NH125, an eukaryotic elongation factor 2 kinase inhibitor, radiosensitizes breast cancer by the abrogation of G2/M checkpoint

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Abstract

Radiotherapy remains an important treatment option for both surgical and non-surgical breast cancer patients; however resistance to radiation results in tumor recurrence and metastasis. eEF2K has been reported to play a critical role in cancer development and progression, but little is related to its radiosensitization effect. This study aimed to evaluate the radiosensitization effect of NH125, an eukaryotic elongation factor 2 kinase inhibitor, in breast cancer MDA-MB-231 and MCF-7. Cell proliferation rate was evaluated by Cell Counting Kit 8 assay. Cell cycle distribution and apoptosis rate were detected by flow cytometry. Radiosensitization ratio was evaluated by clonogenic survival assay. DNA damage repair was detected by immunofluorescent assay. Tumor-bearing xenografts confirmed the radiosensitization effect in vivo. NH125 potentially sensitized MDA-MB-231 and MCF-7 to radiation with sensitization enhancement ratios 1.29 and 1.43 respectively. NH125 enhanced the radiosensitization through abrogation of G2 checkpoint and prolongation of DNA damage repair. Above all, these results demonstrated that NH125 may be a promising radiosensitizer in breast cancer radiotherapy.

Keywords: NH125, eEF2K, Eukaryotic elongation factor 2 kinase, Breast cancer, Radiosensitivity

Introduction

Breast cancer arouses public concern as the leading malignancy among women worldwide [1-3]. Despite chemotherapy and targeted therapy have developed rapidly, radiotherapy remains a critical treatment option for those non-surgical breast cancer patients [4,5]. However, cancer relapse and metastasis often result in treatment failure [6,7]. Thus, increasing the sensitivity of therapy and prolonging patient survival are urgent problems to be solved.

Eukaryotic elongation factor 2 kinase (eEF2K) has aroused interest as an effective target in recent years [8,9]. eEF2K is a calcium/calmodulin (Ca²⁺/CaM) dependent Ser/Thr-kinase which is regulated extensively by posttranslational phosphorylation [10,11]. It phosphorylates elongation factor 2 (eEF2)

and results in decreased global protein translation [12]. eEF2K has been reported that is abundant in breast cancer tissues but is absent in normal specimens [13]. It also has been reported to exist commonly in other malignances such as malignant glioma [14], leukemia [15], *et al.* Starvation, hypoxia and oxidative stress are known to increase eEF2K activity [16]. So eEF2K activation may be a survival strategy for breast cancer cells by regulating protein synthesis to provide their own raw materials and energy [17,18].

NH-125 plays anti-tumor role through decreasing the expression of eEF2K and phosphorylation of eEF2 as an effective inhibitor of eEF2K [19]. eEF2 is located downstream of the eEF2K, and inhibition of eEF2K will decrease the phosphorylation of eEF2. The reduction of eEF2 phosphorylation can inhibit the

cancer proliferation and survival. The anti-tumor effect of NH-125 was closely related to the reduction of phosphorylation of eEF2. In order to clarify the potential mechanisms of eEF2K in cancer progression, we used NH125, an eukaryotic elongation factor 2 kinase inhibitor, to find out its critical role in sensitizing breast cancer to radiotherapy *in vivo* and *in vitro*. Furthermore, we explored the novel molecular mechanisms of NH125 in radiosensitization.

Materials and methods

Reagents and cell lines

The human breast cancer cells MDA-MB-231 and MCF-7 were obtained from the Shanghai Institutes for Biological Sciences, China. MCF-7 cells were cultured in Dulbecco's Modified Eagle medium (Hyclone) containing 10% fetal bovine serum (Invitrogen) and MDA-MB-231 cells were cultured in RPMI-1640 (Gibco) containing 10% fetal bovine serum (Invitrogen). Two cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂. NH-125 (97.45% purity, APExBIO, China) was dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) and stored in a refrigerator at -80°C. Monoclonal antibodies against Cdc2, p-Cdc2, cyclin B1, p-Cdc25 were obtained from Santa Cruz Bio-technology (Santa Cruz, USA) and horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit) were obtained from Bioworld Technology, Inc. (Bioworld, USA).

Cell proliferation assay

CCK8 assay was used to detect cell proliferation. Cells were planted with a density of $4-5 \times 10^3$ cells/well in 96-well plate overnight. Cells were treated with different concentrations of NH-125 for 24 h, 48 h and then 10% CCK8 was added to each well cultured for 1.5 h at 37 °C. Finally, The OD value was detected by a microplate reader at 450 nm wavelengths.

Clonogenic survival assays

Cells were plated at different densities in six-well plates. After cells adhesion, cells were pretreated with the indicated concentrations of NH-125 (0.1 μM, 0.25 μM) for 24 h and with DMSO as control. Then cells were irradiated with 6 MV X-rays with different doses (0, 2, 4, 6, 8 Gy), 4.5 Gy/min. Cells postirradiation continue to be cultured for two weeks in the incubator. Then cells were fixed and stained for each 30 minutes, and more than 50 cells were counted.

Flow cytometry

The adherent cells were pretreated with indicated concentrations of NH-125 (0.5 μM, 1 μM) and with DMSO as control for 24 h. After irradiated after 48 h, breast cells were collected and stained with AV/PI (Keygen). Avoiding light for 15 minutes, apoptotic rates were measured by flow cytometry through using an Annexin V-FITC Apoptosis Kit.

Cell cycle analysis

Cells were incubated in six-well plates (3×10^5 per well) and

allowed to adhere overnight. Then cells were treated with NH-125 (0.5 or 1 μM) and with DMSO as control for 24 h. After 24 h, the cells were given a dose of 8 Gy. Cells were resuspended in 70% ice ethanol at 4°C overnight postirradiation. Then cells were stained with PI and RNase A (5μg/mL) and analyzed by using flow cytometry.

Immunofluorescence

In order to detect DNA double-strand breaks (DSBs) and homologous recombination repair (HRR), we performed immunofluorescence assay to detect phospho-H2AX foci. MDA-MB-231 cells were planted on confocal laser small dish overnight. Then cells were treated with NH-125 (1 μM) and irradiated with 6 Gy. Irradiated after 2 h, 6 h and 24 h, cells were fixed by 4% paraformaldehyde for 30 min at room temperature and anhydrous methanol treated with 30 min at -20°C. Blocked with Immunol Staining Blocking Buffer (Beyotime) for 1 h at room temperature, then cells were incubated with antibody for phospho-H2AX (Ser139) at 4°C overnight followed by staining with Fluorescein (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, PA) for 1 h at room temperature. At last the nuclei were stained with DAPI (Beyotime) and mounting medium viewed by confocal microscopy.

Western blotting analysis

Two breast cancer Cells were pretreated with NH-125 combined with radiation for 24h, and then cells were lysed in RIPA buffer containing PMSF and lysate was collected with a scraper in the EP tube, all the experiments are on the ice. The EP tube was centrifuged at 4°C at 14000g for 15 minutes. Proteins were detected by BCA kit (Beyotime). Proteins were isolated by SDS-PAGE and moved to PVDF membrane. Then membrane was blocked with 5% serum at 4°C for 2h and incubated with main antibodies against Cdc2, p-Cdc2, cyclin B1, cyclin D1, β-actin, GAPDH at 4°C overnight. At last the membrane was washed three times and incubated with secondary antibodies for another 1.5h. Protein imprinting was conducted by using ECL Kit.

Xenografts

MDA-MB-231 cells (1×10^6) were subcutaneously injected into armpit of 6-week old female nude mice (Nanjing, China). When the tumors grew to 150 mm³, the animals were intraperitoneal injection with 1mg/kg NH-125 or physiological saline 6 h and 24 h before irradiation. Then, tumors were given a dose of 6 Gy (2 Gy/min). Body weights and tumor diameters were calculated every other day, and tumor volumes were measured on the basis of the formula: (length [L] × width [W] 2)/2. The tumor doubling time (TD) was calculated as follows: $TD = t \times \lg 2 / \lg (N_t / N_0)$.

Statistical analysis

Data were analyzed with Prism 6.0 software (GraphPad Prism). The mean ± SE from triplicate assays was calculated, and

differences between treatment groups were determined using two-tailed Student's t test when only two groups or one-way ANOVA when more than two groups, followed by Student-Newman-Keuls test as post hoc test. All experiments were repeated three times. $P < 0.05$ was considered statistically significant.

Results

NH-125 reduces the expression of eEF2K in breast cancer cells

We determined the effect of NH-125 on eEF2K in MDA-MB-231 and MCF-7 cells. As shown in **Figure 1**, results of western blotting demonstrated that the expression of eEF2K and p-eEF2 decreased with the increase of NH-125 dose (dose dependent). However, no change was observed in the expression of eEF2. This result indicated that NH-125 could inhibit the expression of eEF2K and p-eEF2 in MDA-MB-231 and MCF-7 cells.

Figure 1. NH-125 suppresses eEF2K expression in human breast cancer cells.

Cells were treated with NH-125 (0, 0.5, 1, 2, 4, 6 μM) or DMSO as control for 24 h. Western blotting demonstrated that NH125 significantly inhibited the expression of eEF2K and p-eEF2, but has no effect on eEF2.

NH-125 radiosensitizes the breast cancer cells

In order to estimate the inhibitory effects of NH-125 on breast cancer cells MDA-MB-231 and MCF-7, we performed CCK8 experiments. Cells were pretreated with different doses of NH-125 (0, 0.5, 1, 2, 4, 6, 8, 10 μM) for 24h and 48h. This result showed that NH-125 caused proliferative inhibition of breast cancer cells in dose dependent manners (**Figure 2A**). We chose NH125 at concentration of 0.1 μM and 0.25 μM which were low cytotoxicity (survival rate $> 80\%$). As shown in **Figure 2B**, clonogenic survival assays results showed that NH-125 combined radiation could inhibit colony formation of MDA-MB-231 and MCF-7 cells. The sensitization enhancement ratios of MDA-MB-231 and MCF-7 reached 1.29 and 1.43 respectively (**Tables 1 and 2**).

Effects of NH-125 on the apoptosis in breast cancer cells

As shown in **Figure 3**, we detected the effects of NH-125 on

Figure 2. NH-125 inhibits the cell viability of breast cancer cells.

A. NH-125 suppressed the proliferation of breast cells in dose and time-dependent manners. **B.** Clonogenic assay showed that NH-125 enhanced the radiosensitivity in MDA-MB-231 and MCF-7 cells (mean $\pm$ SD, $n = 3$).

Table 1. The Radiosensitization Activity of NH-125 in MCF-7 Cells.

	MCF-7			
	D_0	D_q	SF_2	SER
Control	2.10	1.07	0.55	--
NH-125 0.1 μM	1.49	1.05	0.46	1.41
NH-125 0.25 μM	1.21	0.81	0.34	1.43

Table 2. The Radiosensitization Activity of NH-125 in MDA-MB-231 Cells.

	MDA-MB-231			
	D_0	D_q	SF_2	SER
Control	1.76	1.70	0.64	--
NH-125 0.1 μM	1.75	0.60	0.42	1.01
NH-125 0.25 μM	1.36	0.34	0.29	1.29

apoptosis in breast cancer cells. Cells were divided into control group, NH-125 alone group (0.5 μM , 1 μM), simple radiation group, radiation combined NH-125 group. The results showed that the apoptosis rate of the combined group was significantly higher than other groups ($p < 0.05$).

Effects of NH-125 on cell cycle in breast cancer cells

To estimate the effects of NH-125 on cell cycle in MDA-MB-231 and MCF-7 cells, we implemented the cell cycle assays. The results showed that radiation significantly induced G2/M arrest (81.3% for MDA-MB-231 and 73.9% for MCF-7 respectively). However, NH-125 combined radiation group results

showed that NH-125 (1 μM) significantly abolished the G2/M phase arrest (13.4% for MDA-MB-231 and 22.4% for MCF-7) caused by radiation and induced G1/S arrest (Figure 4A). We also found that NH-125 alone slightly decreased G2/M phase and increased G1/S phase. In order to prove that NH-125 abolished the G2/M phase arrest we added Nocodazole. The results showed that NH-125 combined Nocodazole (0.4 μg/mL) caused the G2/M phase arrest (Figure 4B). These results indicated that NH-125 could reduce irradiation induced accumulation of G2/M in breast cancer cells. Detailed data were shown in Table 3.

Effects of NH-125 on the DNA damage repair in the breast cancer cells

H2AX is an important member of the histone H2A family, which located on the DNA double strand. DSBs could induce the rapid phosphorylation of H2AX (Ser-139) into γ-H2AX. γ-H2AX foci had a high specificity for detection of DNA double strand breaks. To explore whether the G2 phase arrest was related to DNA double-strand breaks and homologous recombination repair. We performed immunofluorescence assay to detect the levels of γ-H2AX foci at 2, 6 and 24 h after 6 Gy irradiation combined NH-125 (Figure 5). The peak of the foci appeared

Table 3. NH-125 abolished the G2/M phase arrest in breast cancer cells.

Treatment	MCF-7			MDA-MB-231		
	G ₁ (%)	S (%)	G ₂ /M (%)	G ₁ (%)	S (%)	G ₂ /M (%)
Control	54.5	34.1	11.4	68.8	15.7	15.5
NH-125	64.9	26.7	8.35	70.3	21.0	8.73
IR	9.81	16.3	73.9	6.69	12.0	81.3
IR+NH-125	45.4	32.3	22.4	57.3	29.3	13.4
Treatment	G ₁ (%)	S (%)	G ₂ /M (%)	G ₁ (%)	S (%)	G ₂ /M (%)
Control	68.4	20.9	10.7	68.3	18.1	13.6
IR	5.61	23.3	71.1	11.0	28.6	60.4
IR+NOC	1.58	1.08	97.3	3.17	1.15	95.7
IR+NH-125	64.7	18.2	17.1	68.4	17.2	14.3
IR+NH-125+NOC	21.1	28.0	50.9	17.7	29.5	52.8

Figure 4. Effects of NH125 on cell cycle in the breast cancer cells.

A. The breast cancer cells were treated with NH-125, IR or the combination group and then were determined by flow cytometry. **B.** Breast cancer cells were pretreated NH-125 before IR and then incubated with or without Nocodazole (NOC, 0.4 μ g/mL) for 24 h. All experiments were repeated three times and obtained same results.

Figure 5. Effects of NH125 on cell cycle proteins through western blotting.

The breast cancer cells were treated with NH-125 (1 μ M) and with 8Gy irradiation. Western blotting analysis showed that the expression of Cyclin B1, p-Cdc2 and Cdc2 in combination therapy group. IR could increase the expression of Cyclin B1 and p-Cdc2 and NH125 could decrease the expression of Cyclin B1 and p-Cdc2.

at 6h after irradiation. In addition to, we found that NH-125 combined radiation obviously delayed γ -H2AX signal at 24 h compared with radiation alone group. The results showed that NH-125 could inhibit DSB repair.

Effects of NH-125 on the protein expression

Cdc2/cyclin B1 was a key complex in the regulation of G2/M phase transition. Cyclin B1 reached a peak in G2/M phase. DNA damage induced G2/M arrest may be related to the increase of Cdc2 phosphorylation and accumulation of cyclin B1. In order to explore the potential mechanisms of NH-125 in regulating cell cycle distribution, western blotting experiments were done. As shown in **Figure 6**, the expression of cyclin B1 and p-Cdc2 were significantly increased in irradiation alone group. This phenomenon was completely reversed in combination therapy group. Thus we speculated that NH125 abrogated the G2 checkpoint through decreasing the expression of p-Cdc2 and cyclin B1.

Effects of NH-125 on breast cancer xenografts in nude mice

In order to observe the effect of NH-125 on xenografts *in vivo*, xenograft experiments were performed. We divided the nude mice into four groups: control group; NH-125 alone group; irradiation alone group; combination treatment group. After the different treatments, the body weight and tumor volume were measured. The results showed that the weight and tumor volume of the combined treatment group were significantly less than control group (**Figures 7A-7C**). The tumor doubling times were control group for 5.56 ± 0.57 , NH-125 alone group for 6.22 ± 0.47 , irradiation alone group for 9.62 ± 2.53 , combination treatment group for 14.01 ± 5.88 . These data proved that NH-125 could inhibit the growth of breast cancer xenografts *in vivo*.

Discussion

eEF2K is an unique calcium/calmodulin dependent enzyme,

Figure 6. NH-125 obviously delayed DSB repair in breast cancer cells.

MDA-MB-231 cells were treated with 0.1% DMSO or 1 μ M NH-125 and then exposed to 6Gy of X-ray. At last, immunofluorescence assays were used to detect the γ -H2AX. Representative images showed that NH125 could significantly delay the DNA damage repair postirradiation.

and plays a decisive role in protein elongation, and the mechanisms of protein elongation are closely related to cell cycle distribution [20]. Dysregulation of eEF2K/eEF2 can change cell cycle distribution and contribute to cancer progression. eEF2K is reported to overexpress in the majority of cancer cells and it can tolerate stress such as starvation, hypoxia and oxidative stress. The mechanisms of protein synthesis modification demonstrated that the significance of this system in carcinogenesis [21]. Tekedereli *et al.* found that eEF2K knockdown could inhibit breast cancer cells growth, induce apoptosis, and sensitize to doxorubicin *in vivo* and *in vitro* [22].

In this present study, we found that eEF2K inhibitor NH125 significantly inhibited the cell viability in breast cancer cell lines MDA-MB-231 and MCF-7. As shown in Figure 1, NH125 significantly decreased the expression of eEF2K and p-eEF2, but has no effect on eEF2. Arora *et al.* found that NH125 inhibited the eEF2K activity and blocked the phosphorylation of eEF2 in the glioma cell lines as a potential anticancer drug [23]. Interestingly, Chen *et al.* indicated that NH125 inhibited the growth of H1299 cells through inhibiting the expression of eEF2K and inducing the phosphorylation of eEF2, which were different from most of other studies and what we have found [24]. Further studies are needed to further characterize the radiosensitive mechanisms of NH125 in breast cancer.

It has been reported that progression of G1 phase entry into the S phase (the G1/S transition) is promoted by eEF2K activity, which is regulated by increase in intracellular calcium (Ca²⁺) [25]. We observed that radiation could induce G2/M arrest in MDA-MB-231 and MCF-7 cells, which allowed time for cells to repair DNA damage. The damaged DNA postirradiation can activate the p53 gene and p21 gene [26,27]. Active P21 gene can inhibit the activity of cyclinB1, resulting in G2/M arrest for

DNA damage repair. If this stage of DNA damage cannot be repaired, cells will be induced to apoptosis [28,29]. As shown in Figure 3, NH125 could increase apoptosis, especially when combined with irradiation. Its radiosensitization effect is possible related to abrogating radiation-induced G2/M arrest.

DNA repair following double-stranded breaks induced by irradiation requires a variant histone protein called H2A.X. PI3K-like kinases such as ATR, ATM and DNA-PK can induce rapid H2A.X phosphorylation at Ser139 site after irradiation induced DNA damage. As shown in Figure 6, immunofluorescence detection of phospho-H2AX foci found that NH-125 obviously delayed DNA damage repair after radiation compared with radiation alone group.

Our study found that NH125 selectively reduced the expression of eEF2K in breast cancer cells and abrogated radiation-induced G2/M arrest. The potential mechanism may be through modulating Cdc2/cyclin B1. Cdc2/cyclin B1 was a key complex in the regulation of G2/M phase transition. Cdc2 regulates the entry of cells into mitosis, the phosphorylation of Cdc2 can contribute to inactivate Cdc2 and prevent cells mitosis. As such, dephosphorylation of Cdc2 at Thr14 and Tyr15 contributes to progression into mitosis. Cyclin B1 is a checkpoint regulatory protein and thought to modulate the translocation to nucleus at G2/M checkpoint and initiation of mitosis. The degradation of cyclin B1 is necessary for cells to progression out of mitosis. Although our study demonstrated that NH-125 increased the radiosensitivity of breast cancer cells mainly through abrogating G2 checkpoint. Further studies are expected to clarify the relationships between eEF2/eEF2K and the cell cycle.

The results were further verified by *in vivo* studies in xenografts. The results expanded our understanding of the effects of eEF2K activity in radiation and suggested that targeting eEF2K may be a potential radiosensitizing strategy in breast treatment.

In summary, NH125 increased the radiosensitivity through induction of apoptosis, abrogation of radiation-induced G2/M checkpoint and delay DNA damage repair *in vivo* and *in vitro*. These results provided support that NH125 may be a potentially promising agent in adjuvant therapy to enhance the effects of radiotherapy for breast cancer patients. However, further studies are needed to investigate the molecular mechanisms and confirm the effects before clinical use.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Authors' contributions	HMS	GZC	HCZ	JHM	ZMW	TLH	YDW	XCS	JXM
Research concept and design	✓	--	--	--	--	--	--	--	✓
Collection and/or assembly of data	--	✓	--	✓	--	--	--	--	--
Data analysis and interpretation	--	--	--	--	✓	--	--	✓	--
Writing the article	✓	✓	✓	--	--	--	--	--	--
Critical revision of the article	--	--	--	--	--	✓	--	--	--
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