Effect of RAD51C expression on the chemosensitivity of Eμ-Myc p19Arf−/− cells and its clinical significance in breast cancer

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Abstract. The aim of the present study was to investigate the chemosensitivity to anti-cancer drugs of RAD51 paralog C (RAD51C)-deficient Eμ-Myc p19Arf−/− cells, to detect the expression of RAD51C in breast cancer tissues by immunohistochemistry (IHC), and to explore their association with clinicopathological factors. Eμ-Myc p19Arf−/− cells were stably transfected with retroviruses co-expressing short hairpin-RNA against RAD51C and green fluorescent protein (GFP). A single-cell flow cytometry-based GFP competition assay was used to assess the change in sensitivity to anti-cancer drugs. GFP-negative cells in the same population served as an internal control. In total, tissue samples from 213 cases of breast cancer and 99 adjacent non-cancerous tissue samples were collected to construct tissue microarrays. IHC was used to detect the expression of RAD51C protein. Relevant clinical information was collected for a correlation analysis. Transfection of RAD51C-shRNA was demonstrated to effectively reduce the RAD51C protein expression in the Eμ-Myc p19Arf−/− cells. The sensitivities of the cells to three drugs, camptothecin, cisplatin and olaparib, significantly increased following RAD51C gene knockdown. In breast cancer tissue, RAD51C expression was significantly higher in the Erb‑B2 receptor tyrosine kinase 2 overexpression group. The overall survival time of the patients with RAD51C-negative expression was longer than that of patients with RAD51C-positive expression. RAD51C expression was an independent prognostic factor for survival of breast cancer patients. In summary, the results indicate that silencing of RAD51C may represent a potential therapeutic strategy for malignant tumors, and that measuring RAD51C expression by IHC may have prognostic value for breast cancer patients.

Introduction

DNA double-strand breaks (DSBs) represent the most detrimental form of DNA damage (1,2). Cells rely on two major pathways to repair DSBs: Homologous recombination (HR), which is the low-error mechanism for DNA repair; and non-homologous end joining, which may introduce changes to the DNA sequence at the repair site (1,2). HR is a fundamental cellular process that is conserved in all organisms. It maintains genome integrity by repairing endogenous and exogenous DSBs (2).

The RAD51 paralogs form two identified complexes: BCDX2 (RAD51B-RAD51C-RAD51D-XRCC2) and CX3 (RAD51C-XRCC3) (3). These two complexes act at two different stages of homologous recombinational DNA repair (3). The BCDX2 complex is responsible for RAD51 recruitment or stabilization at damage sites (3). The BCDX2 complex appears to act by facilitating the assembly or stability of the RAD51 nucleoprotein filament (3). The CX3 complex acts downstream of RAD51 recruitment to damage sites (3). RAD51 paralog C (RAD51C) serves an important role in the DNA damage response. It acts as a transducer of the damage signal to ensure that the HR pathway of repair is engaged. RAD51C localizes to the sites of DNA damage min after damage occurs, indicating that RAD51C has a role in the early stage of HR. RAD51C is also required for the phosphorylation of checkpoint kinase 2 by ataxia telangiectasia mutated protein, which is required for checkpoint activation (4). This indicates that RAD51C is required for efficient checkpoint signaling, which delays cell cycle progression in response to DNA damage. RAD51C foci persist long after RAD51 can no longer be detected. Therefore, it is possible that RAD51C is involved in the early and late stages of the HR reaction (4). Liu et al (5) demonstrated that RAD51C is involved in regulating the resolution of Holliday Junctions (HJ) during the later stages of HR. Furthermore, another study reported that RAD51C-deficient hamster cells and mouse embryonic fibroblasts have a reduced level of HJ resolution activity (6).

The HR pathway is a critical repair mechanism for various lethal forms of DNA damage. Mutations in HR-associated
genes have been observed to cause the accumulation of unrepair DSBs, which may lead to carcinogenesis. Previous studies have demonstrated that mutations in RAD51C increase the risk of breast and ovarian cancers (7-9). RAD51C mutation is also associated with Fanconi anaemia-like disorder (10).

Considering the important functions of RAD51C in DNA repair by HR, the present study aimed to investigate the possible correlation between RAD51C expression and drug sensitivity in Eμ-Myc p19ARF−/− lymphoma cells and explore the association between RAD51C expression and clinicopathological factors in breast cancer. Arf negative cells were selected as Arg negatively regulated MDM2, resulting in decreased p53 level which, when combined with Eμ-driven Myc expression, created an immortalized cell line; thus, creating a cell line without point mutations for screening (11). Knockdown of p53 in these cells leads to resistance to DNA damage drugs, indicating these was still adequate p53 to trigger cell arrest (12). A single-cell flow cytometry-based green fluorescent protein (GFP) competition assay was used to assess changes in sensitivity to anti-cancer drugs. Immunohistochemistry (IHC) was used to detect the protein expression of RAD51C in 213 samples from breast cancer tissue and 99 samples from the adjacent, non-cancerous tissues, and relevant clinical information was collected from patients for a correlation analysis.

Materials and methods

Cell culture and drugs. Eμ-Myc p19ARF−/− mouse lymphoma cells were obtained from the Shanghai Institute for Biological Science (Shanghai, China) and cultured in B cell medium [45% Dulbecco's modified Eagle's medium (HyClone; GE Healthcare, Chicago, IL, USA), 45% Iscove's Modified Dulbecco's Medium (HyClone; GE Healthcare), 10% fetal bovine serum (Biochrom; Merck, Germany), L-glutamate and β-mercaptoethanol] at 37˚C in a humidified atmosphere with 5% CO2. All drugs (Table I) were obtained from Selleck Chemicals (Shanghai, China). Short hairpin-RNA (shRNA) vectors were generated as described previously (13,14).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from Eμ-Myc p19ARF−/− mouse lymphoma cells using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. RNA was quantified using the Nanodrop 1000 (Invitrogen; Thermo Fisher Scientific, Inc.,) and, in total, 1 µg RNA from each sample was treated with DNase I (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the sample was reverse transcribed using randomized hexanucleotides and M-MLV reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). The temperature protocol for reverse transcription was as follows: 65˚C for 5 min, cooled on ice for 5 min, 25˚C for 10 min, 50˚C for 50 min (cDNA synthesis) and 85˚C for 5 min (deactivation). The product from reverse transcription (10 µl) was diluted to 150 µl in ddH2O and, subsequently, 3 µl from the 150 µl sample was added to each qPCR system. qPCR was performed in triplicate using a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with SYBR-Green Supermix (Bio-Rad Laboratories, Inc.). The sequences of forward and reverse primers for RAD51C were as follows: Forward 5'-CAACTGCGCTGCA TTACGAC-3' and reverse 5'-TGCCAGAAGTGCAGTATA ATCA-3'. The GAPDH gene primers were as follows: Forward, 5'-CCTGGAGAAACCTGCAAAGTATG-3'; reverse, 5'-AGA GTGGGAGTTGCAGTGA-3'. PCR amplification was performed as follows: 94˚C for 2 min (initial denaturation), followed by 45 cycles of 95˚C for 15 sec, 60˚C for 15 sec, and 72˚C for 20 sec. To ensure that RNA samples were not contaminated with DNA, negative controls were obtained by performing PCR on samples that were not reverse transcribed but were otherwise identically processed. Retroviral pMSCV-IREs-GFP vector serves as negative control. All results were normalized by the Pfaffi method (15) using Bio-Rad CFX Manager 3.1.1517.0823 (Bio-Rad Laboratories, Inc.) to the GAPDH internal control and were expressed as the mean ± standard error of the mean.

Western blot analysis. Equal amounts of protein were extracted from Eμ-Myc p19ARF−/− mouse lymphoma cells. Cells were centrifuged at 1,000 x g at 4˚C for 2 min, cell pellets were weighed and resuspended in PBS, mixed with 10 ml 2X SDS sample buffer [2 ml Tris (1 M, pH 6.8), 4.6 ml glycerol (50%), 1.6 ml SDS (10%), 0.4 ml bromophenol blue (0.5%) and 0.4 ml β-mercaptoethanol] and boiled for 15 min at 100˚C to generate whole-cell lysates. Were separated by SDS-PAGE (12% gel) and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) via electroblotting. The membranes were then blocked with 5% skimmed milk in TBST for 1 h, incubated with specific primary antibodies against RAD51C (dilution, 1:1,000; catalog no. ab2180; Abcam, Cambridge, MA, USA) and β-actin (dilution, 1:200; catalog no. sc-47778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4˚C. ProteinFind goat anti-mouse immunoglobulin G horseradish peroxidase-conjugated secondary antibody (dilution, 1:5,000; catalog no. HS201; TransGen Biotech, Co., Ltd., Beijing, China) was applied and incubated at 37˚C for 1 h. Protein detection was performed by applying Immunobilon Western Chemiluminescent HRP Substrate (WBKLS0500; EMD Millipore, Billerica, MA, USA) and images were captured using Luminescent Image Analyzer (LAS4000; FujiFilm, Tokyo, Japan). β-actin was used as a loading control for normalization. Image J software (version 1.49; National Institutes of Health, Bethesda, MD, USA) was used to quantify western blot results.

Drug treatment and flow cytometry-based GFP competition assay. Eμ-Myc p19ARF−/− cells at a density of 1x106 cells/ml were seeded in 48-well plates, as aforementioned, and treated with various concentrations of distinct drugs (Table 1), according to a previous study (12). Gemcitabine, Lovastatin and Taxol were diluted in ethanol, Pematrexed disodium was diluted in water and the other drugs were diluted in DMSO. Half of the drug-containing medium from each experiment was removed and replenished with fresh medium every 24 h to approximate therapeutic situations in which drug dose decreases over time. Cells were analyzed by fluorescence-activated cell sorting (FACS) using propidium iodide (PI) as a viability marker. The LD80,90 value for each drug was defined as the concentration at which the lowest viability reading out of three FACS time points (24, 48 and 72 h) was between 10 and 20%. After
Table I. Names of the 30 drugs used in the study.

| No. | Drug name       | RI value |
|-----|-----------------|----------|
| 1   | Camptothecin    | 0.46     |
| 2   | Cisplatin       | 0.28     |
| 3   | Doxorubicin     | 0.70     |
| 4   | Gemcitabine     | 1.03     |
| 5   | Methotrexate    | 1.04     |
| 6   | Dexamethasone   | 0.62     |
| 7   | Tioguanine      | 0.69     |
| 8   | Otalarpib       | 0.29     |
| 9   | Erlotinib       | 1.02     |
| 10  | Vismodegib      | 0.92     |
| 11  | Actinomycin D   | 0.86     |
| 12  | Vincristine     | 0.70     |
| 13  | Lovastatin      | 0.77     |
| 14  | Vorinostat      | 1.06     |
| 15  | Taxol           | 1.28     |
| 16  | 5-aza-2’-deoxycytidine | 0.75 |
| 17  | Fluorouracil    | 0.84     |
| 18  | Triludrine      | 0.75     |
| 19  | All-trans-retinoic acid | 0.93 |
| 20  | PP242           | 0.87     |
| 21  | Cytarabine      | 0.58     |
| 22  | Fluorastain sodium | 0.82 |
| 23  | Clofarabine     | 1.00     |
| 24  | Pemetrexed disodium | 1.00 |
| 25  | Axitinib        | 0.85     |
| 26  | Albendazole     | 1.12     |
| 27  | Bortezomib      | 0.73     |
| 28  | Adefovir dipivoxil | 0.63 |
| 29  | Prasugrel       | 1.25     |
| 30  | Vardenafil      | 0.88     |

The numbers associated with each drug correspond to the numbers shown in Fig. 2.

Calculation of RI. To compare the relative level of chemoresistance and sensitization conferred by gene knockdown, the concept of RI was used to analyze the GFP competition assay results more accurately. The biological meaning of RI is that in a mixture of uninfected and infected (knockdown) cells, the infected cells will be RI-fold as likely to survive drug treatment when compared with uninfected cells. By this definition of RI, if 1/m uninfected cells survives a drug treatment, then RI/n infected cells would survive. If the total number of uninfected and infected cells is defined as T and the GFP-containing percentage of the untreated population as G1, then the number of surviving uninfected cells (un) can be defined as n-un=T x (1/G1) x 1/m, and the number of surviving infected cells (in) can be defined as n-in=T x G1 x RI/m. T represents the total number of uninfected and infected cells, whereas m represents the number of uninfected cells which resists drug treatment. Thus, the GFP percentage of the treated surviving population (G2) can be calculated as G2=(n-in)/(n-un) + (n-in). n-in and n-un represent the numbers of infected and uninfected cells, respectively. From these equations, it is derived that RI=(G2/G1 x G2)/(G1-G1 x RI); this equation was used in the present study to calculate the RI values (12).

Patients and tissue samples. A total of 213 patients (median age, 53.4 years) with primary breast carcinomas who had undergone curative surgery at Changhai Hospital, Ruijin Hospital and Central Hospital of Huangpu District (Shanghai, China) between 2001 and 2010 were enrolled for this study. Histological confirmation of primary breast carcinoma was obtained from the Department of Pathology at Changhai Hospital. None of the patients had received preoperative adjuvant chemotherapy. Of the 213 patients, paired non-neoplastic breast tissues sampled from the resection margins were available for 99 patients. All of the tissue specimens were obtained for the present study with informed consent from the patients, and the use of the human specimens was approved by the Ethics Committee of Changhai Hospital. The last follow-up date was 31 December 2012, with a median follow-up time of 72 months (range, 5-115 months). The clinicopathological features of the tumors and patient survival times were recorded.

Tissue microarray (TMA) and IHC. A manual arrayer (Beecher Instruments, Sun Prairie, WI, USA) was used to create paraffin-embedded TMA blocks of normal breast and breast cancer tissue specimens obtained from the patients. Each block had at least one 1.5-mm core of non-neoplastic tissue and two 1.5-mm cores of primary tumor tissue; 4-μm paraffin sections were then produced using a standard technique (16). A RAD51C mouse monoclonal antibody (dilution, 1:100; catalog no. ab2180; incubated for 1 h at 37°C; Abcam) was used to detect RAD51C protein expression in the sections. An EnVision kit (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) was used to visualize antibody binding, and slides were subsequently counterstained with hematoxylin. Expression of RAD51C in the TMAs was evaluated by two individuals (Dr. Shao-Guang Liao and Dr. Lu Liu), who were blinded to the other characteristics of the patients, using an Olympus CX31 microscope (Olympus Corporation, Tokyo, Japan). A positive reaction was indicated by a reddish-brown precipitate in the nucleus of a cell. The

the drug LD_{50,00} was determined, Eμ-Myc p19^{Arf-} cells were infected with retroviruses encoding shRNAs targeting specific genes, according to a previous study (12). Eμ-Myc p19^{Arf-} cells were infected in the presence of polybrene (7 μg/ml), by centrifugation at 700 x g for 5 min at room temperature. The negative control used was the same as previously described (12). Following infection, GFP, which was included in the retroviruses as previously described (12), proportion typically reached 30%, which is desired for the GFP competition assay. Individual infected cell populations were counted and seeded at 1x10^6 cells/ml in 48-well plates and treated with drugs, using the aforementioned protocol. Treated and untreated cells were analyzed with flow cytometry after 72 h. GFP-expressing percentages of live (PI-negative) cells were recorded and used to calculate relative resistance index (RI). To avoid outgrowth of the untreated control cells, cells were typically seeded at 0.25 million per ml, and 75% of the medium was replaced at 24 and 48 h (12).
scores of staining depended on the percentage of positive cells and staining intensity. The percentage of positive cells was divided into five grades (percentage scores): 0, <10%; 1, 10-25%; 2, 25-50%; 3, 50-75%; and 4, >75%. The intensity of staining was divided into four grades (intensity scores): 0, no staining; 1, light brown; 2, medium brown; and 3, dark brown. RAD51C staining positivity was determined by the following formula: Overall score=percentage score x intensity score. An overall score of ≤3 was defined as negative, and >3 as positive. Discrepancies in the scores were resolved by discussion between the evaluators.

**Statistical analysis.** All statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA) software. The associations between RAD51C expression and clinicopathological characteristics of breast cancer were analyzed with Pearson’s correlation and $\chi^2$ tests. Survival was defined as the time between diagnosis and mortality. Survival analysis was performed by the Kaplan-Meier method and the log-rank test. Cox’s regression analysis was used to estimate hazard ratios and their 95% confidence intervals. A log-rank test was performed to conduct survival analysis. $P<0.05$ was considered to indicate a statistically significant difference.

**Results**

**Inhibition of RAD51C expression with RAD51C-shRNA.** Eμ-Myc p19Arf-/- cells were stably infected with retroviruses coexpressing RAD51C-shRNA and GFP. The RT-qPCR results showed that RAD51C mRNA was significantly inhibited ($P<0.001$) by shRNA targeting RAD51C and not by the negative control (Fig. 1A). Western blotting revealed a similar effect of RAD51C shRNA on RAD51C protein levels in the cells (Fig. 1B).

**Effect of knockdown of RAD51C on sensitivity to anticancer drugs.** A total of 30 drugs were selected for this study (listed in Table I). All drugs were used at their LD$_{80-90}$, i.e., the concentration at which 80-90% of uninfected lymphoma cells were killed. A single-cell flow cytometry-based GFP competition assay was used to determine the change in sensitivity of the cells to each drug. Lymphoma cells were infected with retroviruses co-expressing RAD51C-shRNA and GFP, and subjected to 72 h of drug treatment. GFP-negative cells in the same population served as an internal control. The effects of the gene knockdown on chemosensitivity were recorded as the GFP-determined RI values. The results indicated that the sensitivities of the cells to three drugs, camptothecin (CPT), cisplatin (DDP) and olaparib, were significantly increased following RAD51C gene knockdown (Fig. 2). The value of RI for each drug is shown in Tables I and II.

Table II. RI values for the three drugs that were more effective against Eμ-Myc p19Arf-/- cells following RAD51C knockdown by shRNA.

| Drug            | RAD51C-shRNA group | Control group | P-value |
|-----------------|--------------------|---------------|---------|
| Camptothecin    | 0.46±0.02          | 0.91±0.08     | <0.001  |
| Cisplatin       | 0.28±0.01          | 0.94±0.01     | <0.001  |
| Olaparib        | 0.29±0.04          | 0.93±0.12     | <0.001  |

RI=(G2-G1 x G2)/(G1-G1 x G2); where G1 is the surviving proportion of GFP-positive cells in an untreated population; and G2 is the surviving proportion of GFP-positive cells following treatment with the named drug. Control group, empty vector; RI, resistance index; RAD51C, RAD51 paralog C; shRNA, short hairpin-RNA.

**IHC, clinicopathological features, and survival time analysis.** The semi-quantitative results of RAD51C immunohistochemical staining in breast cancer tissue are presented in Table III. As is shown in Fig. 3, RAD51C was predominantly expressed in the nuclei and cytoplasm of cancer and normal cells. As
Table III. Expression of RAD51C in breast cancer tissues and paired non-neoplastic breast tissues.

| Breast tissue type | Total, n | RAD51C expression, n (%) | χ²   | P-value |
|--------------------|----------|--------------------------|------|---------|
|                    |          | Negative | Positive |      |         |
| Cancer             | 213      | 103 (48.4) | 110 (51.6) | 1.72 | 0.22    |
| Non-neoplastic     | 99       | 40 (40.4)  | 59 (59.6)  |      |         |

RAD51C protein functions primarily in the nucleus (2-3), cells expressing RAD51C in their nuclei were considered RAD51C-positive.

RAD51C was positively expressed in 51.6% of the breast cancer tissues and in 59.6% of the paired non-neoplastic breast tissues. There were no significant differences in RAD51C
expression observed between breast cancer and paired non-neoplastic breast tissues (Table III).

RAD51C expression was not significantly correlated with histological type, patient age, tumor size, lymph node metastasis, TNM stage, histological grade or estrogen receptor (ER)/progesterone receptor status of the patients with breast cancer. However RAD51C expression in breast cancer tissues positive for Erb-B2 receptor tyrosine kinase 2 (HER2) was significantly higher compared with that in HER2-negative breast cancer tissues (P=0.009; Table IV).

Cox multivariate regression analysis indicated that high RAD51C expression (P=0.01) and ER-positive status (P=0.02) were significant risk factors that may influence the postoperative survival time of breast cancer patients (Table V). Survival analysis showed that the postoperative survival time of patients with expression of RAD51C was significantly shorter compared with that of patients without RAD51C expression (P=0.03; Fig. 4).

**Discussion**

It has been demonstrated that RAD51 paralogs are involved in two distinct complexes associated with HR: The RAD51B/RAD51C/RAD51D/x-ray repair cross complementing (XRCC)2 complex (BCDX2) and the RAD51C/XRCC3 complex (CX3) (17,18). As the only factor found in both the BCDX2 and CX3 complexes, RAD51C plays an important role in early and late HR. Downregulation of RAD51C expression may sensitize cells to DNA-damaging agents (19). In the present study, it was demonstrated that the sensitivity to three drugs, CPT, DDP and olaparib, was
significantly increased in Eμ-Myc p19Arf−/− cells following RAD51C gene knockdown. This result suggests that silencing RAD51C may represent a potential therapeutic strategy against malignant tumors.

Based on the association between RAD51C expression and drug sensitivity in tumor cells, we hypothesized that cancer patients who lack RAD51C expression may be more sensitive to anticancer drugs and have an improved prognosis. To test this hypothesis, IHC was used to detect the expression of RAD51C protein in breast cancer tissues. The result indicated that RAD51C expression in breast cancer tissue was positively correlated with HER2 expression, which is an effective predictive and prognostic marker for breast cancer patients. Furthermore, the result showed that high RAD51C expression is an important risk factor that may influence the postoperative survival time of breast cancer patients. The Cox multivariate regression analysis result regarding ER status was consistent with a previous study (26). Additionally, survival analysis revealed that the postoperative survival time of patients with RAD51C expression was significantly shorter compared with that of patients without RAD51C expression, which confirmed the hypothesis that the high expression of RAD51C is a marker for an adverse prognosis in breast cancer.

In conclusion, it was identified that the sensitivity to three drugs, CPT, DDP and olaparib, significantly increased following RAD51C gene knockdown. This result suggests that silencing RAD51C may represent a potential therapeutic strategy against malignant tumors.

Cox multivariate regression analysis revealed that ER and RAD51C expression were independent risk factors that may influence the postoperative survival time of patients with breast cancer. ER, estrogen receptor; CI, confidence interval; RAD51C, RAD51 paralog C.
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