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ORIGINAL ARTICLE

Identification of novel NRAGE involved in the radioresistance of esophageal cancer cells

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Abstract Radiotherapy (RT) is one main method for the treatment of esophageal squamous cell carcinoma (ESCC), and the radioresistance is the predominant cause of patients with local recurrence. The previous results of gene microarray and subsequent verification showed that NRAGE might be involved in radiation resistance of ESCC cells. In this study, we reestablished human esophageal carcinoma radioresistant cell lines TE13R120 and ECA109R60 with gradient dose irradiation as previously reported, respectively. NRAGE expression was high in TE13R120 and ECA109R60 cells and was correlative with ionizing radiation (IR) resistance in clinic. However, the radiosensitivity of TE13R120 cells had a remarkable increase detected by colony formation assays after siRNA against NRAGE (siNRG) transfection into TE13R120 cells. Compared with TE13 cells, an increasing number of TE13R120 cells with NRAGE overexpression in S phase and a lower ratio in G2/M were observed by flow cytometry method (FCM). Intriguingly, the above changes were partially reversed in TE13R120 cells treated with siNRG. More importantly, the ectopic subcellular localization of NRAGE mediated nuclear translocation of β-catenin which may be one reason of IR resistance of esophageal carcinoma cell. These data indicate that NRAGE extremely may be a pivotal factor involved in Wnt/β-catenin signal pathway, mediating nuclear translocation of β-catenin and then facilitating the formation of radioresistance of ESCC.

Keywords Esophageal squamous cell carcinoma · Radioresistance · NRAGE · β-catenin · Subcellular localization

Introduction

Esophageal cancer (EC) is the eighth most frequently diagnosed cancer and the sixth most common malignancy caused deaths worldwide [1, 2]. Radiotherapy (RT) is one of the primary treatments of esophageal carcinoma, especially for inoperable and locally advanced esophageal squamous cell carcinoma (ESCC) patients. However, the resistance to ionizing radiation (IR) is the main cause of induced therapeutic failure. Therefore, the radioresistance is the prime restraining factor for the improvement of radiotherapy effect, and there is an increasing focus on the mechanism of esophageal cancer radiation resistance.

NRAGE (Dlxin-1, MAGE-D1), a neurotrophin receptor-interacting melanoma antigen-encoding gene homolog, belongs to type II of melanoma antigen (MAGE) protein family. The expression profile of NRAGE differs apparently from other MAGE family members. There is a broad expression of NRAGE in tissues of adult individuals, especially focusing on the brain with the highest expression [3]. NRAGE protein consists of 200 amino acids of the MAGE homology domain (MHD), a conserved area of MAGE family, and 25 repeats of a WQXPXX hexapeptide domain in the middle region. The former is a common characteristic of MAGE family, but the latter is unique for NRAGE, indicating the complicated role that NRAGE may possess the similar and peculiar functions with other family members [4]. Initially, NRAGE was

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regarded as a tumor suppressor which caused cell apoptosis through interacting with nerve growth factor receptor (p75NTR) [5], anti-apoptotic transcription factor Che-1 [6], nerve growth factor 1 receptor UNC5H1 [7], and the compound of XIAP-TAK1-TAB1 [8]. However, Kumar et al. [9] found that NRAGE as an anti-apoptosis factor can prevent the transcription of anoikis sensitivity key factor P14ARF through interacting with TBX_2, a member of transcription repressor protein family. It is contradictory to what were reported before. Besides, increasing evidence has shown that NRAGE was highly expressed in certain types of cancer [3, 6, 7]. Taken together, it suggests that the complicated NRAGE may have different functions under various stimulations involved in multiple signaling pathways.

Our previous study [10] showed that NRAGE may participate in the formation of radiation resistance in the ESCC cell line TE13R120. In this paper, the purpose of the study is to explore the role of NRAGE in the formation of IR resistance and illuminate the relationship among NRAGE, canonical Wnt pathway, and radioresistance in ESCC. Our results reveal that NRAGE is a critical factor in promoting the generation of IR resistance of EC cells by altering its subcellular localization to the nucleus, then changing the nucleus distribution of β-catenin.

Materials and methods

Cells and cultures

ESCC cell line TE13 was obtained from the department of the first surgery of Okayama University (Okayama, Japan), and ECA109 was purchased from the Institute of Biochemistry and Cell Biology China Academy of Science (Shanghai, China). Both TE13 and ECA109 were cultured in RPMI-1640 (Gibco, Life Technologies) supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin in a humidified incubator at 37 °C, and 5 % CO_2. Cells were subcultured each 3~4 days for exponential growth.

Human biopsy specimen

To detect the expression of NRAGE in clinical specimens, 27 ESCC patient specimens which were histopathologically and clinically diagnosed according to the WHO criteria were collected before radiotherapy at the Second Hospital of Hebei Medical University from November, 2009, to June, 2012. For the study, the patients' informed consent was obtained from the subject and the study was approved by the Ethics Committee of Second Hospital of Hebei Medical University. The characteristics of 27 case patients were shown in Table 1. All 27 patients accepting radical radiotherapy received conventional fractionated radiotherapy by 6MV X-ray linear accelerator with three-dimensional conformal radiation therapy (3D-CRT). The curative effects of the 27 patients were assessed when radiotherapy was completed and 3 months later after radiotherapy by referencing the esophageal barium meal evaluation standard.

Establishment of radioresistant cell lines

Radioresistant cell lines TE13R120 and ECA109R60 were reestablished in our department with gradient dose irradiation as previously reported by Zhang et al. [11]. TE13 and ECA109 cells about 70 % confluences in 25-cm^2 culture flasks were first irradiated with 2 Gy of 6MV X-rays by a linear accelerator (Elekta, Synergy) at a dose rate of 200 cGy/min using a tissue compensation system with a 1.5-cm membrane. The cells were replaced medium and returned to the incubator for future culture immediately irradiated. Reaching approximately 90 % confluences, cells were digested and subcultured into new flasks. After passage (24 h), the cells were re-irradiated three times (4 Gy, 6 Gy, and 8 Gy) under the processes previously described. And then the procedures were repeated four times (10 Gy/time) in ECA109 cells in 4 months and reached a total dose of 60 Gy. Similarly, TE13 cells got additional six repeated exposures with 10 Gy/time up to a total dose of 120 Gy. Radiation resistance levels of radioresistant cell lines TE13R120 and ECA109R60, which were several clones isolated from the two resistant cell populations, were identified through a colony formation assay. Both TE13R120 and ECA109R60 were incubated under the conditions previously described.

MTT assay

After treatment with or without radiation, TE13 and TE13R120 cells were inoculated in 96-well plates at 5000 cells/well. After the first 24 h, cell viability was evaluated by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay every day for seven consecutive days; 20 μL of MTT (5 mg/mL) was added to each well at a final concentration of 0.5 mg/mL, followed by incubation at 37 °C for 4 h. Then, the supernatant was removed, and added with 100 μL DMSO to terminate the reaction. Optical density (OD) at 490 nm was measured by a microplate reader (BioTek, USA). The relative viability of cells was calculated as follows: $OD_{\text{experimental group}}/OD_{\text{control group}} \times 100\ \%$.

Colony formation assay

To compare radioresistance of different cells, colony formation assay was used. Cells in the exponential growth period were seeded in 6-well plates at 200, 500, or 800 cells/well and in 10-mm culture dishes at 3000 or 5000 cells/dish. Cells were

Table 1 The characteristics of the 27 cases of esophageal squamous cell carcinoma patients

Number	Gender	Age	Lesion location	Lesion length (cm)	Lymph node metastasis	Distant metastasis	Tumor stage	Curative effect
1	M	69	Lower	8	N1	M0	III	NR
2	M	66	Middle	10	N2	M1	IV	PR
3	M	77	Middle	3	N0	M0	I	PR
4	M	24	Middle	3	N0	M0	I	NR
5	M	59	Middle	8	N0	M0	III	PR
6	M	78	Middle	7	N0	M0	II	CR
7	F	86	Middle	7	N1	M0	II	CR
8	M	54	Lower	2	N2	M0	II	CR
9	F	79	Middle	2	N1	M0	II	CR
10	M	79	Lower	5	N2	M0	II	CR
11	M	74	Middle	5	N1	M0	II	NR
12	M	79	Middle	2	N0	M0	I	CR
13	M	82	Lower	2	N1	M0	II	PR
14	M	68	Middle	6	N0	M0	II	CR
15	M	78	Upper	2	N0	M0	I	CR
16	F	84	Middle	8	N2	M0	III	PR
17	F	75	Lower	5	N0	M0	I	NR
18	F	48	Middle	9	N0	M0	III	PR
19	F	71	Upper	5	N1	M0	II	NR
20	M	76	Lower	7	N2	M0	IV	CR
21	F	63	Middle	5	N0	M0	III	CR
22	F	59	Upper	7	N0	M0	II	CR
23	M	56	Upper	2	N0	M0	I	CR
24	F	60	Upper	7	N1	M0	II	CR
25	F	60	Lower	5	N1	M0	II	PR
26	M	70	Middle	6	N1	M0	II	CR
27	M	57	Middle	5	N1	M0	II	CR

CR complete response, *PR* partial response, *NR* no response

exposed to X-rays by doses of 0, 2, 4, 6, 8, or 10 Gy according to the procedure described above. After incubation for 10~14 days to form the colony, the cells were fixed with methanol for 20 min and stained with crystal violet for 10 min. Colony counting was done manually by two people, respectively. The colony containing at least 50 cells was counted, and each value was from the two people's average. Every group was repeated in triplicate. Surviving fraction (SF) = (number of colonies formed in experiment group/number of corresponding cells inoculated)/(number of colonies for sham-irradiated group/number of corresponding cells inoculated).

RNA extraction and real-time PCR

Total RNA was extracted with TriZol reagent (Invitrogen). The quality of the total RNA was assessed by a Nanodrop 2000c and agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized from 1.0 μg of total RNA using oligo (dT) primers using PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Shiga, Japan). Expression of NRAGE messenger RNA (mRNA) and β-catenin mRNA were performed by real-time PCR with SYBR Premix Ex Taq™ (Takara, Shiga, Japan) on MxPro real-time PCR system (Agilent Technologies Stratagene Mx3005P, USA). Samples were normalized on the basis of the expression of the gene-encoding human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference. Relative expression levels were calculated as $2^{-[(\mathrm{Ct\ of\ target\ gene}) - (\mathrm{Ct\ of\ GAPDH})]}$. The specific primers used are showed in S1 Table.

Western blotting

To detect the expression of NRAGE and β-catenin protein in different treated groups, Western blotting was used. Cells were harvested and lysed in ice-cold lysis buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.0 % Triton X-100, 20 mM EDTA, 1 mM Na3VO4, 1 mM NaF, and protease inhibitors (Halt™

Protease Inhibitor Cocktail, EDTA-free, and PMSF)]. Protein concentrations were detected using BCA kit (Generay, Shanghai, China); 20 μg of samples were separated on 10 % SDS-PAGE as previously described [10]. Finally, software Quantity One v4.62 for Windows (Bio-Rad, CA, USA) was used to analyze the density of the bands. Antibodies against NRAGE (Proteintech, USA), β-catenin (Proteintech, USA), and β-actin (Sigma-Aldrich) were used in the experiment.

Immunofluorescence

Immunofluorescence was performed to test the subcellular localization of NRAGE and β-catenin in different cells. Cells grown on sterile coverslips overnight were rinsed with PBS for three times and then fixed in acetone for 15 min at 4 °C. After washing again, cells were permeabilized for 10 min using PBS containing 0.30 % Triton X-100. Cells were blocked with 5 % bovine serum albumin for 30 min. Followed by primary antibodies overnight at 4 °C. Washing three 5-min washes in PBS, peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG secondary antibodies (Proteintech, USA) were added for 1 h at room temperature in the dark. After same three washes, 6-diamidino-2-phenylindole (DAPI) (10 μg/mL, Solarbio, Beijing, China) was used to stain cell nuclei. After three washes again, cells were mounted in a vectashield mounting medium. Images were captured using a fluorescence microscope (Leica DMI4000 B) and analyzed with a Leica confocal software.

siRNA transfections

SiRNA against NRAGE was purchased from GenePharma, Shanghai, China. After being inoculated 24 h in 6-well plates at 60~70 % confluences, TE13R120 cells were transfected with 5 μL of Lipofectamine RNAiMax reagent (Invitrogen) at 20 μM of siRNA in Opti-MEM according to the manufacturer's instruction. The primers used are showed in S2 Table.

Cell cycle assay by flow cytometry method

Cells (2×10^5 cells/well) were seeded in 6-well plates, and when cells were adherent, some TE13R120 were transfected with siRNA-NRAGE or scramble-control siRNA. Twenty-four hours later, cell cycle assay was done according to the protocol of the manufacturer (Beyotime, Shanghai, China). Briefly, cells were harvested, washed by ice-cold PBS, and fixed in 70 % of ice-cold ethanol at 4 °C for at least 2 h. Cells were centrifuged, washed with cold PBS, re-centrifuged, and re-suspended in 50 μL PBS. Then, cells were stained with propidium iodide (PI) staining solution (staining buffer + PI (20×) + RNase A (50×)) at 37 °C for 30 min. Then, cells were kept at 4 °C for detecting DNA content by flow cytometry (BD FACSCalibur) and calculating the percentage of cells in G0/G1, S, and G2/M phases with FlowJo software.

Immunohistochemistry

A total of 27 cases of ESCC paraffin-embedded samples were collected before radiotherapy which were confirmed by fiber optic esophageal speculum biopsy. The expression of NRAGE protein by the streptavidin-peroxidase (SP) immunohistochemical staining method was detected as described in the instruction of ZSGB-BIO (Beijing, China).

Cytoplasm and nuclear protein extraction

Cells were harvested when growing at 80~ 90 % confluences. Cytoplasm and nuclear protein were extracted as described in the instruction of Vazyme Biotech (Nanjing, China).

Statistical analysis

All experiments were repeated at least three times. All data were presented as means ± SD. To analyze relative cell viability difference after irradiation and the expression difference of NRAGE or β-catenin in different groups, statistical Student's *t* test was applied. Differences were considered significantly existent when $P < 0.05$.

Results

TE13R120 obtained different characteristics from the parental cell line TE13

In morphological distinction, TE13R120 cells were visibly different from the parental cell lines (Fig. 1a). Observed under a microscope, TE13R120 cells displayed more irregular, elongated spindle-shaped, and with the disappearance of polarity compared with TE13.

In addition to morphological distinction, TE13R120 cell line showed significant radiation resistance compared with the parental cell line TE13 with a significantly increased growth rate from MTT assay, enhanced cell viability after irradiated from MTT assay, and increased clonogenic survival from Colony formation assay (Fig. 1b, c). As showed in Fig. 1b, the difference of cell viability generally starts from the third day after irradiation. Then, we further analyzed cell viability difference of the two cells after irradiated with doses of 2, 4, 6, 8, and 10 Gy 3 or 4 days using MTT assay (Fig. 1d). It displayed similar difference that TE13R120 cells had stronger cell viability after irradiation, which further demonstrates the enhanced radioresistance of TE13R120 cells.

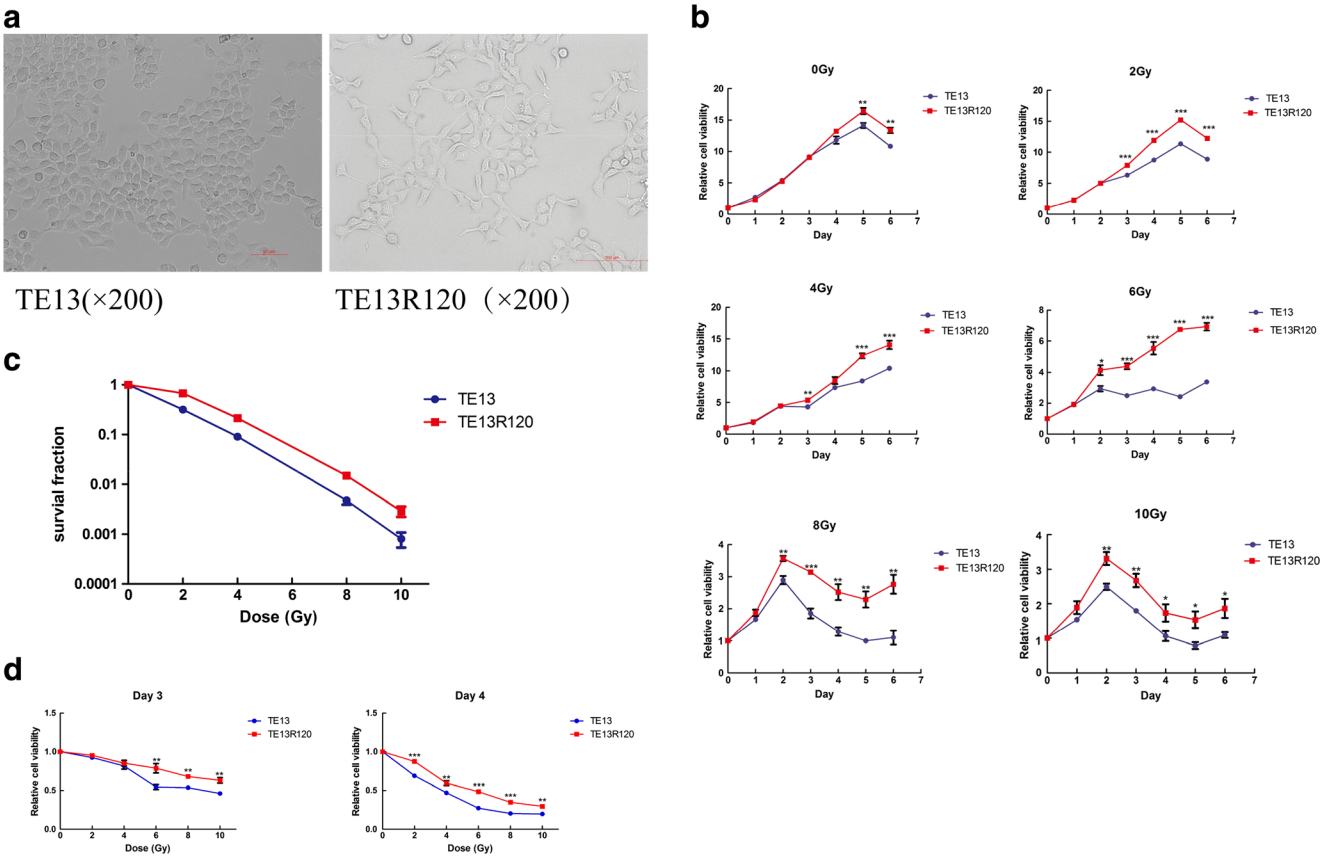

Fig. 1 The characteristics of TE13R120 cells differ from its parental cell line TE13. **a** The TE13R120 cells were morphologically distinct from the parental cell line. **b** MTT assays of TE13R120 cells and the parental cells after treated with doses of 0, 2, 4, 6, 8, and 10 Gy. **c** Clonogenic survival assay of TE13R120 cells and the parental cells. **d** MTT assays of the two cells after irradiated with doses of 2, 4, 6, 8, 10 Gy 3 and 4 days

Upregulation of NRAGE in esophageal cancer cells correlates with their resistance to IR

Previous studies found that human NRAGE was strongly expressed in human esophageal carcinoma radioresistant cell line TE13R120 through cDNA microarray detection and late experimental verification [10, 11], which suggested that NRAGE might play a pivotal role in the formation of radioresistance in esophageal carcinoma cells. We detected the expression of NRAGE in another human esophageal carcinoma radioresistant cell line ECA109R60 (cell morphology and colony formation assay were shown S1 Fig A, B) by real-time PCR and Western blot and found remarkable overexpression of NRAGE in ECA109R60 in comparison with ECA109 (S1 Fig C, D). It hints that NRAGE overexpression extremely likely involved in the IR resistance's acquisition of esophageal cancer cells. To verify the hypothesis, we tested whether knockdown NRAGE by siRNA transfection could enhance the radiosensitivity of TE13R120 or not. Real-time PCR and Western blotting showed that the expression of NRAGE was significantly inhibited in TE13R120 cells treated with siNRG (Fig. 2a, b). Then, we measured the single-cell viability in TE13R120 cells with or without NRAGE depletion by colony-forming assays after irradiation with a single dose (5 Gy) (Fig. 2c, d). The experimental data showed a significant decrease of single-cell viability in TE13R120 cells with NRAGE depletion ($P = 0.0148$). Altogether, these data reveal that overexpression of NRAGE in esophageal cancer cells could confer resistance to IR treatment.

NRAGE expression is clinically relevant to radioresistance of esophagus cancer

To determine if the expression of NRAGE correlates with IR resistance of esophagus cancer in clinic, we tested the expression levels of NRAGE protein in a total of 27 cases of esophageal cancer biopsy specimens by IHC-P. According to the curative effect of radiotherapy, 27 patients was divided into complete response (CR; 15 cases), partial response (PR; 7 cases), no response (NR; 5 cases). The effective ratio is 81.5 % (CR + PR). The density value of NRAGE in the effective group (CR + PR) (0.048 ± 0.002) was dramatically lower than that in the invalid group (NR) (0.065 ± 0.005). The difference was statistically significant ($P = 0.0029$, Fig. 3a–c, Table 2). These results reveal that the high expression of

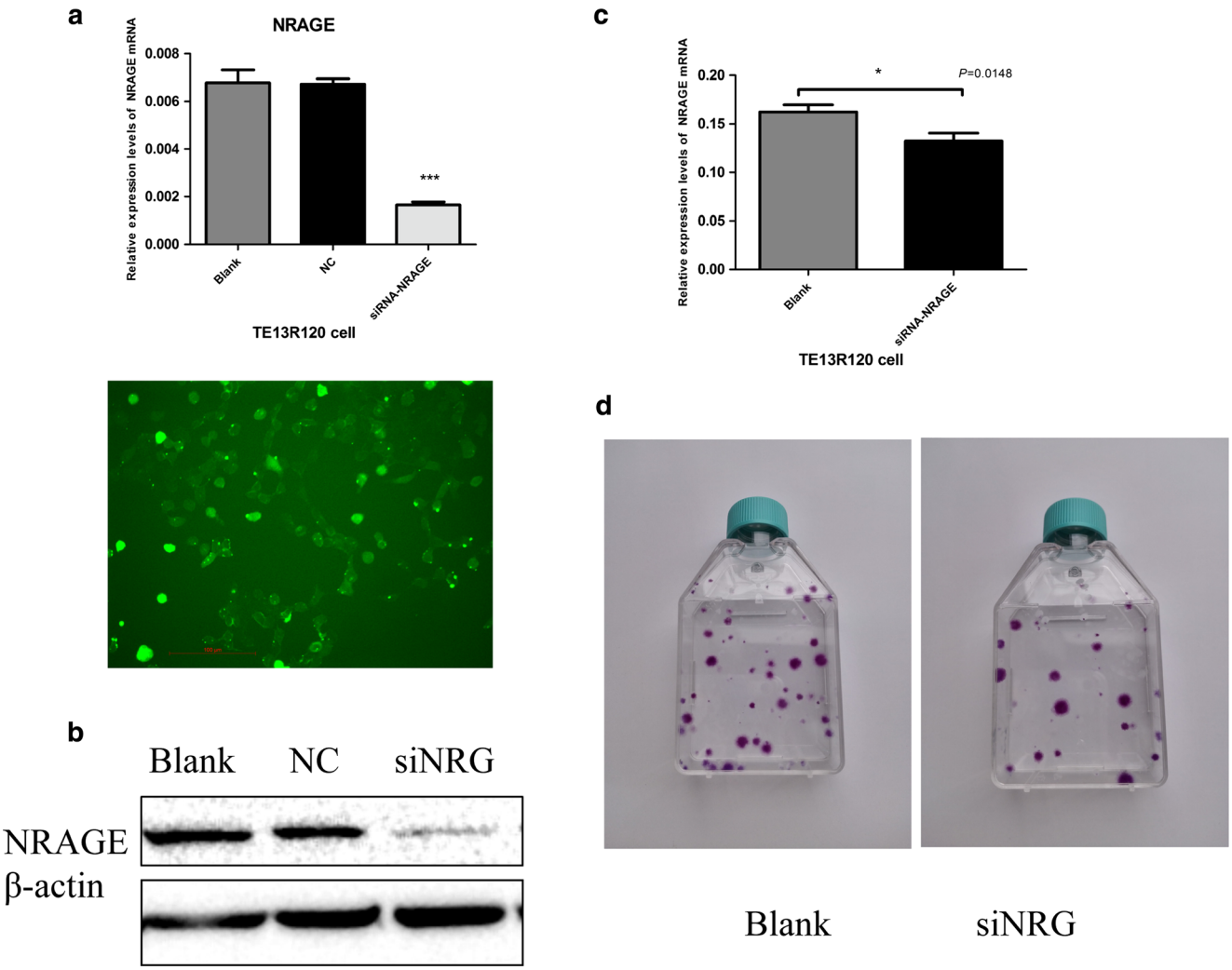

Fig. 2 Overexpression of NRAGE in esophageal cancer cells correlates with their resistance to IR. **a** Real-time PCR and **b** Western blotting analyses of the efficiency of the siRNA against NRAGE was significantly inhibited in TE13R120. **c**, **d** The colony-forming assays of TE13R120 cells and TE13R120 cells with siNRG

NRAGE in esophagus cancer is clinically relevant to IR resistance.

Among the multiple limited factors, changes in cell cycle were one of the chief factors. To determine the effect of NRAGE on cell cycle, we analyzed the phases of cell cycle in TE13 cells, TE13R120 cells, and TE13R120 cells treated with siNGR. In comparison with TE13 cells, TE13R120 cells with NRAGE overexpression had an increasing number of

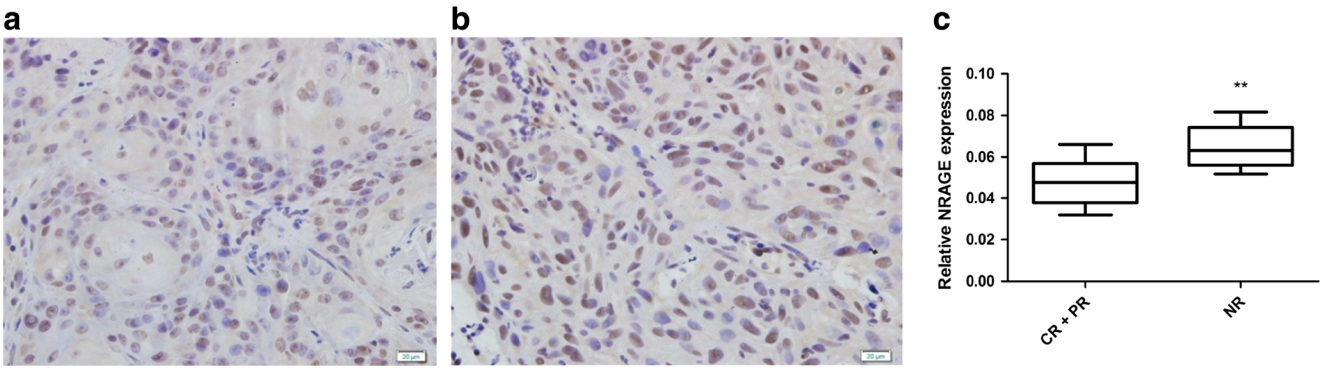

Fig. 3 The association between NRAGE expression and radioresistance is clinically relevant. Representative images (×400) of immunohistochemical analysis of NRAGE protein expression in the **a** effective group and the **b** invalid group. **c** The comparison in the density value of NRAGE in IF between the two groups, $^{**}P < 0.01$

Table 2 Mean density value of NRAGE in the esophageal carcinoma tissue from effective and invalid patients tested by IHC-P

Group	Number	Value	*P*
The effective group	22	0.048 ± 0.002	<0.01
The invalid group	5	0.065 ± 0.005	

NRAGE contributed to enhanced division of human esophageal cancer cells

cells in S phase (TE13 vs TE13R120: 24.33 vs 44.51 %), the most radioresistant cell stage, and a lower ratio in the most radiosensitive cell stage G2/M (TE13 vs TE13R120: 19.66 vs 10.07 %) (Fig. 4a). However, after transfecting the siNRG in TE13R120 cells, the cells in S phase was reduced (NC vs siNRG: 44.32 vs 38.96 %), more of NRAGE downregulated cells were arrested in G2/M phase (NC vs siNRG: 23.35 vs 29.94 %) (Fig. 4b). This indicated that NRAGE contributed to enhanced cell division of human esophageal cancer.

NRAGE displays changes in subcellular localization in human esophageal cancer radioresistant cells TE13R120

Aiming to investigate the possible mechanism of NRAGE in esophageal IR resistance's acquisition, we initially examined the location of NRAGE in the parental cells and radioresistant cells by Western blot and immunofluorescence analysis. It showed a significant excess of protein levels of NRAGE in TE13R120 cells (Fig. 5a, b) and ECA109R60 cells (S2 Fig). Especially, it was much clearer that an enhancement of nuclear distribution occurred in radioresistant cells (Fig. 5b, c, S2 Fig). Taken together, those results suggest that subcellular

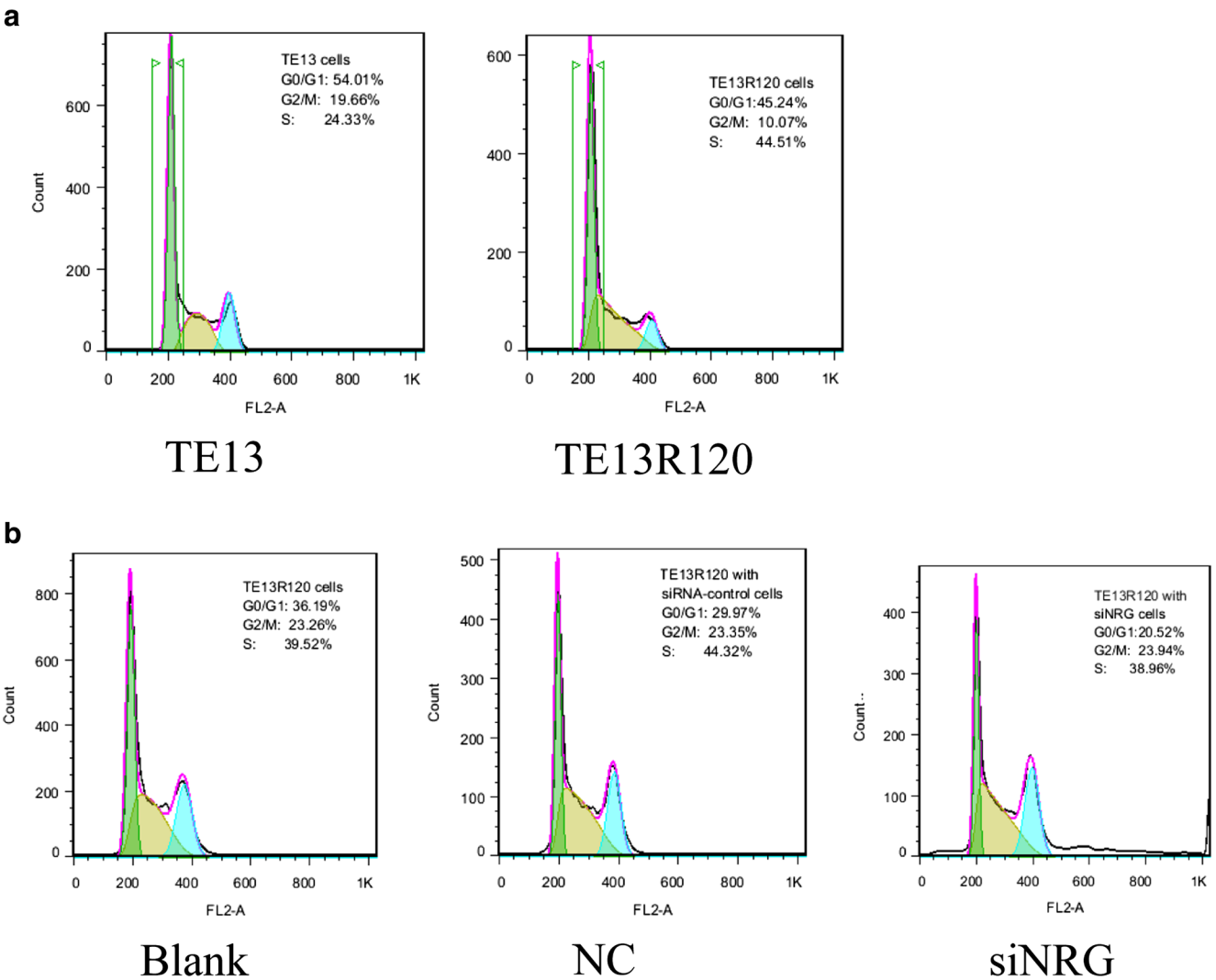

Fig. 4 The influence of NRAGE overexpression on human esophageal cancer cell cycle. **a** Analysis of the difference between TE13 and TE13R120 cells on cell cycle; **b** cell cycle detection of TE13R120 cells transfected with siRNA-NRAGE or siRNA-control by means of flow cytometry

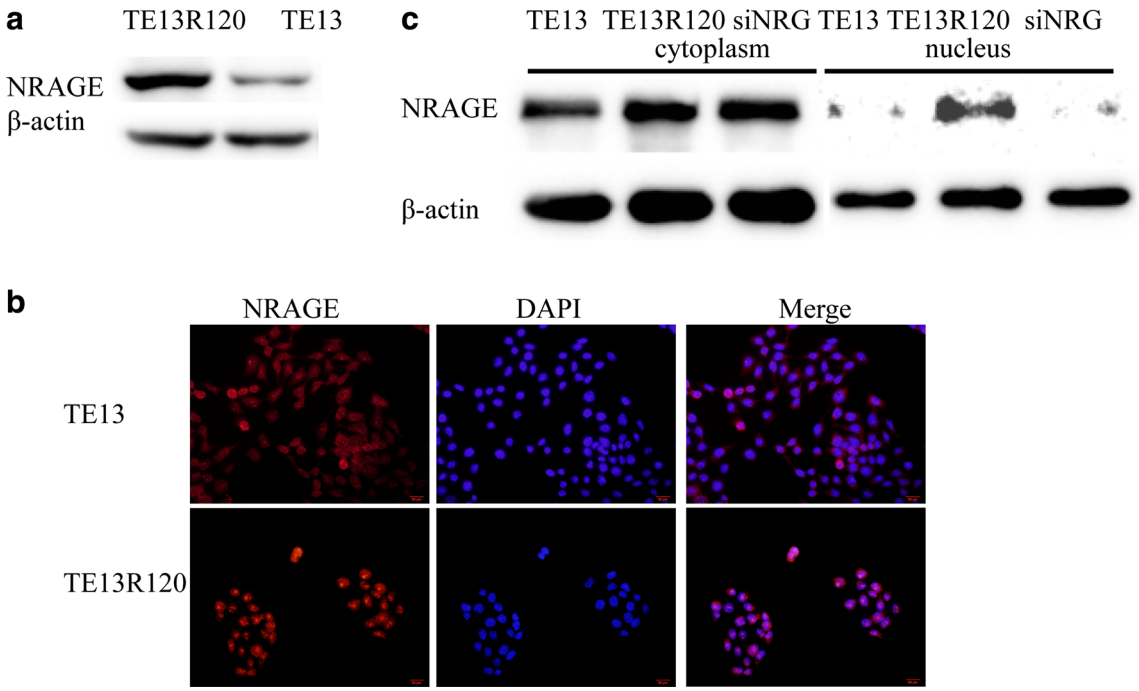

Fig. 5 NRAGE displays the changes in subcellular localization in human esophageal cancer radioresistant cells TE13R120. **a** Western blot showed the difference of NRAGE protein in total protein. **b** TE13 cells and TE13R120 cells were stained by NRAGE antibody. **c** Western blot showed the distribution difference of NRAGE protein cytoplasm and nucleus

localization of NRAGE may be critical to radioresistance of the esophageal cancer cells.

NRAGE affects expression and subcellular localization of β-catenin

Many investigations showed that a close-knit association between radioresistance and Wnt/β-catenin signaling pathway. Li et al. [12] and Su et al. [13] found respectively that based on gene microarray between different parental cells and corresponding radioresistant cells, the classic Wnt/β-catenin signaling pathway was involved in the generation of esophageal cancer cell radiation resistance. Especially, Xue et al. [14] discovered that NRAGE can disrupt E-cadherin/β-catenin to regulate homotypic cell-cell adhesion and induce subcellular localization of β-catenin into cytoplasm and nucleus from cytomembrane in U2OS cells, a human osteosarcoma cell line. Hence, we speculated that NRAGE may affect subcellular localization and expression of β-catenin to regulate esophageal cancer cells radioresistance by Wnt/β-catenin signaling pathway. To verify the hypothesis, we detected the expression of β-catenin using real-time PCR, Western blot, and IF in TE13 and TE13R120 cells. It showed that β-catenin was remarkably increased in both mRNA and protein levels in TE13R120 in comparison with TE13 cells (Fig. 6a–c). Moreover, the evident differential of β-catenin expression was also found between ECA109 and ECA109R60 (S3 Fig A, B, C), which suggests the expression of β-catenin is relevant to human radioresistant esophageal carcinoma cells with overexpression of NRAGE.

The distribution of the β-catenin was mainly in the cell membrane in the parent cell TE13. By contrast, β-catenin was mainly localized in the cytoplasmic and nuclear of TE13R120 cells. Western blot against β-catenin antibody in extracted cytoplasm and nuclear protein of TE13R120 showed the accordant result (Fig. 6e). There was a similar tendency discovered in ECA109R60 cells in comparison with ECA109 cells (Fig. S3 C). These results indicate the ectopic subcellular localization of β-catenin may be relevant to human radioresistant esophageal carcinoma cells with NRAGE overexpression.

To confirm whether the high expression and abnormal subcellular localization of β-catenin in TE13R120 cells are caused by NRAGE or not, we examined β-catenin expression in TE13R120 cells treated with siRNA against NRAGE. After knocking down NRAGE, β-catenin displayed a significant decline both in mRNA and total protein levels (Fig. 6b–d). In addition, there was an evident decrease of cytoplasmic and nuclear protein β-catenin in TE13R120 cells with siNRG (Fig. 6c, e). Taken together, these data show that the upregulation of β-catenin and the changes of its subcellular localization were caused by high expression of NRAGE in TE13R120 cells.

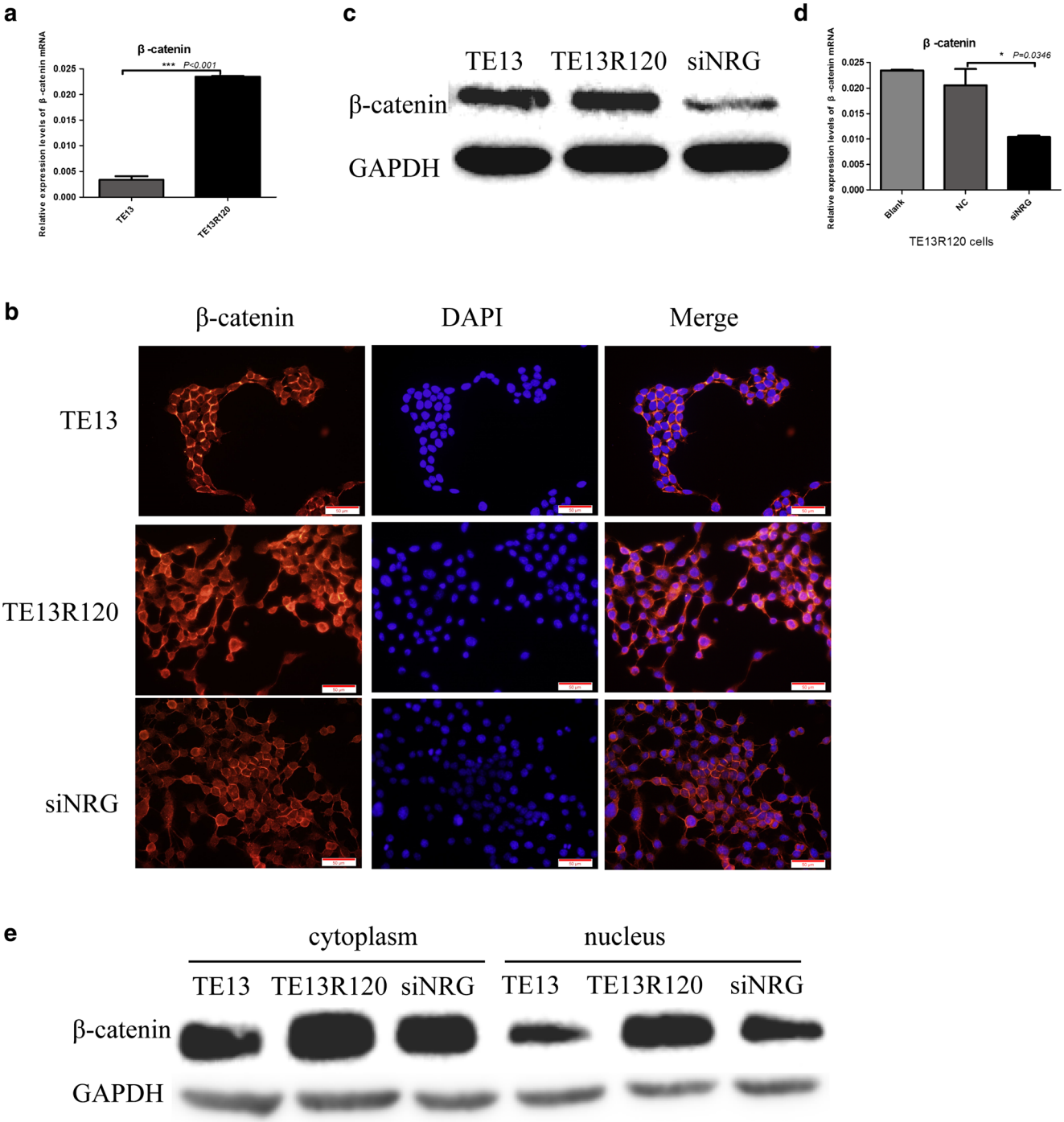

Fig. 6 Human NRAGE affects expression and subcellular localization of β-catenin. **a** The β-catenin mRNA expression detected by Real-time PCR in TE13 and TE13R120 cells. **b**, **c** The changes of NRAGE protein among the three groups: TE13, TE13R120, and TE13R120 with siNRG. **d** Real-time PCR analyzed the β-catenin mRNA expression difference after knocking down the NRAGE in TE13R120 cells. **e** The distribution changes of β-catenin in total, cytoplasm, and nucleus proteins of the three groups: TE13, TE13R120, and TE13R120 with NRAGE deficiency tested by Western blot

Discussion

Radiation therapy is a key component of standard therapy for EC patients. The radioresistance of EC is mainly the restraining factor for improving radiotherapy effect. Here, we show for the first time that NRAGE, as a novel mediator which was found to be highly expressed in IR resistant cells and EC tissues of radiotherapy invalid group, may participate in the formation of resistance to IR in EC cells by changing subcellular localization itself and nucleus distribution of β-catenin.

NRAGE has been reported as a tumor suppressor or a cell apoptosis inducer in nervous system development, cell differentiation, apoptosis, cell cycle regulation, and so on. It can bind to a variety of signaling molecules, including cell surface receptors, such as P75NTR [5], UNC5H1 [7], Ror2 [15], transcription factors, such as Che-1 [6], Dlx/Msx [16], cell apoptosis regulating factors, and protein modification factors, such as XIAP [8], and Praja1 [17]. However, in the researches of Kumar et al. [9] and Yang et al. [4], the NRAGE has been found to be an anti-apoptosis factor and tumor promoter. Kumar et al. [9] showed that after nuclear translocation, NRAGE could interact with TBX2, a member of transcription repressor protein family, to inhibit the expression of tumor-suppressor p14ARF, thereby inhibiting apoptosis, and inducing an aggressive tumor. In Yang's [4] study, NRAGE was discovered to be an oncotarget. Its upregulation could availably accelerate cellular growth through the interaction of its DNAPIII domain with proliferating cell nuclear antigen (PCNA) and post-translationally control PCNA stability via a proteasome-dependent pathway. It is implied that NRAGE could be a bridge among the molecules with complex functions, participating in variable signaling pathways to commit different biological functions.

Recently, studies focused on the mechanism of IR resistance by establishing radioresistant cell lines [18, 19]. In our previous study [10, 11], we established radioresistant esophageal carcinoma cell line TE13R120. Based on the result of gene microarray and subsequent verification, we found that NRAGE was unexpectedly upregulated in the radiation resistance cells of esophageal carcinoma. Here, we firstly compared the characteristics of IR resistant TE13R120 cells with its parental TE13 cells and found TE13R120 cells with more irregular morphology, faster growth, and enhanced radioresistance markedly differed from TE13 cells.

Overexpression of NRAGE was also detected by real-time PCR [10] (S1 Fig C), Western blot (Fig. 5a, S2 Fig A), and IF (Fig 5b, S2 Fig B) in both of IR resistant cells TE13R120 and ECA109. Intriguingly, our data showed that an increasing radiation sensibility occurred in TE13R120 cells when knockdowning NRAGE by transfecting temporarily siNRG via colony formation analysis. Subsequently, we further measured the correlation between expression level of NRAGE and radiotherapy effect in clinic through IHC-P, which manifested a striking enhancement of NRAGE staining in the invalid group than the effective group. Taken together, these findings indicated that the overexpression of NRAGE is a critical factor of the response of esophageal cancer cells to IR. In addition, to investigate the possible mechanisms about radioresistance of esophageal cancer cells induced by NRAGE, we assessed the difference among TE13 cells and TE13R120 cells with or without siNRG on cell cycle by FCM. Consequently, an evident accumulation of S, the most insensitive to IR cell stage, was observed in TE13R120 cells, which can be reversed by NRAGE depletion in TE13R120 cells. The result revealed that overexpression of NRAGE may induce radioresistance of EC cells through influencing cell cycle. In turn, upregulation of NRAGE is a mediator for formation of IR resistance in EC cells.

Takeru et al. [15] held an idea that NRAGE colocalized normally with the cytoplasmic C-terminal region of Ror2, a mammalian Ror family receptor tyrosine kinase in the membranous compartments. When Ror2 was missing, however, NRAGE translocated into nucleus associating with Msx2 and affecting its transcriptional function. In Kumar's research [9], downregulation of Ankrin-G could accelerate nuclear translocation of NRAGE causing the low expression of p14ARF, a tumor suppressor, to reduce apoptosis. Therefore, as a cancer-related gene, NRAGE is orientated differently in the cells under the different stimulus, and then performs diversified biological functions. We speculated that the radiation resistance of TE13R120 cells may be generated by nuclear translocation of NRAGE. Through observing the subcellular localization by IF, we verified the hypothesis that the novel function of NRAGE inducing IR resistance extremely was endowed by the change of subcellular localization in EC cells.

Wnt signaling pathway, an evolutionarily conserved pathway, is of the essence for cell growth, migration, and differentiation, especially for human tumorigenesis [20–22]. Recently, a growing studies focused on the correlation between Wnt signaling pathway, particularly the canonical Wnt pathway, and radiation resistance. Lots of scholars have identified the feasibility of targeting the major components in the pathway to overcome radioresistance as the therapeutic strategy in many human cancers [23–25]. β-catenin, as a signal molecule in genetic transcription, is a key concern of Wnt signaling. Zhang et al. [26] discovered that the nuclear transport of β-catenin was greatly related to the poor prognosis and chemo-/radioresistance of human cervical squamous cell cancer patients. Similar discoveries with nuclear β-catenin overexpression were demonstrated in gliomas [27], breast cancer [28], colon cancer [29], and rectal adenocarcinoma [30]. Interestingly, Su et al. [31] showed an evident correlation between β-catenin and IR resistance of radioresistant esophageal cancer cell line KYSE-150R. Xue et al.[14] has previously pointed out that NRAGE could partly translocate β-catenin into nucleus to disrupt the cell-cell adhesion in a human osteosarcoma cell line U2OS. Furthermore, in Zhang's [26] study, they determined the nuclear localization of β-catenin is related to poor survival and chemo-/radioresistance in human cervical squamous cell cancer. Our data showed that NRAGE not only induced the nucleus translocation of β-catenin but also upregulated the expression in radioresistance ESCC TE13R120 cells, which revealed the β-catenin nuclear expression has a high correlation with the IR resistance of EC, but the clinical verification in EC remains to be done further. Most importantly, accumulation of nuclear β-catenin is a symbol for

activation of the canonical Wnt pathway. Wnt/β-catenin pathway was involved in IR resistance [24, 32]. Taken together, it suggests that the formation of IR resistance in EC may be caused by NRAGE through activating the canonical Wnt pathway, which indicated that NRAGE is extremely involved in the Wnt/β-catenin pathway. Further experiments for verifying the precise mechanism need to be done.

In conclusion, our data demonstrated that NRAGE may serve as a vital mediator of the response of EC cells to IR. Moreover, we found that the role of NRAGE regulating radioresistance in esophagus cancer may be achieved by mediating β-catenin nuclear transport and activating the Wnt/β-catenin pathway. It shows a promise that the clinical application of NRAGE is not only as a valuable predictor of radioresistance but also a novel therapeutic target for improving the radiotherapy efficiency of EC. In addition, we found for the first time that NRAGE nuclear translocation may be the upstream signal of canonical Wnt pathway.

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Compliance with ethical standards

Conflicts of interest None

Ethical approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. And the study was approved by the Ethics Committee of Second Hospital of Hebei Medical University.

Informed consent Informed consent was obtained from all individual participants included in the study.

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