RESEARCH ARTICLE

Effects of Rad51 on Survival of A549 Cells

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Abstract

Rad51, a key factor in the homologous recombination pathway for the DNA double-strand break repair, plays a vital role in genesis of non-small-cell lung cancer (NSCLC). In recent years, more and more studies indicate that high expression of Rad51 is of great relevance to resistance of NSCLC to chemotherapeutic agents and ionizing radiation. However, the underlying molecular mechanisms are poorly understood. In this study, we investigated the role of single Rad51 on cell viability *in vitro*. Our results show that depletion of endogenous Rad51 is sufficient to inhibit the growth of the A549 lung cancer cell line, by accumulating cells in G1 phase and inducing cell death. We conclude that independent Rad51 expression is critical to the survival of A549 cells and can be an independent prognostic factor in NSCLC patients.

Keywords: Rad51 - A549 cell - cell survival

Asian Pac J Cancer Prev, **16 (1)**, 175-179

Introduction

The Rad51 recombinase is an essential factor in the homologous recombination (HR) DNA repair process, which plays a key role in the maintenance of genomic integrity, as well as in error-free repair of DNA dam¬ages including double strand breaks (DSBs) and interstrand crosslinks (Liu and Maizels., 2000). Expression of Rad51 is tightly controlled in normal cells to avoid aberrant chromosomal recombination, but is highly upregulated in a variety of tumors (Maacke et al., 2000; Hine et al., 2008). The upregulation of Rad51 has been shown to promote tumor progression through increasing the number of recombination events and inducing genome instability (Richardson et al., 2004). Several studies have shown that HR-defective tumors displayed more sensitivities to cyto¬toxic drugs which can lead to DNA damage or inhibition of DNA damage repair pathways (Farmer et al., 2005; Feng et al., 2011).

Lung cancer, the most common cancer all over the world, is a major public health problem. It is estimated that about 1.6 million new cases of lung cancer are diagnosed each year. Among those, almost 85% are non-small-cell lung cancers (NSCLCs), and the average 5-year survival rate only accounts for 10% to 15% (Gajra et al., 2003; Ferlay et al., 2010). Although there are several treatment options such as surgery, chemotherapy and radiotherapy for lung cancer, the low rate of complete tumour resection and the resistance to therapeutic agents are still the main barriers for cancer therapy. Thus, understanding the molecular mechanisms that promote NSCLC development is of importance for improving cancer treatment.

Rad51 overexpression not only promotes cancer development, but also allows cancer cells to evade treatment, like radiation, and is linked to tumor metastasis (Collis et al., 2001; Kauffmann et al., 2007; Lee et al., 2014). However, the underlying mechanisms of such correlations are poorly understood. Tumorigenesis of NSCLC and its resistance to anticancer treatments have also been linked with a high Rad51 expression (Ko et al., 2008a). Moreover, high-level Rad51 expression is significantly associated with a shorter median survival time of NSCLC patients (Qiao et al., 2005; Allera-Moreau et al., 2012; Nogueira et al., 2013). In addition, Ko et al. showed that reduc¬ed endogenous Rad51 activity can result in an increased lung cancer cell death, following treatment with gefitinib, a EGFR tyrosine kinase inhibitor (Ko et al., 2008b).

To date, studies have focused on the effects of Rad51 activity on cytotoxic drug or other treatments, but little information is available regarding the influence of Rad51 itself in NSCLC cell proliferation and survival. Therefore, understanding the effects of Rad51 on NSCLC cells may further help to reveal the role of independent Rad51 in the development of NSCLC. Here we explored the function of endogenous Rad51 protein in the lung cancer cell line, A549 cells, and found that knockdown of Rad51 by siRNA impaired the colony-forming ability of these cells. Furthermore, our data show that endogenous Rad51 is required for the cell viability and cell cycle progression. Taken together, our study reveals a critical role of Rad51 in the survival of cancer cells and this may

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explain the correlation of treatment-resistance with Rad51 overexpression in cancer cells, like NSCLS cells.

Materials and Methods

Cell culture

Human bronchioloalveolar cell carcinoma A549 cells (purchased from BOSTER) were cultured in DMEM/high glucose medium (HyClone) supplemented with 10% Fetal Bovine Serum (HyClone), 1%L-glutamine, 50 μg/ml streptomycin and 100 U/ml penicillin in a humidified atmosphere containing 5% CO_2 at 37 °C

Rad51 siRNA design and plasmid construction

The human Rad51 cDNA sequence (Genebank accession number: NM_002875.4) was searched for compatible siRNA target sequences, and CCGGCCACAACCCATTTCACGGTTACTCGAGT AACCGTGAAATGGGTTGTGGTTTTT was chosen. After chemical synthesis and double digestion with HpaI and XhoI the target sequence were inserted into the GV112 expression vector, and were ligated with T4 DNA ligase according to the manufacturer's guidelines. Then the ligated DNA was transformed into frozen competent Escherichia coli DH5 cells. Restriction enzyme analysis and DNA sequencing were used to identifie the correct transformant. A corresponding random siRNA sequence TTCTCCGAACGTGTCACGT was selected as a control for Rad51 siRNA.

Lentivirus production and transduction

Lenti-Rad51si and Lenti-NC virus were manufactured by plasmid cotransfection of A549 cells, as described previously (Segura et al., 2013). ViraPower packaging mix were transfected into 293T cells using 100 μl Lipofectamine 2000 reagent in accordance with the manufacturer's instructions. At 48h after transfection, the viral supernatant was harvested, and passed through 0.45μm filters following concentrated. Finally, the viral titer was determined. A549 cells were infected with the viral supernatant to acquire stably transfected Lenti-Rad 51si cell and Lenti-NC cells cells.

Real-time quantitative RT-PCR analysis

RNA was extracted from cultured cells by using TRIzol (purchased from Invitrogen) as detailed by the manufacturer. The RNA purity was determined by an acceptable OD260/280 ratio utilizing an ultraviolet spectrophotometer (Shimadzu, Japan). cDNA was reverse transcribed according to the protocol of the manufacturer supplied by the RT-PCR reverse transcription kit (purchased from Invitrogen). The SYBR green PCR master mix (TAKALA) and StepOnePlusTM Real-Time PCR System (ABI, United Kingdom) were used for real-time quantitative RT-PCR. The primers were as follows: Rad51 (forward: 5'-CTTTGGCCCACAACCCATTTC-3'; reverse: 5'-ATGGCCTTTCCTTCACCTCCAC-3') and human GAPDH (Rad51 values were normalized against human GAPDH; forward: 5'-CTACATGGTTTACATGTTCC-3'; reverse: 5'-GTGAGCTTCCCGTTCAGCTCA-3'). The PCR conditions consisted of 40 cycles, with 10 s denaturation at 95ºC, 30 s annealing at 72ºC, and 30s primer extension at 72ºC. Expression of target gene was normalized to the housekeeping gene GAPDH expression of for each sample. Data were evaluated using the 2-ΔΔCt method. q-PCR was performed in triplicate for all reactions.

Western blot

The cells were rinsed with cold PBS and lysed in SDS-PAGE protein loading buffer including 5% 2-mercaptoethanol. Equal amounts of total proteins from each set of samples were subjected to Western blot analysis as described previously (Maher et al., 2011). The specific Rad51 and actin antibody were purchased from Cell Signaling Technology.

Cell survival analysis

The cell viability of the A549 cells was analyze by a 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 2×103 of the cells were plated into 96-well cell culture plates with DMEM/high glucose medium containg 10% Fetal Bovine Serum in a final volume of 0.2 ml, and then incubated for 24 h (repeat at 48h, 72h and 96h). 20 μl of MTT solution was directly adding to the medium for 4 h incubation, DMSO (150 μl/well) was then added and all the plates were shaken at room temperature for 15 min. Cell survival was evaluated by measuring the absorbance at 490 nm by using a Biorad Technologies Microplate Reader (Hercules, CA).

Colony-forming ability assay

Cells were washed twice with PBS and trypsinized to determine the cell numbers. About 300 cells were plated on a 6-well cell culture plates in triplicate for each treatment. After 14 days of culture, the cell colonies were fixed and stained with 1% cresyl violet solution. Cytotoxicity was decideed by the number of colonies in the Lenti-Rad 51si and Lenti-NC transfected cells divided by the colony number in the untreated control. All experiments were carried out three times and accounted by Student's t tests.

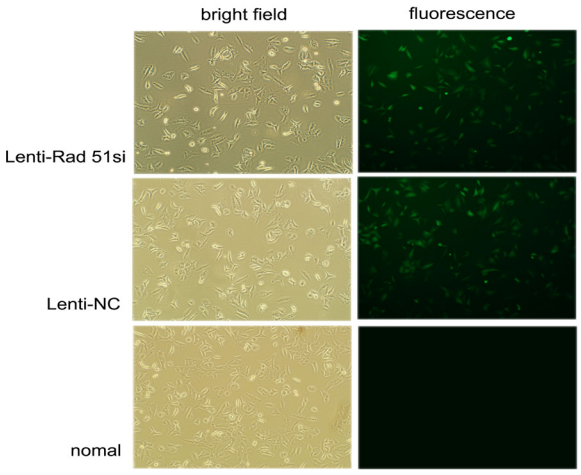

Figure 1. Transfection Efficiency of Lenti-Rad 51si and Lenti-NC Cells. A549 cells were transfected with Lenti-Rad 51si and Lenti-NC. Three days after transfection the percentage of lentiviral vector transduction was determined by fluorescence microscope

Cell cycle and fluorescence-activated cell sorter (FACS) analysis

When cells were dissociated and cultured for 48 h, propidium iodide (PI) (containg 0.1% trisodium citrate, 9.65 mM NaCl, 0.3% NP40, 50 μg/ml PI and RNase A 200 μg/ml) were used to stain those cells for 30 min at room temperature. Cell cycle profile was obtained by a FACSCanto flow cytometer (Becton Dickinson, USA) and assayed with Summit software.

Statistical analysis

Each experiment was carried out at least 3 times, and data are expressed as the mean±SD where applicable. In each assay, statistically significant differences between groups were defined by ANOVA. $p<0.05$ was considered statistically significant.

Results

Rad51 siRNA reduces endogenous Rad51 expression in A549 cells

To study the function of endogenous Rad51 in A549 cells, we used Rad51 siRNA to knock down the endogenous Rad51 expression. First of all, we checked the transfection efficiency of the lentiviral vector by monitoring the expression of GFP, which is within the vector cassette, 3 days after A549 cells were transfected with Lenti-NC (control) and Lenti-Rad51si (Rad51 siRNA). The transduction percentages were 98.9% for Lenti-NC and 98.5% for Lenti-Rad51si, suggesting an efficient transduction with this method (Figure 1).

Then, we examined the mRNA and protein levels of Rad51 in the Lenti-Rad51si treated cells. The mRNA expression was tested by quantitative RT-PCR after three days transduction of Lenti-Rad51si and Lenti-NC. The melting peaks and melting curves showed that there was no non-specific amplification product or primer dimer. Rad51 mRNA expression was significantly reduced in Lenti-Rad51si treated A549 cells compared with control cells (Figure 2). The protein level was determined by western blot. Consistent with the result of Rad51 mRNA, the Rad51 protein level was also significantly decreased after three days transfection with Lenti-Rad51si (Figure 3). Thus, our data indicate that transfection with Lenti-Rad51si that we performed in this study can efficiently reduce the endogenous Rad51 expression.

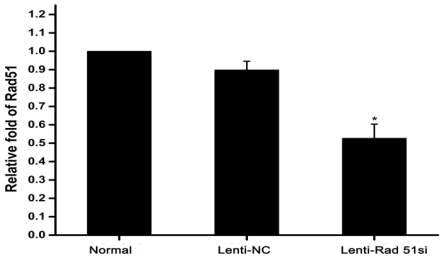

Figure 2. Suppression of Rad 51 mRNA Expression by Lenti-Rad 51si. The mRNA expression level of Rad 51 was tested use quantitive RT PCR after three days transduction of Lenti-Rad 51si and Lenti-NC. Rad 51 mRNA inhibition rate was 53.5%

Knockdown of Rad51 reduces the colony formation of A549 cells

Next, we set to examine the effect of reducing Rad51 level on the A549 cell function by checking the colony formation ability. Rad51 protein expression was knocked down using Lenti-Rad51si as described above, and colony-forming assay was performed. We found that transfection with Rad51 siRNA resulted in a significant reduction of the colony-forming number of A549 cells compared to cells

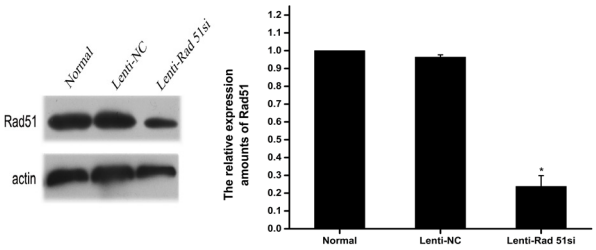

Figure 3. Western Blot Analysis for Rad51 Protein Levels. After three days transfection total proteins from each set of samples were subjected to Western blot analysis as described previously. The expression of Rad 51 protein was significantly decreased

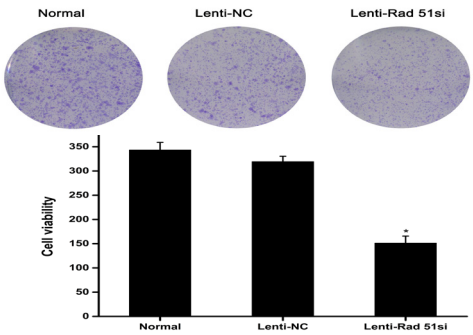

Figure 4. Colony-Forming Ability Assay of NSCLC Cells. After 14 days of culture, the cell colonies were fixed and stained with 1% cresyl violet solution. Cytotoxicity was decideed by the number of colonies in the Lenti-Rad 51si and Lenti-NC transfected cells divided by the colony number in the untreated control

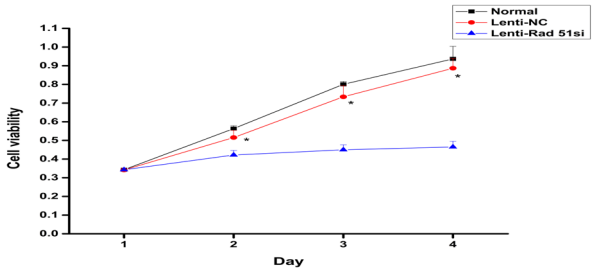

Figure 5. Suppression of Rad51 by Lenti-Rad 51si Transfection Lead to an Enhanced Cell Death Determined by MTT Assay. 2×10^3 of the cells were plated into 96-well cell culture plates with DMEM, and then incubated for 24 h (repeat at 48h, 72h and 96h) . 20μl of MTT solution was directly adding to the medium for 4 h incubation, DMSO (150μl /well) was then added and all the plates were shaken at room temperature for 15 min. Cell survival was evaluated by measuring the absorbance at 490 nm by using a Biorad Technologies Microplate Reader

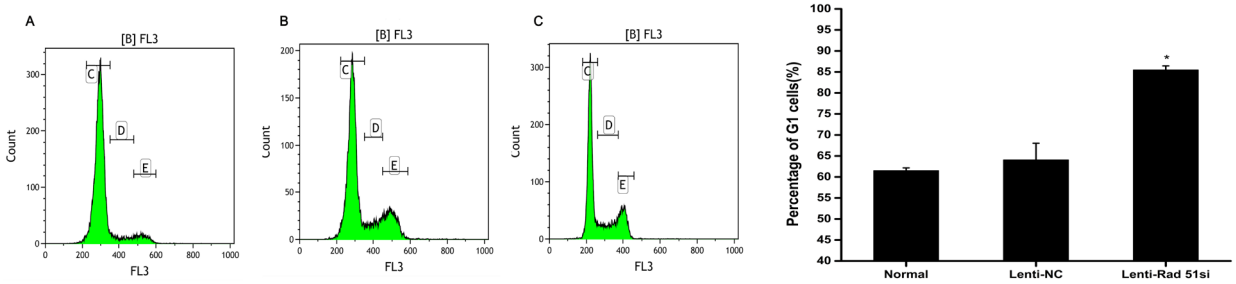

Figure 6. Cell cycle Analysis. When cells were dissociated and cultured for 48 h, propidium iodide (PI) (containg 0.1% trisodium citrate, 9.65 mM NaCl, 0.3% NP40, 50 μg/ml PI and RNase A 200 μg/ml) were used to stain those cells for 30 min at room temperature. Cell cycle profile was obtained by a FACSCanto flow cytometer. A substantial accumulation in G1 phase was observed in response to Lenti-Rad 51si

transfected with Lenti-NC or non-treated cells (Figure 4). Although we noticed a slight decrease of colony-forming number of Lenti-NC treated cells compared to normal cells, the difference is not statistically significant.

Knockdown of Rad51 affects the viability and cell cycle of A549 cells

Then we asked the question how Rad51 reduction affects the colony formation of the A549 cells. To answer this question, we checked the cell viability and cell cycle of Lenti-Rad51si treated A549 cells. The MTT assay was applied to assess the cell viability, and our data showed that Lenti-Rad51si transfection induced significant cell death compared to Lenti-NC treated or non-treated cells (Figure 5).

Another possibility is that the Rad51 reduction inhibits the cell proliferation by affecting the cell cycle. To test this hypothesis, we performed cell cycle analysis using flow cytometry. A549 cells, treated with Lenti-Rad51 and Lenti-NC, and non-treated cells were fixed and then subjected to flow cytometry analysis. About 85.49% cells were found in G1 phase in response to Rad51 siRNA, while 61.53% and 72.52% were observed in G1 phase from Lenti-NC treated and non-treated cells, respectively. This data suggests that reducing Rad51 expression leads to a substantial accumulation in G1 phase of A549 cells. Taken together, our data indicate that repression of endogenous Rad51 protein will induce cell death and impact the cell cycle progression of A549 cells, thus impairing the ability of colony formation.

Discussion

A line of studies have revealed the role of Rad51 overexpression in the cancer progression, cancer metastasis and treatment resistance (Tennstedt et al., 2013), but little is known about how endogenous Rad51 affects cell survival, cell proliferation or cell cycle. Here we used HIV-1-based lentiviral vectors to deliver Rad51 siRNA into A549 cells to suppress the Rad51 gene expression, and found that endogenous Rad51 is required for the survival and cell cycle progression of lung cancer cells *in vitro*. However, we still do not know whether reduction of Rad51 expression *in vivo* is also sufficient to induce these changes in cancer cells. To test this, knockdown of Rad51 in the animal model is needed and will be of interest for future study.

Constitutive up-regulation of Rad51 is an important determinant in DNA repair and cell survival, whereas Rad51 antisense inhibition sensitizes cells to DNA-damage-induced apoptosis (Raderschall et al., 2002). In addition, Rad51-deficient chicken lymphocytes with an inactivated human Rad51 transgene displayed an increase in chromosome breaks as well as cell death (Yamamori et al., 2013). Cells with inactivated HR components display hypersensitivity to DNA-damaging agents, such as ionizing radiation (Thompson and Schild, 2002). Consistent with these findings, our *in vitro* experiments also identified a key role of Rad51 in cell survival of the lung cancer cells. There is direct evidence that the RAD51 down-regulation shows great relevance with a substantial and prolonged decrease in the capacity of cells to perform HR (Bindra et al., 2004), and HR-deficiency will perturb the genome integrity (Mukherjee and Karmakar, 2013). Hence, our results suggest that reduction in Rad51 expression has a major impact on the capability of tumor cells to maintain genome integrity, which is important for the cell survival.

Numerous studies have indicated that overexpression of Rad51 can lead to an accumulation of cells in G2 phase (Franchitto et al., 2003). Interestingly our *in vitro* study suggests a substantial accumulation of A549 cells in G1 phase when treated with Rad51 siRNA. Several studies performed in Saccharomyces cerevisiae show that, when single unrepaired DNA double-strand break occurs, the cells undergo division-arrest due to the effects of MEC1 on the G1 checkpoints (Clémenson and Marsolier-Kergoat, 2009; Putnam et al., 2009). However, these cells eventually escape from the arrest and return to the cell cycle in a process named adaptation. And Rad51 is one of the proteins for this adaptation (Petermann et al., 2010). Therefore, Rad51 reduction-induced A549 cell division-arrest in G1 phase is likely through affecting checkpoint adaptation.

It has been well known that Rad51 expression is up-regulated during tumorigenesis (Fayaz et al., 2013), but what factors are regulating Rad51 is still not clear and needs to be explored. One recent study has characterized different regions of the Rad51 promoter that are responsible for activation or repression of Rad51 expression, and has identified EGR1 transcription factor as a positive regulator of Rad51 (Hine et al., 2014). With

this information, more positive and negative regulators of Rad51 can be identified by gene or drug screen. Then, inhibiting Rad51 protein by manipulating the identified factors or drugs will be helpful to provide ideas to improve the treatment of high Rad51-related cancers, like NSCLC.

Acknowledgements

This work was supported by the Natural Science Foundation of Jiangxi Province (No.20122BAB205004). The authors state no conflict of interest.

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