Selective nuclear export inhibitor KPT-330 enhances the radiosensitivity of esophageal carcinoma cells

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Abstract. Although the concurrent application of definitive chemoradiation has improved the prognosis of patients with esophageal cancer, resistance to therapy poses a major threat to treatment. The present study aimed to investigate whether the use of KPT-330, a selective inhibitor of nuclear export (SINE), enhances the radiosensitivity of esophageal cancer cells. Immunohistochemical staining assays were employed to evaluate the expression and prognostic significance of chromosome maintenance protein-1 (CRM1) in 111 esophageal carcinoma (ESCA) tissues collected from patients with esophageal squamous cell carcinoma. The data showed that the expression of CRM1 in the ESCA tissues was significantly upregulated compared with that in the normal adjacent tissues. Furthermore, patients with higher CRM1 expression had significantly decreased overall survival compared with those with lower CRM1 expression. The effects of KPT-330 and/or radiation on ECA109 human ESCA cells were also evaluated. KPT-330 suppressed the viability of the ECA109 cells. A colony formation assay demonstrated that a combination of KPT-330 and radiation significantly decreased ECA109 cell proliferation. Flow cytometric analysis showed that KPT-330 increased the arrest of the ECA109 cells at the G_2/M phase and induced apoptosis. In addition, western blotting revealed that the inhibitory effect of KPT-330 on cell viability was associated with the increased expression of p53 and promotion of the nuclear accumulation of the p53 protein. In conclusion, the present study demonstrated that CRM1 expression is associated with the prognosis of patients with ESCA following radiotherapy. The inhibition of CRM1 expression by the SINE inhibitor KPT-330 increases radiosensitivity and is potentially useful in a combination treatment strategy for esophageal cancers.

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Introduction

Esophageal carcinoma (ESCA) is the eighth most frequently occurring cancer and the sixth most common cause of cancer-associated mortality worldwide (1,2). Definitive chemo-radiation therapy has become the standard therapeutic scheme for advanced ESCA (3-5), which has been demonstrated to result in a median survival time of 14 months and 5-year survival rate of 27% (6). However, radio-resistance threatens treatment efficacy and leads to a poor prognosis. The most lethal radiation-induced lesions are DNA double-strand breaks, which can induce cellular DNA damage responses, some of which help cells recover from radiation injury. These responses include cell cycle arrest, DNA repair and the activation of DNA damage sensing and early transduction pathways, and it is considered that these protective DNA damage responses induce tumor-associated radio-resistance (7). It is urgently necessary to identify novel sensitizers that are able to improve the radiosensitivity of the cells.

The appropriate subcellular location of proteins in normal cells defines the physiology and homeostasis of the cells. Chromosome maintenance protein-1 (CRM1) is a nuclear export protein, which has >200 cargo proteins. Most nuclear export molecules act solely as tumor suppressors (8-10). CRM1 in the nucleus binds with RAs-related nuclear protein (Ran)-GTP and cargo proteins to form a triplet-complex that undergoes shuttling via the nuclear pore complex to the cytoplasm (11,12). Ran-GTP is hydrolyzed into Ran-GDP by Ran-GTPase in the cytoplasm, which releases CRM1 and cargo proteins, after which CRM1 returns to the nucleus. It has been shown that CRM1 is often upregulated in hematologic carcinomas and numerous solid tumors (13-15). The increased expression or activation of CRM1 can cause tumor suppressor protein (TSP) dysfunction. The excessive transportation of TSP protein to the cytoplasm triggers its degradation (14,16). p53 is a multifunctional TSP, and its mutations constitute the most common genetic alterations in human tumors. Studies have demonstrated that the p53 protein mainly plays a role in the nucleus and participates in a variety of antitumor processes, including apoptosis, cell cycle arrest and DNA damage repair (17,18). It has been identified that CRM1 is

dysregulated in esophageal squamous cell carcinoma, and the inhibition of CRM1 can disturb the expression of TSPs and inhibit NF-κB activity in esophageal squamous cell carcinoma cell lines (19). Therefore, the proper sub-localization of p53 protein in the cells is important for its efficient antitumor functions. p53 is a nuclear transport cargo protein of CRM1, and its ability to execute tumor suppressor functions normally is closely associated with its nuclear transport (20,21).

The use of a selective inhibitor of nuclear export (SINE) is a novel strategy in the treatment of numerous tumors. KPT-330, also known as selinexor, is a SINE that inhibits CRM1 with high affinity and low toxicity. A previous study reported that KPT-330 inhibits CRM1 and promotes the accumulation of p53 protein in the nucleus of colorectal cancer cells (22). As a result, it has a synergistic therapeutic effect with inhibitors of protein kinases such as BRAF (23). Furthermore, studies have demonstrated that the inhibition of CRM1 by KPT-330 increases the radiosensitivity of rectal cancer and non-small cell lung cancer cells (24,25). The potential mechanism may be associated with the role of KPT-330 as an inhibitor of the accumulation of DNA repair proteins (26). Phase I/II clinical trials of KPT-330 have been carried out in non-Hodgkin's lymphoma (NHL) (27) and other hematological tumors (28). KPT-330 is approved by the FDA and EMA in combination with dexamethasone for the treatment of patients with relapsed and refractory multiple myeloma (29).

Genomic studies on esophageal cancer have demonstrated mutations and the abnormal upregulation of CRM1 (30). The postoperative immunohistochemical staining of pathological sections of esophageal cancer tissue has demonstrated that CRM1 expression is associated with the poor prognosis of esophageal cancer (19,31). However, data on the relationship between CRM1 and the prognosis of patients with esophageal cancer undergoing radical radiotherapy remains scarce. Therefore, the present study investigated the association between CRM1 inhibition and radiosensitivity in esophageal cancer. Furthermore, the study assessed the ability of KPT-330 to increase the radiosensitivity of esophageal cancer cells and explored its sensitization mechanisms.

Materials and methods

Clinical data and patient samples. The data and tissues of 111 patients with esophageal squamous cell carcinoma were collected from the First Affiliated Hospital of China Medical University (Shenyang, China) from January 2009 to December 2012. As the present research was a retrospective study, approval was obtained from the ethics committee of the First Affiliated Hospital of China Medical University (approval no. AF-SOP-07-1.1-01) and the requirement for informed consent from all patients was waived. The enrolled patients were diagnosed with primary esophageal squamous cell carcinoma by pathological examination. The patients had not received any other treatments prior to undergoing definitive radiotherapy or concurrent radio-chemotherapy. Due to the challenges of total surgical excision, samples were collected by endoscopy. In addition, 10 pairs of tumor tissues and adjacent normal tissues (located <5 cm from the cancer tissues and without neoplasm invasiveness) were collected to evaluate the CRM1 expression cut-off point for high and low expression. The clinical and pathological characteristics of these patients were analyzed (Table I).

Bioinformatics analysis. The GEPIA database (http://gepia.cancer-pku.cn/index.html) was used to profile CRM1 expression in 33 malignancies and compare it with that in matched normal tissues in The Cancer Genome Atlas (TCGA) datasets. The transcriptome data of different tumor tissues and matched normal tissues were downloaded from TCGA database (https://cancergenome.nih.gov). The Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/gds/) was utilized to find gene expression datasets for patients with esophageal squamous cell carcinoma (GSE20347, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20347; GSE23400, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23400) (32,33). The CRM1 expression levels between esophageal squamous cell carcinoma and adjacent normal tissues were compared.

Immunohistochemical assays. The tissues collected from the patients were stained using the streptavidin-peroxidase method using a commercially available SP-kit (SP-9001, OriGene Technologies, Inc.). Paraffin-embedded tissues were dewaxed at 65˚C for 4 h and hydrated by passing the tissues through a series of solutions: Xylene I for 20 min, Xylene II for 10 min, 100% ethanol for 10 min, 95% ethanol for 5 min, 80% ethanol for 5 min and 75% ethanol for 5 min at room temperature. Tissues were incubated in 3% hydrogen peroxide at room temperature for 10 min and then incubated with 5% sheep serum (reagent A in the SP-kit) at room temperature for 10 min. The slides were incubated with polyclonal rabbit anti-human CRM1 antibody (1:180; cat. no. ab24189; Abcam) overnight at 4˚C. According to SP kit instructions, the tissues were incubated with biotin-labeled goat anti-rabbit IgG secondary antibody (reagent B in the SP-kit) at 37˚C for 15 min and streptavidin/peroxide complex (reagent C in the SP-kit) at 37˚C for 15 min. Then, immunostaining was developed using a DAB kit (PV-8000, OriGene Technologies, Inc.). An Eclipse Ni microscope (Nikon Corporation) with a CCD camera (Ds-Qi1Mc, Nikon Corporation) was used for imaging.

The percentage of positive cells was evaluated and graded as the % stained cells among the total cells in cancer nests as follows: 0, <1% stained cells; 1, 2-25% stained cells; 2, 26-50% stained cells; 3, 51-75% stained cells; and 4, >75% stained cells. In addition, staining intensity was graded as follows: 0+, no color; 1+, light yellow; 2+, light brown; and 3+, brown. The expression of CRM1 was defined as the arithmetic product of the positive percentage score and intensity grade. A receiver operating characteristic (ROC) curve was built to obtain the cut-off value for the high and low CRM1 expression groups.

Cell lines. The human ESCA cell line ECA109 was obtained from the pathology laboratory of China Medical University. The ECA109 cells were cultured in RPMI-1640 (Hyclone; Cytiva) with 10% fetal bovine serum (Clark Bioscience) in a humidified atmosphere with 5% CO_2 at 37˚C.

Reagents. KPT-330 was purchased from Selleck Chemicals, dissolved in dimethylsulfoxide (DMSO) to a concentration

Table I. Association of the relative expression of CRM1 expression with clinical parameters.

Characteristic	Low CRM1	High CRM1	P-value
Number	49	62	
Sex, n (%)			0.395
Female	5 (4.5)	11 (9.9)	
Male	44 (39.6)	51 (45.9)	
Age, years, n (%)			1.000
≤65	25 (22.5)	32 (28.8)	
>65	24 (21.6)	30 (27.0)	
Tumor site, n (%)			0.269
Cervical esophagus and proximal third of the esophagus	15 (13.5)	15 (13.5)	
Distal third of esophagus and EGJ	13 (11.7)	11 (9.9)	
Middle third of the esophagus	21 (18.9)	36 (32.4)	
Tumor diameter, cm, n (%)			0.192
>5 to ≤7	8 (7.2)	19 (17.1)	
>7	15 (13.5)	18 (16.2)	
≤5	26 (23.4)	25 (22.5)	
Radiation dose, Gy, n (%)			0.468
60	21 (18.9)	32 (28.8)	
66	28 (25.2)	30 (27.0)	
Therapeutic method, n (%)			0.283
Concurrent chemoradiation therapy	19 (17.1)	24 (21.6)	
Radiation therapy alone	28 (25.2)	30 (27.0)	
Sequential chemoradiation therapy	2 (1.8)	8 (7.2)	
T stage, n (%)			0.891
T1	1 (0.9)	2 (1.8)	
T2	5 (4.5)	8 (7.2)	
T3	10 (9.0)	9 (8.1)	
T4	33 (29.7)	43 (38.7)	
N stage, n (%)			0.450
N0	19 (17.1)	19 (17.1)	
N1	29 (26.1)	39 (35.1)	
N2	1 (0.9)	4 (3.6)	
Pathologic stage, n (%)			0.876
Stage I	4 (3.6)	4 (3.6)	
Stage II	8 (7.2)	12 (10.8)	
Stage III	37 (33.3)	46 (41.4)	
Survival status, n (%)			0.560
Dead	39 (35.1)	52 (46.8)	
Alive	10 (9.0)	10 (9.0)	

Indices in the low and high CRM1 expression groups were analyzed by Chi-square test, with the exception of T stage, N stage and pathologic stage which have >20% of expected counts <5 and were analyzed using Fisher's exact test. CRM1, chromosome maintenance protein-1; EGJ, esophagogastric junction.

of 1 mmol/l and stored at -80˚C. The working solution was diluted in RPMI-1640.

Western blot analysis. The cells were treated with specific concentrations (0.1 and 0.3 μmol/l) of KPT-330 for 12 h at 37˚C and then subjected to a 0- or 4-Gy dose of radiation at a rate of 300 cGy/min using a Siemens Accelerator (Siemens AG). After 24 h, the cells were harvested and washed twice in phosphate-buffered saline. The cells were then lysed in RIPA lysis buffer (Beyotime Institue of Biotechnology) for 30 min on ice and centrifuged at 13,618 x g for 15 min at 4˚C to collect the total protein extract. Nuclear and cytoplasmic proteins were separated using a Nuclear and Cytoplasm Protein Extraction Kit (cat. no. P0028; Beyotime Institute of Biotechnology)

according to the manufacturer's instructions. The proteins from each sample were quantified and normalized using a bicinchoninic acid protein assay.

The proteins (10 μg/lane) were separated by 6-12% SDS-PAGE gel and blotted onto a PVDF membrane. Each membrane was then blocked with 5% non-fat milk at room temperature for 1 h and incubated with primary antibodies targeting CRM1 (dilution 1:180; ab24189; Abcam), lamin a/c (dilution 1:10,000; ab133256; Abcam), β-actin (dilution 1:1,000; 3700; Cell Signaling Technology, Inc.), p53 (dilution 1:1,000; sc-126; Santa Cruz Biotechnology, Inc.) and GAPDH (dilution 1:1,000; sc-47724; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. Thereafter, the blots were washed three times with Tris-buffered saline with 0.5% Tween-20, peroxidase conjugated goat or rabbit IgG antibody (1:5,000, cat. nos. ab6721 and ab6728, Abcam) were used as secondary antibodies and incubated at room temperature for 1 h. Finally, chemiluminescent working solution (cat. no. P0018A, Beyotime Institute of Biotechnology) was introduced to the membrane, the membrane was exposed using Tanon-5200 ECL film for 1-30 min.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. Cells were seeded into 96-well plates at a density of $4x10^3$ cells/well and incubated overnight until attachment occurred. The cells were then treated with increasing concentrations of KPT-330 (0.01-50 μmol/l) or with the DMSO vehicle as the control. After incubation for 72 h at 37˚C, 20 μl 1 μg/ml MTT was added to each well and the plate was incubated for another 4 h at 37˚C. Thereafter, all the solution was aspirated and 150 μl DMSO was added for 15 min to dissolve the purple formazan crystals. The optical density (OD) at 570 nm was detected using a microplate reader (Bio-Rad Laboratories, Inc.). Cell viability was calculated using the following formula: Cell viability (%)=OD (experimental group)/OD (control group) x100. The half-maximal inhibitory concentration (IC_{50}) values of KPT-330 were obtained using GraphPad Prism 5.0 (GraphPad Software; Dotmatics).

Colony formation assay. The cell survival fraction (SF) was calculated and cell survival curves were delineated for cells treated with KPT-330 and/or radiation. Cells ($4x10^3$ per well) were seeded in 6-well plates and incubated until cell attachment occurred. The cells were pretreated with KPT-330 (0.1 μmol/l) for 12 h at 37˚C followed by irradiation with different doses of radiation (0, 2, 4, 6 and 8 Gy) according to the aforementioned method for 24 h. Cells without KPT-330 treatment and subjected to these radiation doses served as controls. The incubation of the cells was continued for 10-14 days until colonies formed. Thereafter, the cell colonies were stained with 0.1% crystal violet. The plating efficiency (PE) was calculated as follows: PE (%)=(number of colonies/number of plated cells) x100. The SF was calculated as follows: SF=experimental group colonies ratio/PE. Survival curves were plotted using the equation: $SF=1-[1-e(-k*D)]^N$ (34). For each experiment, K was the slope of the straight part of cell survival curve, the lethal dose D_0 was the inverse of K ($D_0=1/K$), N was the section of the cell survival curve after the extension of the straight line intersected the ordinate. Radiation sensitivity enhancement ratio (SER) is the ratio of D_0(radiation group)/D_0(KPT-330+radiation) and SF at 2 Gy (SF2) were calculated.

Apoptosis assay. Cells were pretreated with 0.1 or 0.3 μmol/l KPT-330 for 12 h at 37˚C and then treated with 0 or 4 Gy radiotherapy. After 48 h, the cells were harvested, 5 μl Annexin V and 5 μl propidium iodide (PI) from a cell apoptosis detection kit (cat. no. KGA107, Nanjing KeyGen Biotech Co., Ltd.) were added, and the cells were incubated for 15 min at room temperature in darkness. The proportion of apoptotic cells was then analyzed using a FACSAria flow cytometer (BD Biosciences) and FlowJo (version, 7.6; FlowJo, LLC.).

Cell cycle assay. To determine whether KPT-330 alone or in combination with radiation therapy influenced the cell cycle distribution of the cells, the ECA109 cells were treated with KPT-330 (0.1 or 0.3 μmol/l) for 12 h at 37˚C prior to irradiation with 4 Gy. Cells were also treated with KPT-330 alone, without irradiation. After 24 h, the cell cycle was assessed by flow cytometry (according to the aforementioned method) using a cell cycle analysis kit (cat. no. DKW41-CCK-010, Dakewe Biotech Co., Ltd.).

Statistical analysis. Continuous variables are presented as the mean ± standard deviation (SD). Paired t-tests were used to identify differences in matched tumor/normal sample expression. Survival analysis was performed using Kaplan-Meier survival curves. The significance of the survival differences between high- and low-CRM1 expression groups was assessed with the log-rank test. Univariate and multivariate analyses of the risk factors for OS were performed using the log-rank test and Cox proportional hazards model, respectively. Multiple groups were analyzed using two-way ANOVA followed by Bonferroni's post-hoc test using GraphPad Prism software. $P<0.05$ was considered to indicate a statistically significant result. The experiments were performed in triplicates.

Results

CRM1 is universally upregulated in human cancers. To characterize the expression of CRM1 in tumor tissues, GEPIA was used to compare CRM1 expression in 33 malignancies with that in matched normal tissues in datasets from TCGA. Results from the GEPIA analysis showed the upregulation of CRM1 protein in six cancer types, including cholangiocarcinoma, ESCA, pancreatic adenocarcinoma, sarcoma, stomach adenocarcinoma and thymoma ($P<0.05$; Fig. 1).

Upregulation of CRM1 is associated with poor patient survival in esophageal squamous cell carcinoma. Differential CRM1 expression in esophageal squamous cell carcinoma was validated in two GEO datasets. The analysis demonstrated significant upregulation of CRM1 in the tumor tissues compared with the adjacent normal tissues in the GSE20347 ($P=1.53x10^{-5}$) and GSE23400 ($P=1.23x10^{-13}$) datasets (Fig. 2A and B). To explore the expression of CRM1 protein, immunohistochemical analysis was performed in 10 pairs of esophageal squamous cell carcinoma and matched adjacent tissues. CRM1 expression was detected in the tumor and normal tissues. However, in the normal tissues, CRM1

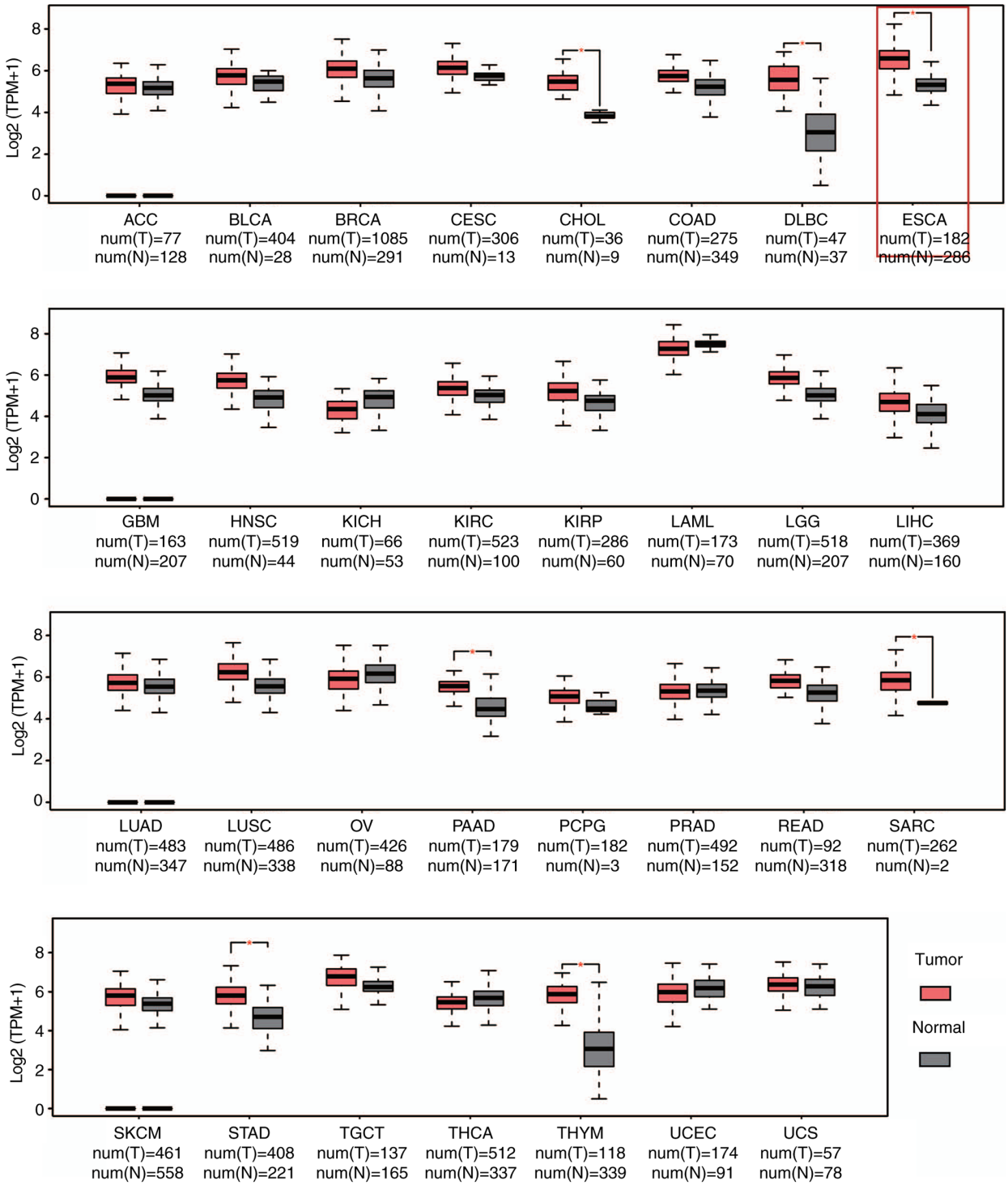

Figure 1. CRM1 is upregulated at the transcriptional level in various cancers according to the GEPIA database. GEPIA analysis showed that CRM1 was significantly elevated in six types of cancer as compared with the respective normal tissue. *P<0.05. CRM1, chromosome maintenance protein-1; ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; T, tumor; N, normal; TPM, transcripts per million.

protein was localized in the nucleus (Fig. 2C), while in the tumor tissues, it was distributed in the nucleus and cytoplasm (Fig. 2D).

Based on CRM1 staining in the 10 pairs of esophageal squamous cell carcinoma and adjacent tissues, a ROC curve was constructed to determine the cut-off point for the

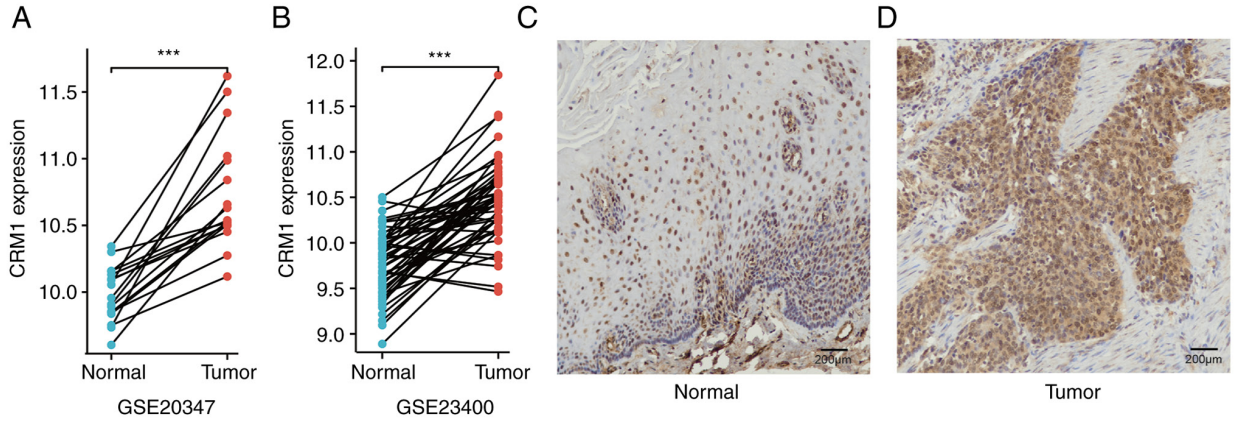

Figure 2. Comparison of CRM1 expression in esophageal squamous cell carcinoma and normal tissue. CRM1 was upregulated at the transcriptional level in the Gene Expression Omnibus cohorts (A) GSE20347 and (B) GSE23400. ***P<0.001. Representative images of the immunohistochemical staining of CRM1 in (C) normal tissue adjacent to esophageal squamous cell carcinoma tissue and (D) esophageal squamous cell carcinoma tissue. Scale bar, 200 μm. CRM1, chromosome maintenance protein-1.

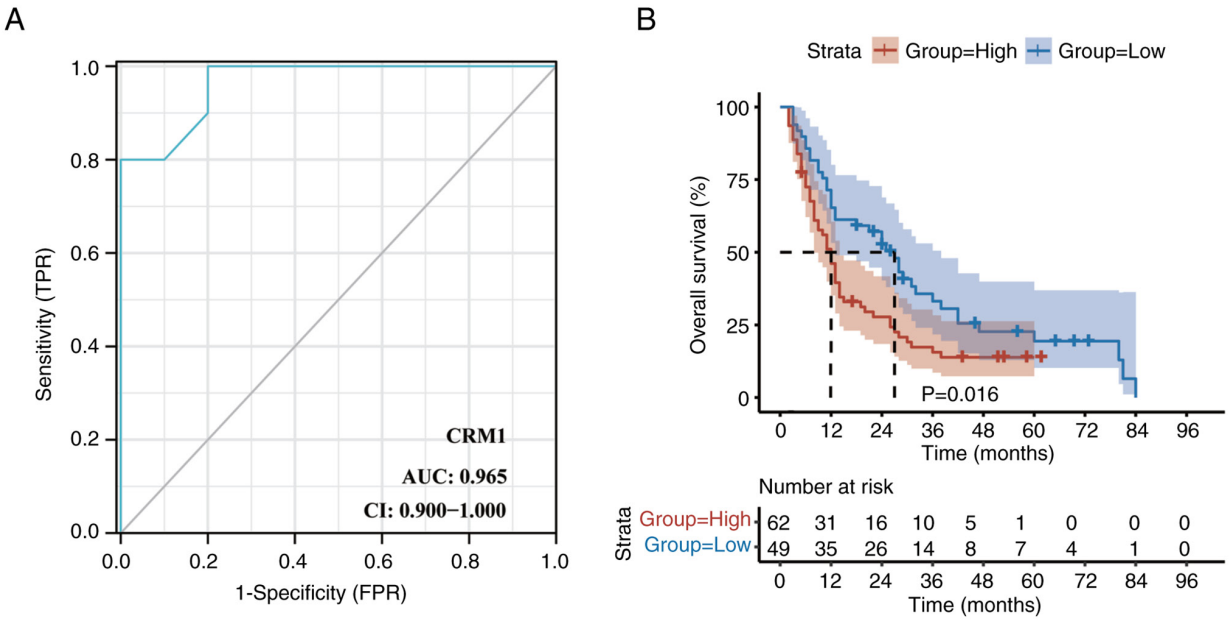

Figure 3. Effect of CRM1 expression on the prognosis of patients with esophageal squamous cell carcinoma. (A) Receiver operating characteristic curve for CRM1 expression in 10 pairs of esophageal squamous cell carcinoma and adjacent tissues. (B) Kaplan-Meier survival analysis of the effect of CRM1 expression on the overall survival of the patients. CRM1, chromosome maintenance protein-1; high, high CRM1 expression; low, low CRM1 expression; AUC, area under the curve; TPR, true positive rate; FPR, false positive rate.

immunohistochemical staining score (Fig. 3A). A cut-off point of 4.5 provided the maximum Youden index, with a specificity and sensitivity of 100 and 80%, respectively (area under the ROC curve, 0.965; P<0.05). Therefore, a cut-off point of 4.5 was defined as the threshold for distinguishing between the high- and low-expression states of CRM1. High expression of CRM1 was detected in 62/111 (55.86%) of the esophageal squamous cell carcinoma specimens. The basic clinicopathological characteristics of the 111 patients are presented in Table I. The analysis demonstrated that the CRM1 expression was not associated with sex, age, clinical stage, the diameter of the tumor or the tumor site (P>0.05).

In addition, Kaplan-Meier analysis revealed that the high expression of CRM1 was significantly associated with poor OS (P=0.016; Fig. 3B). The results of univariate analyses indicated that the OS of the patients was significantly influenced by sex, therapeutic method, radiation dose and CRM1 expression (P<0.05; Table II). In addition, multivariate analyses showed that the OS was significantly associated with gender, CRM1 expression, clinical stage and therapeutic method (P<0.05; Table II). Patients with high CRM1 expression had a 2.38-fold increased risk of death compared with those with low CRM1 expression.

KPT-330 inhibits cell viability. The aforementioned results demonstrate that the CRM1 protein is upregulated in ESCA. To evaluate whether KPT-330 inhibits cell viability, ECA109 cancer cells were incubated with various concentrations of KPT-330 in RPMI-1640 for 72 h. The inhibitory effect of the treatment on cell viability was then assessed using an MTT assay. The data showed a reduction in cell viability as the

Table II. Univariate and multivariate analysis of the risk factors for overall survival in patients with esophageal squamous cell carcinoma.

	Univariate analysis		Multivariate analysis	
Variable	HR	P-value	HR	P-value
Age, years				
≤65	Reference			
>65	0.96 (0.63, 1.46)	0.85		
Sex				
Female	Reference		Reference	
Male	2.22 (1.14, 4.31)	0.02	3.10 (1.39, 6.95)	0.01
Tumor site				
Upper	Reference			
Middle	1.30 (0.77, 2.17)	0.33		
Distal	1.03 (0.55, 1.94)	0.92		
Diameter of the tumor, cm				
>5 to ≤7	Reference			
>7	0.87 (0.50, 1.54)	0.64		
≤5	0.66 (0.39, 1.09)	0.11		
T stage				
T1	Reference			
T2	1.27 (0.27, 5.98)	0.76		
T3	1.44 (0.33, 6.36)	0.63		
T4	2.58 (0.63, 10.57)	0.19		
N stage				
N0	Reference			
N1	1.58 (1.00, 2.49)	0.05		
N2	0.44 (0.10, 1.85)	0.26		
Stage				
Stage I	Reference		Reference	
Stage II	2.10 (0.69, 6.40)	0.19	1.62 (0.53, 4.99)	0.40
Stage III	3.23 (1.18, 8.88)	0.02	2.88 (1.04, 8.01)	0.04
Therapeutic method				
Concurrent chemoradiation therapy	Reference		Reference	
Radiation therapy alone	1.78 (1.14, 2.80)	0.01	2.15 (1.34, 3.43)	<0.01
Sequential chemoradiation therapy	1.49 (0.68, 3.25)	0.31	2.09 (0.83, 5.28)	0.12
Radiation dose, Gy				
54-60	Reference		Reference	
>60	1.66 (1.09, 2.54)	0.02	NA	NA
CRM1 expression				
High	Reference		Reference	
Low	0.59 (0.39, 0.91)	0.02	0.42 (0.26, 0.67)	<0.01

Univariate analysis and multivariate analysis were applied to the risk factors for overall survival using the log-rank and Cox proportional hazards models. CRM1, chromosome maintenance protein-1.

concentration of KPT-330 increased. The IC_{50} of KPT-330 in the ECA109 cell line was 0.9 μmol/l (Fig. 4A).

Combination of KPT-330 and radiation suppresses cell proliferation and decreases the colony formation ability of ECA109 cells. To evaluate our hypothesis that the CRM1 inhibitor is able to suppress cell proliferation and increase their radiation sensitivity, 0.1 μmol/l KPT-330 was used in combination with radiation to treat the ECA109 cells in a colony formation assay. Cells treated with radiation alone served as controls. Images of the plates were captured and cell SF curves were constructed (Fig. 4B and C). Radiobiological

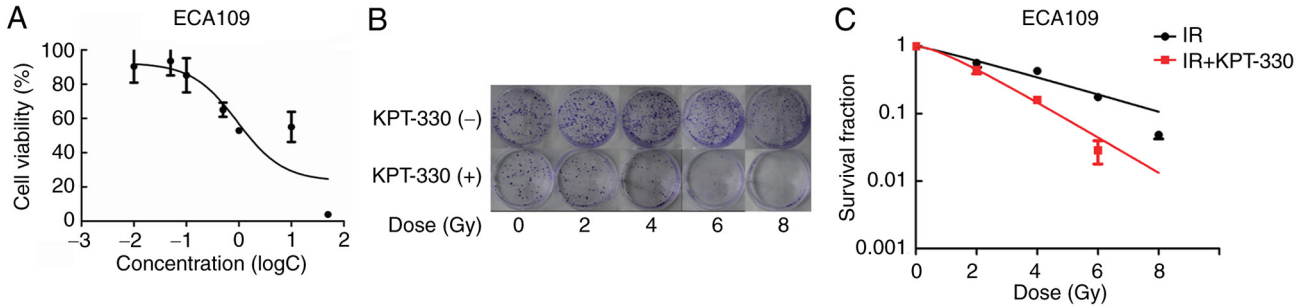

Figure 4. Effect of the CRM1 inhibitor KPT-330 on the viability of ECA109 esophageal carcinoma cells. (A) KPT-330 decreased cell viability and its inhibitory effect increased as its concentration increased. (B) Representative images from a colony formation assay in which the ECA109 cell line was pretreated with KPT-330 (0.1 μmol/l) prior to the application of radiation (0, 2, 4, 6 and 8 Gy) or treatment with radiation alone for 24 h. (C) Survival curves for IR with and without KPT-330. KPT-330 decreased proliferation and increased radiosensitivity. CRM1, chromosome maintenance protein-1; IR, irradiation.

parameters (Table III) were also obtained. The mean D_0 was 3.36 Gy for the irradiation group and 1.65 Gy for the combined KPT-330 and irradiation group, while the SF2 values were 56.71 and 44.89%, respectively. The SER was 2.04. Due to the insensitivity of the ECA109 cell line to radiotherapy, relatively small doses of radiotherapy including 2, 4 and 6 Gy were not able reach a favorable treatment outcome. However, following the application of KPT-330, the number of colonies formed by the ECA109 cells was markedly reduced, which may indicate an increase in the sensitivity of the ECA109 cell line to radiotherapy. Thus, the data demonstrated an improved inhibitory effect on proliferation and lower SF in the cells treated with a combination of KPT-330 and irradiation compared with those treated with radiation alone.

KPT-330 in combination with radiation therapy induces apoptosis in ESCA cells. To assess the combined effect of KPT-330 and radiation in the induction of apoptosis, ECA109 cells were pretreated with KPT-330 for 12 h prior to being subjected to irradiation. After 48 h the ECA109 cells were stained with Annexin-V and PI, and apoptosis was analyzed using flow cytometry. The results illustrated that the ECA109 cells were sensitive to the pro-apoptosis effect of KPT-330 to a certain extent, especially when used in combination with radiation therapy (Fig. 5A and B); however, the effect of KPT-330 was not statistically significant.

KPT-330 arrests the cell cycle in the G_2/M phase. To determine the mechanisms by which the inhibition of cell growth occurred, changes in the cell cycle after treatment with KPT-330 alone or in combination with irradiation were evaluated in the ECA109 ESCA cells. The cell cycle was evaluated using flow cytometry. KPT-330 induced cell cycle arrest and increased the proportion of cells in the G_2/M phase in the ECA109 cell line ($P<0.05$; Fig. 5C and D), The cell cycle distribution of cells treated with a combination of radiation and KPT-330 was significantly different from that of cells treated with radiation alone ($P<0.01$).

KPT-330 decreases CRM1 expression and increases p53 expression. To elucidate the mechanism of apoptosis and cell cycle arrest in the ECA109 cell line, western blot assays were used to evaluate the CRM1-p53 signaling pathway in the whole cell extracts. The results showed that CRM1 was highly expressed in the ESCA cells and was downregulated following treatment with KPT-330, while p53 was upregulated, particularly in the 0.3 μmol/l KPT-330 group ($P<0.01$). Upregulation of p53 expression was also observed in the radiation plus KPT-330 combination group compared with radiation alone ($P<0.05$), but the upregulation was not significantly different from that achieved using KPT-330 alone (Fig. 6).

KPT-330 plus radiation induces p53 nuclear accumulation. Since CRM1 is a nuclear export protein, the nuclear and cytoplasmic distribution of the known CRM1 cargo protein p53 was evaluated. KPT-330 significantly induced the nuclear accumulation of p53 ($P<0.05$) as the concentration of KPT-330 increased, especially in the combination group. This effect was more obvious when ECA109 cells were treated with radiation and KPT-330 (Fig. 7). Although cytoplasm p53 was also increased, p53 high-expression and its nuclear location could perform its function. Nuclear p53 plays a tumor suppressor role and participates in apoptosis and cell cycle control (17,20,35).

Discussion

CRM1 is a member of the surface factor β family of nuclear transport receptor karyopherins and the only nuclear export protein of most TSPs (36-38). CRM1 causes a variety of TSPs such as p53, p21 and FOXO1 to be transported to the cytoplasm in excessive quantities, resulting in the degradation of the TSPs. In the present study, the upregulation of CRM1 in ESCA tissues was identified, and high expression levels of CRM1 were found to be associated with decreased OS. In addition, the study demonstrated that the application of the CRM1 inhibitor KPT-330 increased the radiosensitivity of ECA109 cells, indicating that this may be regarded as an alternative treatment approach for patients with esophageal cancers.

The upregulation of CRM1 has been reported in several types of solid tumor. For example, it was reported that the high expression of CRM1 has a positive correlation with serum CEA and CA19-9, and CRM1 protein expression is an independent prognostic factor for OS and progression-free survival in patients with pancreatic cancer (39). In addition, Liu *et al* demonstrated that the expression of CRM1 was significantly increased in glioma tissues and associated with the poor prognosis of glioma (40). Furthermore, a previous study reported that patients with gastric cancer and high CRM1 expression

Table III. Radiobiological parameters of ECA109 cells in the IR and IR + KPT-330 treatment groups.

	K value	N value	D_0 (Gy)	SER	SF_2 (%)
IR	0.2977	1.169	3.36	-	56.71
IR + KPT-330	0.6063	1.705	1.65	2.04	44.89

IR, irradiation; K value, the slope of the straight part of cell survival curve; N value, the section of the cell survival curve after the extension of the straight line intersects the ordinate. D_0, lethal dose; SER, radiation sensitivity enhancement ratio; SF_2, survival fraction at 2 Gy.

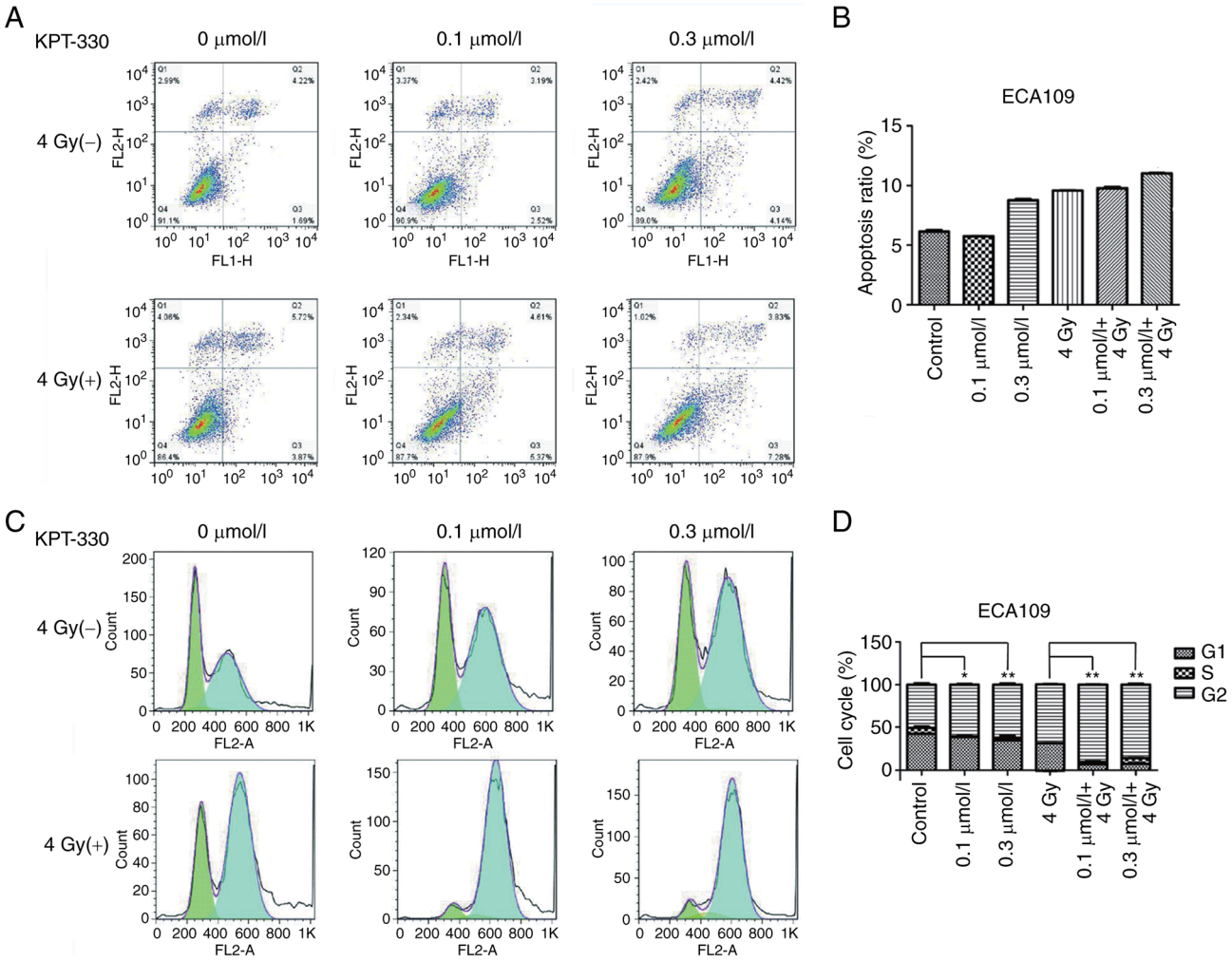

Figure 5. KPT-330 increased the apoptosis of ECA109 cells and arrested the cell cycle at the G_2/M phase. (A) Flow cytometry plots showing the apoptosis level in six treatment groups 48 h after treatment: KPT-330 (0, 0.1 and 0.3 μmol/l) with or without radiation treatment. (B) Quantitative analysis showing the apoptosis level in the six groups. Images are representative of three independent experiments. (C) Flow cytometry plots showing the cell cycle distribution of the six groups 24 h after treatment. (D) Quantitative analysis showing the cell cycle data. Images are representative of three independent experiments. *P<0.05; **P<0.01. Control, untreated cells.

have poor postoperative prognoses (41). The results of the present study demonstrated that the upregulation of CRM1 expression is associated with poor survival in patients with esophageal squamous cell carcinoma. In line with the present study, a study by Yang *et al* (19) demonstrated that increased expression of CRM1 in esophageal cancer is associated with a poor postoperative prognosis. This previous study also found that deletion of the CRM1 gene in ECA109 cells increased 5-fluorouracil-induced cell apoptosis, while also increasing the expression levels of c-poly(ADP-ribose) polymerase and caspase-3. Collectively, these findings indicate that CRM1 may play a key role in the resistance of tumors, and result in a poor prognosis.

KPT-330 is the most studied and widely-applied CRM1 inhibitor. Compared with leptomycin B, the first CRM1 inhibitor, KPT-330 has been shown to be less toxic and have improved tolerability (42). However, whether KPT-330 synergizes with radiation and its underlying mechanisms are yet to be studied. Radiation resistance is a major problem in advanced ESCA, which is mainly mediated by mechanisms

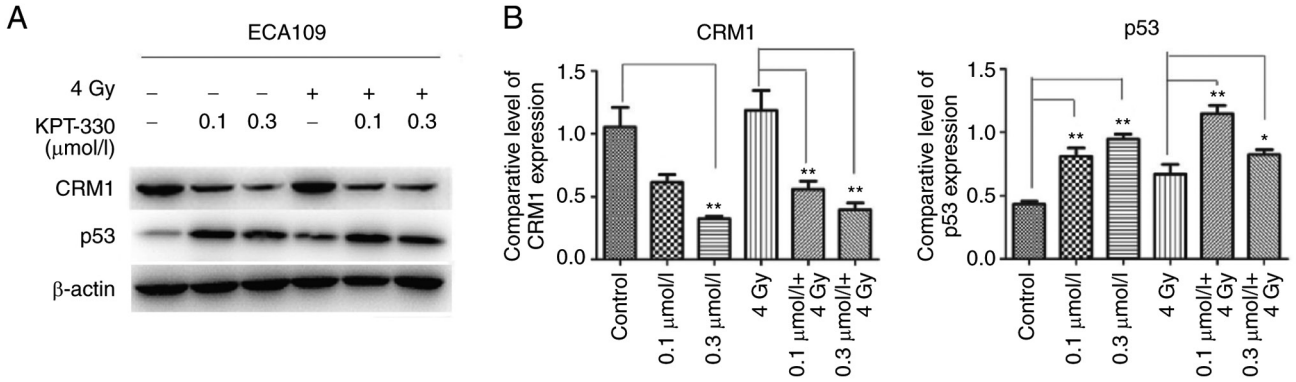

Figure 6. Expression of CRM1 and p53 in ECA109 cells after exposure to various treatments. (A) Representative western blots for cells treated with KPT-330 (0, 0.1 and 0.3 μmol/l) with or without radiation. (B) Comparison of CRM1 and p53 expression among the six groups. All experiments were conducted three times independently. *P<0.05; **P<0.01. CRM1, chromosome maintenance protein-1; control, untreated cells.

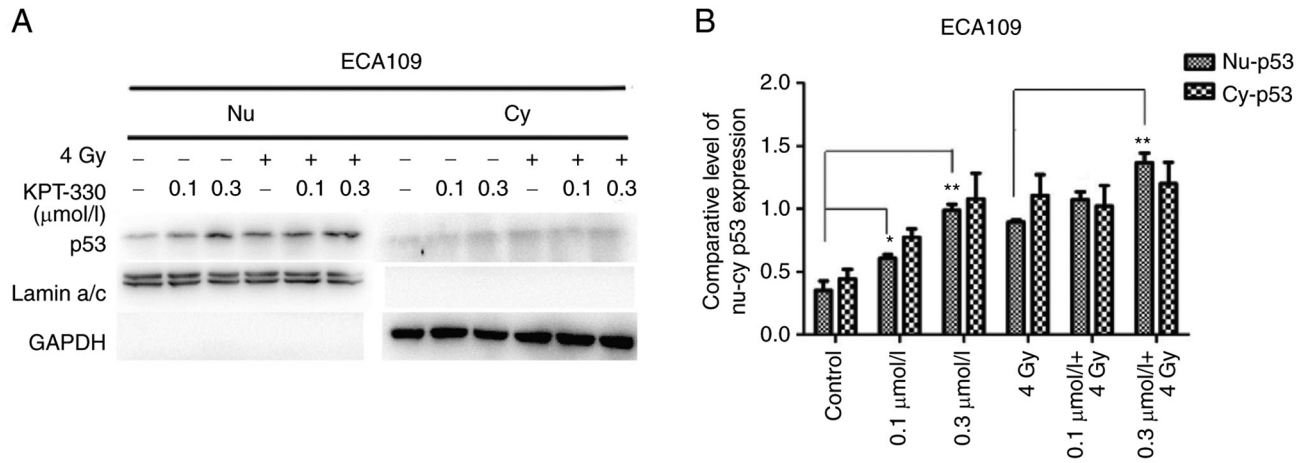

Figure 7. Nuclear and cytoplasmic expression of p53 in ECA109 cells. (A) ECA109 cells were treated with radiation and/or KPT-330, after which nuclear and cytoplasmic proteins were isolated and analyzed by western blotting. Representative western blots showing CRM1 expression in six groups: KPT-330 (0, 0.1 and 0.3 μmol/l) with or without radiation. (B) Comparison of p53 expression among the six groups. *P<0.05; **P<0.01. Nu, nucleus; Cy, cytoplasm; CRM1, chromosome maintenance protein-1; control, untreated cells.

associated with apoptosis, the cell cycle and DNA damage repair. In the present study, the *in vitro* results showed that KPT-330 decreased the viability of ECA109 cells and increased their radiosensitivity, and suggested that the elevated radiosensitivity may be associated with the elevated level of apoptosis and cell cycle arrest at the G_2/M phase detected in the cells. Consistent with this, other studies have demonstrated that the inhibition of CRM1 can increase the apoptosis of malignant melanoma cells, induce the cell cycle arrest of liver cancer and renal cell carcinoma cells, and reduce DNA damage repair (43-45). In addition, Arango *et al* (46) showed that KPT-330 used alone or in combination with chemotherapeutics such as paclitaxel or carboplatin was able to increase the apoptosis of triple-negative breast cancer cells and reduce cell colony formation. Furthermore, Ranganathan *et al* (47) showed that a combination of KPT-330 and topoisomerase II (topo II) inhibitors increased the nuclear accumulation of topo IIα and suppressed DNA damage repair, thereby reducing the chemotherapy resistance of acute myeloid leukemia. Currently, KPT-330 is in phase I/II clinical trials for the treatment of hematological and some solid tumors, including NHL (27) and metastatic triple-negative breast cancer (48). Recently, KPT-330 has been approved by the FDA as a novel therapy for treatment in multiple myeloma and diffuse large B-cell lymphoma (29,49). However, the large-scale application of KPT-330 in patients with esophageal cancer has not yet been reported. Such application appears to be worthy, based on the findings of the present study. KPT-330 has the potential to be evaluated as a novel type of radiosensitizer in the treatment of esophageal cancer.

The underlying mechanism of the enhanced radiosensitivity of ECA109 cells induced by KPT-330 was explored in the present study. The results demonstrated that KPT-330 induced G_2/M phase arrest in the ECA109 cells, particularly when used in combination with radiation. Previous studies have indicated that the G_2/M phase is the most sensitive to radiotherapy, followed by the G_1 phase, while the S phase is the most resistant (50,51). The present findings suggest that KPT-330 increases the G_2/M phase arrest and radiosensitivity of esophageal cancer cells. KPT-330 has been shown to change the cell cycle distribution, reduce DNA damage repair protein and sensitize cells to radiotherapy in non-small cell lung cancer cells (24). Inoue *et al* (52) demonstrated that KPT-185, another CRM1 inhibitor, increased the G_2/M phase arrest of

von Hippel-Landau (VHL)-wild-type renal cell carcinoma cell lines, but increased G_1 phase block in the VHL-negative cell line 786-O. A previous study reported that the treatment of p53-deficient H1299 cells with KPT-330 and irradiation increased G_1 phase arrest, while p53 wild-type A549 cells underwent G_2 phase arrest following the same treatment (24). Thus, both cell type and p53 gene serve an important role in the cell cycle.

p53 is a typical TSP, which participates in a variety of important biological processes, including apoptosis, the cell cycle and DNA damage repair (21,53). A previous study has shown that p53 inhibits CRM1 promoter activity, and CRM1 inhibits the expression of p53 (54). The present study showed that following the treatment of ECA109 ESCA cells with KPT-330 alone, CRM1 expression was suppressed and the expression of the p53 protein in the nucleus was upregulated. Furthermore, compared with the cells that were only irradiated, the cells treated with a combination of KPT-330 and radiation exhibited significantly suppressed CRM1 protein expression, upregulated expression of p53 protein, and increased expression of p53 protein in the nucleus. These results demonstrate that KPT-330 promoted nuclear accumulation of the p53 protein.

The intracellular sub-localization of p53 plays an important role in the regulation of tumorigenesis and development. A previous study showed that KPT-330 combined with bortezomib, a protein kinase inhibitor, altered the localization of the p53 protein in p53 wild-type colorectal cancer cells and increased the expression of p53 in the nucleus (22). It has also been reported that in p53 mutant colorectal cancer cell lines, transfection with a plasmid expressing the wild-type p53 gene reduced the expression of cyclin B mRNA, thereby increasing G_2/M phase arrest; this effect was not observed in cells transfected with a plasmid expressing p53 mutations in the cyclin B DNA junction site (55). Intriguingly, different levels of p53 protein may display different functions (56-58). For instance, low levels of p53 expression have been shown to cause cell cycle arrest, while high levels of p53 expression lead to cell apoptosis.

In the present study, the CRM1 inhibitor KPT-330 was shown to increase the rate of radiotherapy-mediated apoptosis and induce G_2/M cell cycle arrest through the signal transduction pathway protein p53. Since CRM1 is involved in the nuclear export of a variety of proteins, it might also affect other molecular pathways in CRM1 transportation. Kazim *et al* (59) showed that KPT-330 plus gemcitabine increased the apoptosis of pancreatic cancer cells by inducing the expression of p27 and reducing the expression of the anti-apoptotic protein survivin at the transcriptional level. In a study of mantle cell lymphoma cells (60), the inhibition of CRM1 increased the nuclear expression of cyclin D1 protein and reduced the level of cyclin D1 in the cytoplasm. The study also showed that the upregulation of cyclin D1 in the cytoplasm promoted the invasion and metastasis of the cells. Therefore, the inhibition of CRM1 expression may slow the development of tumors and change the radiosensitivity of tumor cells through multiple signal transduction pathways.

In summary, in the current study it was identified that KPT-330 inhibited CRM1 expression and increases p53 expression in ESCA cells, which impacted the cell cycle distribution and apoptosis, thereby improving radiosensitivity. However, due to the large number of CRM1 cargo proteins, it is necessary to perform additional experiments to explore the involvement of other proteins in the mechanism of KPT-330 radio-sensitization. Furthermore, only a single cell line and squamous cell carcinoma were explored in the present study; further verification of the findings in other esophageal squamous cell carcinoma cell lines such as TE1, KYSE-30, EC-9706 and KYSE-70 and in other differentiated types of ESCA is required.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JX, SW and GL contributed to conception and design, the collection and assembly of data, data analysis and interpretation, and writing the manuscript. GL provided administrative support, funded the project and provided study materials and patients. All authors read and approved the final manuscript. JX and SW confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki as revised in 2013. The study was approved by the ethics committee of the First Affiliated Hospital of China Medical University (approval no. AF-SOP-07-1.1-01) and the requirement for individual patient consent for this retrospective analysis was waived.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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