Supplementary Materials and Methods section

ESCC patients

In this study, a total of 140 ESCC patients treated with definitive CRT were consecutively selected from The First Affiliated Hospital of Anhui Medical University (Hefei, China) and Cancer Center of Sun Yat-Sen University (Guangzhou, China) between March 2007 and August 2015. In addition, 25 samples of normal esophageal mucosa were used for controls. The cases selected were based on the following criteria: a) all the cases have reliable biopsy specimens and follow-up date; b) patients without previous treatment, malignant disease or a second primary tumor; c) no previous treatment or severe complications; d) no distant metastases except for supraclavicular or celiac lymph nodes; e) Karnofsky>=70; f) all the samples were endoscopic biopsy specimens obtained before CRT. 14 pairs of cancer tissues and adjacent normal esophageal specimens were snap-frozen in chilled liquid nitrogen and stored at -80℃ until further processing. The study was approved by the medical ethics committee of our institutes.

Chemoradiotherapy

All the 140 patients received the same concurrent chemoradiotherapy with PF (Cisplatin/5-fluorouracil) regimen. Cisplatin was administered as i.v. drip at a dose of 60-80mg/m^2 on days 1 to 2; 5-fluorouracil 3000mg/m^2 was administered as a continuous i.v. infusion for 48 hours on days 1–2. Two cycles of chemotherapy were done during radiotherapy at 4-week intervals.

Radiotherapy was performed by three-dimensional conformal radiotherapy or

intensity-modulated radiotherapy with 6 MV X-ray. The gross tumor volume (GTV) was defined as the primary tumor and positive lymph nodes. The clinical target volume (CTV) included the GTV with a 3-4cm margin in the cephalad and caudal directions, and a radial margin of 0.5-1.0 cm. CTV also comprised the regional lymphatic regions. The planning target volume (PTV) included the CTV with a uniform 0.8cm expansion margin in all directions. A total prescription dose of 60-70Gy was delivered in 1.8-2.0 Gy fractions over 6-7 weeks.

Clinical response evaluation and follow-up

The response to CRT was evaluated clinically for primary lesions based on esophagography, endoscopy and CT two months after CRT according to the following criteria. Complete response (CR) was defined as no evidence of disease on imaging and complete resolution of all assessable lesions by endoscopic biopsy. Partial response (PR) was defined as a 30% or greater reduction in tumor maximum dimension and no progression of assessable lesions. Stable disease (SD) was defined by a reduction by <30% or increase of <20% in tumor size. All these conditions had to last for at least 4 weeks and there was no appearance of new lesions. Progressive disease (PD) was defined as an increase $\geqslant$20% in tumor size or the appearance of new lesions. We divided these categories into two groups: CR and non-CR (PR/SD/PD).

The patients were followed every 3 months for the first year and then every 6 months for the next 2 years and finally annually. The diagnostic examinations consisted of esophagography, CT, chest X-ray, abdominal ultrasonography and bone

scan when necessary to detect recurrence and/or metastasis. The disease-specific survival (DSS) was defined as the time from diagnosis to the date of cancer-related death or when censured at the latest date if patients were still alive.

Immunohistochemistry (IHC) staining

IHC analysis was performed to examine DAB2IP expression levels in ESCC specimens. The staining protocol used in this study has been described previously(1). DAB2IP antibody(1:200 dilution, Abcam) was used in IHC staining. In our study, we divided the cell staining percentage into 10-scale system ranging from 0 to 9; a score of 0 indicated staining was<10%，a score of 1 indicated staining was >10% but ≤ 20%,，a score of 2 indicated staining was >20% but ≤30%, and so forth. Meanwhile we divided the staining intensity into 4 degrees, ranging from 0 to 3, 0 indicated negative, 1 indicated weak intensity, 2 indicated moderate intensity, and 3 indicated strong intensity.

The two individual parameters were added, resulting with total score ranging from 0 to 12. The stained tissue sections were reviewed and scored separately by two pathologists blinded to the clinical parameters. The concordance rate of 88.9% among the evaluated results from the pathologists demonstrated that this scoring method was highly reproducible. The value was selected until at least two pathologists reported consistent results. In cases where scoring was completely different, pathologists worked to reach a consensus on the score.

X-tile

Camp *et al*. (2)previously developed a graphical method, named X-tile plot, which

can present a new tool for the evaluation of biological relationships and discover cut-points based on marker expression. The X-tile plots allows the determination of an optimal cutoff score while correcting for the use of minimum P statistics by Miller-Siegmund P-value correction. According to the X-tile plots, we categorized the samples into low (IHC score ≤5) and high (IHC score >5) expression subgroups based on a cut-point determined by X-tile software related to survival status.

Knocking down of DAB2IP by lentiviral short hairpin RNA (shRNA)

The packaging protocol of shRNA lentivirus was performed as described previously (3) with slight modification. Briefly, the vector pLLU2G (kindly gifted by Professor Peng Xiang, Center for Stem Cell Biology and Tissue Engineering, Sun Yat-Sen University) used in this study was derived from pLL3.7 and contained separate green fluorescent protein (GFP) and short hairpin RNA (shRNA) expression elements, as well as elements required for lentiviral packaging. The target sequences of DAB2IP and control Renilla luciferase (luc) for constructing lentiviral shRNA is 5′-GTAATGTAACTATCTCACCTA-3′(4) and 5′-GTAGCGCGGTGTATTATAC-3′ respectively. The ASK1 shRNA lentiviral particles were obtained from Santa Cruz Biotech (sc-29748-V). Packaging of viruses was performed by transient transfection of 293FT cells with a transfer plasmid and three packaging vectors: pMDLg/pRRE, pRSV-REV and pCMV-VSVG. Seventy-two hours after transfection, the lentiviral particles were collected and filtered, then concentrated by ultracentrifugation at 50000g for 2.5 hours at 4°C. Subsequently, we infected the ESCC cancer cell lines with the lentivirus in a 6-well plate. Four days after infection, the knockdown

efficiency of DAB2IP or ASK1 was examined by Western blotting.

Mammalian expression plasmids construction and transfection

DAB2IP fragment was amplified by PCR and cloned into pcDNA3.1 plasmid. Cells were transfected with pcDNA3.1-DAB2IP or the control plasmid pcDNA3.1 (+) using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. For the establishment of DAB2IP stably expressed Kyse150 ESCC cells, 48 h after transfection, the cells were split at a ratio of 1:10, and subsequently maintained in selective medium containing 250μg/ml of G418 (Invitrogen). After 6 weeks of selection, resistant colonies stably transfected with pcDNA3.1-DAB2IP or pcDNA3.1(+) were pooled for further experiments.

Determination of half maximal inhibitory concentration, IC50

Briefly, cells were seeded in 96-well plates and cultured. Cell viability was determined by A Queous One Solution MTS kit (Promega) according to the manufacturer's instructions, and the absorption was read at 490 nm. Curves were fitted using the GraphPad Prism 5.0 (Graph Pad Software Inc., San Diego, CA, USA) and IC50 was calculated. Data was presented as means ± standard deviation (SD) from three independent experiments.

Annexin V-fluorescein isothiocyanate (FITC) / propidium iodide (PI) apoptosis detected by flow cytometry

To quantify the apoptotic cells, the occurrence of apoptosis was determined by staining cells with both Annexin V-FITC and PI. The apoptosis assay was conducted using the protocol according to manufacturer's instructions (Vazyme Biotech, China).

Each sample was then subjected to analyses by flow cytometry (BD Biosciences, San Jose, CA, USA).

Clonogenic assays after radiation and Linear-Quadratic Model

Kyse150-DAB2IP, EC109-shDAB2IP and their corresponding control cells (Kyse150-vector, EC109-shluc) were seeded in 60 mm dishes with different cell numbers for each dose group (500–8000 cell/dose). At 24 hours after seeding, cells were subjected to radiation (0, 2, 4, 6, 8, or 10 Gy) using a linear accelerator with 6 MV photons (Varian Associates Inc., Palo Alto, California). The field size was 30×30 cm^2 and source surface distance was 100 cm. Then the cells were incubated in complete medium to allow colony growth for a period of 14-21 days. The surviving colonies (>50cells/colony) were stained with Giemsa (Invitrogen) and manually counted. Data from radiation exposure cells were normalized to the untreated cells (scored as 100% colony forming ability). Plating efficiencies and survival fractions were calculated to obtain survival parameters and plot cell survival curves. All experiments were performed in triplicate and data were presented as means ± SD. The radiation survival curves were fitted according to the linear quadratic (LQ) model by GraphPad Prism 5.0 (GraphPad, SanDiego, CA): as Survival Fraction $(SF)=\exp(-\alpha D-\beta D^2)$. We calculated the Sensitization Enhancement Ratio (SER) as follows(5): SER=SF2 of experimental cells/SF2 of the corresponding control cells. SF2 means the surviving fraction at 2Gy (SF2).

Immunofluorescence.

Cells were grown on glass coverslips up to 80% confluency. Then, cells were washed

twice with PBS, fixed in 4% paraformaldehyde and processed for immunofluorescence staining. The cells were double immunostained with mouse anti-human antibody Phospho-Histone H2AX(γ-H2AX) and rabbit anti-human 53BP1 antibody in a humidified chamber overnight at 4°C. Cells were washed with PBS twice and primary antibodies were visualized by DyLight 549 conjugated Goat anti-Mouse IgG and Alexa Fluor 488 conjugated Goat anti-Rabbit IgG. DNA were stained with DAPI. Images were collected on the Olympus FluoView confocal microscopes and analyzed with FV10-ASW viewer software (Olympus, Tokyo, Japan). The number of 53BP1 and γ-H2AX fusion co-positive (yellow) focal lengths were counted at 0h and 36h, respectively, and then statistical analyzed. All experiments were repeated at least three times.

In vivo experiments

We used 6- to 8-week-old female BALB/c nude mice. Kyse150-vector and Kyse150-DAB2IP cells at the concentration of 1×10^6 per site were subcutaneously injected into the right flanks of mice. When xenograft tumors had grown to the volume of 180 mm^3, tumors were treated with 6Gy of radiation. Tumor diameters were measured every 3 days by calipers, and tumor volumes were calculated using the formula (length× $width^2$ /2). All experimental procedures were approved by the Institutional Animal Care and Use Committee.

Immunoprecipitation (IP)

Cells were lysed by lysis buffer (50mM Tris, pH 7.5, 150mM NaCl, 1mM EDTA,1% Triton X-100) containing 1mM sodium orthovanadate (New England Biolabs, Beverly, MA) plus a mixture of protease inhibitor cocktail (Sigma) for 10min on ice, then

sonicated and the cell debris was removed by centrifugation (18000×g for 30 min). Bicinchoninic acid (BCA, Pierce, Rockford, IL) assay was preformed to determined protein concentrations. Cells lysates was precleared by incubation with Protein A/G beads (Sigma) for 1 hour at 4°C to eliminate nonspecific binding. The lysates were then subjected to inmmunoprecipitation overnight at 4°C with indicated antibodies or purified mouse/rabbit immunoglobulin G (Sigma), followed by incubation with protein A/G beads for 2 hours. Precipitates were washed 4 times with a lysis buffer before resuspending with a 2× protein sample buffer, and were boiled for 5 min to release the bound proteins. Subsequently, the proteins were analyzed by Western blot analysis with the indicated antibodies.

Statistical analysis

Statistical analysis was performed with SPSS software (SPSS Standard version 23.0, SPSS, Chicago, IL). The Chi-square test was used to evaluate the relationship between DAB2IP expression and clinicopathological variables. Pearson contingency coefficient (r_p) was used to analyze the relationship between DAB2IP expression and CRT response. Survival curves were plotted by the Kaplan–Meier method and compared by the log-rank test. Multivariate survival analysis was performed on all parameters that were found to be significant on univariate analysis using the Cox regression model. *P*-values of <0.05 were considered as independent influence factors.

Supplementary references

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