Overexpression of Rad51 predicts poor prognosis and silencing of Rad51 increases chemo-sensitivity to doxorubicin in neuroblastoma

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Abstract: Outcome for children with high-risk neuroblastoma (NB) remains suboptimal. Recurrence and metastasis caused by chemo-resistance is an underlying mechanism contributing to the poor prognosis. Aberrant expression of Rad51 is implicated in both radio- and chemo-sensitivity in many human malignancies. However, its clinical significance and relationship with chemo-sensitivity in NB remain undefined. In this study, Rad51 expression was first evaluated in 70 surgically resected NB specimens by immunochemistry using tissue microarray and the correlation with clinic-pathologic features including survival was assessed. We then conducted microarray-based search with the Tumor Neuroblastoma public datasets to validate the immunochemistry results. Furthermore, the role of Rad51 in drug sensitivity was studied by using short hairpin RNA in the human NB SK-N-BE(2) and SH-SY5Y cells with treatment of doxorubicin. Our findings demonstrated for the first time that Rad51 is a prognostic marker in NB and down-regulation of Rad51 can lead to chemo-sensitizing effect in human NB cells.

Keywords: Rad51, neuroblastoma, drug resistance, chemo-sensitivity, doxorubicin

Introduction

Neuroblastoma (NB) is the most common extra-cranial malignant tumor in early childhood. In the United States, the 5-year survival rate of neuroblastoma increased from 46% in 1974-1989 to 71% in 1999-2004 [1]. In Europe, the 5-year overall survival (OS) rate for children treated has risen from 37% in the late 1970s to 70% in the last decade [2]. However, the 5-year OS rates in children with high-risk NB still show only modest improvement, less than 40%, although comprehensive treatments such as surgery, chemotheraphy, radiotherapy and bone marrow transplantation are used [3-5].

Studies showed that approximately half of high-risk patients did not respond sufficiently to commonly employed therapeutic agents (e.g. cyclophosphamide, etoposide, cisplatin, vincristine) for neuroblastoma treatments [6, 7], especially to doxorubicin (DOX) therapy [8]. Drug resistance is the major obstacle of chemotherapy and results in treatment failure, characterized by tumor recurrence and metastasis [9-11]. The drug resistance mechanisms are complex and multifactorial. Identified resistance mechanism consists in: 1) Increased efflux and decreased uptake of drugs. For example, the overexpression of P-glycoprotein (P-gp), an adenosine 5-triphosphate (ATP)-binding cassette (ABC) transporters pumping the anticancer drug out of tumor cells [12]. 2) Various intrinsic changes that diminish the capacity of cytotoxic drugs to kill cells, including reduced apoptosis, increased DNA repair, and altered metabolism of drugs [13, 14]. Drug resistance is a difficult problem, which not only has adverse effects on traditional chemotherapy, but also leads lifelong health issues, such as hepatic and renal injury, myelosuppression and hearing loss, to survivors due to the toxic side effects of high-dose chemotherapy [15, 16]. Therefore, it is important to solve the problem of chemo-resistance in neuroblastoma.
Rad51 is a nuclear protein that catalyzes homologous recombination (HR), one of the DNA damage repair pathways [17]. Recent findings have indicated Rad51 protein overexpression in a variety of tumors, such as pancreatic adenocarcinoma [18], non-small-cell lung cancer [19], breast cancer [20] and esophageal squamous cell carcinoma [21]. Meanwhile, it has received much attention for its involvement in several cellular processes, such as genomic integrity, cell cycle regulation and apoptosis [22]. Interestingly, evidence also showed the association of abnormal Rad51 expression with tumor resistance to chemotherapy [23-25]. Furthermore, suppression of Rad51 expression through RNA interference or small molecule inhibitor directly enhanced the sensitivity of tumor cells to chemotherapy [24, 26, 27]. Yet, expression of Rad51 and its association with NB chemo-resistance remain unexplored. In this study, we sought to elucidate this issue.

Materials and methods

Tissue specimens

In our study, there were 75 primary pediatric NB patients, who were histologically diagnosed in Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine during September 2012 and February 2015. All tumor samples from the patients enrolled in the study were surgically removed. Each was frozen by liquid nitrogen and stored at -80°C for tissue microarray analysis. The study and consent procedure were approved by the Ethics Committee of the Xinhua hospital affiliated to Shanghai Jiaotong University School of Medicine.

Tissue microarray (TMA) preparation and immunohistochemistry (IHC)

Tissue specimens were separated out a small part and shaped in the special mold for microarray preparation. After fixed in the 4% paraformaldehyde overnight, they were trimmed and embedded in paraffin as a planned array. Then, samples were sectioned (5 μm) and attached to poly-L-lysine coated slides. Immunohistochemical staining was performed as previously described [28, 29], with antibody specific for Rad51 (1:300 dilutions, Cat. #ab133534, Abcam, UK). The immunoreactivity in tissue sections were viewed in three random microscopic fields and then assessed by at least two pathologists without knowledge of the clini-

copathological features of tumors. According to the percentage of positive cells (number of positive cells/number of total cells × 100%), the staining intensity was graded as follows: “0” represented negative expression (tumors without any detectable staining), “1” represented weakly positive expression (1-10% positive cells), “2” represented mildly positive expression (11-30% positive cells), “3” represented moderately positive expression (31-50% positive cells), “4” represented strongly positive expression (51-100% positive cells).

Validation patient datasets analysis

Three publicly available datasets: Kocak (GEO: GSE45547) [30], SEQC (GEO: GSE49710) [31], and Oberthuer (ArrayExpress: E-TABM-38) [32], which included comprehensive information on neuroblastoma relevant clinical and prognostic factors, were obtained from R2: microarray analysis and visualization platform (http://r2.amc.nl) and selected for analysis. All Kaplan-Meier analyses were conducted online, and the best p value and corresponding cutoff values for separating high and low expression groups were selected by median.

Cell culture

The human neuroblastoma cell lines SK-N-Be(2) and SH-SY5Y were obtained from ATCC (Manassas, USA) and maintained in a 1:1 mixture of Eagle’s Minimum Essential Medium and Ham’s nutrient mixture F12 Medium with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin (All from Gibco, USA), and cultured at 37°C in a 5% CO₂ humidified incubator.

Lentivirus-mediated silence for Rad51

Oligonucleotides with the nucleotide sequences (Table 1) and a non-targeting control shRNA (scrambled control) were used for the cloning of shRNA-encoding sequences into a lentiviral vector GV248 obtained from GeneChem (Shanghai, China). The lentiviral constructs were co-transfected into 293T cells with viral packaging plasmids (psPAX2 and pMD2.G) using Lipofectamine 2000 (Life Technologies) in Opti-MEM medium (Gibco, USA). Virus-
containing supernatants were collected at 48 h post transfection and were used to infected SK-N-BE(2) and SH-SY5Y cells according to the manufacturer’s protocol of GeneChem. Finally, cells with stable lentiviral transfection were screened in the presence of 1 μg/ml puromycin (Cat. #ST551, Beyotime, China) for 3 days, and the puromycin-resistant cells were pooled.

Western blotting

Whole-cell lysates were harvested for protein analysis. Briefly, cells were lysed in the RIPA buffer (Cat. #P0013B, Beyotime, China) containing PMSF (Cat. #ST506, Beyotime, China) for 30 min on ice, and centrifuged at 20,000 g for 15 min, with the supernatant used for western blotting. Protein concentration was determined by the BCA assay (Cat. #20201ES76, Yeasen, China). Equal amounts of protein concentration were diluted in 5 × SDS loading buffer (Yeasen, China) and heated to 99°C for 5 min and separated by 12% SDS-PAGE. Proteins were electrotransferred onto PVDF membranes (Millipore, Sigma Aldrich, USA) and blots were blocked with 5% bovine serum albumin dissolved in Tris buffered saline (TBS) containing 0.1% Tween-20 (TBST) at room temperature for 1 h, and incubated with primary antibodies overnight at 4°C. After washing the blots 3 times with TBST, the membranes were incubated with appropriate HRP-conjugated secondary antibody (Cell Signaling Technology) for 1 h at room temperature, and washed again with TBST. Protein immunoblots were finally visualized by electrogenerated chemiluminescence (ECL, Pierce Biotechnology, USA) with the Bio-Rad ChemiDoc XRS imaging system. Primary antibody to Rad51 (Cat. #ab133534) and β-actin (Cat. #ab8227) were purchased from Abcam. Cleaved PARP (Cat. #5625), Bcl-2 (Cat. #4223) and AIF (1:1000, #5318) were purchased from Cell Signaling Technology (CST). The blots were quantitative analyzed through the software of Image J (X64, v. 2.1.4).

Drug treatment and cell proliferation assay

Common chemotherapeutic drug doxorubicin (Sigma, USA) for clinic neuroblastoma therapy was chosen here for drug resistance studies. Doxorubicin was dissolved in dimethyl sulfoxide (DMSO). ShRad51 cells and shNC cells were plated at a density of 5,000 cells per well in 96-well plates, 24 h later, cells at logarithmic phase were treated with proper dosage of different doxorubicin (0-2 μM) for 48 h as our previously study described [28]. Cell proliferation assay was detected by Cell Counting Kit-8 (CCK-8) reagent (Yeasen, China) according to the manufacturer’s instructions. The absorbance of the samples at a wavelength of 450 nm was measured using a microplate reader (BioTek, USA). Drug-response curve was draw according the result of CCK-8 assay.

Statistical analysis

GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, USA) and SPSS version 25.0 software (SPSS, Chicago, IL, USA) for Windows was applied for statistical analysis. The χ² analysis was used for association between the expression of Rad51 and individual clinicopathological characteristics. The Kaplan-Meier analysis was used to assess survival rates, and the log-rank test was used to estimate survival difference. For 2-group comparisons, data were analyzed with 2-tailed t-test. Results are expressed as mean ± standard error of the mean (SEM). P < 0.05 was considered a statistically significant difference. Significance was expressed as: *P < 0.05, **P < 0.01, and ***P < 0.001.

Results

The mRNA expression levels of Rad51 in different types of human cancers

We first analyzed different Rad51 expression between various cancer types and their normal...
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The Rad51 mRNA levels were analyzed using Oncomine database with P-value of 0.0001, fold change of 2, and gene ranking of all. This analysis revealed that Rad51 expression was higher in most of the cancers compared to the normal tissues, such as kidney, breast, lung, bladder, cervical, ovarian, colorectal and esophageal cancers, etc. (Figure 1A). Data from Tumor Immune Estimation Resource (https://cistrome.shinyapps.io/timer/) also demonstrated that, Rad51 expression was significantly higher in almost the TCGA tumors compared with adjacent normal tissues (Figure 1B). The results were consistent with previous findings and implied RAD51 as a promising and novel target for cancer therapy.

Elevated expression of Rad51 is correlated with decreased survival in neuroblastoma

To determine the potential clinic implication of Rad51 in NB, the expression level of Rad51 was determined by TMA-based IHC analysis. Of all the 75 NB patients, 5 patients were lost to follow-up, so the remaining 70 patients were used for the following analysis. Of all the patients, there were 41 boys and 29 girls. 25 cases were diagnosed as daganglioneuroblastoma (GNB) and 45 cases as neuroblastoma (NB). Median age at diagnosis was 29.5
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months. INSS stage analysis revealed that there were 10 cases with stage I, 18 cases with stage II, 6 cases with stage III, 35 cases with stage IV, 1 case with stage IVs. Primary tumors were localized in the retroperitoneum (n = 56) and at the postmediastinum (n = 14). As shown in Figure 2A-C, among the 70 NB samples, Rad51 protein could be detected in 65 cases (92.9%), with 42 of patients show strong intensity staining (IHC staining grade 3-4). Meanwhile, the association between Rad51 expression with clinical pathologic parameters and three-year overall survival (OS) rates were evaluated. We found that strong Rad51 staining were markedly correlated with some indicators of NB progression, such as advanced stage (P < 0.001) and bone marrow metastasis (P < 0.001). We also found elevated expression of Rad51 in neuroblastoma compared with ganglioneuroblastoma (P = 0.011). The association between Rad51 expression and clinical characteristics are displayed in Table 2. Finally, Kaplan-Meier analysis demonstrated that patients with low Rad51 expression showed significantly favorable 3-year overall survival (OS) than those with high expression (P = 0.041, Figure 2D).

Rad51 is a potential prognostic factor in NB

To further evaluate and confirm the possibility of Rad51 as a prognostic marker in NB, we conducted Kaplan-Meier analysis of OS and event-free survival (EFS) using three different Tumor Neuroblastoma public datasets, which are available from the online R2: microarray analysis and visualization platform. In the Kocak dataset, patients with high Rad51 mRNA levels showed worse OS (P = 8.6e-19) and EFS (P = 3.1e-20) (Figure 3A). Moreover, Rad51 expression was different in various tumor stages, with significantly increased expression in stage 4 tumors compared to stage 1 and stage 2 (Figure 3A). We also confirmed high Rad51 expression as poor prognosis in the SEQC and Oberthuer datasets (Figure 3B and 3C).
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Table 2. The association between Rad51 with clinical pathologic characteristics in 70 TMA cohort

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Low: IHC staining grade 0–2, High: IHC staining grade 3–4; GNB: ganglioneuroblastoma, NB: neuroblastoma.

Together, our data demonstrated that high level of Rad51 expression is prognostic for poor outcome in neuroblastoma.

Rad51 expression was induced by doxorubicin

To investigate how Rad51 responds to treatment with doxorubicin in neuroblastoma cells, western blotting was carried out to assess Rad51 expression in SK-N-BE(2) and SH-SY-5Y cells after exposure to the agent (0 μM–0.6 μM for 48 h). The result showed that Rad51 expression exhibited a relatively positive response with increasing concentration of doxorubicin. In SK-N-BE(2) cells, Rad51 protein level increased with incremental doxorubicin concentrations (0.6 μM/DMSO = 5.24, P < 0.0001; 0.4 μM/DMSO = 2.95, P = 0.0116; 0.2 μM/DMSO = 1.58, P = 0.0116) (Figure 4A). In SH-SY5Y cells treated with doxorubicin, Rad51 protein level was also up-regulated, but Rad51 protein reached its peak in the group of cells treated with 0.4 μM doxorubicin (Figure 4B).

Our results suggest that Rad51 might play an important role in process of NB cells response to chemotherapy.

Rad51 expression was inhibited in cells infected with the lentivirus

After shRNA interference Rad51 for 48 hours in SK-N-BE(2) cells, Rad51 protein were measured by western blotting. In our assay, three Rad51 shRNAs (sh-1, sh-2, sh-3) were used to suppress Rad51 expression, compared with sh-1 and sh-2, sh-3 could better efficiently suppress Rad51 expression at protein levels (Figure 5). Therefore, sh-3 was used in all the subsequent experiments.

Inhibition of Rad51 expression increase sensitivity of cells to chemotherapy

Using a CCK-8 cell viability assay, we found that the drug response curve was left-shifted in Rad51 knockdown SK-N-BE(2) cells, suggesting that these cells were more sensitive to doxorubicin (Figure 6A). Specifically, the IC50 of doxorubicin in Rad51 knockdown SK-N-BE(2) cells was 182.8 nM, 2.48-fold lower than that of control cells (IC50 value of control cells was 453.3 nM, P = 0.0008). On microscopic examination, we found more extensive cytotoxic effects in Rad51 knockdown cells compared with control cells, following treatment with doxorubicin at concentration of 0.5 μM for 48 h, as showed by cell shrinkage, disappearing connections, and apoptotic body formation (Figure 6B). Besides, we also examined the effect of Rad51 repression on chemosensitivity of SH-SY5Y cells, and showed the similar phenomenon (Figure 6C and 6D). IC50 value of Rad51 knockdown SH-SY5Y cells was 84.99 nM, while IC50 value of control cells was 192.5 nM, 2.26-fold lower than that of control cells (P = 0.0238). What’s more, Rad51 knockdown led to a significant increase in apoptosis following doxorubicin therapy compared to control group, as evidenced by expression of apoptotic proteins, such as the increased cleavage of poly (ADP-ribose) polymerase (PARP) and AIF (Apoptosis Inducing Factor) and decreased Bcl-2 in SK-N-BE(2) cells (Figure 6E and 6F). These results indicate that Rad51 may play an important role in promoting NB cells drug resistance and Rad51 suppression conferred chemo-sensitivity by augmenting apoptosis.
Discussion

Resistance to chemotherapy is an element contributing to the recurrence and metastasis of neuroblastoma, a problem that must be overcome to improve the prognosis of NB patients, especially the high-risk ones. In recent years, several efforts have been taken to identify genes and proteins associated with drug resistance. Studies have suggested that overexpression of multidrug resistance protein 1 (MRP1) correlates with chemoresistance in several biological processes, including tumor relapse and metastasis [33, 34]. Study has also shown that p53 mutations and loss-of-function was observed in some recurrent NB with high-level drug resistance [35]. Our previous study showed that knocking down ANXA2 could attenuate multidrug resistance in NB cells through upregulation of apoptotic genes [28]. Recently, NF1 loss was identified as the major resistance mechanism of neuroblastoma to PIM kinase inhibitors [36]. Even though considerable efforts are being made to use tumor genomics, expression profiling and proteomic studies to better understand this malignancy and to develop more effective therapies, current outcome of high-risk NB remains unacceptable. It is therefore essential to conduct further study to find the candidate genes and proteins that involved in drug resistance of NB.
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Estimation Resource (TIMER) site, indicated that higher levels of the Rad51 in most tumors than in the normal tissues. Interestingly, high expression of Rad51 is reported to be associated with enhanced resistance to DNA damage induced by chemical agents and/or ionizing radiation [37]. In addition, some small molecule inhibitors of Rad51 was studied for potential cancer treatment [38]. However, the role of Rad51 in NB drug resistance remain elusive.

Consistent with excessive expression of Rad51 in breast cancer, pancreatic cancer and non-small lung cancer, Rad51 overexpression was also seen in neuroblastoma. Using TMA cohort of neuroblastic tumors and three independent microarray databases we found that high Rad51 expression correlated significantly with some clinicopathologic parameters, such as stage and bone marrow metastasis. Meanwhile, patients with high expression of Rad51 held significantly worse three-year OS both in the TMA cohort of neuroblastic tumors and microarray databases. Notably, higher expression of Rad51 was seen in neuroblastoma than ganglioneuroblastoma, stage 4 than stage 1 and stage 2, indicating that Rad51 expression might associate with the NB neural differentia-

Rad51 is the key protein of DNA homologous recombination repair. Our results, based on the Oncomine database and Tumor Immune

Figure 4. Dose-response analysis of Rad51 expression in cells exposed to doxorubicin. Cells were exposed to doxorubicin for 48 hours. The protein expression of Rad51 were measured by immunoblotting analysis. The densitometry of the bands was quantified using ImageJ software, and β-actin was used as controls. A. Dose-response analysis of Rad51 expression in SK-N-BE(2) cells exposed to doxorubicin. B. Dose-response analysis of Rad51 expression in SH-SY5Y cells exposed to doxorubicin. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 5. Specific downregulation of Rad5 expression by Rad51 short hairpin RNA. Cells were transfected with RAD51 shRNA or scrambled shRNA (shNC) for 48 hours. The relative Rad51 protein level was determined by western blotting, and data was normalized using β-actin as an internal standard. Compared with Rad51 sh-1 and sh-2, sh-3 mostly silenced Rad51 expression. *P < 0.05, **P < 0.01.
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Importantly, our data indicate that knockdowning Rad51 with shRNA enhanced the sensitivity of SK-N-BE(2) and SH-SY5Y cells to doxorubicin treatment. SK-N-BE(2), which is reported to be a kind of drug resistant neuroblastoma cell line [28, 35], seemed to be more sensitive to doxorubicin after silence of Rad51. Taken together, our results suggest that Rad51 could be a prognostic biomarker in NB and may play an important role in chemoresistance.

Genome sequencing studies of neuroblastoma have revealed a markedly lower mutation rate in genes than in adult’s [39], suggesting that overexpression of Rad51 in neuroblastoma might be due to transcriptional and/or epigenetical increased but not due to amplification.

**Figure 6.** Rad51 knockdown enhanced drug sensitivity of NB cells to doxorubicin. A. CCK-8 cell viability assay showed drug response curve was left-shifted in Rad51 knockdown SK-N-BE(2) cells. B. Under the microscope, more extensive cytotoxic effects in Rad51 knockdown SK-N-BE(2) cells following treatment with doxorubicin: cell shrinkage and apoptotic body formation. C, D. Rad51 knockdown enhanced drug sensitivity of SH-SY5Y cells to doxorubicin. *P < 0.05, **P < 0.01. E, F. Rad51 knockdown led to a significant increase in the expression of cleavage of PARP and AIF with decrease in the expression of BCL-2 compared to control group following 0.5 μM doxorubicin treatment for 48 h. The plotted error bars represent mean ± SEM. ***: P < 0.001, **: P < 0.01.
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of the Rad51 gene. Study showed that the absence of Rad51C in gastric tumor is due to DNA methylation [40]. Data from CCLE (Cancer Cell Line Encyclopedia) database showed hypo-methylation of Rad51 in neuroblastoma cell lines. Meanwhile, in our study of the molecular mechanism of LMO1 in neuroblastoma, we found that ISL1 could interact with LMO1, and ISL1 could regulate the expression of RAD51 (data not show), consistent with a previous report [41]. However, further study is needed to understand the underlying mechanism of Rad51 overexpression in neuroblastoma.

In summary, our present study is the first report describing the role of Rad51 in the prognosis of NB patients and providing a shRNA-mediated Rad51 silencing strategy to enhance drug sensitivity in neuroblastoma. Further work is warranted to validate our results and to understand the underlying mechanism.

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Disclosure of conflict of interest

None.

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