGFR activation in 468 TNBC cells (Fig. 2). We further demonstrated that inhibition of PRMT1 reduced cell proliferation (Fig. 3), and that inhibition of PRMTs by AMI-1 sensitized 468 cells to cetuximab (Fig. 4). Thus, similar to colorectal cancer, PRMT1-mediated EGFR arginine methylation may play a role in cetuximab resistance in TNBC.

We examined EGFR arginine methylation in both several breast cancer cell lines and TNBC patient tissues (Fig. 1). We observed high EGFR methylation in 19.8% of TNBC patient tissues. It has previously been reported about 60% of tumor tissues from colorectal cancer patients have high EGFR methylation [18]. Although the ratio of EGFR methylation in TNBC is lower than colorectal cancer, these results suggest that EGFR methylation plays a role in EGFR function and cetuximab resistance in TNBC as well. In contrast to TNBC patient samples, we detected EGFR methylation only in 468 cells among the TNBC cell lines we examined. It may be partly attributed to the relatively low ratio of methylated EGFR in breast cancer cell lines compared to that in TNBC tissues. The methylation levels in proteins are controlled by both methyltransferases and demethylases. It has been reported that several lysine demethylases may also function as arginine demethylases [22], and some of these lysine demethylases may serve as EGFR R198/200 demethylases. Thus, such EGFR demethylases may highly express in the cells including BT549 and HCC38, which does not show EGFR methylation but express relatively high EGFR and PRMT1 (Fig. 1a). Also, it is plausible that the antibody used in this study may be more suitable for IHC staining than Western blot. Therefore, we may need to develop monoclonal antibodies more suitable for detecting EGFR methylation by Western blot in the future.

Consistent with colorectal cancer cells, EGF-induced activation of EGFR and downstream signaling was attenuated in 468 cells when PRMT1 was silenced. These results further support the notion that EGFR methylation is also crucial in TNBC cells. Interestingly, EGF-induced STAT3 phosphorylation was strongly inhibited compared to AKT and ERK phosphorylation in PRMT1-knockdown cells. Therefore, PRMT1 may also be involved in STAT3 activation independently of EGFR signaling. STAT3 is preferentially activated in TNBC and involved in cancer stem cell maintenance in breast cancer [23], and our data showed the reduced cancer sphere formation in PRMT1-

knockdown cells (Fig. 3b). Thus, PRMT1 may regulate cancer stem cell properties via activation of STAT3 in TNBC. Indeed, inhibition of STAT3 has been demonstrated to downregulate cancer stem cell population, cell migration and invasion in 468 cells [24]. Furthermore, PRMT1 has been also shown to contribute to breast cancer stem cells through the upregulation of ZEB1, a regulator of epithelial mesenchymal transition and cancer stem cells. Because EGFR-ERK signaling upregulates ZEB1 [25], PRMT1-mediated EGFR upregulation may also contribute to ZEB1 upregulation and ZEB1-mediated cancer stem cell regulation.

Monoclonal antibodies targeting EGFR have been approved by the US Food and Drug Administration to treat colorectal (cetuximab and panitumumab), head and neck (cetuximab) and squamous non-small-cell lung cancer (necitumumab) cancers but not breast cancer because of unsatisfactory responses seen in multiple clinical trials [7, 26]. Although the overall response rate to cetuximab is too low to be approved for TNBC patients, a small number of TNBC patients still respond to cetuximab [27]. Thus, it is particularly important to identify the subpopulation of TNBC patients that will respond to treatment with cetuximab. Our present study suggests that EGFR is methylated in TNBC and EGFR methylation contributes to cetuximab sensitivity in TNBC. Therefore, EGFR methylation may be used as a predictive biomarker for cetuximab treatment of TNBC patients, with immediate benefits for those who have tumors with low EGFR methylation. PRMTs have been considered as drug targets for various cancers, and multiple PRMT inhibitors have been developed [9]. Thus, the combination of PRMT1 inhibitors and cetuximab may be a potential therapeutic approach for TNBC patients whose tumors have high EGFR methylation. Alternatively, if EGFR methylation significantly contributes to resistance to cetuximab and EGFR oncogenic function, the antibody that specifically target methylated EGFR may be a potential therapeutic strategy for TNBC with high EGFR methylation. Therefore, further clinical studies are necessary to validate role of EGFR methylation in response of TNBC patients to cetuximab.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest to declare.

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