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RESEARCH ARTICLE

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CPNE1 predicts poor prognosis and promotes tumorigenesis and radioresistance via the AKT singling pathway in triple-negative breast cancer

Zhihong Shao[1] | Xiaolong Ma[2] | Yufeng Zhang[3] | Yuanyuan Sun[3] | Wenjuan Lv[3] | Kuigang He[3] | Rui Xia[3] | Peijun Wang[2] | Xiaolong Gao[3]

[1]Department of Radiology, Shibei hospital of Jing'an District of Shanghai, Shanghai, China

[2]Department of Radiology, Tongji Hospital, Tongji University School of Medicine, Shanghai, China

[3]Department of Radiology, Luodian Hospital, Shanghai, China

Correspondence
Peijun Wang, Department of Radiology, Tongji Hospital, Tongji University School of Medicine, No.389 Xincun Road, Putuo Districa, 200065 Shanghai, China.
Email: tjpjwang@sina.com

Xiaolong Gao, Department of Radiology, Luodian Hospital, Baoshan District, No. 88 Yongshun Road, Baoshan District, 201908 Shanghai, China.
Email: 13916780898@sohu.com

Abstract

Elevated expression of Copine 1 (CPNE1) has been observed in multiple cancers; however, the underlying mechanisms by which it affects cancer cells are unclear. We aimed to study the effect of CPNE1 on the tumorigenesis and radioresistance of triple-negative breast cancer (TNBC). Quantitative real-time polymerase chain reaction was used to detect the expression of CPNE1 in TNBC tissues and cell lines. Western blot, immunohistochemistry, and immunofluorescence were used to investigate the levels of CPNE1, p-AKT, AKT, cleaved caspase-3, cleaved PARP1, and γ-H2AX. Cell viability and apoptosis were measured by CCK-8 and flow cytometry, respectively. CPNE1 was overexpressed in TNBC tissues and cell lines and was associated with tumor size, distant metastases, and survival rates of patients with TNBC. Moreover, function study shows that CPNE1 promoted cell viability and inhibited cell apoptosis in vitro and inhibited the radiosensitivity of TNBC. Importantly, inactivation of AKT signaling inhibited the tumorigenesis and radioresistance mediated by CPNE1 in TNBC cells. In vivo xenograft study also shows that CPNE1 knockdown inhibited tumor growth and promoted cell apoptosis. Overall, our findings suggest that CPNE1 promotes tumorigenesis and radioresistance in TNBC by regulating AKT activation and targeted CPNE1 expression may be a strategy to sensitize TNBC cells toward radiation therapy.

KEYWORDS

AKT, CPNE1, DNA damage, radioresistance, triple-negative breast cancer

1 | INTRODUCTION

Breast cancer (BC) is the most leading cause of cancer mortality and morbidity among women worldwide and is the second most common cancer overall, with an estimated 2.4 million new cases diagnosed and 523 thousand deaths per year.[1] Although early BC patients have a better prognosis, 20% to 40% of patients eventually develop distant metastases.[2] Current treatment for BC includes surgery, a core clinical management, and systemic treatment, such as hormone and target therapies, radiotherapy, and chemotherapy, which decreases both the local and distant recurrence rate.[3] Although radiotherapy is an important locoregional treatment modality commonly used to destroy cancer cells by inducing irreparable DNA damage,[4,5] some of patients develop tumor growth and spread, resulting in progression or recurrence due to the radioresistant tumor cells.[6] Triple-negative breast cancer (TNBC) is an aggressive and invasive subtype of BC,

Zhihong Shao and Xiaolong Ma contributed equally to this study.

accounting for approximately 15% to 20% of all cases, which does not express estrogen receptor (ER), progesterone receptor, human epidermal growth factor receptor-2 (HER2), remains refractory to current traditional HER2-targeted, and endocrine therapies,[7] and is correlated with a high risk of locoregional recurrence after radiotherapy.[8] Consequently, it is urgent to identify new biomarkers and to elucidate the mechanism of radioresistance.

Copine 1 (CPNE1) is one of the calcium-dependent membrane- and phospholipid-binding proteins that regulate molecular events at the interface of the cell membrane and cytoplasm, containing two N-terminal type II C2 domains and an integrin A domain at the C-terminus but not a predicted signal sequence or transmembrane domains. It was previously reported that CPNE1 expression is upregulated in lung cancer, prostate cancer, and osteosarcoma; correlates with the survival of lung cancer and prostate cancer patients; and regulates tumorigenesis and chemoresistance.[9-12] AKT pathway has been found to be associated with various cellular functions, including cell survival, motility, cell cycle progression, chemoresistance, and radioresistance.[13,14] CPNE1 can directly induce the differentiation of hippocampal progenitor cells via AKT phosphorylation.[15] However, whether CPNE1 can regulate tumorigenesis and radiosensitivity in TNBC is not well understood.

In this study, the expression level of CPNE1 and relationship between CPNE1 and clinical parameters including survival of patients with TNBC were detected. Meanwhile, TNBC stable cell lines with CPNE1 knockdown or overexpression were established to investigate the tumorigenesis and radioresistant effect of CPNE1 on TNBC cells. Our data will be of great significance for the future prevention and control strategy of TNBC.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue samples

This study was approved by the ethics committee of Luodian Hospital. Two cohorts of patients treated at Luodian Hospital were enrolled in this study after written consent informed was collected. Cohort 1 includes non-TNBC (n = 60) and TNBC (n = 60) patients treated between 2010 and 2014 with clinical and prognosis information and specimens of the tumor and nontumorous normal tissues were collected from these patients. Patients in cohort 1 had not received hormone therapy, radiotherapy, or neoadjuvant chemotherapy before the surgery. Cohort 2 contains 100 patients with TNBC who had gone through radiotherapy between 2016 and 2017. The paraffin-embedded specimens from cohort 1 and 2 were available for immunohistochemical (IHC) staining using anti-CPNE1 antibody (Abcam; ab66898; 1:100) as previously described.[16] Protein expression was quantified using a visual grading system based on the intensity of staining (graded on a scale of 0-4: 0,<5%; 1, 5%-25%; 2, 25%-50%; 3, 50%-75%; 4, >75%). Score more than equal to 2 was defined as CPNE1 high-expression group otherwise defined as CPNE1 low-expression group.

2.2 | Bioinformatics analysis

The gene expression data were obtained from the UALCAN database (http://ualcan.path.uab.edu/analysis.html) for breast invasive cancer based on the TCGA samples, including 1097 cases of tumor tissues and 114 cases of normal breast tissues. Survival rate data were obtained from Kaplan Meier-plotter database (access id: GSE19615 and GSE31519). Overall survival was determined by Kaplan-Meier survival analysis and log-rank test.

2.3 | Cell culture

BC cell lines ZR-75-1, MCF-7, BT-474, MDA-MB-231, MDA-MB-468, BT-549, HCC-1937 and normal human breast epithelial cell line MCF-10A were obtained from the American Type Culture Collection (Manassas, VA). These cell lines and the cells isolated from primary tumor site of patients with TNBC in Hospital cohort 1 as previously described[17] were cultured in RPMI-1640 with 10% fetal bovine serum under a humidified atmosphere at 37°C.

2.4 | RNA interference and plasmid construction

The RNA interference sequence targeting the human CPNE1 gene (shRNA#1 GCTACGCTTTGGAATCTAT; shRNA#2 GGTGCAATGCTC CGATTAT; shRNA#3 CCAACTTTGCACCCATCAT) or scramble shRNA as a negative control (NC) was inserted into the pLKO.1 lentiviral vectors. Full-length human CPNE1 cloned into the lentiviral expression vector pLVX-Puro (Addgen, Cambridge, MA) was constructed for CPNE1 overexpression or blank pLVX-Puro as a NC. Transfection was performed using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). At 48 hours after transfection, the recombined vectors were collected and then transduced into TNBC cells.

2.5 | Radiation therapy

The TNBC cells were seeded into 6-cm culture dishes and exposed to irradiation dosages of 4 Gy by Elekta AxesseTM linear accelerator (Elekta AB, Stockholm, Sweden) at a dose rate of 8 Gy/min when cells reached to 80% confluence. After irradiation, the dishes were replaced with fresh media, and the cells were cultured at 37°C incubator.

2.6 | Cell viability assay

MDA-MB-231 and HCC-1937 cells were transduced with pLKO.1-CPNE1 shRNA (shCPNE1) or pLKO.1-scramble shRNA (shNC) and irradiated with 4 Gy X-ray. MDA-MB-468 cells infected with CPNE1 expression vector or blank vector were irradiated with 4 Gy X-ray in the absence or presence of 10 μM LY294002. Primary isolated TNBC

cells were irradiated with 4 Gy X-ray. After treatment, cell viability was analyzed using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Tokyo, Japan) in accordance with the manufacturer's protocol. Optical density (OD) value of each well was recorded at 450 nm and detected by a microplate reader (Tecan, Grödig, Austria).

2.7 | Cell apoptosis assay

MDA-MB-231 and HCC-1937 cells were transduced with pLKO.1-CPNE1 short hairpin RNA (shRNA) (shCPNE1) or pLKO.1-scramble shRNA (shNC). MDA-MB-468 cells were infected with CPNE1 expression vector or blank vector in the absence or presence of 10 μM LY294002. After treatment, cells were incubated in annexin V fluorescein isothiocyanate and propidium iodide (Biovision Inc., Mountain View, CA). FACScan flow cytometry (Becton Dickinson, Franklin Lakes, NJ) using Cell Quest software (Becton Dickinson) was then performed to examine apoptosis of cells.

2.8 | RNA isolation and quantitative real-time polymerase chain reaction

We extracted total RNA from BC tissues and cell lines by using Trizol reagent (Invitrogen). Then Reverse Transcription System Kit (Takara, Dalian, China) was used to synthesize the first-strand from 1 μg of total RNA. The messenger RNA (mRNA levels were quantified by SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA) and detected by CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). The expression level of β-actin was used as an internal control and each assay was performed in triplicate. The primers used for PCR were as follows: CPNE1 forward: 5′-GGTGTGGGTGGTGCTGACTTTG-3′; CPNE1 reverse: 5′-TCC TTGGCTGAGGGTGGAAGTG-3′; β-actin forward: 5′-GATGACCCAG ATCATGTTTGAG-3′, CPNE1 reverse: 5′-TAATGTCACGCACGATT TCC-3′.

2.9 | Western blot analysis

Cells were harvested and lysed and the supernatants were collected. Tweenty-five micrograms protein was loaded into each well of 10% or 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Separated proteins were then transferred onto Polyvinylidene fluoride membrane followed by blocking for 1 hour at room temperature in 5% nonfat milk. The blots were then incubated with primary antibody overnight at 4°C (CPNE1; Abcam, Cambridge, MA; ab155675, 1:1000; cleaved caspase-3; Abcam, ab2302, 1:500; cleaved PARP1; Abcam, ab32064, 1:500; AKT; CST, Danvers, MA, #9272, 1:1000; p-AKT; CST, #9271, 1:000; β-actin; CST, #4970, 1:1000). The HRP-labeled secondary antibody (Beyotime Biotechnology Inc., Shanghai, China; A0208; 1:1000) was incubated with the membrane at 37°C for 1 hour. Finally, the membranes were incubated with an enhanced chemiluminescence detection kit (Millipore, Billerica, MA) for image scanning. β-actin was used as a loading control.

2.10 | Immunofluorescence staining

MDA-MB-231 and HCC-1937 cells were transduced with pLKO.1-CPNE1 shRNA (shCPNE1) or pLKO.1-scramble shRNA (shNC) and irradiated with 4 Gy X-ray. MDA-MB-468 cells infected with CPNE1 expression vector or blank vector were irradiated with 4 Gy X-ray. After treatment, cells were incubated overnight at 4°C with the primary antibody (γ-H2AX; Abcam; ab2893, 1:500) and 1 hour at 25°C with secondary goat antihuman IgG conjugated to AlexaFluor-488 (Beyotime Biotechnology Inc.; A0423, 1:500). Nuclei were stained with 4′, 6-diamidino-2-phenylindole (Millipore).

2.11 | Xenograft study

MDA-MB-231 cells stably transduced with lentivirus knockdown CPNE1 or control (shCPNE1 or shNC) were subcutaneously injected into the right armpit of 6-week-old male nude mice (n = 6 peer group). On the day 33 day after inoculation, the tumors were collected, photographed, weighed, and analyzed by Western blot and TUNEL staining as previous described.[18] All animal studies were approved by Luodian Hospital Ethics Committee.

2.12 | Statistical analysis

Data were expressed as mean ± standard deviations. Statistical comparisons were conducted through one-way analysis of variance in SPSS software (version 18.0), followed by LSD test. Statistical significances were defined as $P < .05$.

3 | RESULTS

3.1 | CPNE1 expression was increased in TNBC and correlated with TNBC patient survival

We first examined the CPNE1 mRNA expression in breast invasive cancer tissues and nontumorous normal breast tissues from TCGA samples. BC tissues showed significantly higher expression of CPNE1 than normal breast tissues (Figure 1A). Moreover, CPNE1 expression was significantly increased in TNBC tissues compared with normal breast and HER2+ BC tissues (Figure 1B). Transcript assessment results by quantitative real-time polymerase chain reaction (qRT-PCR) in Hospital cohort 1 show that CPNE1 mRNA levels were markedly increased in TNBC tissues compared with normal breast and non-TNBC tissues (Figure 1C). CPNE1 expression levels were markedly increased in TNBC cell lines compared with MCF-10A cells

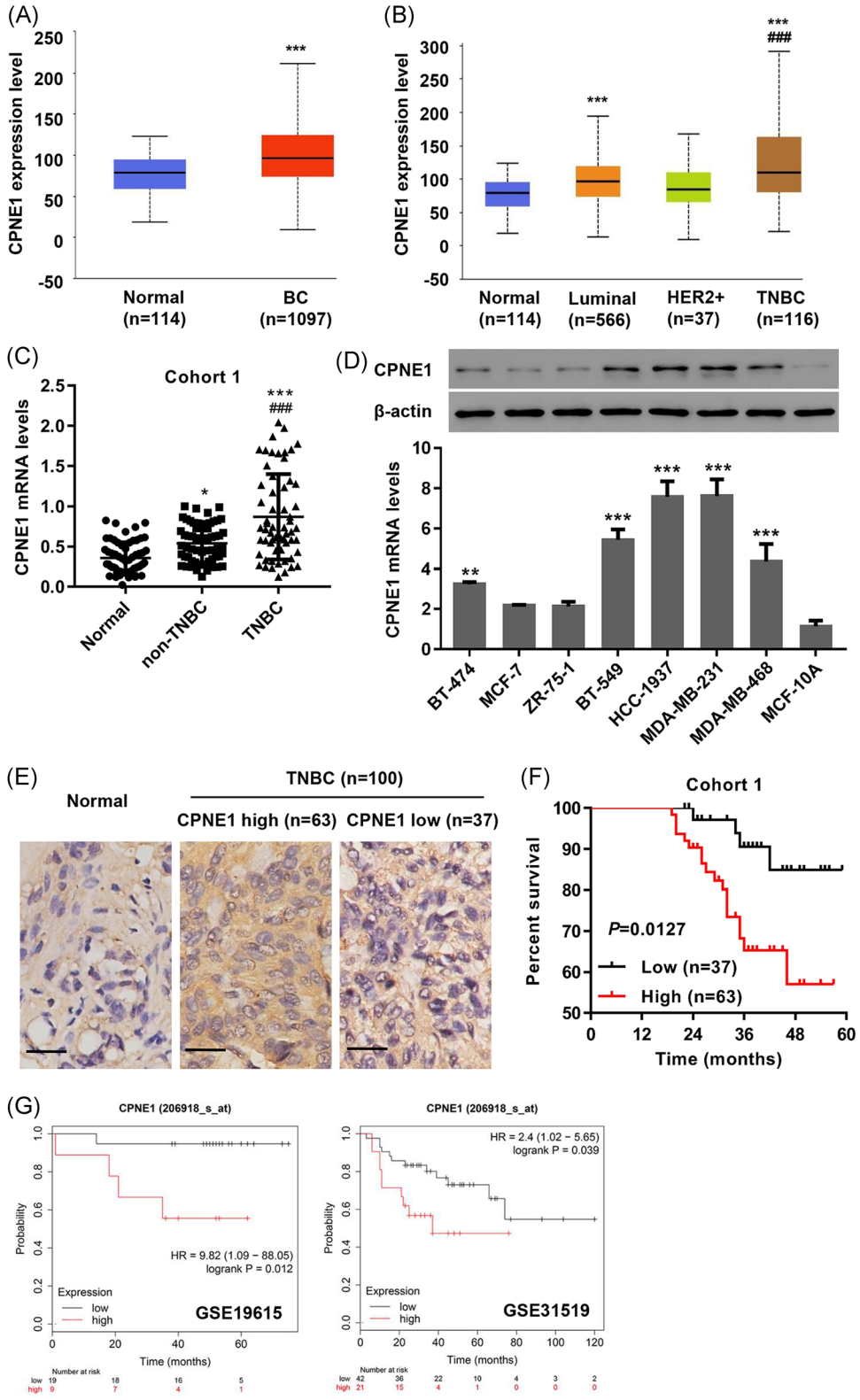

FIGURE 1 CPNE1 was overexpressed in TNBC and correlated with overall survival rates of patients with TNBC. A,B, CPNE1 expression levels were measured in normal breast tissues and BC tissues with different subtypes from TCGA samples. C, CPNE1 expression levels were measured in normal breast tissues, non-TNBC, and TNBC tissues (n = 60 per group) from cohort 1. D, CPNE1 expression levels were measured in BC cell lines and human breast epithelial cell line MCF-10A. E, IHC for CPNE1 in TNBC and normal tissues from cohort 1. Scale bar: 20 μm. F,G, Kaplan-Meier curves for overall survival rates of patients with TNBC according to CPNE1 expression levels. ***$P < .001$ compared with normal or MCF-10A group. ###$P < .001$ compared with HER2+ or non-TNBC group. BC, breast cancer; IHC, immunohistochemical; TNBC, triple-negative breast cancer [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Relationship between CPNE1 expression and clinical parameters in patients with TNBC

Clinical parameter	Number of cases	CPNE1 expression		
		High (%)	Low (%)	*P* value
Age, years				.0927
≥60	38	20 (52.6)	18 (47.4)	
<60	62	43 (69.4)	19 (30.6)	
Tumor size				.0216
≤2 cm	34	19 (55.9)	15 (44.1)	
>2≤5 cm	36	19 (52.8)	17 (47.2)	
>5 cm	30	25 (83.3)	5 (16.7)	
Histopathology				.3694
Ductal	53	30 (56.6)	23 (43.4)	
Lobular	33	23 (69.7)	10 (30.3)	
Other	14	10 (71.4)	4 (28.6)	
Histologic grade				.3656
1	18	12 (66.7)	6 (33.3)	
2	45	25 (55.6)	20 (44.4)	
3	37	26 (70.3)	11 (29.7)	
Distant metastasis				.0279
Absent	40	20 (50.0)	20 (50.0)	
Present	60	43 (71.7)	17 (28.3)	
TNM stage				.8626
I	17	11 (64.7)	6 (35.3)	
II	59	38 (64.4)	21 (35.6)	
III	24	14 (58.3)	10 (41.7)	

Differences between groups were done by the Chi-square test.
Abbreviation: TNBC, triple-negative breast cancer.

and non-TNBC cell lines (Figure 1D). Additionally, 63 of 100 TNBC specimens demonstrated higher CPNE1 expression and 37 of 100 TNBC specimens demonstrated lower CPNE1 expression, detected by IHC (Figure 1E). According to the IHC data, 100 patients with TNBC were categorized into two groups (CPNE1 high vs. low). The Chi-square test demonstrated that CPNE1 protein expression was correlated with tumor size and distant metastases, but not with age, histopathology, histologic grade, and TNM stage (Table 1). Survival analysis using Kaplan-Meier and log rank test revealed worse overall survival in TNBC patients characterized with high CPNE1 expression as compared with patients with low CPNE1 expression (Figure 1F). The similar results were also found in patients with TNBC corrected from GSE19615 and GSE31519 data set (Figure 1G). We next performed univariate analysis of prognostic factors for overall survival with the Cox regression model (Table 2). CPNE1 expression ($P = .002$, hazard ratio [HR] = 0.7215, 95% confidence interval [CI] = 0.587–0.869) and TNM stage ($P = .036$, HR = 1.326, 95%CI = 1.016–1.962) were independent prognostic indicators of patients with TNBC. We then performed multivariate analysis with Cox regression model (Table 2). CPNE1 expression ($P = .038$, HR = 0.801, 95%CI = 0.652–0.986) was independent prognostic factor in multivariate analysis. All of these data demonstrate that CPNE1 is a prognostic factor, and high CPNE1 expression is associated with poor overall survival.

3.2 | CPNE1 knockdown inhibited viability and induced apoptosis in TNBC cells

To further investigate the role of CPNE1 in BC progression, HCC-1937 and MDA-MB-231 cells which demonstrated higher CPNE1 expression were transduced with lentivirus knockdown CPNE1. As shown in Figure 2A,B, shCPNE1#1, shCPNE1#2, and shCPNE1#3 significantly reduced the expression of CPNE1 by 84.3%, 84.2%, and 78.1% at mRNA levels and by 79.0%, 77.3%, and 61.7% at protein levels in MDA-MB-231 cells compared with shNC, respectively. Moreover, CPNE1 knockdown significantly inhibited the viability of MDA-MB-231 and HCC-1937 cells compared with shNC (Figure 2C). Flow cytometry analysis demonstrated that CPNE1 knockdown significantly induced the apoptosis of MDA-MB-231 and HCC-1937 cells compared with shNC (Figure 2D). Furthermore, CPNE1 knockdown in HCC-1937 and MDA-MB-231 cells also reduced p-AKT level and increased cleaved caspase-3 and PARP1 levels, but did not affect the AKT level compared with shNC (Figure 2E). These data indicate that downregulation of CPNE1 inhibits cell viability and contributes to cell apoptosis in TNBC.

TABLE 2 Univariate and multivariate analysis of overall survival in patients with TNBC

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age (<60 vs ≥60)	0.914 (0.704–1.132)	.415		
Tumor size (cm) (<5 vs ≥5)	0.907 (0.744–1.171)	.399		
Histopathology (Ductal vs Lobular + other)	0.895 (0.740–1.294)	.452		
Histologic grade (1 + 2 vs 3)	1.245 (0.997–1.659)	.054		
Distant metastasis (absent vs present)	1.221 (0.989–1.509)	.061		
TNM stage (I + II vs III)	1.326 (1.016–1.962)	.036		
CPNE1 expression	0.7215 (0.587–0.869)	.002	0.801 (0.652–0.986)	.038

Abbreviations: CI, confidence interval; HR, harzard ratio; TNBC, triple-negative breast cancer.

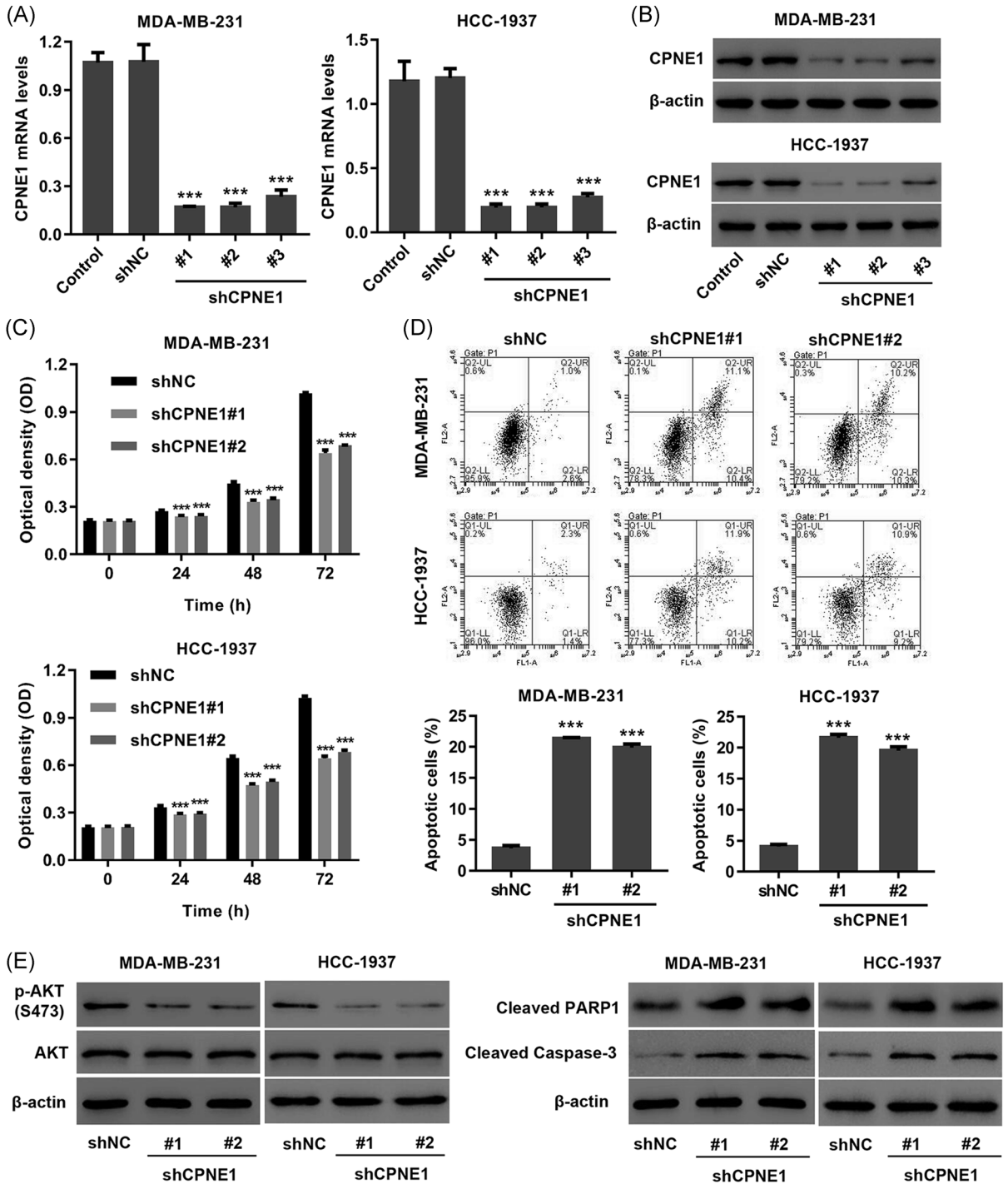

FIGURE 2 CPNE1 knockdown inhibited viability and induced apoptosis in TNBC cells. The levels of CPNE1 (A,B), p-AKT, AKT, cleaved caspase-3, and cleaved PARP1 (E), cell viability (C), and apoptosis (D) were measured in pLKO.1-CPNE1 shRNA (shCPNE1) or pLKO.1-scramble shRNA (shNC) transduced MDA-MB-231 and HCC-1937 cells. ***$P < .001$ compared with shNC group. shRNA, short hairpin RNA; TNBC, triple-negative breast cancer

3.3 | CPNE1 knockdown inhibited tumor growth and induced apoptosis in vivo

To determine the effect of CPNE1 on tumor growth in vivo, MDA-MB-231 cells transduced with lentivirus knockdown CPNE1 were injected into nude mice. As shown in Figure 3A, CPNE1 expression in xenograft tumors was significantly decreased in CPNE1 knockdown group compared with shNC group. CPNE1 expression also significantly reduced tumor weight and volume, and induced apoptosis compared with shNC group (Figures 3B-D). These data indicate that downregulation of CPNE1 inhibits the tumor growth in vivo.

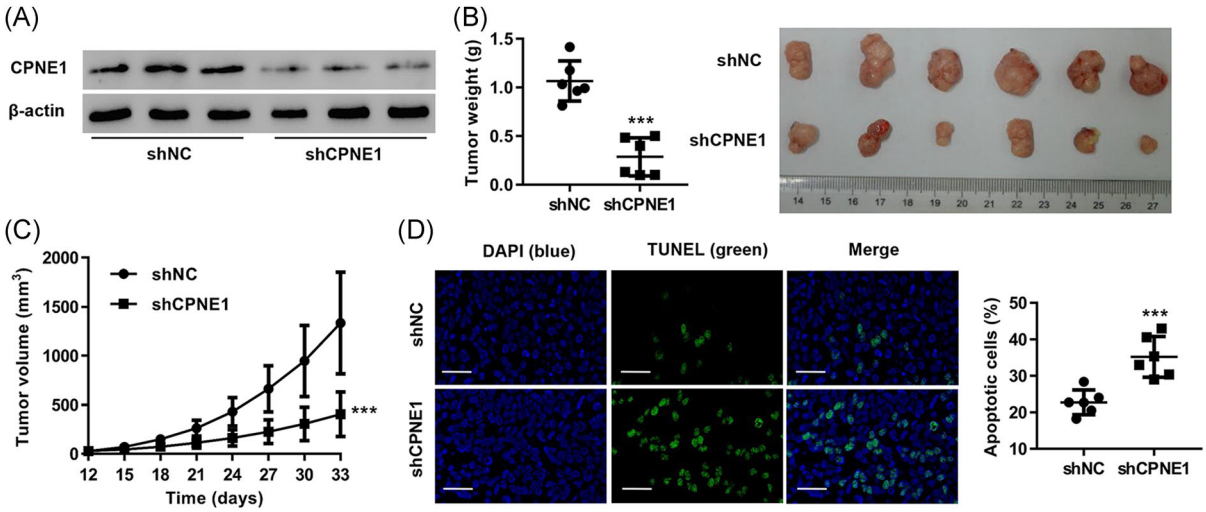

FIGURE 3 CPNE1 knockdown inhibited tumor growth in vivo. MDA-MB-231 cells transduced with pLKO.1-CPNE1 shRNA (shCPNE1) or pLKO.1-scramble shRNA (shNC) were subcutaneously injected into the right armpit of nude mice. Thirty-three days after injection, CPNE1 expression in xenograft tumors (A), tumor weight (B), and volume (C), and xenograft tumors with TUNEL staining (D) were measured. Scale bar: 50 μm. ***$P < .001$ compared with shNC group. shRNA, short hairpin RNA [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | CPNE1 overexpression promoted viability and reduced apoptosis in TNBC cells by regulating AKT activation

To examine the molecular mechanism through which CPNE1 regulates TNBC cell viability and apoptosis, MDA-MB-468 cells, which demonstrated a lower CPNE1 expression, were transduced with lentivirus expressing CPNE1 in the absence or presence of AKT signaling inhibitor LY294002. As shown in Figure 4A, CPNE1 overexpression in MDA-MB-468 cells markedly increased the expression of CPNE1 by 3.81-fold at mRNA levels and by 1.85-fold at protein levels compared with vector, respectively. CPNE1 overexpression also promoted cell viability and inhibited cell apoptosis compared with vector (Figures 4B-D). Meanwhile, CPNE1 overexpression significantly induced AKT activation and decreased cleaved caspase-3 and PARP1 levels compared with vector (Figure 4E). Importantly, LY294002 treatment in MDA-MB-468 cells significantly inhibited CPNE1 overexpression-mediated cell viability and apoptosis. These data indicate that CPNE1 contributes to the increased cell viability and decreased cell apoptosis in TNBC by activating AKT signaling pathway.

3.5 | CPNE1 was correlated with radiosensitivity in patients with TNBC

To explore the role of CPNE1 in radiosensitivity in TNBC, the expression of CPNE1 in radiosensitive (including complete and partial response) and radioresistant (stable and progressive disease) patients with TNBC from Hospital cohort 2 was measured by IHC staining. As shown in Figure 5A, the expression of CPNE1 was higher in radioresistant patients with TNBC than radiosensitive patients with TNBC. The data are statistically significant (Figure 5B). Moreover, the expression of CPNE1 in the cells isolated from primary tumor site of patients with TNBC in Hospital cohort 1 was measured by qRT-PCR and categorized into CPNE1 high and low groups (Figure 5C). In addition, the primary TNBC cells with high CPNE1 expression were more resistant to radiation than that with low CPNE1 expression (Figure 5D). These data suggest that CPNE1 may involve in radiosensitivity of TNBC.

3.6 | CPNE1 regulated radiosensitivity in TNBC cells

To further investigate the role of CPNE1 in the regulation of TNBC radiosensitivity in vitro, γ-H2AX foci formation and cell viability were performed by immunofluorescence staining and CCK-8 assay, respectively. γ-H2AX foci is regarded as an indicator for DNA damage, especially double strand breaks, and intimately associated with DNA damage repair. As shown in Figure 6A,B, the cell with γ-H2AX foci formation was markedly increased at 4 hours post irradiation in HCC-1937 and MDA-MB-231 cells with CPNE1 knockdown, but decreased in MDA-MB-468 cells with CPNE1 overexpression. In addition, HCC-1937 and MDA-MB-231 cells with CPNE1 knockdown were more sensitive to radiation than shNC cells, while MDA-MB-468 cells with CPNE1 overexpression were more resistant to radiation than shNC cells (Figures 6C-E). Importantly, the radioresistance of MDA-MB-468 cells with CPNE1 overexpression was significantly inhibited by LY294002 (Figure 6E). These results indicate that CPNE1 may enhance the radioresistance of TNBC cells through AKT signaling and be of great importance in the DNA damage response.

4 | DISCUSSION

Compared with other subtypes of BC, TNBC is associated with larger tumor sizes, increased likelihood of recurrence, distant metastasis,

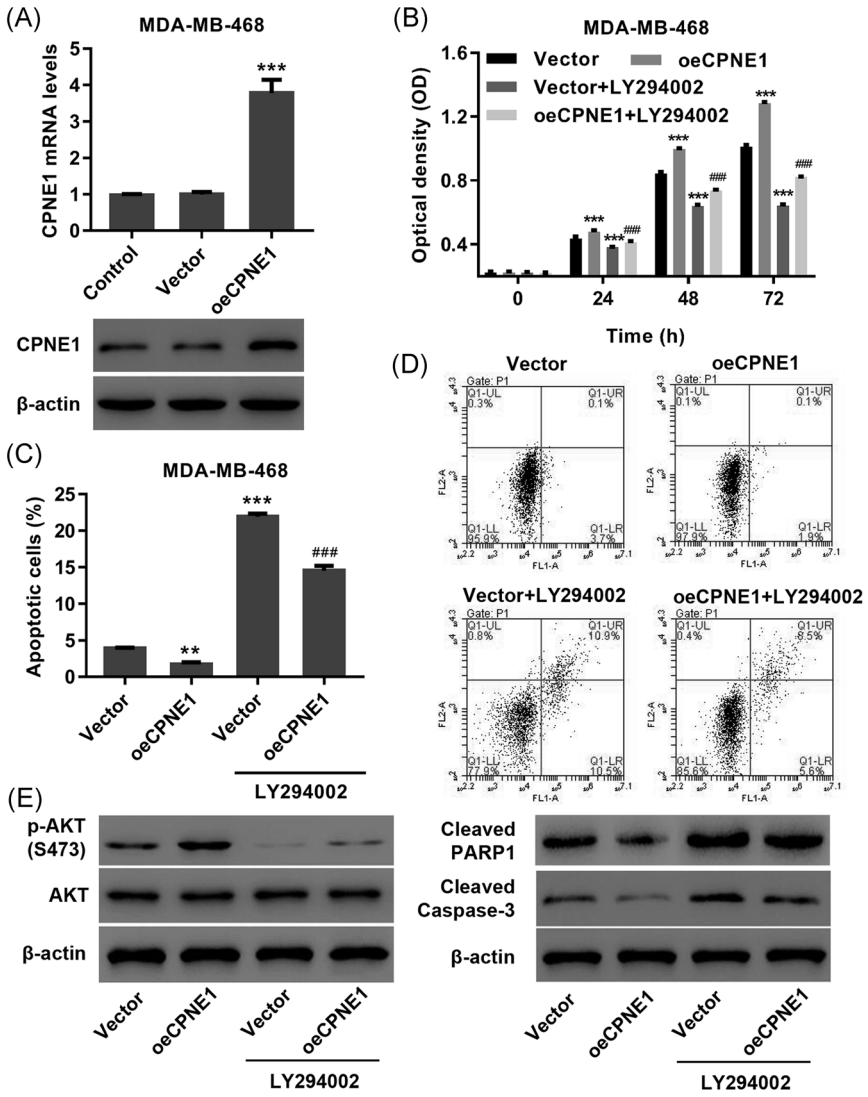

FIGURE 4 CPNE1 overexpression promoted viability and inhibited apoptosis in TNBC cells by activating AKT signaling. The levels of CPNE1 (A), p-AKT, AKT, cleaved caspase-3 and cleaved PARP1 (E), cell viability (B), and apoptosis (C,D) were measured in pLVX-Puro-CPNE1 (oeCPNE1) or blank pLVX-Puro (vector) transduced MDA-MB-468 cells in the absence or presence of 10 μM LY294002. $^{**}P < .01$, $^{***}P < .001$ compared with vector group. $^{\#\#\#}P < .001$ compared with oeCPNE1 group. TNBC, triple-negative breast cancer

and decreased overall survival.[19] Explaining the molecular mechanism of TNBC tumorigenesis and, especially, identifying the therapeutic targets contribute to TNBC cell survival and susceptibility, may be of importance to improve the prognosis of TNBC. CPNE1 is a recently discovered calcium-dependent membrane-binding protein with a broad tissue distribution and may regulate signal transduction and membrane trafficking. Dysregulation of CPNE1 is related to various pathologies such as osteoarthritis, neuronal disorders, and tumorigenesis.[9-12,15,20] However, its role in TNBC tumorigenesis and radioresistance is not understood. In this study, CPNE1 expression was increased in BC tissues, especially in TNBC tissues, and correlated with tumor size, distant metastasis, and overall survival of patients with TNBC. CPNE1 regulated TNBC cell viability, apoptosis, and radiosensitivity through the AKT signaling pathway in vitro. Moreover, the effects of CPNE1 on tumor growth of TNBC-bearing mice and radioresistance of patients with TNBC were also confirmed, respectively.

Consistent with the previous studies that demonstrated the increased CPNE1 expression in lung cancer, prostate cancer, and osteosarcoma,[9-12] we also found increased CPNE1 expression in BC and TNBC compared with normal controls. CPNE1 expression was correlated with lymph node metastasis, distant metastasis, TNM stage, and overall survival, but not with age, tumor size, histology, clinical stage, and differentiation of patients with nonsmall cell lung cancer,[9,12] whereas it was correlated with tumor stage, histological grade, biochemical recurrence, and recurrence-free survival, but not with age of patients with prostate cancer.[10] However, CPNE1 expression was herein correlated with tumor size, distant metastasis, and overall survival, but not with age, histology, histological grade, and TNM stage of patients with TNBC, and other clinical parameters were unavailable. Moreover, the overall survival rate of patients with TNBC was lower than that of patients with BC, which is consistent with the characteristic of patients with TNBC with a poorer prognosis and suggests the involvement of CPNE1 in the prognosis of TNBC.

Consistent with the increased expression of CPNE1 in TNBC tissues, TNBC cell lines also demonstrated higher CPNE1 levels than normal breast cell line MCF-10A and BC cell lines, suggesting that

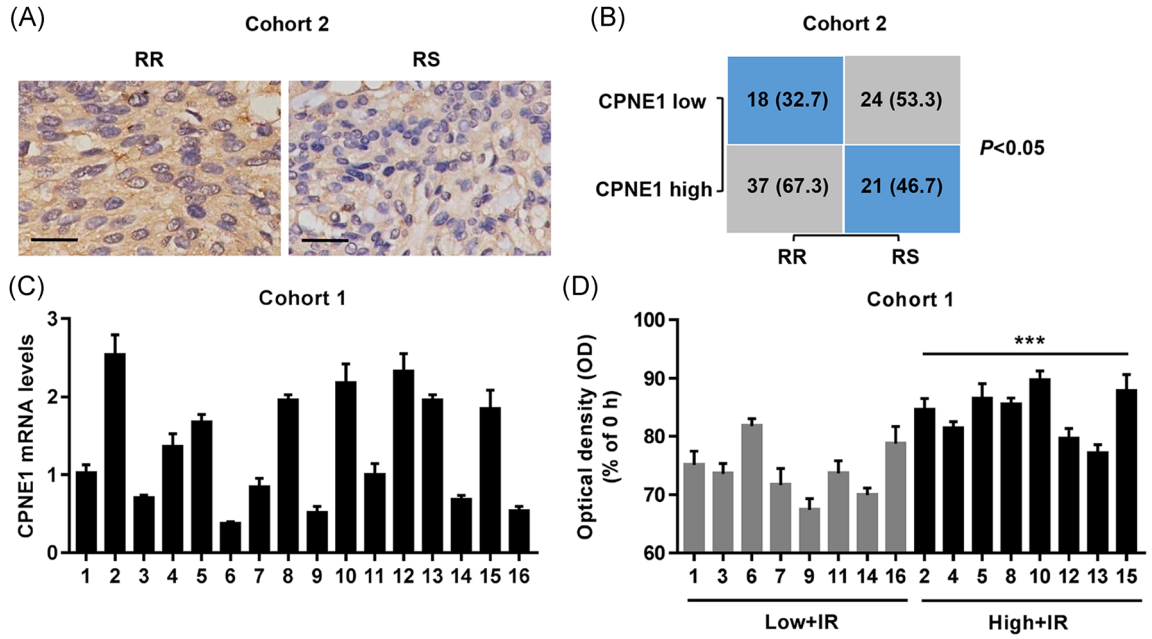

FIGURE 5 CPNE1 inhibited the radiosensitivity in patients with TNBC. A, IHC for CPNE1 in radiosensitive (RS; including complete and partial response) and radioresistant (RR; stable and progressive disease) patients with TNBC (n = 100) from cohort 2. Scale bar: 20 μm. B, Statistical analysis of TNBC tissues under different staining conditions in cohort 2. C, CPNE1 expression levels were measured in the tumor cells isolated from primary patients with TNBC (n = 16) from cohort 1. D, After primary isolated TNBC cells (n = 16) with different CPNE1 expression levels (high: case 2, 4, 5, 8, 10, 12, 13, 15 and low: case 1, 3, 6, 7, 9, 11, 14, 16) were irradiated with 4 Gy X-ray, the cell viability was measured at 0 and 24 hours. ***$P < .001$ compared with low + IR group (TNBC cells with lower CPNE1 expression levels were irradiated with 4 Gy X-ray). IHC, immunohistochemical; IR, irradiation; TNBC, triple-negative breast cancer [Color figure can be viewed at wileyonlinelibrary.com]

CPNE1 may involve in the tumorigenesis of BC, especially in TNBC. Previous studies have shown that CPNE1 promoted viability and inhibited apoptosis in lung cancer and osteosarcoma cells,[9,11,12] which is in line with our findings in TNBC in vitro and in vivo. It has shown that the PI3K/AKT signaling pathway mediates multiple cellular processes, including cell differentiation, apoptosis, and viability. CPNE1 can directly induce the differentiation of hippocampal progenitor cells via AKT phosphorylation.[15] Inhibiting PI3K/Akt signaling pathway resulted in decreased cell proliferation and increased cell apoptosis in TNBC.[21,22] In line with these studies, our results also demonstrated the pro-proliferative and antiapoptotic effects of AKT signaling in TNBC cells and that inhibition of AKT signaling by LY294002 significantly inhibited viability and induced apoptosis of TNBC cells induced by CPNE1 overexpression, suggesting that CPNE1 regulates TNBC tumorigenesis by activating AKT signaling pathway. It was recently reported that the C2 domain of CPNE1 is associated with the increased phospho-Akt (S473) levels induced by CPNE1.[15] The C2 domain is a calcium-dependent phospholipid-binding motif that was originally identified in conventional protein kinase C isoforms.[23] We are trying to find a possible upstream Akt activator, whose activity can be regulated by the C2 domain of CPNE1. However, additional studies are needed to further validate whether C2 domains or other domains of CPNE1 play an important role in regulating TNBC progression.

Increasing evidence has suggested that cell proliferation, apoptosis, cell cycle progression, and DNA damage are associated with cell radiosensitivity.[24,25] As it was found out, CPNE1 expression levels were higher in radioresistant patients with TNBC than radiosensitive patients with TNBC, and the cell viability was also inhibited significantly in primary isolated TNBC cells with lower CPNE1 expression after radiation therapy. Measurement of γ-H2AX is a sensitive and specific assay for unrepaired DNA damages.[26,27] Consistent with this notion, our data show that CPNE1 knockdown promoted DNA damage-induced γ-H2AX foci formation in TNBC cells, while the opposite effect was demonstrated when CPNE1 was upregulated. Compared with CPNE1 higher expressed HCC-1937 and MDA-MB-231 cells, MDA-MB-468 cells with lower CPNE1 expression were more radiosensitive. These data suggest that CPNE1-high-expressing TNBC cells are resistant to DNA-damaging radiotherapy. Consistent with our results, other studies reported that MDA-MB-231 cells demonstrated a higher survival fraction than MDA-MB-468 cells after 2 or 4 Gy.[28,29] However, the differences in colony formation between MDA-MB-468 and MDA-MB-231 cells after 4 Gy were not significant.[30] It was recently reported that PI3K/AKT inhibitor inhibited the resistance to endocrine or DNA-damaging radiotherapy in ER-positive early patients with BC.[31] Besides, activation of the AKT pathway might also contribute to the radioresistance in BC and glioblastoma cells.[13,26] Consistent with this notion, our data show that the viability was increased in cells treated with LY294002 and radiation than in cells treated with radiation alone in the absence or presence of CPNE1 overexpression, suggesting that CPNE1 regulates TNBC radiosensitivity by regulating

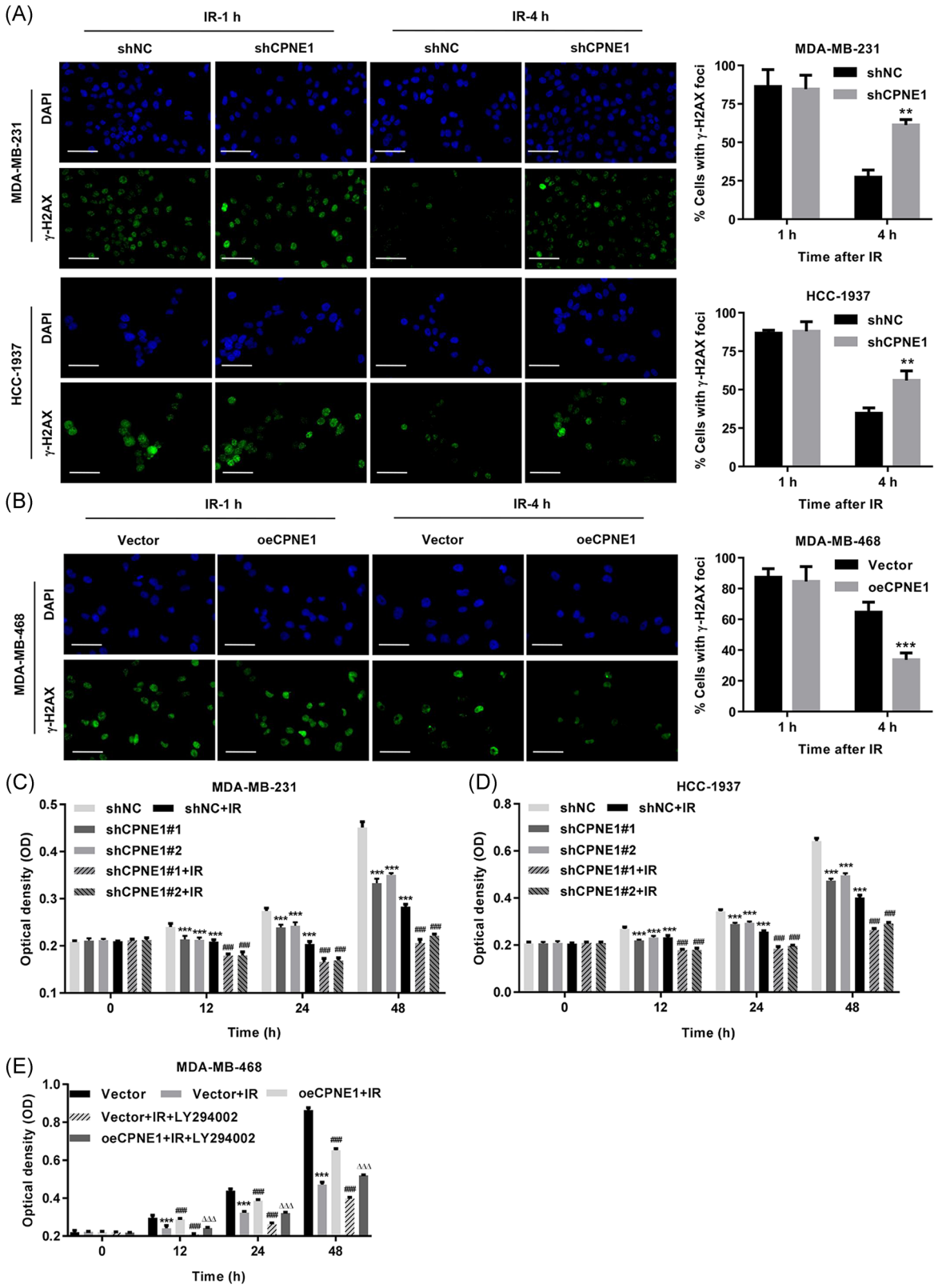

FIGURE 6 CPNE1 inhibited the radiosensitivity in TNBC cells. pLKO.1-CPNE1 shRNA (shCPNE1) or pLKO.1-scramble shRNA (shNC) transduced MDA-MB-231 and HCC-1937 cells (A), and pLVX-Puro-CPNE1 (oeCPNE1) or blank pLVX-Puro (vector) transduced MDA-MB-468 cells (B) were irradiated with 4 Gy X-ray. Representative γ-H2AX foci formation at indicated times after IR was presented. Scale bar: 50 μm. After MDA-MB-231 (C) and HCC-1937 cells (D) were transduced with pLKO.1-CPNE1 shRNA (shCPNE1) or pLKO.1-scramble shRNA (shNC), while MDA-MB-468 cells (E) were transduced with CPNE1 expression vector or blank vector in the absence or presence of 10 μM LY294002, and irradiated with 4 Gy X-ray. Cell viability was measured at 0, 12, 24 36, and 48 hours. $^{**}P < .01$, $^{***}P < .001$ compared with shNC or vector group. $^{\#\#\#}P < .001$ compared with shNC + IR or Vector + IR group. $^{\Delta\Delta\Delta}P < .001$ compared with oeCPNE1^{+}IR group. IR, irradiation; shRNA, short hairpin RNA; TNBC, triple-negative breast cancer [Color figure can be viewed at wileyonlinelibrary.com]

AKT activation. However, whether DNA-damaging radiotherapy depends on the AKT signaling is still unknown.

5 | CONCLUSION

In conclusion, our results indicate that CPNE1 can be considered as a prognostic factor for patients with TNBC. Moreover, increased CPNE1 expression was associated with TNBC tumorigenesis and radioresistance. Targeting CPNE1 may be a potential new therapeutic strategy for TNBC and contributes to the prediction of radiosensitivity of specific patients and to the sensitization of tumor cells, thus improving the outcome of radiotherapy to patients with TNBC.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ORCID

Xiaolong Gao http://orcid.org/0000-0002-9911-1776

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