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Original Research

EGFR-independent Elk1/CIP2A signalling mediates apoptotic effect of an erlotinib derivative TD52 in triple-negative breast cancer cells

Chun-Yu Liu [a,b,j], Tzu-Ting Huang [a,j], Chun-Teng Huang [b,e], Ming-Hung Hu [c,d], Duen-Shian Wang [a], Wan-Lun Wang [a], Wen-Chun Tsai [a], Chia-Han Lee [a], Ka-Yi Lau [a], Hsiu-Ping Yang [a], Ming-Huang Chen [a,b], Chung-Wai Shiau [f], Ling-Ming Tseng [b,g,j,**], Kuen-Feng Chen [h,i,*]

[a] *Division of Medical Oncology, Department of Oncology, Taipei Veterans General Hospital, No. 201, Sec. 2, Shih-Pai Road, Taipei 112, Taiwan*
[b] *School of Medicine, National Yang-Ming University, No. 155, Sec. 2, Li-Nong Street, Taipei 112, Taiwan*
[c] *Division of Hematology and Oncology, Department of Medicine, Cardinal Tien Hospital, No. 362, Zhong-zheng Road, New Taipei City 231, Taiwan*
[d] *School of Medicine, Fu Jen Catholic University, No. 510, Zhong-zheng Road, Xin-zhuang Dist., New Taipei City 24205, Taiwan*
[e] *Division of Hematology & Oncology, Department of Medicine, Yang-Ming Branch of Taipei City Hospital, No.145, Zhengzhou Road, Datong Dist., Taipei 10341, Taiwan*
[f] *Institute of Biopharmaceutical Sciences, National Yang-Ming University, No. 155, Sec. 2, Li-Nong Street, Taipei 112, Taiwan*
[g] *Department of Surgery, Taipei Veterans General Hospital, No. 201, Sec. 2, Shih-Pai Road, Taipei 112, Taiwan*
[h] *Department of Medical Research, National Taiwan University Hospital, No. 7, Chung-Shan South Road, Taipei 100, Taiwan*
[i] *National Taiwan University College of Medicine, No.1 Sec. 1, Jen-Ai Road, Taipei 100, Taiwan*
[j] *Comprehensive Breast Health Center, Taipei Veterans General Hospital, No. 201, Sec. 2, Shih-Pai Road, Taipei 112, Taiwan*

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Abstract ***Objectives:*** Cancerous inhibitor of protein phosphatase 2A (CIP2A) has emerged as a therapeutic determinant mediating the anti-cancer effects of several new agents. We

* *Corresponding author*: Department of Medical Research, National Taiwan University Hospital, No 7, Chung-Shan South Road, Taipei, Taiwan. Fax: +886 2 23225329.
** *Corresponding author*: Department of Surgery, Taipei Veterans General Hospital, No. 201, Sec. 2, Shih-Pai Road, Taipei 112, Taiwan. Fax: +886 2 28757537.
E-mail addresses: lmtseng@vghtpe.gov.tw (L.-M. Tseng), kfchen1970@ntu.edu.tw (K.-F. Chen).

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CIP2A;
Erlotinib;
EGFR;
Triple-negative breast cancer

investigated the efficacy and mechanism of TD52, an erlotinib derivative with minimal p-EGFR inhibition but significant CIP2A downregulation, in triple-negative breast cancer (TNBC) cells.

Methods: TNBC lines were used for *in vitro* studies. Cell apoptosis was examined by flow cytometry and Western blot. Signal transduction pathways in cells were assessed by Western blot. *In vivo* efficacy of TD52 was tested in xenograft nude mice.

Results: We explored the CIP2A mRNA expression in a publically available database and found that higher levels of CIP2A mRNA is associated with worse recurrence-free survival in patients with TNBC. TD52-enhanced apoptosis accompanied with CIP2A downregulation and CIP2A overexpression protected cells from TD52-mediated apoptosis. The activity of protein phosphatase 2A (PP2A) was also increased in TD52-treated cells. TD52-induced apoptosis and p-Akt downregulation was attenuated by PP2A antagonist okadaic acid. Furthermore, TD52 indirectly downregulated CIP2A transcription via disturbing the binding of Elk1 to the CIP2A promoter. Importantly, TD52 showed anti-tumour activity in mice bearing TNBC xenograft tumours and downregulated CIP2A and p-Akt in these xenografted tumours. Interestingly, higher Elk1 mRNA expression was also associated with worse recurrence-free survival in TNBC patients by Kaplan–Meier survival analysis.

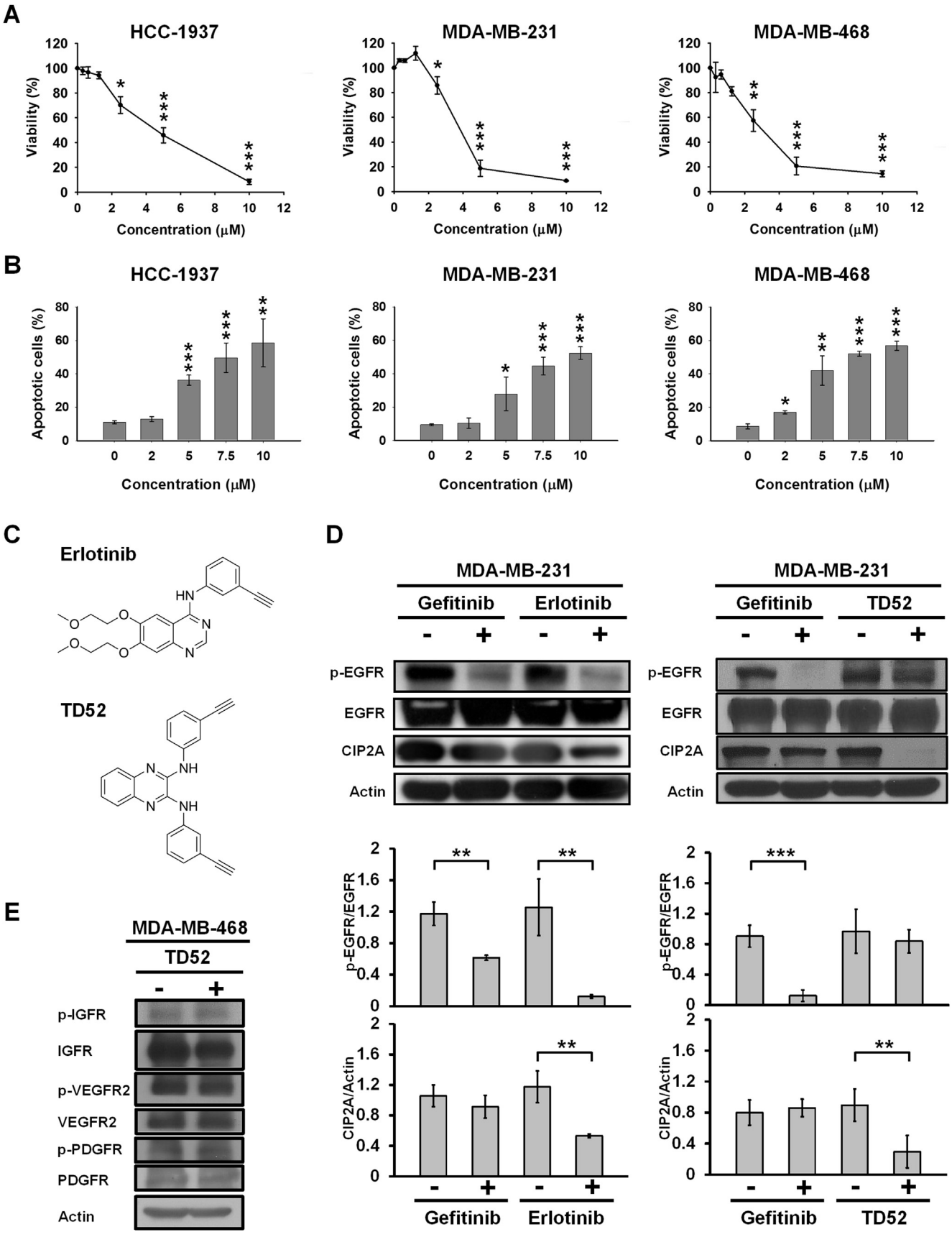
Conclusion: Our findings indicated that EGFR-independent pharmacological modulation on Elk1/CIP2A signalling mediates the apoptotic effect of TD52 in TNBC cells, suggesting the potential therapeutic strategy.

1. Background

Triple-negative breast cancer (TNBC) remains a difficult-to-treat breast cancer subtype not only because of the lack of typical breast cancer targets, hormone receptors and human epidermal growth factor receptor 2, but also due to the intrinsic heterogeneous and aggressive tumour behaviour. Patients with metastatic TNBC generally have a poorer outcome compared with those with non-TNBC, with reported median survival time around 18–19 months [1]. Therefore, this unmet medical need in patients with TNBC remains a great clinical challenge.

Protein phosphatase 2A (PP2A) is one of the few serine/threonine phosphatases that can function as a tumour suppressor through dephosphorylation of onco-kinases such as p-Akt and p-ERK [2]. Cancerous inhibitor of protein phosphatase 2A (CIP2A) is an endogenous inhibitor of PP2A and has been shown to promote tumourigenesis by suppressing PP2A [3]. CIP2A over-expression is widely found across various cancer types, such as breast cancer, non-small cell lung cancer (NSCLC), hepatocellular carcinoma (HCC), etc [4–6]. Recently, De *et al*. proposed a comprehensive review of an interconnected regulatory network (oncogenic nexus) of CIP2A [7]. This nexus is established through either the direct interactions of CIP2A or the indirect interactions of CIP2A-PP2A with either multiple key cellular proteins/transcription factors or with components of the key oncogenic pathways. For example, through suppressing the serine/threonine phosphatase function of PP2A, CIP2A activates oncogenic proteins such as c-Myc, extracellular-signal regulated kinase (ERK) and Akt [2,7,8]. Moreover, as an oncogenic protein in the transformation of cells and cancer progression, CIP2A has been shown to play a role in mediating several drug effects in cancer [7]. In our previous work, we demonstrated that pharmacological downregulation of CIP2A increased PP2A activity and subsequent inactivation of p-Akt signalling, inhibited proliferation and induced apoptosis in breast cancer cells [9,10]. Together, these findings suggest that targeting CIP2A could be an ideal therapeutic approach for breast cancer.

Quinazoline is an aromatic bicyclic organic compound (with the formula $C_8H_6N_2$) that has been used as a major chemical backbone for medicine such as anti-cancer and antimalarial agents [11–13]. Studies on structure–activity relationship have demonstrated that quinazoline derivatives can be designated as tyrosine kinase inhibitors of epidermal growth factor receptor (EGFR) [14]. These agents, such as gefitinib, erlotinib and lapatinib, have been approved for clinical use in cancer patients. Erlotinib, an EGFR tyrosine kinase inhibitor, is currently one of the standard treatments for advanced EGFR mutation-positive NSCLC [15,16]. In our previous study, we found that CIP2A is a molecular determinant of erlotinib-induced apoptosis in HCC cells [6]. Given the pharmacophore importance of quinazoline, we developed disubstituted-quinazoline derivatives based on the quinazoline-like drug erlotinib to expand candidate anticancer agents with therapeutic potentials [17]. Importantly, to generate new chemical entities without patent violation, these erlotinib derivatives were designated to be devoid of EGFR tyrosine kinase inhibition in comparison with erlotinib.


A
HCC-1937
MDA-MB-231
MDA-MB-468
Viability (%)
Concentration (μM)
B
HCC-1937
MDA-MB-231
MDA-MB-468
Apoptotic cells (%)
Concentration (μM)
C
Erlotinib
TD52
D
MDA-MB-231
Gefitinib
Erlotinib
TD52
p-EGFR
EGFR
CIP2A
Actin
p-EGFR/EGFR
CIP2A/Actin
E
MDA-MB-468
TD52
p-IGFR
IGFR
p-VEGFR2
VEGFR2
p-PDGFR
PDGFR
Actin

Interestingly, these erlotinib derivatives exhibit potent CIP2A-repressing ability [17]. Subsequently, a series of erlotinib derivatives acting as CIP2A-ablating agents were generated [17]. TD52, one of those derivatives, showed enhanced anticancer efficacy through EGFR-independent CIP2A inhibition compared with erlotinib in HCC cells [18]. Here, we report the *in vitro* and *in vivo* efficacy of TD52 in TNBC experiment models and delineate its mechanism of CIP2A inhibition.

2. Methods

A full description of materials and methods was described in the Supplementary data.

2.1. Cell culture

The HCC-1937, MDA-MB-231 and MDA-MB-468 TNBC cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). All TNBC cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 0.1-mM non-essential amino acids, 2-mM L-glutamine, 100 units/mL penicillin G, 100 μg/mL streptomycin sulphate and 25 μg/mL amphotericin B in a 37 °C humidified incubator and an atmosphere of 5% CO_2 in air.

2.2. Xenograft tumour growth

Female NCr athymic nude mice (5–7 weeks of age) were obtained from the National Laboratory Animal Center (Taipei, Taiwan, ROC). All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Taipei Veterans General Hospital. Each mouse was inoculated orthotopically to the mouse mammary pads with 5×10^6 breast cancer cells suspended in 0.1-mL serum-free medium containing 50% matrigel (BD Biosciences, Bedford, MA, USA) under isoflurane anaesthesia. Tumours were measured using calipers and their volumes calculated using a standard formula: $\text{width}^2 \times \text{length} \times 0.52$. When tumours reached around 100 mm^3, mice were administered orally with TD52 at 10 mg/kg daily by oral gavage. Controls received vehicle (1 × PBS). On termination of treatment, mice were sacrificed and xenografted tumours were harvested and assayed for tumour weight, PP2A activity and molecular events by Western blot analysis.

2.3. Statistical analysis

Data are expressed as mean ± SD or SE. Statistical comparisons were based on nonparametric tests, and statistical significance was defined as a P value less than 0.05. For survival analysis, progression-free survival curves of patients were generated by the Kaplan-Meier method and compared by log-rank test. All statistical analyses were performed using SPSS for Windows software, version 12.0 (SPSS, Chicago, IL, USA).

3. Results

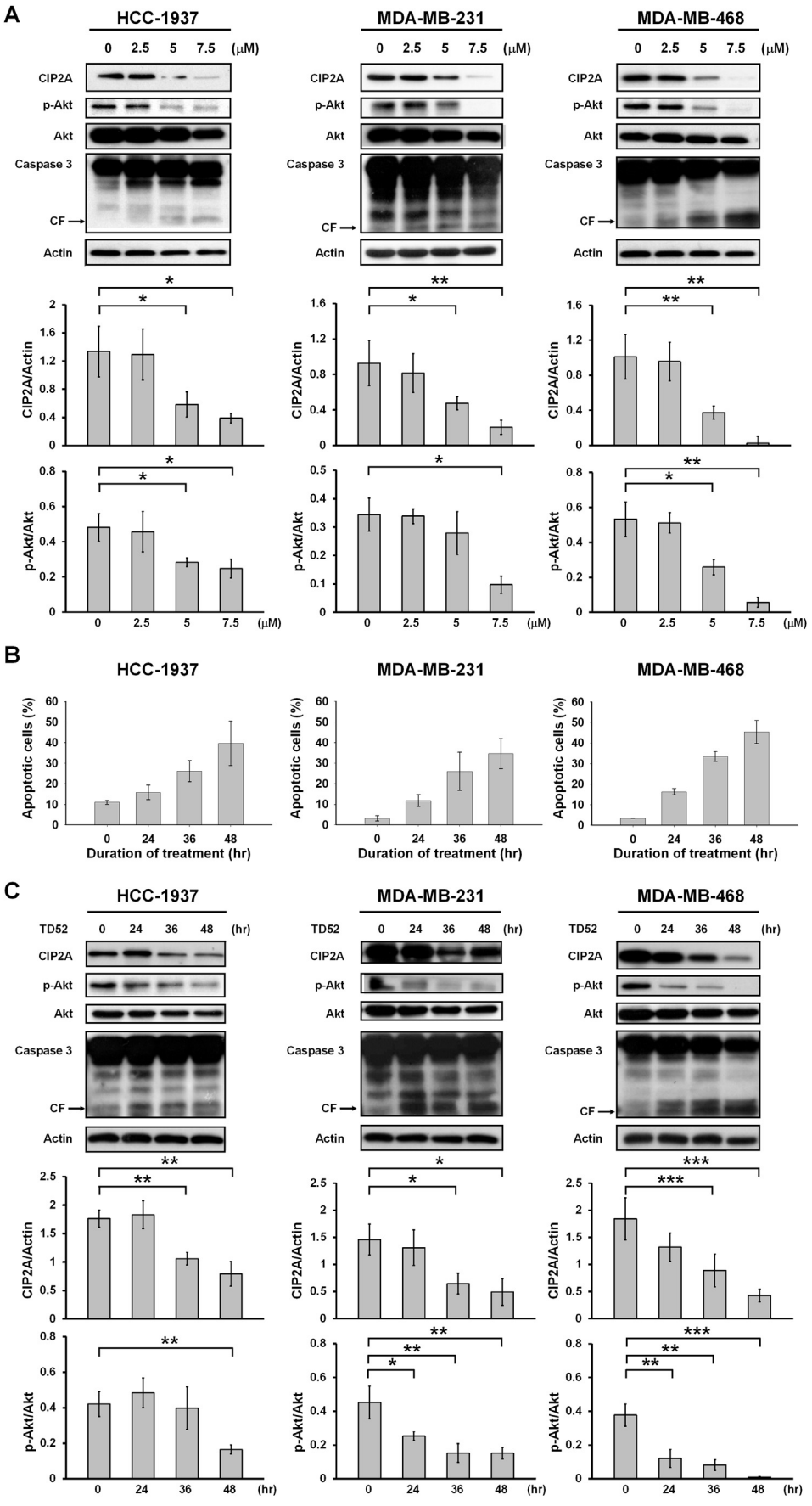
3.1. TD52-enhanced cell apoptosis in TNBC cells

To investigate the apoptosis effect induced by TD52, a panel of three TNBC cell lines including HCC-1937, MDA-MB-231 and MDA-MB-468 cells was treated with TD52. TD52 showed anti-proliferative ability and induced differential apoptotic effects in these cell lines (Fig. 1A and B). The comparative chemical structures of erlotinib and TD52, which have been disclosed in previous literature [6], are shown in Fig. 1C. Previous studies have shown that erlotinib also downregulates CIP2A in HCC and NSCLC cells [6,19]. Here, we performed a side-by-side comparison of the effects of EGFR kinase inhibitors and TD52 on CIP2A and p-EGFR (Fig. 1D and Supplementary Fig. 1). Both erlotinib and gefitinib, well-known EGFR kinase inhibitors, downregulated p-EGFR expressions. In contrast, TD52 had minimal effects on p-EGFR or EGFR expression but downregulated CIP2A expression. Consistent with previous studies [6,19], erlotinib also downregulated CIP2A in TNBC cells (Fig. 1D and Supplementary Fig. 1). We also checked whether TD52 affects other RTKs and found that TD52 had no obvious effects on other common RTKs, such as IGFR, PDGFR and VEGFR2 (Fig. 1E).

3.2. TD52 induces apoptosis by downregulating CIP2A/p-Akt signalling in sensitive TNBC cells

Next, we examined whether CIP2A is also a major target in regulating TD52-induced apoptosis in TNBC. TD52 was demonstrated to dose-dependently downregulate CIP2A and p-Akt (Fig. 2A). Moreover, TD52 time-dependently induced apoptosis accompanied with downregulating CIP2A and p-Akt (Fig. 2B and C). To further validate whether TD52-mediated cell death is

Fig. 1. TD52 exerts anti-proliferative and apoptotic-inducing effects in TNBC cells. (A–B) Cells were exposed to TD52 at the indicated doses for 48 h. (A) Cell viability was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and (B) apoptotic cells were determined by flow cytometry. (C) Structure of erlotinib (upper) and TD52 (lower). (D) Cells were treated with 5 μM of gefitinib, erlotinib or TD52 for 48 h and cell lysates were assayed by Western blotting. (E) Cells were treated with 5 μM of TD52 or dimethyl sulfoxide (DMSO) for 48 h, and protein levels of p-IGFR, IGFR, p-VEGFR2, VEGFR2, p-PDGFR and PDGFR were analysed by Western blot. Values are expressed as mean ± S.D., and the results are representative of at least three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.


A
HCC-1937
MDA-MB-231
MDA-MB-468
0 2.5 5 7.5 (μM)
CIP2A
p-Akt
Akt
Caspase 3
CF
Actin
CIP2A/Actin
p-Akt/Akt
B
Apoptotic cells (%)
Duration of treatment (hr)
C
TD52 0 24 36 48 (hr)

due to downregulate CIP2A, we also checked the TD52-mediated apoptotic effects at earlier time points. As shown in Supplementary Fig. S2, TD52 significantly decreased CIP2A expressions with no obvious cell death at 6-, 12- and 18-h time points in MDA-MB-468 cells.

3.3. CIP2A/p-Akt pathway is a molecular target of TD52-induced TNBC apoptosis

To validate the role of CIP2A/p-Akt signalling in regulating the apoptotic effects with TD52 treatment, the stable CIP2A-expressing MDA-MB-468 cell line was generated. TD52-induced apoptosis was reduced significantly in CIP2A-expressing TNBC cells (Fig. 3A and Supplementary Fig. 3). Because CIP2A is an inhibitor of PP2A [3,20], we further checked the activity of PP2A in cells treated with TD52. Results showed that the phosphatase activity of PP2A was significantly increased in TNBC cells with TD52 treatment (Fig. 3B). In addition, phosphatase activity of PP2A was decreased with the PP2A inhibitor okadaic acid, whereas it was increased with PP2A agonist forskolin in these cells (Fig. 3B). Furthermore, PP2A inhibition by okadaic acid pretreatment retarded the TD52-mediated apoptotic effects and CIP2A/p-Akt inhibition (Fig. 3C). Taken together, TD52 mediates the apoptotic effect in TNBC cells *via* regulating the CIP2A/PP2A/p-Akt signalling pathway.

3.4. TD52 suppresses CIP2A transcription via Elk1 in TNBC cells

To explore the mechanism regarding the regulation of TD52-mediated CIP2A expression, we first determine whether TD52 affected the stability of cellular CIP2A proteins by a specific protein synthesis inhibitor cycloheximide. We found that TD52 combined with cycloheximide-induced CIP2A degradation, which is similar to cells treated with cycloheximide only (Fig. 4A). We also use a proteasome inhibitor bortezomib (Velcade®) to examine the effect of CIP2A inhibition induced by TD52. As shown in Supplementary Fig. 4, TD52 reduced CIP2A proteins with no significant proteasome inhibition activity. Moreover, cells treated with TD52 in combination with bortezomib did not further affect the accumulation of ubiquitinated proteins, supporting that TD52 reducing the expression of CIP2A is not due to ubiquitin mediated downregulation. These results suggested that TD52 may reduce the expression of CIP2A at the level of pre-translation. Thus, we next explored whether TD52 inhibited CIP2A mRNA levels using real-time quantitative polymerase chain reaction analysis. As a result, TD52 dose-dependently decreased CIP2A transcripts in sensitive TNBC cell lines (Fig. 4B).

To further evaluate whether TD52 suppressed CIP2A expression through inhibiting its promoter activity *via* transcriptional regulators, because previous reports have clarified many transcription factors interacting with CIP2A promoter [21,22], we transfected different length segments of human CIP2A promoter into MDA-MB-468 cells and treated with DMSO and TD52 (Fig. 4C). The results indicated that TD52 significantly reduced the activities of the CIP2A promoter at the region of −2000 bp to −1 bp and −400 bp to −1 (Fig. 4C). Based on the previous studies [21,22], Ets1 and Elk1 mediate high CIP2A expression by interacting with the proximal −110 to −400 CIP2A promoter. Therefore, to further examine whether TD52 downregulates the CIP2A transcripts by Ets1 or Elk1, the ChIP assay was performed. We found that TD52 not only reduced the nuclear expression of Elk1 (Fig. 4D) but also interrupted the interaction between Elk1 and CIP2A promoter (Fig. 4E). Previous studies have reported that Elk-1 is activated by phosphorylation in a cell-specific manner in response to MAPKs (mitogen-activated protein kinases) such as ERK signalling [23]. Accordingly, we checked whether TD52 affected the expression of ERK. As shown in Fig. 4F, TD52 decreased p-ERK as well as p-Elk1 and Elk1-regulated molecules Egr1 (early growth response protein 1) and c-Fos, suggesting that TD52 might decrease ERK-mediated Elk1 phosphorylation to decrease its binding to CIP2A promoter. Taken together, TD52 may downregulate CIP2A through Elk1.

3.5. Effect of TD52 on TNBC xenograft tumour growth in vivo

To confirm that TD52-mediated CIP2A inhibition has potential clinically relevant implications in TNBC, we next used a TNBC xenograft model to assess the effect of TD52 *in vivo*. TD52 significantly inhibited MDA-MB-468 xenograft tumour size (Fig. 5A) and tumour weight (Fig. 5B). In addition, TD52 increased the activities of PP2A compared with the control group (Fig. 5C). We then checked whether TD52 also affected the CIP2A/p-Akt signalling *in vivo*. Results showed that the protein expressions of CIP2A and p-Akt were decreased by TD52 in the three MDA-MB-468 xenograft tumours, whereas no reduction in the control group (Fig. 5D). Most importantly, the treatments were tolerated well without observable toxic effects. Besides, the body weights were stably maintained throughout the whole treatments (Fig. 5E). The molecular mechanism of TD52

Fig. 2. TD52 induces apoptosis in association with downregulation of CIP2A and p-Akt in TNBC cells. (A–B) Cells were exposed to TD52 at the indicated (A) doses and (B) time points. Apoptotic cells were determined by flow cytometry. (C) Cells were exposed to TD52 (5 μM) at the indicated times. Cell lysates were assayed by Western blotting. Values are expressed as mean ± S.D., and the results are representative of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

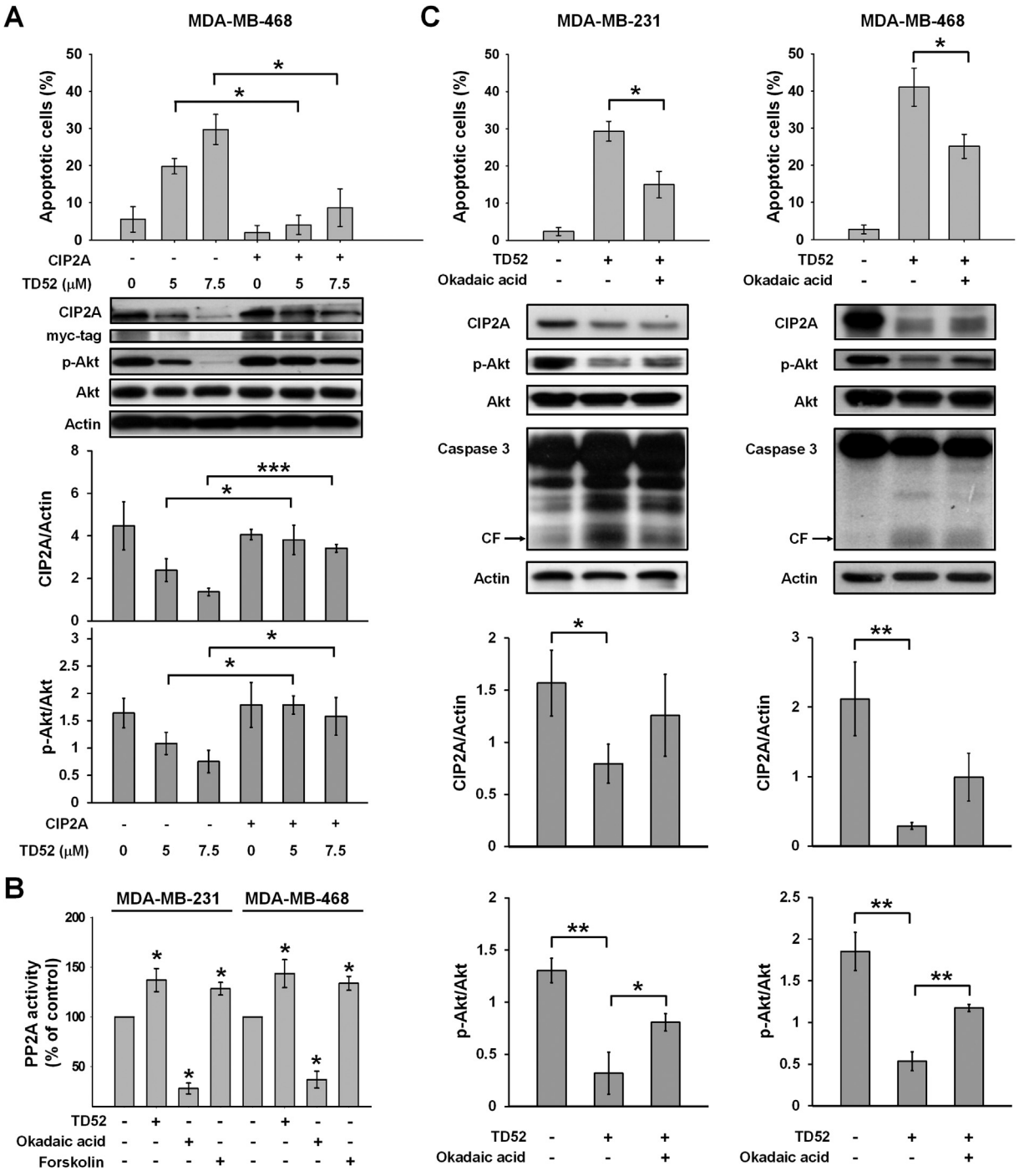


Fig. 3. CIP2A/PP2A/p-Akt mediates TD52-induced apoptosis in TNBC cells. (A) Ectopic expression of CIP2A reduced the apoptotic effect of TD52 in MDA-MB-468 cells. (B) Cells were treated with DMSO or TD52 (5 μM) or a PP2A inhibitor okadaic acid (20 nM) as a negative control, or a PP2A agonist forskolin (40 μM) as a positive control for 24 h. Cell lysates were assayed for PP2A activity. (C) Cells were pretreated with okadaic acid (20 nM) for 1 h; then washed and treated with DMSO or TD52 (5 μM) for 24 h. Cell lysates were separated and assayed for sub-G1 analysis and Western blotting. Values are expressed as mean ± S.D., and the results are representative of at least two independent experiments. $*P < 0.05$ and $**P < 0.01$.

in sensitive TNBC cells is summarised in Fig. 5F; TD52 indirectly downregulates CIP2A transcripts through interrupting the binding of Elk1 to CIP2A promoter. Then the activity of PP2A is recovered to reduce p-Akt expressions and led to cancer cell apoptosis.

3.6. Clinical relevance of CIP2A and Elk1 mRNA by exploration of a publically available database

In our previous studies, expressions of Elk1 and CIP2A mRNA were higher in tumour compared with adjacent

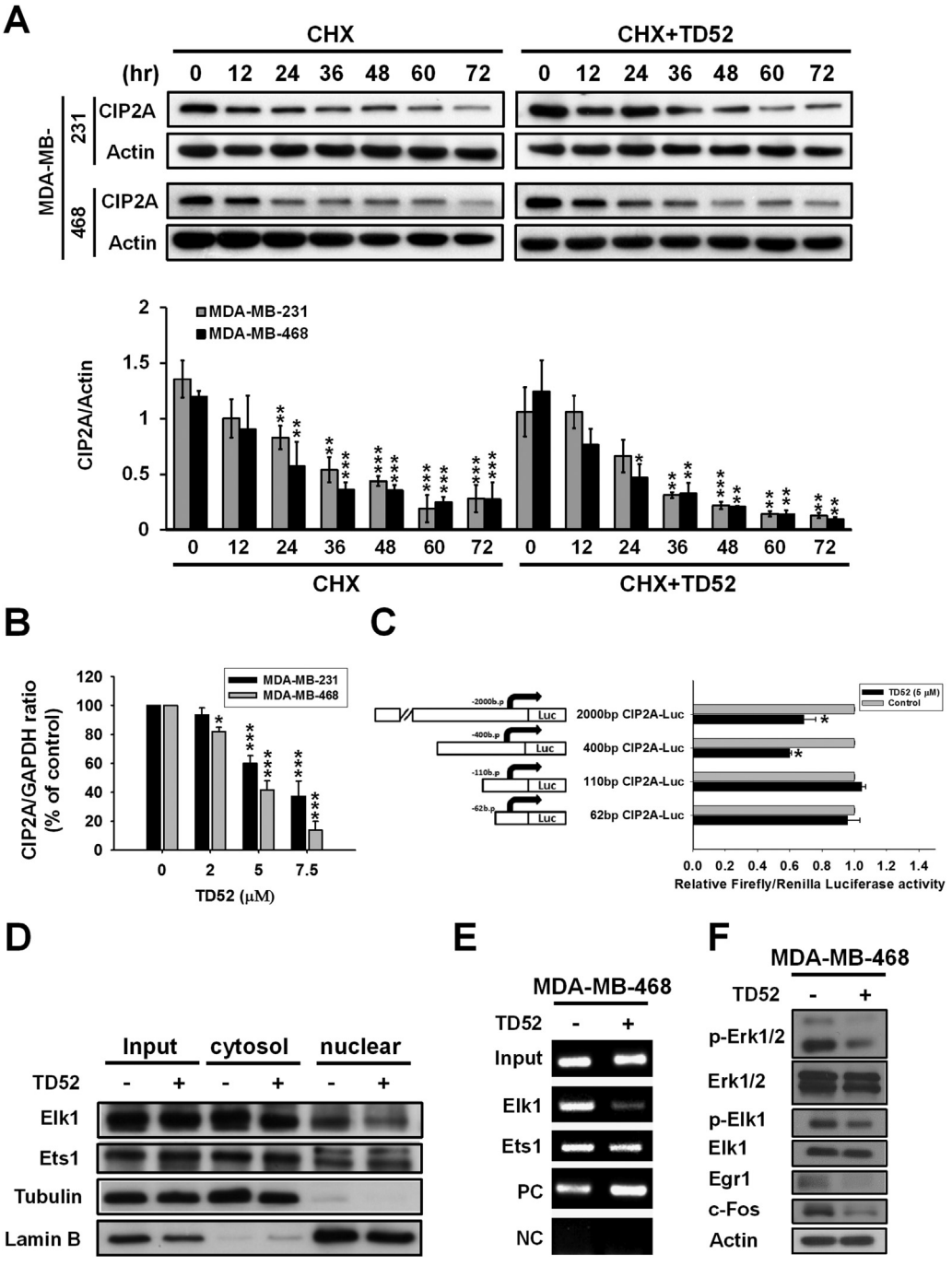


Fig. 4. TD52 indirectly downregulates transcription of CIP2A. (A) Cells were treated with 100 μg/mL pan-translation inhibitor cycloheximide (CHX) in the presence (right) or absence (left) of TD52 (5 μM) at the indicated times, then the stability of CIP2A protein was assessed by Western blot. (B) Cells were treated with TD52 at the indicated doses for 24 h, and CIP2A mRNA was assayed by real-time quantitative polymerase chain reaction. (C) MDA-MB-468 cells were transfected with firefly luciferase reporter vectors carrying CIP2A promoters of different lengths as indicated, and Renilla vectors for 24 h and then treated with TD52 (5 μM) or DMSO for 24 h. Cell lysates were assayed for dual luciferase activity. (D) Nuclear and cytoplasmic extracts were prepared from MDA-MB-468 cells treated with TD52 (5 μM) or DMSO for 24 h. Cell lysates were Western blotted for Elk1 and Ets1. Lamin B and Tubulin were used as a loading control. (E) ChIP assays of the CIP2A promoter were performed as described in Methods. (F) Cell lysates were prepared from MDA-MB-468 cells treated with TD52 (5 μM) or DMSO for 48 h. Protein levels of p-ERK, ERK, p-Elk1, Elk1 and it downstream targets Egr-1 and c-Fos were analysed by Western blot. β-actin was used as a loading control. Values are expressed as mean ± S.D., and the results are representative of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

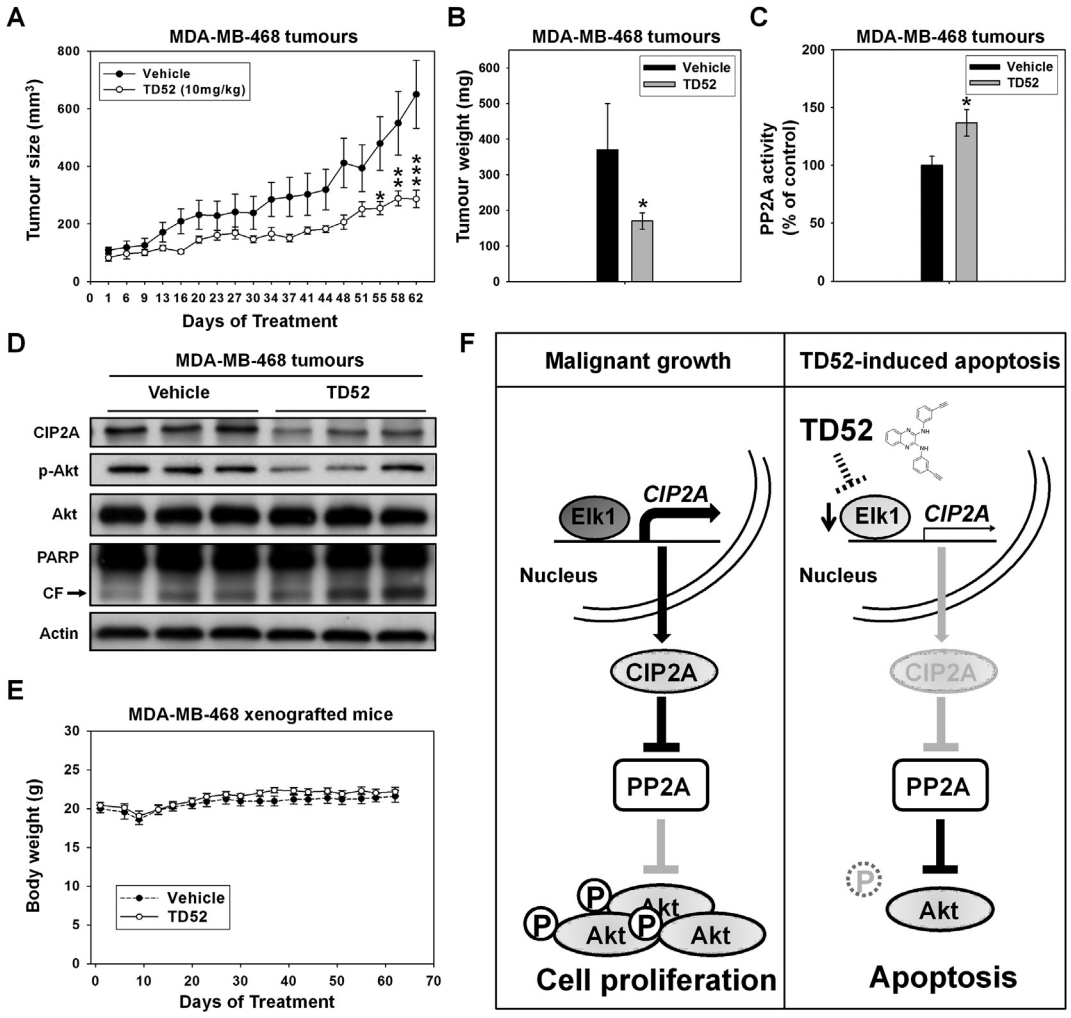


Fig. 5. *In vivo* effect of TD52 on MDA-MB-468 xenograft nude mice. (A) TD52 decreased the size of MDA-MB-468 tumours. Points, mean (n = 6); bar, SE. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. Mice were administered as described in Methods. (B) Tumour weight and (C) PP2A activity in MDA-MB-468 xenografts treated with control or TD52. Columns, mean (n = 3); bars, SD; *, $P < 0.05$. (D) Western blot analysis of the expression level of CIP2A, p-Akt and Akt in MDA-MB-468 xenografts treated with control or TD52. (E) Body weights of xenograft mice bearing tumours. Points, mean (n = 6); bar, SD. (F) Schema of the molecular mechanism of the action of TD52 on the CIP2A/PP2A pathway. By indirectly inhibiting CIP2A transcription, TD52 restores PP2A activity downregulating p-Akt and leading to subsequent cell apoptosis.

normal tissue from all breast cancer patient samples, especially in TNBC subpopulation [24]. To further explore the clinical relevance of CIP2A and Elk1, we examined the clinical association of CIP2A and Elk1 mRNA expression by Kaplan-—Meier plotter, which is an online survival analysis software (accessible at http://kmplot.com/analysis/) that can be used to assess the prognostic value of biomarkers using transcriptomic data [25–27]. In all breast cancer, patients with high CIP2A or Elk1 expression were significantly associated with poorer relapse-free survival than patients with low CIP2A or Elk1 expression (Supplementary Fig. 5A). Interestingly, the relapse-free survival of Her2+ patients with high CIP2A or Elk1 expression was longer than for those with low CIP2A or Elk1 expression (Supplementary Fig. 5B). In Her2−/ER+ population, the relapse-free survival was shorter in high CIP2A expression group than in low CIP2A expression group, whereas there was no significant difference between high and low Elk1 expression groups (Supplementary Fig. 5C). TNBC patients with high CIP2A expression showed a trend of shorter relapse-free survival compared with those with low CIP2A expression (Supplementary Fig. 5D, left). Moreover, TNBC patients with high Elk1 expression were significantly associated with poorer relapse-free survival than patients with low Elk1 expression (Supplementary Fig. 5D, right).

4. Discussion

In this study, we introduced an erlotinib derivative, TD52, with potential antitumour effects in TNBC. We found that CIP2A was a major determinant in TD52-

mediated apoptosis. Through inhibiting CIP2A/PP2A/p-Akt pathway, TD52 revealed a dose-dependent, anti-proliferative effect in TNBC. Furthermore, TD52 indirectly reduced CIP2A by disturbing Elk1 binding to the CIP2A promoter. These results not only increase the current understanding of CIP2A but also support the rationale for a potential therapeutic role of CIP2A in TNBC.

CIP2A expression is correlated with aggressiveness and is a poor prognostic factor in breast cancer [3,7]. Recently, bioinformatics pathway analyses demonstrated that the CIP2A signature clustered with basal-like breast cancer, one of the TNBC subtypes [4]. Our data suggest that CIP2A inhibition is crucial for TD52-mediated TNBC apoptosis through EGFR-independent pathway. Pretreated with Okadaic acid, a PP2A inhibitor, TD52-enhanced apoptotic effects and CIP2A/p-Akt inhibition were abolished (Fig. 3C).

Previously, we have demonstrated that erlotinib induced HCC cell apoptosis through an off-target CIP2A inhibition pathway [6]. CIP2A has been reported as a major determinant regulating bortezomib-induced apoptosis in hepatoma cells, which is independent of proteasome inhibition [28,29]. Moreover, afatinib decreased CIP2A to inhibit NSCLC cell proliferation [30]. A recent study has mentioned that rabdocoetsin B, a traditional Chinese medicinal herbal extract of *Rabdosia coetsa*, inhibits cell growth and induces apoptosis of lung cancer cells through CIP2A-dependent p-Akt inhibition [29]. Based on these studies, our current data strengthened that CIP2A is a potential therapeutic target.

Elk1, a member of the ETS family of transcription factors, is involved in many important cellular processes in normal tissues as well as in many malignancies [31]. In previous studies, Elk1 has been implicated in regulation of the immediate early genes including c-Fos and Egr1 that regulate cell growth, differentiation and survival in tumourigenesis [32–34]. Both Elk1 and Ets-1 are required to coordinate regulating CIP2A transcription in cervical, endometrial and liver carcinoma cell lines [22]. It has been suggested that different transcription factors regulate CIP2A expression in a cell type–specific manner. Therefore, we propose that any attempt to therapeutically decrease CIP2A through inhibiting specific transcription factors will need to be guided by the type of cancer that is being treated. These studies also suggested that CIP2A regulation is quite complex and needs further investigation.

In this study, we further investigated whether the transcription of CIP2A was affected by TD52, and found that TD52 decreased the CIP2A transcripts and its promoter activity (Fig. 4B–C). Moreover, TD52 interrupted the interaction between Elk1 and the proximal promoter region of CIP2A (Fig. 4E). Because MAPK/ERK activation has been reported as necessary for Elk1 phosphorylation [35], we also checked whether TD52 affected ERK activation and found that TD52 significantly decreased p-ERK and p-Elk1 (Fig. 4F). These results suggested that TD52 might decrease Elk1 phosphorylation to decrease its binding to CIP2A promoter through inhibiting ERK activation. However, whether other pathways are affected by TD52 is unclear, and further studies are needed to delineate the mechanism of TD52-induced CIP2A inhibition.

Compared with other breast cancer subtypes, TNBC exhibits much higher levels of EGFR expression [36]. EGFR has been reported to exist in 45%–76% of cases of TNBC [36,37]. The potential of EGFR inhibition in TNBC, therefore, has been previously investigated. Cetuximab is a chimeric monoclonal antibody targeting EGFR. However, cetuximab elicits only a minor response to advanced TNBC, either as a single-agent or a combination therapy [38–40]. Similarly, erlotinib inhibited a limited degree of cancer cell proliferation and metastasis in TNBC [41]. Gefitinib, another EGFR kinase inhibitor, exerted some anticancer ability in TNBC only when combined with other agents [42]. In brief, the effect of targeting EGFR in TNBC is disappointing. However, inhibiting the PI3K/AKT pathway potentiates the cytotoxicity of gefitinib in TNBC, suggesting that other molecules regulate the pharmacological inhibition of EGFR in TNBC [43]. By using TD52, which is an erlotinib derivative with limited anti-EGFR ability, we demonstrated that CIP2A is a major target in TNBC cells. The interaction between CIP2A and EGFR is not well understood. A previous study has indicated that CIP2A transcription can also be upregulated *via* EGFR-MEK-ERK signalling pathway [21]. However, our data suggest that EGFR kinase inhibition does not necessarily correlate with CIP2A inhibition.

In conclusion, CIP2A-dependent p-Akt down-regulation mediates TD52-induced apoptosis in TNBC. This study strengthens that CIP2A is a major molecular determinant of the sensitivity of TNBC and that targeting CIP2A may be an attractive anti-cancer strategy. Future studies to explicate the mechanism by which TD52 inhibits Elk1 may lead to further progress in the development of molecular-targeted therapies for TNBC.

Authors' contributions

LMT and KFC were responsible for coordination and manuscript editing as well as acting as corresponding authors. CYL drafted the manuscript. CYL, MHH, DSW, CTH, WCT, CHL, KYL and SPY conducted *in vitro* experiments. DSW, WLW, WCT and CTH conducted animal experiments. CYL, MHC and TTH performed *in silico* data analysis. CWS, LMT, and KFC helped in data interpretation and statistical analysis. CYL, MHH, TTH, DSW, CTH, WLW, WCT, and CWS

prepared the figures. CWS and KFC designed and synthesised TD52. All authors had substantial contributions to the conception or design of the work. All authors have read, revised critically for the intellectual content, and approved the final manuscript. All authors agreed with the accuracy and integrity of any part of the work.

Conflict of interest statement

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejca.2016.11.012.

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