RNAi-mediated *ERBB2* gene knockdown sensitizes human colorectal cancer cells to radiation

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Abstract. This study aimed to investigate the involvement of c-erbB-2, encoded by the receptor tyrosine kinase *ERBB2* gene, in the pathogenesis of colorectal cancer and to validate its potential as an anticancer target. Immunohistochemical and histopathological analyses were applied in tissue samples derived from 80 colorectal cancer patients. *ERBB2* stable small hairpin RNA (shRNA) knockdown in HT29 human colorectal cancer cells was confirmed by RT-PCR and western blotting. Cell cycle profile and apoptosis were measured using PI or Annexin V-PI dual staining. A significant correlation between *ERBB2* levels and Dukes' stage of colorectal cancer, in both the primary malignancy and lymph node metastatic tissues, was observed. *ERBB2*-depleted HT-29 cells exhibited increased sensitivity to radiation compared to control cells, likely due to enhanced G0/G1 phase cell cycle arrest and apoptosis. *ERBB2* may be involved in the malignancy and metastasis of colorectal cancer. Overexpressed *ERBB2* may constitute a potential target for colorectal cancer therapy.

Introduction

Colorectal cancer is the second most common malignancy in the developed world (1,2) and persists as a significant cause of cancer mortality despite advances in treatment strategies. Currently, prognosis and treatment options for colorectal cancer therapy are based solely on the stage of the disease, which may not accurately predict disease outcome. Post-operative adjuvant chemotherapy improves the outcome in stage III (Dukes' stage C) colon cancer and is now widely accepted as standard therapy (3). Many patients with stage II (Dukes' stage B) disease are considered at a high risk for recurrence and receive adjuvant therapy, although the benefit of this in such cases is still uncertain.

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Recently, an awareness that decisions regarding cancer care could be more closely tailored to the individual patient has led to the development of several potential prognostic and predictive markers in colon cancer (4,5). Colorectal carcinogenesis is associated with complex and massive genetic alterations. Some of these alterations are used as prognostic markers in colorectal cancer (6). For example, loss of heterozygosity at chromosome 18q has been shown to indicate a poor prognosis (7,8). Studies identifying biomarkers in order to individualize chemotherapy decisions and predict clinical outcome have discovered widespread genetic mutations on chromosomes 17, 18 and 5 associated with colorectal carcinogenesis (9). The tumor-suppressor genes *TP53* and *NME1* and the proto-oncogene *ERBB2* are all located on these chromosomes (10). Among these, *ERBB2* has attracted considerable interest in colon cancer research of the last decade (11,12).

The *ERBB2* proto-oncogene on chromosome 17q21 produces a transmembrane glycoprotein, known as c-erbB-2 or HER-2/neu, which belongs to the epidermal growth factor receptor tyrosine kinase family. It forms functional receptors by heterodimerization with other members of the same receptor family. To date, no ligand has been indentified that binds the c-erbB-2 homodimer. Instead, such homodimerization is thought to occur in a ligand-independent manner upon overexpression (13,14). c-erbB-2 protein has been known to interfere in a number of cellular proliferations under normal circumstances (15), and evidence suggests that it is frequently expressed at low levels in a variety of adult epithelial cells. However, aberrant activation of *ERBB2* due to amplification or overexpression can contribute to unrestrained proliferation and tumor development or progression (16). Clinically, *ERBB2* overexpression has been associated with poor prognosis in breast (17,18), ovarian (19) and gastric carcinomas (20). To date, it has not been recognized as a prognostic indicator in large cohorts of colorectal tumors.

A part from its utility as a prognostic biomarker and risk factor, *ERBB2* has also become the target of novel anti-cancer therapies, either in the form of antibody-based therapy or tyrosine kinase inhibitors (21). Therefore, it is essential to accurately assess the frequency of *ERBB2* overexpression in colorectal tumors compared to normal tissues, and also to evaluate *ERBB2* expression in secondary lesions, in order to gauge the likely efficacy of anti-*ERBB2* therapy in such tumors.

In this study, we analyzed the frequency of overexpression of *ERBB2* and its association with clinicopathological factors

in a large series of well-characterized colorectal tumors, and in a subset of paired lymph node metastases. Moreover, we observed that the knockdown of *ERBB2* expression using a RNA interference (RNAi) approach sensitized human colon cancer cells to radiotherapy.

Materials and methods

Characteristics of the patients. Eighty patients (45 males and 35 females) of the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China) who underwent surgery for colorectal cancer from 2006 to 2007 were enrolled in this study. The ages of the patients ranged from 41 to 75 years, with an average age of 58 years. Thirty of these were diagnosed as papillary adenocarcinoma and the remainder had tubular adenocarcinoma. Of the 80 patients, 42 had highly differentiated tumors and the remaining 38 were moderately differentiated or lower. All of the patients were subjected to radiotherapy before enrollment. Hepatitis B, human immunodeficiency virus and cytomegalovirus infections were not detected in any of the patients. Precancerous mucosal tissues were collected from a subset of patients with lymph node metastases, and were paired with 30 noncancerous subjects composed of 19 males and 11 females, aged 46 to 99 years. Noncancerous colonic mucosal epithelial tissues were collected from these control subjects.

Immunohistochemical analysis. Formalin-fixed, paraffin-embedded sections (4 μm) were dewaxed and rehydrated, and then endogenous peroxidase activity was blocked using 3% H_2O_2 in a methanol solution. Antigen was retrieved by microwaving sections for 20 min in 10 mM pH 6.0 citrate buffer. Endogenous biotin activity was blocked to prevent non-specific background staining. Following incubation with anti-c-erbB-2 monoclonal antibody, biotinylated rabbit immunoglobulin and the streptavidin/biotin/horseradish peroxidase complex were visualized using diaminobenzidene (DAB). Slides were then counterstained with hematoxylin and analyzed using light microscopy.

Sections were scored semi-quantitatively according to the following United States Food and Drug Administration-approved scoring system (22): 0, no immunostaining; 1, complete, light brown membranous immunostaining of <30% of tumor cells; 2, weak, complete, brown membranous staining of 30 to 70% of tumor cells; 3, complete, dark brown membranous staining of >70% of tumor cells. A score of 0 indicated a lack of *ERBB2* expression. Scores of 2 and 3 were regarded as a weakly positive expression of *ERBB2*, and scores of 5 and 6 were considered a strongly positive expression of *ERBB2*. Only cases with a value of 4 or above were considered positive for *ERBB2*.

Small hairpin RNA (shRNA) construction. The plasmid vector pGenesil-1, which contains the human U6 promoter and the green fluorescent protein (GFP) reporter gene, was purchased from Wuhan Genesil Biotechnology (Wuhan, China). The shRNA targeting *ERBB2* was constructed by targeting the nucleotide sequence 5'-ATTCCAGTGGCCATCAAAG-3'. A vector containing a scrambled shRNA sequence was designed as a negative control with the sequence 5'-AAGCTTCATAA GGCGCATAGC-3'. The constructs were sequence verified.

Generation of stable shRNA cells. Cells of the human colon adenocarcinoma cell line HT29 were transfected with pGenesil-*ERBB2* or pGenesil-scramble shRNA constructs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfection efficiency was verified by monitoring the GFP expression 24 h after transfection. After selection with 600 μg/ml G418 for 21 days, single colonies were picked and cultured in RPMI-1640 containing 600 μg/ml G418 for amplification. The knockdown of *ERBB2* was verified at both the mRNA and protein levels using reverse transcription (RT)-PCR and western blotting, respectively.

RT-PCR. Total-RNA was isolated using a TRIzol total-RNA isolation system (Invitrogen). One microgram of total-RNA was reverse transcribed into first-strand cDNA using a PrimeScript™ 1st Strand cDNA Synthesis kit (Takara Biotechnology, Dalian, China). PCR was carried out in a 25-μl final volume reaction containing specific primers synthesized by Sangon (Shanghai, China). The sequences of the human *ERBB2* gene primer were: forward, 5'-AAC TCA CCT ACC TGC CCA CCA ATG C-3' and reverse, 5'-CAA AGA GCT GGG TGC CTC GCA CAA TCC-3'. Human β-actin, forward, 5'-ACC CCC ACT GAA AAA GAT GA-3' and reverse, 5'-ATC TTC AAA CCT CAT GAT G-3' were used as an internal control. After 94˚C for 5 min, the experimental reaction was subjected to 30 cycles of 94˚C for 30 sec, 52˚C for 30 sec, and 72˚C for 30 sec, and the PCR products were analyzed by gel electrophoresis.

Western blotting. Cell lysate containing 20 μg of protein was separated by SDS-PAGE on a 10% acrylamide gel and transferred onto a nitrocellulose membrane. After blocking with 5% non-fat milk for 2 h at room temperature, the membrane was incubated with anti-c-erbB-2 antibody overnight at 4˚C. The membrane was washed in Tris-buffered saline with Tween-20 (TBST) and then incubated with horseradish peroxidase (HRP)-linked secondary antibodies (Cell Signaling Technology) for one hour at room temperature. A hybridized protein band was detected using chemiluminescence reagents (Cell Signaling Technology) according to the manufacturer's instructions. The relative expression of *ERBB2* vs. β-actin was quantified by densitometry.

Cell survival assay. Cell viability was determined using methylthiazolyl-tetrazolium (MTT) and clonogenic assays. For the MTT assay, cells were plated in 96-well plates at $5x10^4$ cells/well and irradiated with γ-radiation at doses of 2, 4, 6 or 8 Gy. After 5 days of culture, 20 μl of MTT solution (5 mg/ml; Sigma) was added to each well for a 4-h incubation. The MTT solution was removed and 150 μl of dimethyl sulfoxide (DMSO; Sigma) was added to dissolve the crystals. Optical density was measured at a wavelength of 600 nm using an ELISA reader. Cell survival was calculated according to the formula:

$$\text{Survival fraction } \% = \frac{\bar{A}_{treated}}{\bar{A}_{untreated}} \text{ x } 100,$$

where $\bar{A}$ is the mean absorbance.

For the clonogenic assay, cells were plated in single-cell suspensions, treated with different doses of radiation and incubated until single colonies were visible to the unaided eye. The number of single colonies containing >50 cells were

Table I. *ERBB2* expression levels in tumoral, peritumoral and normal tissues.

Groups	Patients (n)	*ERBB2* expression		Positive ratio	P-value
		Positive	Negative		
Tumoral tissues	80	50	30	62.5%	0.001
Peritumoral tissues	30	8	22	26.7%	
Normal tissues	30	2	28	6.7%	

Table II. Analysis of the correlation between tumoral *ERBB2* expression level and clinicopathological factors.

Clinicopathological factors	Patients (n)	*ERBB2* expression		χ^2	P-value
		Positive	Negative		
Gender					
Male	45	30	15	0.762	0.383
Female	35	20	15		
Age (years)					
≤50	26	14	12	1.231	0.267
>50	54	36	18		
Tumor size (dimension)					
≤5 cm	41	28	13	1.204	0.273
>5cm	39	22	17		
Histological types					
Tubular adenocarcinoma	50	32	18	0.128	0.721
Papillary adenocarcinoma	30	18	12		
Differentiation					
High	42	30	12	3.008	0.083
Moderate or low	38	20	18		

counted and the cell survival rate was calculated according to the formula:

$$\text{Survival fraction } \% = \frac{\bar{C}_{treated}}{\bar{C}_{untreated}} \text{ x } 100,$$

where $\bar{C}$ = mean number of colonies.

Cell cycle analysis. Cell-cycle distribution was measured using flow cytometry (FACSCalibur, Becton-Dickinson, USA). Briefly, cells were harvested, washed with phosphate-buffered saline (PBS), and fixed in 70% ethanol. Fixed cell pellets were resuspended in 0.5 ml PBS containing 20 μg/ml RNase A (Sigma) for 30 min at 37˚C and then stained with propidium iodide (PI) solution (20 μg/ml; Sigma) for 30 min at room temperature. The DNA content was analyzed using a flow cytometric system and CellQuest and ModFit LT3.0 software (Becton-Dickinson).

Apoptosis analysis. Double staining with Annexin V and PI was used to detect apoptotic cells, using the Annexin V-FLUOS Staining kit from Roche Applied Science (Indianapolis, IN), according to the manufacturer's instructions.

Statistical analysis. Results are shown as the mean ± standard deviation (SD). Statistical comparisons were made by analysis of variance (ANOVA). A probability (P)-value of $P<0.05$ was considered to denote statistically significant. All statistical analyses were carried out with SPSS 11.5 statistical software.

Results

ERBB2 expression is correlated with malignancy and metastasis of colorectal cancer. To reveal the potential role of c-erbB-2 in the malignancy of colorectal cancer, we firstly compared its expression level in tumoral, peritumoral and normal tissues derived from colorectal cancer and noncancerous patients. It was observed that 62.5% of samples from tumoral tissues showed positive c-erbB-2 staining, which was significantly higher than that for either peritumoral (26.7%) or normal (6.7%) tissues (Table I). Representative immunohistochemical staining of c-erbB-2 is shown in Fig. 1a. The data suggest that c-erbB-2 may be implicated in the malignancy of colorectal cancer. However, no significant correlation was observed between c-erB-2 expression levels and the clinicopathological factors examined, which included tumor size, histological subtypes and extent of differentiation (Table II).

Table III. Correlation between tumoral *ERBB2* expression levels and staging and metastasis of colorectal cancer.

Pathological factors	Patients (n)	*ERBB2* expression		χ^2	P-value
		Positive	Negative		
Dukes' stages					
A and B	47	23	24	8.944	0.003
C and D	33	27	6		
Lymph node metastasis					
Yes	33	27	6	8.944	0.003
No	47	23	24		

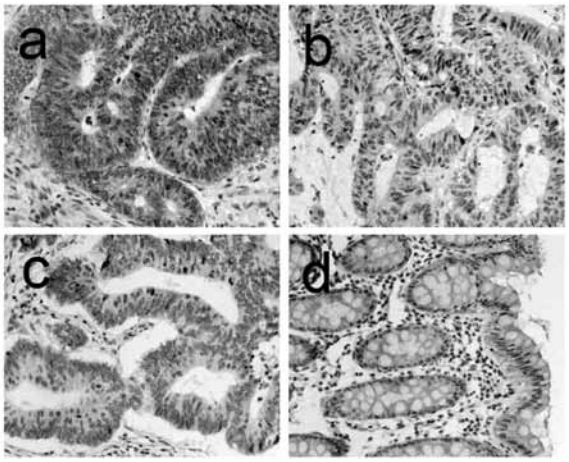

Figure 1. Increased intratumoral expression of *ERBB2* in colorectal cancer. Intratumoral, peritumoral or normal tissue sections derived from cancerous or noncancerous patients were subjected to immunohistochemical staining of *ERBB2* expression. (a) Colorectal cancer at adenocarcinoma grade II; (b) colorectal cancer at adenocarcinoma grade III; (c) negative control; (d) colonic mucosal epithelium from noncancerous patients; magnification, x200.

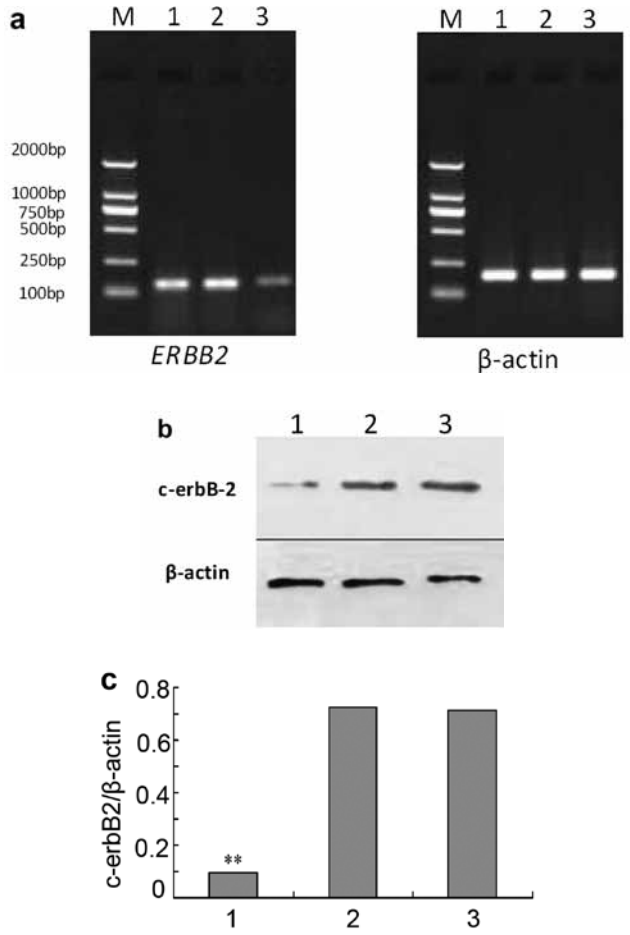

Figure 2. shRNA-mediated knockdown of *ERBB2* in HT29 colon cancer cells. HT29 stable cells were transfected with shRNA constructs harboring either the *ERBB2*-targeting sequence or a scrambled sequence. The knockdown of *ERBB2* in the shRNA was verified at both the mRNA and protein levels. (a) RT-PCR showing the mRNA level of indicated cells. Lane 1, non-transfected cells; lane 2, scrambled shRNA-transfected cells; lane 3, *ERBB2* shRNA-transfected cells; lane M, 1-kb DNA ladder. (b) Western blot analysis indicating the protein levels of the indicated cells: lane 1, *ERBB2* shRNA-transfected cells; lane 2, non-transfected cells; lane 3, scrambled shRNA-transfected cells. (c) Semi-quantification of the *ERBB2* protein level. The intensity of the *ERBB2* band was quantified using densitometric analysis and normalized to that of β-actin. **P<0.01 vs. scrambled shRNA-transfected control cells.

We also investigated the correlation between ERBB2 expression and the colorectal cancer stage (Table III). Colon cancer was staged by the Dukes' system. A total of 82% (27/33) of patients in stages C and D were positive for c-erbB-2 staining compared to 49% (23/47) of patients in stages A and B. The c-erbB-2 positive rate is significantly higher in late stage than in early stage. The incidence of nodal metastasis was compared between the c-erbB-2-positive and -negative patients with colorectal cancer. A total of 82% (27/33) of patients with nodal metastasis were positive for c-erbB-2, whereas only 49% (23/47) of patients without nodal metastasis were positive for c-erbB-2 (P=0.003).

Altogether, these data strongly implicate c-erbB-2 in the malignancy and metastasis of colorectal cancer, which encourages us to further explore the potentials of targeting *ERBB2* in colon cancer cells.

shRNA-mediated stable knockdown of ERBB2 in HT29 colon cancer cells. shRNA-mediated stable knockdown was applied to deplete *ERBB2* in HT29 colon cancer cells. Non-transfected cells and cells transfected with shRNA constructs harboring a scrambled sequence were used as negative controls. G418-selected single colonies were expanded and the knockdown efficiency was confirmed using both RT-PCR (Fig. 2a) and western blotting (Fig. 2b and c). Compared to the control cells,

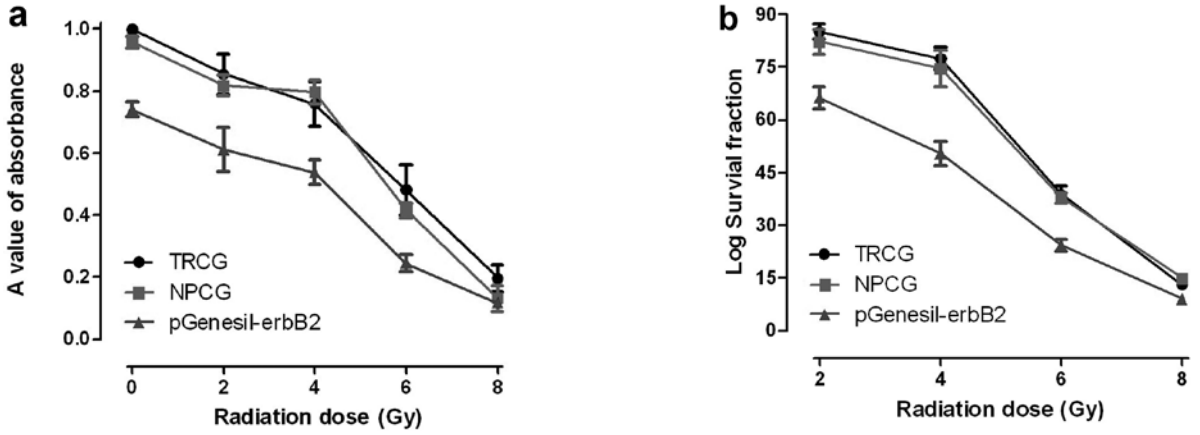

Figure 3. shRNA-mediated knockdown of *ERBB2* sensitizes HT29 colon cancer cells to radiation. *ERBB2* stably disrupted HT29 cells and control cells were irradiated at indicated doses and then subjected to (a) MTT or (b) clonogenic assay. TRCG, non-transfected cells; NPCG, scrambled shRNA construct-transfected cells; pGenesil-erbB2, *ERBB2* shRNA-transfected cells.

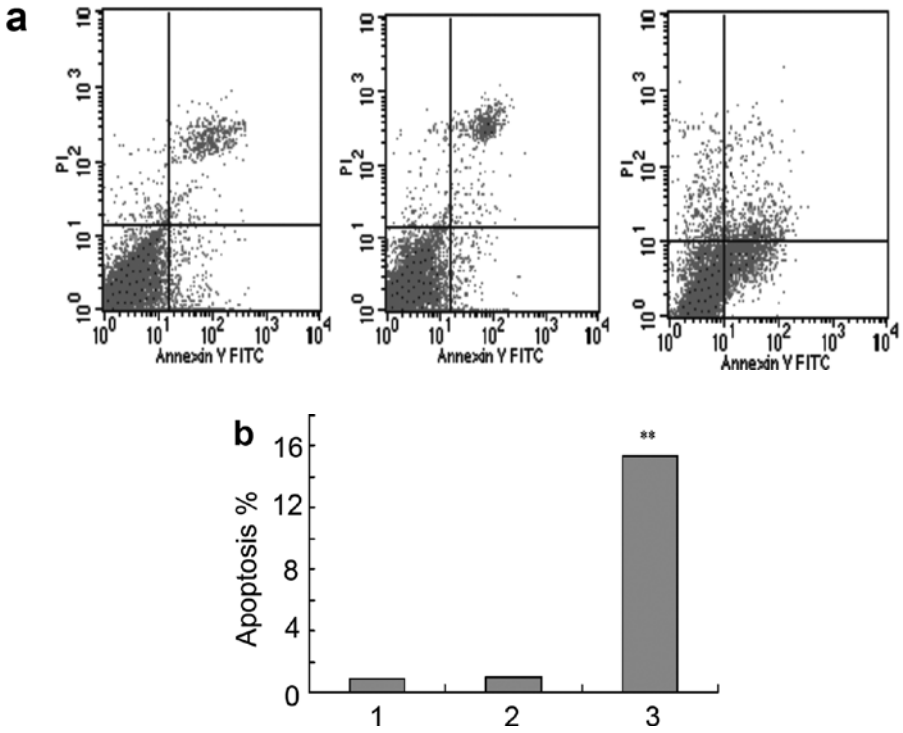

Figure 4. shRNA-mediated stable knockdown of *ERBB2* enhances radiation-induced cell cycle arrest in HT29 colon cancer cells. *ERBB2* stably disrupted HT29 cells and control cells were irradiated at indicated doses. Cell cycle stage distribution was detected using PI staining and then subjected to flow cytometry for analysis. (a) Representative images showing the cell cycle profile of non-transfected (left), scrambled shRNA-transfected (middle) and *ERBB2* shRNA-transfected (right) cells after radiation. (b) Quantification of a: 1, non-transfected cells; 2, scrambled shRNA-transfected cells; 3, *ERBB2* shRNA-transfected cells; $^{**}P<0.01$ vs. scrambled shRNA-transfected control cells.

both the mRNA and protein levels of *ERBB2*-encoding product were substantially reduced in *ERBB2* shRNA-transfected cells. These cells were therefore validated as a reliable model with which to study the targeting of *ERBB2* in colon cancer therapy.

Depletion of ERBB2 sensitizes HT29 colon cancer cells to radiotherapy. Radiation therapy is a major strategy for the medical management of colorectal cancer. Given the increasing occurrence of resistance, we were interested in testing whether knockdown of *ERBB2* could be applied as an approach to adjuvant radiotherapy.

To this end, stable *ERBB2*-knockdown cells were treated with radiation and subjected to the MTT assay (Fig. 3a) for cell viability. As shown in Fig. 3a, cells with stable knockdown of *ERBB2* exhibited considerably reduced cell survival after radiation treatment. We next plotted a cell survival curve based on the results of the clonogenic assay (Fig. 3b). By calculating the sensitizing enhancement ratio (SER), we evaluated the sensitization efficiency of *ERBB2* depletion to radiotherapy. The survival fraction of control cells at a dosage of 2 Gy to that of *ERBB2*-depleted cells was calculated (85.14/66.31 and 82.37/66.31). The values of SER were >1, suggesting that

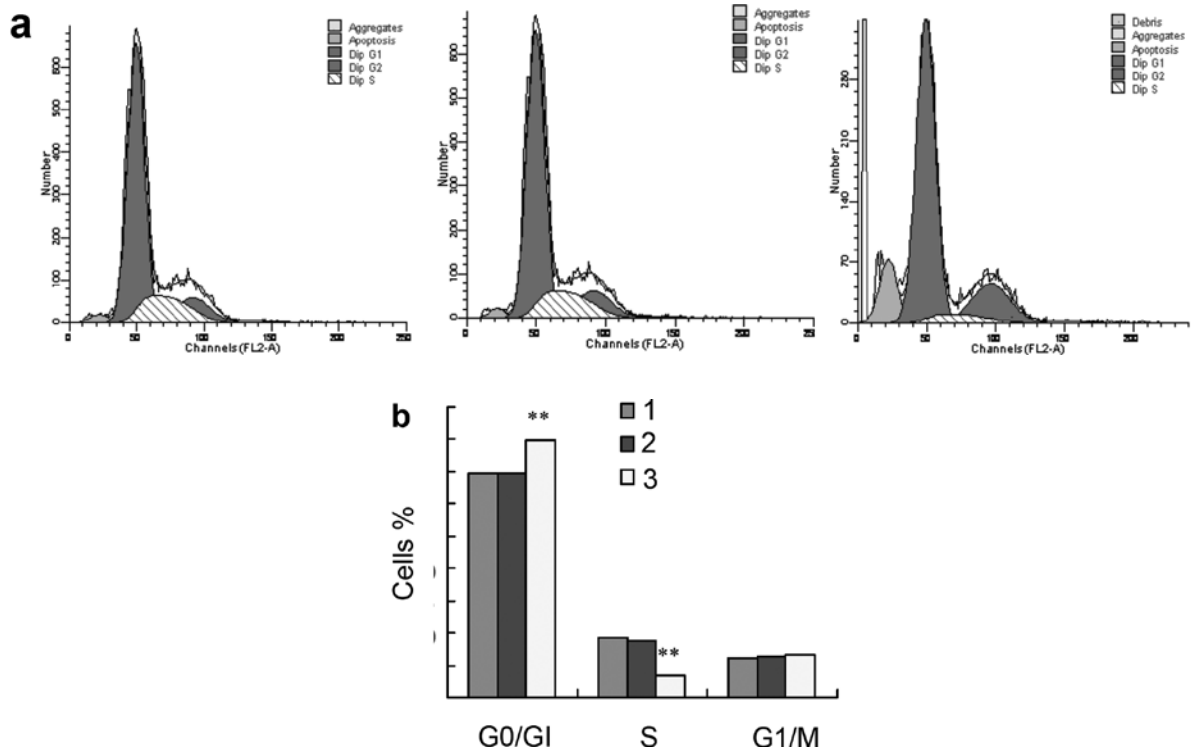

Figure 5. shRNA-mediated stable knockdown of *ERBB2* enhances radiation-induced apoptosis in HT29 colon cancer cells. *ERBB2* stably disrupted HT29 cells and control cells were irradiated at indicated doses. Apoptotic cells were detected using Annexin V-PI dual staining and then subjected to flow cytometry for analysis. (a) Representative images showing Annexin V-PI staining of non-transfected (left), scrambled shRNA-transfected (middle) and *ERBB2* shRNA-transfected (right) cells after radiation. (b) Quantification of a: 1, non-transfected cells; 2, scrambled shRNA-transfected cells; 3, *ERBB2* shRNA-transfected cells; **P<0.01 vs. scrambled shRNA-transfected control cells.

depletion of *ERBB2* or disrupting the function of its expression product could sensitize cancer cells to radiotherapy.

shRNA-mediated stable knockdown of ERBB2 enhances radiation-induced cell cycle arrest and apoptosis in HT29 colon cancer cells. The arrest of the cell cycle and apoptosis constitute the two major mechanisms of cell growth retardation and cell death of cancer cells, respectively. We, therefore, analyzed the degree of cell cycle arrest and apoptosis induced by radiation in *ERBB2*-depleted cells.

After irradiating cells at 4 Gy, *ERBB2*-knockdown cells exhibited an increase in the G0/G1 cell population (80%) compared to either the non-transfected (69%) or scrambled shRNA-transfected cells (69%). This consequently resulted in a decrease in S phase cells in the *ERBB2*-depleted cells (Fig. 4). These data indicate an increase in G0/G1 arrest compared to control cells, which might account for the suppressed cell growth of *ERBB2*-depleted cells after radiation. Similarly, 19% of the shRNA-transfected cells were observed to undergo apoptosis after exposure to 4 Gy of radiation, which was more than 2-fold that of either the non-transfected (7.6%) or scrambled cells (6.8%; Fig. 5).

In summary, our results suggest that radiotherapy in combination with *ERBB2* disruption caused an increase in both G0/G1 arrest and apoptosis and therefore diminished cell survival in colon cancer cells. This implies that targeting *ERBB2* could be an approach to enhance the efficiency or overcome resistance to radiotherapy of colorectal cancer.

Discussion

This study analyzed the association between *ERBB2* expression levels and clinicopathological factors related to cancer malignancy and metastasis in a well-characterized population of colorectal cancer patients. Our results indicate that: i) the c-erbB-2 protein level is higher in colorectal tumors compared to peritumoral or normal tissues; ii) the expression of *ERBB2* correlates with malignancy but not with the extent of tumor differentiation of colorectal cancer; iii) knockdown of the *ERBB2* gene results in increased sensitivity to radiation.

Our findings add to the broad literature regarding the potential significance of *ERBB2* in colorectal cancers. However, whether c-erbB-2 could be considered a reliable prognostic marker for colorectal cancers remains highly controversial and requires extensive study. In agreement with our findings, a recent study (23) of 137 colorectal cancer patients suggested that HER-2/neu (c-erbB-2) overexpression constitutes an independent prognostic factor in colorectal cancer patients. Park *et al* (23) also suggest that patients exhibiting HER-2/neu overexpression might constitute potential candidates for a new adjuvant therapy. Similarly, Kapitanovic *et al* (24) found that the intensity of *ERBB2* staining was independently related to survival in 155 colorectal tumors. Another study of colorectal adenocarcinomas in Taiwan characterized the role of HER2/neu as stage-dependent, i.e., that HER2/neu plays a crucial role in colorectal cancer tumorigenicity, with gene-amplification-independent transcriptional activations early in

carcinogenesis, and gene-amplification-dependent overexpression in the advanced stages (25).

Other researchers appear to contradict these findings. One study (26) showed that *ERBB2* expression does not have prognostic value in colorectal cancer, while another by McKay *et al* (27) of 249 patients with colorectal tumors and a minimum 25-month follow-up concluded that *ERBB2* is not a major factor in the development of colorectal cancer. It is still unclear what accounts for these discrepancies. We speculate that they could, at least partially, be due to procedural disparities.

Notably, a more recent study (28) carried out on 44 Chinese patients who had undergone liver resection for colorectal liver metastases identified an association between *ERBB2* and vascular endothelial growth factor (VEGF) expression in colorectal liver metastases. Indeed, a univariate analysis showed that VEGF was a prognostic factor, which was not the case upon multivariate analysis. Patients with both negative expression of *ERBB2* and *VEGF* had a better outcome than others. These findings suggest that the discrepancies observed before may be at least partly due to the gene expression profiles of certain associated genes.

In addition to a putative role in tumor development, *ERBB2* overexpression is also a target of novel anti-cancer therapies (29). In our laboratory, the specifically overexpressed *ERBB2* in tumor tissues provided the rationale for our further study which carried out the depletion of *ERBB2* in colon cancer cells. We utilized shRNA-mediated RNAi, instead of a small-molecule inhibitor or monoclonal antibodies, to disrupt the function of c-erbB-2. Although the value of RNAi in clinical treatment is still debatable, our study provides a sound scientific foundation to stimulate interest for such investigations in the future.

In conclusion, the protein levels of c-erbB-2 appear to be highly important for the carcinogenesis and metastasis of colorectal carcinoma. shRNA-mediated knockdown of *ERBB2* in colorectal cancer cells enhanced radiation treatment, apparently by inducing cell cycle arrest and apoptosis. Our findings imply that the overexpression of *ERBB2* could provide a potential target for a new adjuvant therapy for colorectal adenocarcinoma.

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