Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology) *2023 24(3):262-268*
www.jzus.zju.edu.cn; www.springer.com/journal/11585
E-mail: jzus_b@zju.edu.cn

Correspondence

https://doi.org/10.1631/jzus.B2200436

Estrogen upregulates DNA2 expression through the PI3K-AKT pathway in endometrial carcinoma

Xinyan WANG[1*], Xiuling XU[2*], Ting ZHANG[1], Yang JIN[2], Sheng XU[3], Lifeng CHEN[3], Yucheng LAI[3], Ling ZHANG[4,5], Ruolang PAN[4,5]✉, Yan YU[3]✉

[1]*Department of Gynecology, Zhejiang Provincial Hospital of Traditional Chinese Medicine, the First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou 310006, China*
[2]*Department of Gynecology and Obstetrics, Hangzhou Hospital of Traditional Chinese Medicine, Affiliated Hangzhou TCM Hospital of Zhejiang Chinese Medical University, Hangzhou 310005, China*
[3]*Department of Gynecology, Zhejiang Provincial People's Hospital, Affiliated People's Hospital of Hangzhou Medical College, Hangzhou 310014, China*
[4]*Key Laboratory of Cell-Based Drug and Applied Technology Development in Zhejiang Province, Hangzhou 311122, China*
[5]*Institute for Cell-Based Drug Development of Zhejiang Province, S-Evans Biosciences, Hangzhou 311122, China*

Endometrial cancer is the most common gynecological malignancy, affecting up to 3% of women at some point during their lifetime (Morice et al., 2016; Li and Wang, 2021). Based on the pathogenesis and biological behavioral characteristics, endometrial cancer can be divided into estrogen-dependent (I) and non-estrogen-dependent (II) types (Ulrich, 2011). Type I accounts for approximately 80% of cases, of which the majority are endometrioid carcinomas, and the remaining are mucinous adenocarcinomas (Setiawan et al., 2013). It is generally recognized that long-term stimulation by high estrogen levels with the lack of progesterone antagonism is the most important risk factor; meanwhile, there is no definite conclusion on the specific pathogenesis. The incidence of endometrial cancer has been on the rise during the past two decades (Constantine et al., 2019; Gao et al., 2022; Luo et al., 2022). Moreover, the development of assisted reproductive technology and antiprogestin therapy following breast cancer surgery has elevated the risk of developing type I endometrial cancer to a certain extent (Vassard et al., 2019). Therefore, investigating the influence of estrogen in type I endometrial cancer may provide novel concepts for risk assessment and adjuvant therapy, and at the same time, provide a basis for research on new drugs to treat endometrial cancer.

DNA replication adenosine triphosphate (ATP)-dependent helicase/nuclease (DNA2) is an evolutionarily conserved, essential enzyme that plays critical roles in multiple DNA metabolic pathways, including Okazaki fragment maturation, DNA replication, end resection, and homologous recombination repair (Thakar et al., 2020). Moreover, it participates in mitochondrial genome maintenance. Thus, the uncontrolled regulation of DNA2 may lead to a cascading effect on genomic instability and telomere imbalance, resulting in DNA damage in particular cell phases. Indeed, researchers have proved that the abnormal activity of DNA2 nuclease is correlated with the growth of various cancer cells, such as in breast and ovarian cancers (Jia et al., 2017; Han et al., 2021). DNA2 is highly expressed in estrogen receptor (ER)-rich cells like those in breast cancer and ovarian cancer. Under an increased DNA2 expression level, the malignancy of tumors and incidence of metastasis will be elevated, indicating that DNA2 may be regulated by the ER pathway. However, the relevant mechanism remains unclear. Furthermore, DNA2 has recently been described as a potential marker and an attractive target in cancer therapy. Therefore, our study investigated

✉ Ruolang PAN, panrl@zju.edu.cn
Yan YU, m05yuyan1@zju.edu.cn
* The two authors contributed equally to this work
Ruolang PAN, https://orcid.org/0000-0002-0810-3392
Yan YU, https://orcid.org/0000-0003-0723-5876

Received Aug. 29, 2022; Revision accepted Dec. 21, 2022;
Crosschecked Feb. 10, 2023

the expression of ER and DNA2 in samples from patients with endometrial cancer and analyzed the relationship between them.

A total of 32 patients with a mean age of (44.3±4.1) years were selected from the Zhejiang People's Hospital, Hangzhou, China. They were diagnosed based on pathological examination and received surgical resection, including 16 ER-positive cases, whereas the others were ER-negative. Another 16 normal tissue samples were collected from control subjects with a mean age of (42.1±4.4) years who underwent laparoscopic surgery after informed consent. All patients, including control subjects, were free from hormonal therapy for three months and presented no serious liver or kidney disease. This study has been carried out in accordance with the Code of Ethics of the World Medical Association. The human endometrial adenocarcinoma cell lines JEC, Ishikawa, and HEC1 were obtained from the American Type Culture Collection.

As mentioned above, a total of 32 patients and 16 control subjects were enrolled in this study. We observed that the DNA2 expression was significantly higher in the patient group ((1.671±0.661)-fold increase, $P<0.05$; Fig. 1). To further explore the correlation between ER and DNA2, we divided the patients into two groups based on ER expression: ER-negative and ER-positive. The expression of ER in the endometrial cancer tissue samples was detected by immunohistochemical analysis. A nuclear staining of >10% was considered to indicate positive ER status. Interestingly, we observed that DNA2 expression in the ER-negative patient group was significantly lower than that in the ER-positive patient group. In addition, there was no significant difference in DNA2 expression between the ER-negative patient group and the control group. In contrast, the ER-positive patient group exhibited a (2.084±0.575)-fold increase in DNA2 messenger RNA (mRNA) expression compared to the control group. These results indicated that ER positivity correlates with DNA2 expression in patients with endometrial cancer.

Next, we investigated the expression of DNA2 between the two types of endometrial cancer cell lines. As depicted in Figs. 2a and 2b, the Ishikawa cell line positively responded to β-estradiol (E2) treatment. After E2 administration, both the mRNA and the protein levels of DNA2 expression were significantly upregulated. The JEC cell line, which is characterized

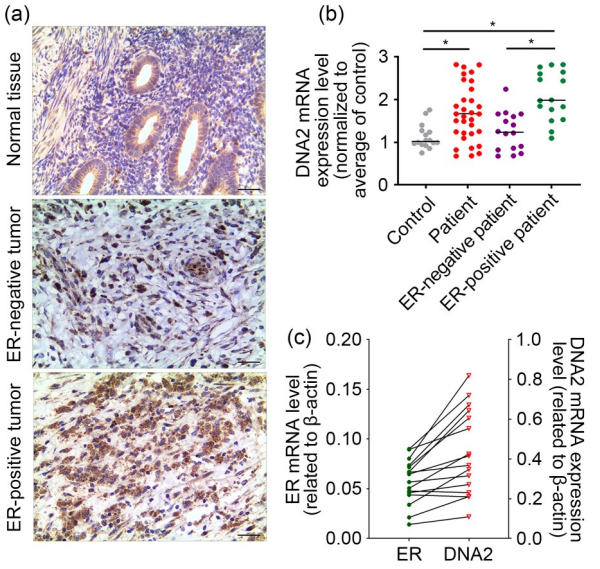

Fig. 1 Correlation between DNA2 expression and ER in patients with endometrioid-type endometrial carcinoma. (a) Staining distribution of DNA2 in representative tissue sections of normal tissue, ER-negative and ER-positive endometrioid-type endometrial carcinoma tissues. The brown precipitate indicates the antigen site. Scale bar= 50 μm. (b) DNA2 mRNA expression levels were significantly higher in ER-positive patients. A total of 32 patients with endometrioid-type endometrial carcinoma were divided according to ER expression. Samples from ER-positive patients presented significantly higher DNA2 expression than those from ER-negative patients (* $P<0.05$; $n=16$). (c) ER-positive samples showed a positive correlation trend between ER and DNA2 mRNA expression levels. Data for the same sample were connected by a line. DNA2: DNA replication adenosine triphosphate (ATP)-dependent helicase/nuclease; ER: estrogen receptor; mRNA: messenger RNA.

by negative ER expression, was used as control. Remarkably, the DNA2 expression levels were relatively stable in the JEC cells before and after E2 treatment, with no significant differences. Subsequently, we investigated whether treatment with raloxifene, an ER modulator, influences the DNA2 expression in endometrial cancer cells. As shown in Fig. 2c, raloxifene treatment led to a significantly reduced expression of DNA2 mRNA levels in Ishikawa cells, which exhibited high levels of ER in a concentration-dependent manner. A similar trend in the effect of raloxifene on DNA2 mRNA levels was observed in an ER^{medium} cell line, HEC1. However, the reduction rates were less than those in Ishikawa cells. Notably, DNA2 mRNA levels in JEC cells that were negative for ER expression were not affected. Then, total proteins were extracted from cells treated with 1×10^{-7} mol/L

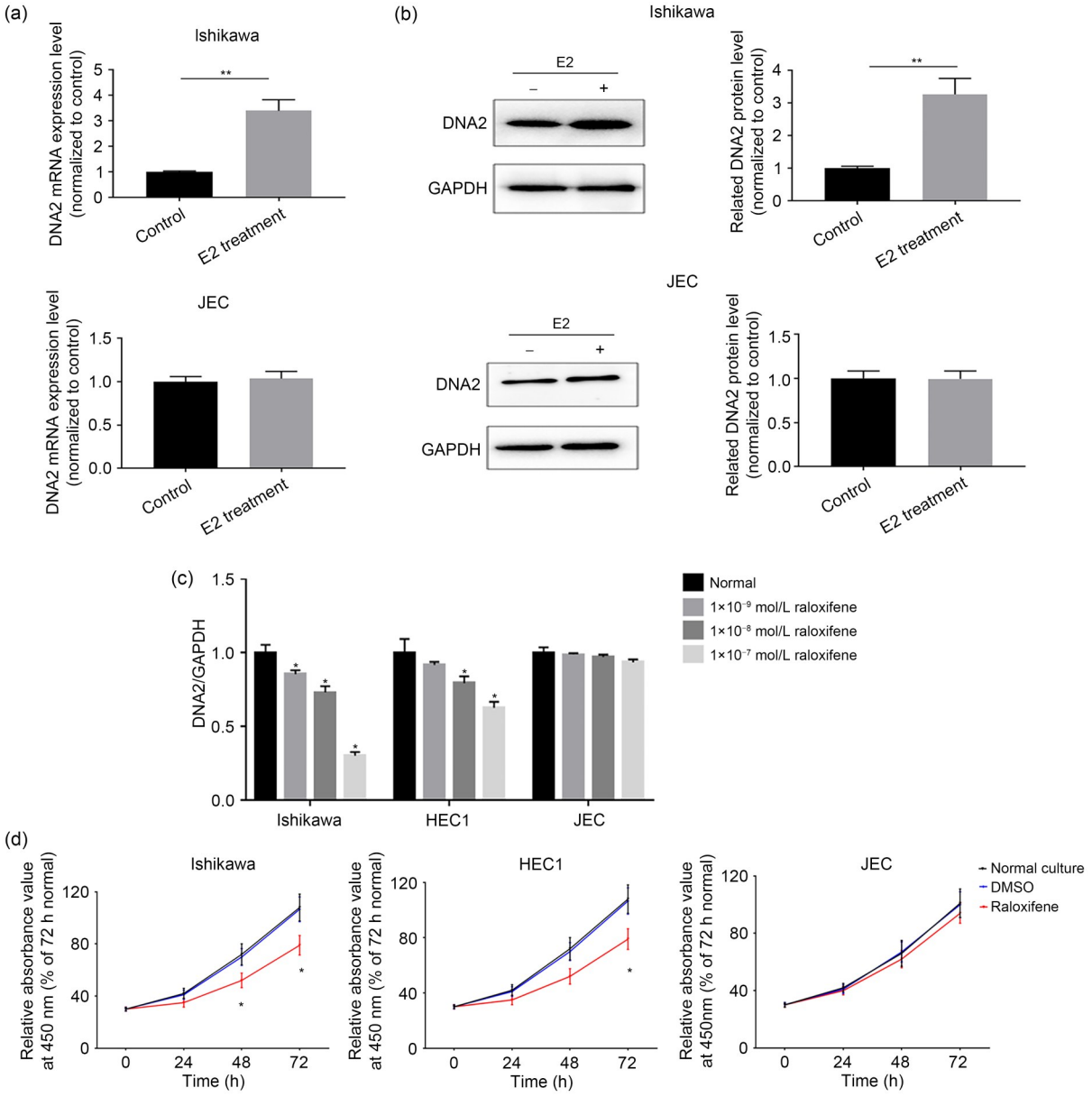

Fig. 2 Increase of DNA2 expression level in Ishikawa but not in JEC cells after exposure to E2. ER-positive Ishikawa and ER-negative JEC cells were treated with E2 and collected to evaluate the DNA2 protein and mRNA expression levels, separately. (a) A 3-fold increase in DNA2 mRNA expression was found in Ishikawa cells after E2 treatment. ** *P*<0.01. (b) The WB analysis results were consistent with those of qRT-PCR assays. ** *P*<0.01. (c) Ishikawa, HEC1, and JEC cells were exposed to different concentrations of raloxifene for 48 h. The DNA2 mRNA levels were determined via qRT-PCR assays. * *P*<0.05 vs. the normal group. (d) The CCK8 assay showed the effects of raloxifene on the proliferation of different endometrial cancer cells. * *P*<0.05 vs. the normal group. Data are expressed as mean±SD (*n*=3). DNA2: DNA replication adenosine triphosphate (ATP)-dependent helicase/nuclease; E2: β-estradiol; mRNA: messenger RNA; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; WB: western blot; qRT-PCR: quantitative real-time reverse transcription-polymerase chain reaction; CCK8: cell counting kit-8; DMSO: dimethyl sulfoxide; SD: standard deviation.

raloxifene for 48 h. The results of western blotting assays were consistent with the changes in mRNA levels (Fig. S1). Cell counting kit-8 (CCK8) assays revealed that raloxifene resulted in approximately 24% and 29% reduction in the proliferation of Ishikawa cells within 48 and 72 h, respectively, compared to those in control cells. A similar trend was found in HEC1 cells, with approximately 13% and 19% reduction within 48 and 72 h, respectively. No significant changes were found in JEC cells (Fig. 2d). We also

confirmed that treatment with tamoxifen, another ER modulator, could also downregulate DNA2 expression in Ishikawa cells, and resulted in approximately 47% reduction in the proliferation of Ishikawa cells within 72 h compared to that in control cells (Fig. S2).

The phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway has been implicated in endocrine resistance. Studies have proved that the upregulation of PI3K/AKT pathway plays a critical role in endocrine treatment resistance through extranuclear ER signaling (Hasson et al., 2013; Bean et al., 2015; Duan et al., 2021). In the present study, the cultured Ishikawa cells were divided into four groups and treated with or without E2, GDC-0810 (an ER inhibitor), and Ly294002 (a PI3K-AKT inhibitor), separately. As shown in Fig. 3, E2 treatment significantly activated the PI3K-AKT signaling pathway. The expression levels of phosphor-AKT (pAKT/Ser473) and PI3K were upregulated by approximately 3.0- and 2.6-fold, respectively. GDC-0810, one of the most commonly used selective ER modulators, can directly antagonize ER in cells. Consequently, PI3K-AKT signaling was not activated after E2 treatment in these Ishikawa cells pretreated with GDC-0810. Moreover, we observed that the DNA2 expression levels were slightly inhibited. We next examined whether the PI3K pathway inhibitor could abolish the effect of E2 treatment on DNA2 expression in Ishikawa cells. Ly294002 was able to inhibit both PI3K/AKT activity and AKT phosphorylation in these cells, and a reduced level of DNA2 expression was noticed. Altogether, these findings suggested that estrogen regulates DNA2 expression through the PI3K-AKT pathway in endometrial cancer, which can be blocked using either ER inhibitors or PI3K-AKT inhibitors.

In order to further explore the role of DNA2 in the proliferation of endometrial cells, we established a DNA2-knockdown Ishikawa cell line with specific DNA2 short hairpin RNA (shRNA) (Fig. 4). The knocked down level of the DNA2 protein was approximately 70%, as determined by western blotting assay

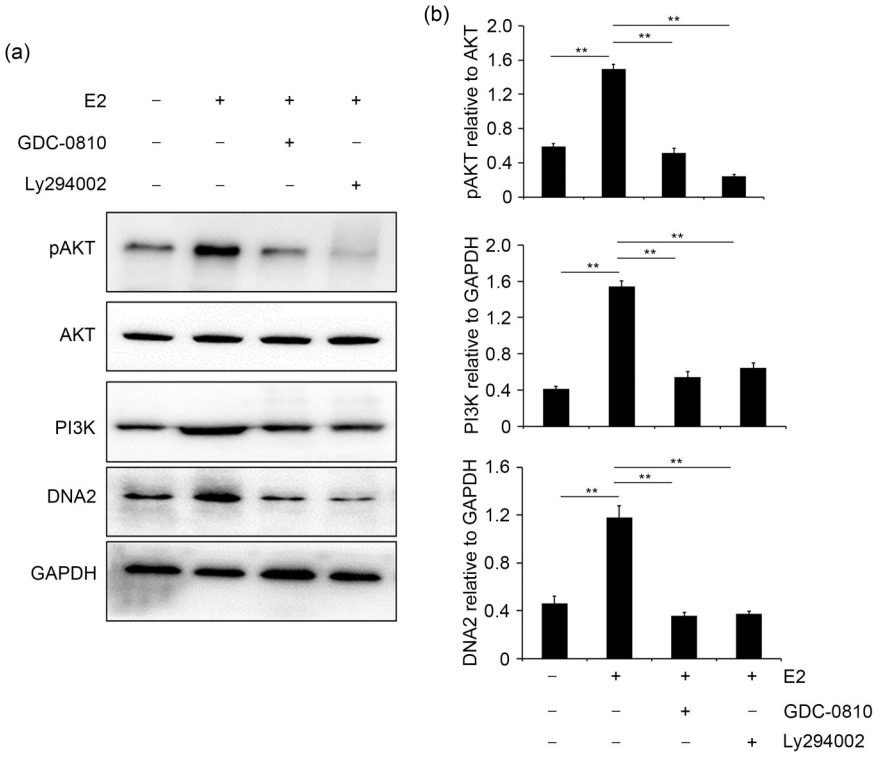

Fig. 3 Estrogen mediation of the PI3K-AKT pathway to improve DNA2 expression in Ishikawa cells, treating with E2, GDC-0810, and Ly294002, separately. (a) The protein expression levels of DNA2, pAKT, AKT, and PI3K in Ishikawa cells were determined by WB analysis. (b) The quantification of protein levels was performed using ImageJ software. Data are expressed as mean±SD (n=3). ** P<0.01. PI3K: phosphoinositide-3-kinase; AKT: protein kinase B; pAKT: phosphor-AKT; DNA2: DNA replication adenosine triphosphate (ATP)-dependent helicase/nuclease; E2: β-estradiol; WB: western blot; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SD: standard deviation.

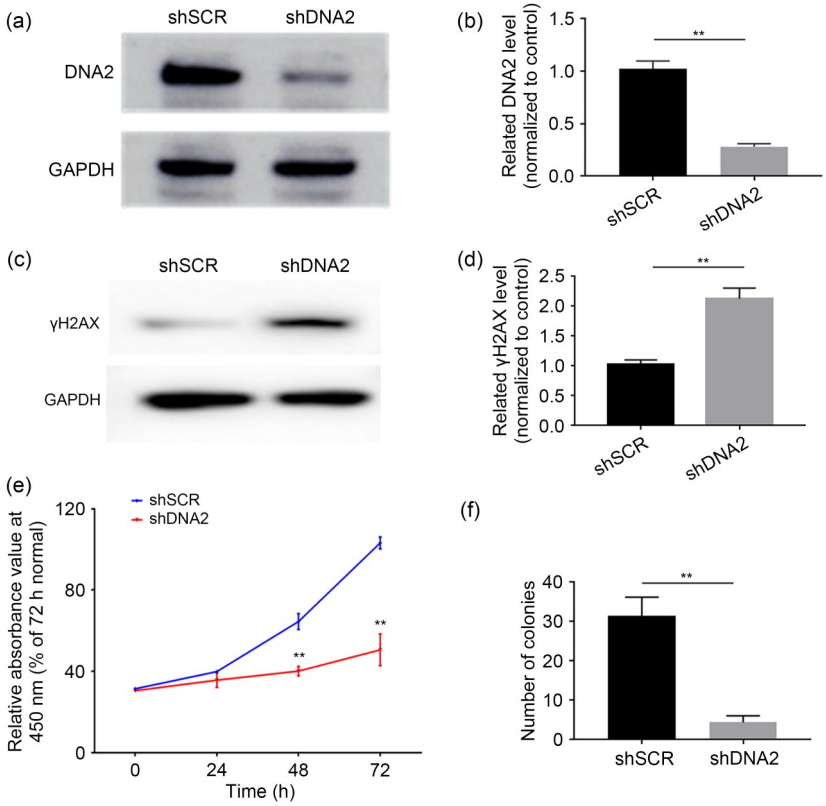

Fig. 4 Reduction of cell proliferation by knockdown of DNA2 in Ishikawa cells, obtaining stably transduced Ishikawa cells (shDNA2 and shSCR) by selection in a medium with puromycin. (a) The DNA2 protein levels were determined by WB assays. GAPDH was used as internal control. (b) The DNA2 mRNA levels were evaluated by qRT-PCR analysis. Values obtained from three independent experiments are shown for DNA2 mRNA following standardization to GAPDH. Data were analyzed using the unpaired *t*-test. (c) The γH2AX protein levels were determined by WB assays. GAPDH was used as internal control. (d) The quantification of γH2AX protein levels was performed using ImageJ software. (e) The CCK8 assay showed that Ishikawa-shDNA2 cell growth was slower than that of control cells (Ishikawa-shSCR). (f) CFE was determined as the variable numbers of Ishikawa cell colonies on soft agar. Data are expressed as mean±SEM (*n*=3). ** *P*<0.01 vs. corresponding control group (shSCR). DNA2: DNA replication ATP-dependent helicase/nuclease; shRNA: short hairpin RNA; shDNA2: cells transfected with DNA2-shRNA; shSCR: cells transfected with non-silencing shRNA; WB: western blot; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; mRNA: messenger RNA; qRT-PCR: quantitative real-time reverse transcription-polymerase chain reaction; SEM: standard error of the mean; γH2AX: gamma histone family member X; CCK8: cell counting kit-8; CFE: colony-forming efficiency.

(Figs. 4a and 4b). Consequently, a significant induction of DNA damage, indicated by increase in the DNA damage marker γ histone family member X (γH2AX), was observed in DNA2 shRNA cells (Figs. 4c and 4d). Subsequently, cell growth was evaluated using the CCK8 and colony-forming efficiency (CFE) assays. As anticipated, the proliferation and colony-forming ability declined when cells were transfected with DNA2 shRNA (Figs. 4e and 4f).

Next, we assessed whether decreased DNA2 expression had any functional consequences for cellular chemoresistance. As depicted in Fig. 5a, the knockdown of DNA2 increased the sensitivity to camptothecin (CPT) in Ishikawa cells (3 nmol/L, (35.8±8.1)% vs. (9.6±2.1)% Annexin V^+; P<0.01). Similarly, a very strong synergy was detected when we combined the treatment with CPT and C5, which is a specific inhibitor of DNA2 activity (Fig. 5b).

DNA replication failure and DNA damage response are the two major factors leading to genome instability. DNA2 is a conserved protein involved in the maintenance of mitochondrial and nuclear DNA stability, and it possesses both endonuclease/exonuclease and helicase activity (Zheng et al., 2008). It can play a vital role in DNA replication and repair at the same time, thereby maintaining genome stability. It has been confirmed that DNA2 is expressed at high levels in breast cancer, ovarian cancer, and other cells that

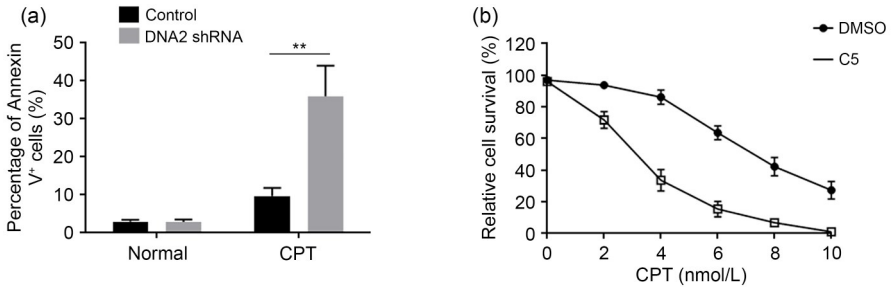

Fig. 5 Sensitivity improvement of Ishikawa cells to CPT by decreased DNA2 expression. (a) When exposed to CPT (3 nmol/L, 48 h), the DNA2 shRNA-transfected cells exhibited a >3-fold increase in apoptosis ((35.8±8.1)% vs. (9.6±2.1)% Annexin V^+). (b) The survival rate of cells treated with different concentrations of CPT in the absence or presence of C5 (1 μmol/L). Data are expressed as mean±SD (n=3). ** P<0.01. CPT: camptothecin; DNA2: DNA replication adenosine triphosphate (ATP)-dependent helicase/nuclease; shRNA: short hairpin RNA; DMSO: dimethyl sulfoxide; SD: standard deviation.

are rich in ERs, and the increase in DNA2 expression correlates with the malignancy and metastasis ability of tumors (Strauss et al., 2014). In the present study, we selected the ER-negative JEC endometrial cancer cell line and the ER-positive Ishikawa endometrial cancer cell line, stimulated them with E2, and detected the expression of DNA2. We observed that the expression of DNA2 in ER-positive cells was significantly increased, indicating that E2 binds to ER in endometrial cancer cells and can regulate DNA2 transcription. Reducing the expression of DNA2 in endometrial cancer can slow down cell proliferation and reduce its CFE activity, which was further confirmed by the in vivo tumor growth assay (Fig. S3). Meanwhile, we observed a positive correlation between DNA2 expression levels and chemoresistance in vivo (Fig. S4). Therefore, we proposed that DNA2 plays a vital role in the estrogen-stimulation-induced DNA damage of endometrial cells, which is linked to the development of cancer.

Overall, the present study confirmed the feasibility of DNA2 as a therapeutic target, and demonstrated that inhibiting DNA2 sensitizes Ishikawa cells to chemotherapy using CPT. Our findings may provide novel insights into the potential mechanisms of DNA2, which assists in developing novel approaches pertaining to the diagnosis and treatment of endometrial cancer.

Data availability statement

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Acknowledgments

This work was supported by the General Projects of Medical and Health Science and Technology Plan in Zhejiang Province (No. 2020KY433), China.

Author contributions

All authors contributed to the study conception and design. Xinyan WANG and Xiuling XU designed the experiment. Ting ZHANG and Yang JIN performed the study. Sheng XU and Lifeng CHEN drafted the manuscript. Yucheng LAI and Ling ZHANG analyzed data. Ruolang PAN and Yan YU designed the experiment, revised the manuscript, and monitored the project progression. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Xinyan WANG, Xiuling XU, Ting ZHANG, Yang JIN, Sheng XU, Lifeng CHEN, Yucheng LAI, Ling ZHANG, Ruolang PAN, and Yan YU declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. Additional informed consent was obtained from all patients for whom identifying information is included in this article. This study was granted by the Ethics Committee of Zhejiang Provincial People's Hospital (November 9, 2019; No. 2019KY204).

References

Bean JR, Hosford SR, Symonds LK, et al., 2015. The PI3K/mTOR dual inhibitor P7170 demonstrates potent activity against endocrine-sensitive and endocrine-resistant ER+ breast cancer. *Breast Cancer Res Treat*, 149(1):69-79. https://doi.org/10.1007/s10549-014-3201-6

Constantine GD, Kessler G, Graham S, et al., 2019. Increased incidence of endometrial cancer following the women's health initiative: an assessment of risk factors. *J Womens Health (Larchmt)*, 28(2):237-243. https://doi.org/10.1089/jwh.2018.6956

Duan L, Calhoun S, Shim D, et al., 2021. Fatty acid oxidation and autophagy promote endoxifen resistance and counter

the effect of AKT inhibition in ER-positive breast cancer cells. *J Mol Cell Biol*, 13(6):433-444. https://doi.org/10.1093/jmcb/mjab018

Gao CX, Shi JM, Zhang JX, et al., 2022. Chemerin promotes proliferation and migration of ovarian cancer cells by upregulating expression of PD-L1. *J Zhejiang Univ-Sci B (Biomed & Biotechnol)*, 23(2):164-170. https://doi.org/10.1631/jzus.B2100392

Han YY, Zhang ZY, Wang Z, et al., 2021. Increased expression of DNA2 was linked to poor prognosis in breast cancer. *Dis Markers*, 2021:8860728. https://doi.org/10.1155/2021/8860728

Hasson SP, Rubinek T, Ryvo L, et al., 2013. Endocrine resistance in breast cancer: focus on the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin signaling pathway. *Breast Care (Basel)*, 8(4):248-255. https://doi.org/10.1159/000354757

Jia PP, Junaid M, Ma YB, et al., 2017. Role of human DNA2 (hDNA2) as a potential target for cancer and other diseases: a systematic review. *DNA Repair (Amst)*, 59:9-19. https://doi.org/10.1016/j.dnarep.2017.09.00

Li CH, Wang CH, 2021. LG-ESSs and HG-ESSs: underlying molecular alterations and potential therapeutic strategies. *J Zhejiang Univ-Sci B (Biomed & Biotechnol)*, 22(8):633-646. https://doi.org/10.1631/jzus.B2000797

Luo J, Song ZM, Zhang T, et al., 2022. Upregulation of h-TERT and Ki-67 in ectopic endometrium is associated with recurrence of endometriosis. *J Zhejiang Univ-Sci B (Biomed & Biotechnol)*, 23(2):158-163. https://doi.org/10.1631/jzus.B2100502

Morice P, Leary A, Creutzberg C, et al., 2016. Endometrial cancer. *Lancet*, 387(10023):1094-1108. https://doi.org/10.1016/s0140-6736(15)00130-0

Setiawan VW, Yang HP, Pike MC, et al., 2013. Type I and II endometrial cancers: have they different risk factors? *J Clin Oncol*, 31(20):2607-2618. https://doi.org/10.1200/JCO.2012.48.2596

Strauss C, Kornowski M, Benvenisty A, et al., 2014. The DNA2 nuclease/helicase is an estrogen-dependent gene mutated in breast and ovarian cancers. *Oncotarget*, 5(19):9396-9409. https://doi.org/10.18632/oncotarget.2414

Thakar T, Leung W, Nicolae CM, et al., 2020. Ubiquitinated-PCNA protects replication forks from DNA2-mediated degradation by regulating Okazaki fragment maturation and chromatin assembly. *Nat Commun*, 11:2147. https://doi.org/10.1038/s41467-020-16096-w

Ulrich LSG, 2011. Endometrial cancer, types, prognosis, female hormones and antihormones. *Climacteric*, 14(4):418-425. https://doi.org/10.3109/13697137.2010.550974

Vassard D, Schmidt L, Glazer CH, et al., 2019. Assisted reproductive technology treatment and risk of ovarian cancer—a nationwide population-based cohort study. *Hum Reprod*, 34(11):2290-2296. https://doi.org/10.1093/humrep/dez165

Zheng L, Zhou M, Guo ZG, et al., 2008. Human DNA2 is a mitochondrial nuclease/helicase for efficient processing of DNA replication and repair intermediates. *Mol Cell*, 32(3):325-336. https://doi.org/10.1016/j.molcel.2008.09.024

Supplementary information

Figs. S1–S4