Impact of germline *RAD51D* mutations on breast cancer: susceptibility to DNA-damaging agents

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Impact of germline *RAD51D* mutations on breast cancer: susceptibility to DNA-damaging agents

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ABSTRACT

Impact of germline *RAD51D* mutations on breast cancer: susceptibility to DNA-damaging agents

BRCAness refers to a deficiency in homologous recombination (HR)-mediated DNA repair, and numerous repair effector proteins are associated with HR. Germline mutations in HR-related genes significantly increase the lifetime risk of breast, ovarian, and other cancers; thus, the concept of BRCAness has been extensively studied. Understanding the impact of a variant in HR genes is crucial for developing appropriate treatment strategies for hereditary breast cancer. Recent studies have shown that RAD51D plays an important role in maintaining genomic integrity.

In this study, we aimed to investigate the association between germline *RAD51D* mutations and breast cancer (BC) in experimental and clinical settings. We observed a decrease in RAD51 expression in RAD51D-deficient triple-negative breast cancer (TNBC) cells *in vitro*. We analyzed the impact of RAD51D-deficiency on drug sensitivity *in vitro* to identify targetable vulnerabilities in RAD51D-deficient tumors. The efficacy of DNA-damaging agents was evaluated in RAD51D-deficient tumors using TNBC cell lines. RAD51D-deficient TNBC cells were significantly more sensitive to cisplatin, and marginally more sensitive to olaparib than wild-type TNBC cells.

In a clinical setting, fourteen cases of BC with germline pathogenic or likely pathogenic mutations in RAD51D were identified through multigene panel testing in the PLEASANT study. Clinicopathological features of germline *RAD51D*-mutated BC were identified by statistically comparing the clinical data of mutation carrier and non-carrier groups. Compared to 1446 wild type cases of BC, they appeared to be more aggressive. The proportion of patients with TNBC was higher and larger tumors (≥2 cm) were more common. RAD51D-deficient tumors showed better pathological complete responses, defined as ypT0 ypN0, to neoadjuvant chemotherapy (NAC), consistent with our experimental data.

RAD51D contributes to protecting genomic integrity, and RAD51D-deficiency confers susceptibility to aggressive BC. RAD51D-deficient tumors were shown to have a phenotype similar

to that of BRCA1-deficient tumors; therefore, they can be targeted by DNA-damaging agents such as platinum-based chemotherapy or PARP inhibitors.

Key words : BRCAness, homologous recombination, RAD51D, genomic integrity, pathological complete responses, cisplatin, olaparib

1. INTRODUCTION

The DNA damage response (DDR) is essential for protecting cells from genotoxic stress and preventing potentially mutagenic genomes from transforming into oncogenes[1]. Specifically, when DNA-double strand breaks (DSBs) occur, cells employ the HR, non-homologous end-joining (NHEJ), or microhomology-mediated end-joining (MMEJ) pathways to maintain genomic stability[2-4]. HR is the most reliable repair pathway because it uses a homologous template to accurately repair DNA. In contrast to HR, NHEJ, which predominately occurs in the G0 or G1 cell cycle, can rapidly ligate DSB ends and introduce genomic insertions, deletions, and rearrangements[5]. In other words, DNA damage in HR-deficient cells is repaired by error-prone mechanisms such as NHEJ and MMEJ, which can cause oncogenic transformation. Therefore, individuals with HR-deficiency are predisposed to develop several cancers, including breast, ovarian, and pancreatic cancers[6].

BRCA1 and BRCA2 play key roles in HR to protect cells from DNA damage. The concept of BRCAness, which represents tumors with defective HR, has been expanded to provide appropriate therapeutic approaches for tumors with a phenotype similar to BRCA-deficient tumors[7]. Recent studies have shown that RAD51D plays an important role in maintaining genomic integrity (Figure 1)[8]. RAD51D suppresses tumorigenesis by mediating HR to prevent genetic alterations[9,10]. RAD51D is one of six paralogs that shares a conserved domain with RAD51 in humans[11]. The BCDX2 complex, which contains RAD51B, RAD51C, RAD51D, and XRCC2, promotes RAD51 filament assembly to stimulate HR-mediated DNA repair[12]. Disruption of RAD51 paralogs impairs RAD51 foci formation and leads to HR deficiency[13,14]. These paralogs are also involved in replication fork protection and restart[15,16]. Although all of these paralogs are required for HR, pathogenic germline variants of *RAD51C* and *RAD51D* are strongly associated with a predisposition to BC[17]. However, there are insufficient reports on *RAD51D* mutations, and the association between germline *RAD51D* mutations and BC, including its clinicopathological features, remains unclear.

Previous studies have revealed that genomic instability induced by HR defects can confer sensitivity to DNA-damaging agents[18-20]. Platinum-based chemotherapy causes DNA damage in proliferating tumor cells by forming crosslinks with DNA, resulting in apoptosis. Poly (ADP-ribose) polymerase (PARP) inhibitors have been developed to specifically target DDR-deficient tumors based on synthetic lethality, which can minimize the off-target toxicity of conventional

chemotherapeutic agents[21-23]. PARP inhibitors block the catalytic activity of PARP family members, causing PARP-trapping and inhibition of single-strand break repair[24,25]. In BC, olaparib and talazoparib have been approved for treatment of germline *BRCA* mutation carriers with metastatic HER2-negative BC[26,27]. PARP inhibitor monotherapy resulted in a significant improvement in progression-free survival compared with standard chemotherapy. These DNA-damaging agents effectively act on cancer cells, which commonly lack DNA repair capacity[28]. Nevertheless, the relatively low frequency of *BRCA1/2* mutations limits their applicability in patients[29]. Therefore, further research is needed to identify potential biomarkers that can increase their application.

In the current study, we demonstrated that RAD51D-deficient tumors could benefit from platinum-based chemotherapy or PARP inhibitors *in vitro.* Furthermore, we determined the clinicopathological characteristics of patients with germline *RAD51D*-mutated BC in the Republic of Korea. This can help to understand the association between germline *RAD51D*-mutation and BC and establish more appropriate treatment strategies for patients with *RAD51D* mutations, beyond *BRCA1/2*.

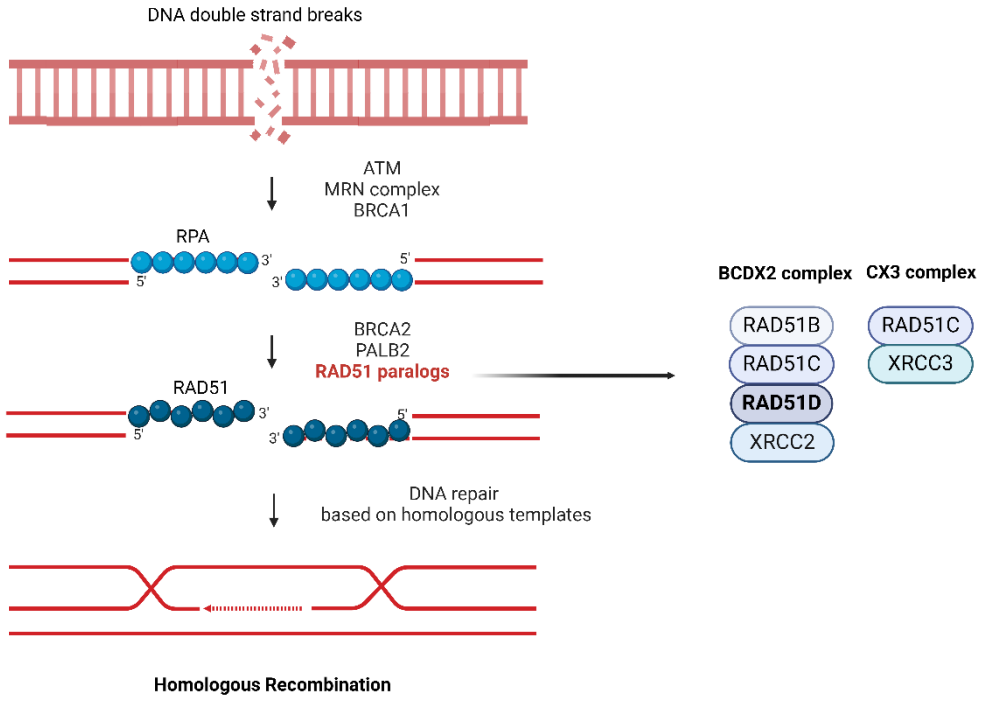

Figure 1. Role of RAD51 paralogs in homologous recombination. When DNA double strand breaks occur, RAD51 coils the single strand of the 3′ overhang. RAD51 paralogs mediate HR by promoting RAD51 filament formation at damage sites.

2. MATERIALS AND METHODS

2.1. Cell culture

The human TNBC cell lines MDA-MB-231 (ATCC, HTB-26) and MDA-MB-468 (ATCC, HTB-132), were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) and Dulbecco's modified Eagle's medium (Thermo Fisher Scientific), supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin and streptomycin (Thermo Fisher Scientific). All cells were maintained at 37 ℃ and 5% CO_2 in a humidified incubator.

2.2. Cell proliferation assay

Cisplatin (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in phosphate buffered saline containing 140 mM NaCl prior to culture treatment. Olaparib (Selleckchem, Houston, TX, USA) was dissolved in dimethyl sulfoxide (Duchefa Biochemie, Amsterdam, Netherlands) to yield a 10 mM stock solution, and aliquots were stored at −80 °C for drug treatment. Cells were seeded in 96-well plates (Corning, Corning, NY, USA) and transfected with small interfering RNA (siRNA) according to the manufacturer's instructions. Olaparib was serially diluted by 1/3 from 100 μM, and cisplatin was serially diluted by 1/3 from 50 μM, and used to evaluate the sensitivity of RAD51D-deficient BC cells. Cell viability was determined using an MTT assay. After 48 hours of treatment, the medium was replaced with fresh medium containing diluted MTT (10%), and incubated for 2 hours at 37 ℃. Formazan crystals were dissolved in methanol and the absorbance was measured at 570 nm using a VersaMax Microplate Reader (Molecular Devices, San Jose, CA, USA).

2.3. siRNA transfection

RAD51D gene silencing was achieved by siRNA transfection using the RNAiMAX Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were seeded in six well plates at a density of 5×10^5/well, and transfected with siRNA after 24 hours incubation. Pre-designed siRNA purchased from Bioneer (AccuTarget Negative Control siRNA, Bioneer Corporation, Daejeon, Republic of Korea) and Thermo Fisher Scientific (SilencerSelect siRNA against RAD51D, assay ID: S11743) was used to induce RAD51D-deficieny in BC cells. The

samples were analyzed 48 hours after transfection.

2.4. Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions, and the RNA concentration was determined using a Nanodrop 2000/2000x spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed using PrimeScript RT Master Mix (Takara, Kyoto, Japan) in an Applied Biosystems Veriti 96-well Thermal Cycler (Thermo Fisher Scientific). Real-time PCR was performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Target gene expression levels were determined by normalizing to housekeeping gene levels. The following primers were used in this study: RAD51D (forward-AATGGCGCTGATCTCTACGA, reverse-TGTCAGCCCTCCATTGGAAT) and GAPDH (forward- CAACGGATTTGGTCGTATTGG, reverse-GCAACAATATCCACTTTACCAGAGTTAA).

2.5. Western blot analysis

Parental TNBC cell lines and their transfected cells were lysed in a cell extraction buffer (Thermo Fisher Scientific) containing a protease inhibitor cocktail (Roche, Basel, Switzerland). The protein concentration in each sample was measured using a Quick-Start Bradford protein assay kit (Bio-Rad). Sodium dodecyl sulfate (SDS, 5X) sample buffer was added to the cell lysates and the samples were heated at 100 °C for 7 minutes. From each sample, 10 μg of protein was loaded onto 10% SDS-polyacrylamide gel electrophoresis gels and then transferred to a nitrocellulose membrane (Bio-Rad) for 2 hours. The membranes were blocked with 5% skim milk in Tris-buffered saline-Tween 20 (TBST) for 1 hour, and then incubated with primary antibodies diluted in 5% skim milk overnight at 4 °C. The membranes were washed with TBST and incubated with secondary antibodies in 5% skim milk for 1 hour at room temperature. After washing the membranes, the proteins were visualized using enhanced chemiluminescence substrate (Thermo Fisher Scientific) and imaged using an Amersham ImageQuant 800 (Cytiva, Marlborough, MA, USA). The following primary and secondary antibodies were used: RAD51D rabbit (ab202063, Abcam, 1:1000), RAD51 rabbit (8875S, Cell Signaling Technology, 1:1000), β-actin rabbit (AbC-2002, Abclonal, 1:5000), and

horseradish peroxidase-conjugated anti-rabbit IgG (ADI-SAB-300, Enzo Life Sciences, 1:5000). Quantification of each protein expression level was performed using Image J[30].

2.6. Clinical data collection

In a previous study called the PLEASANT study (IRB approval number: 4-2015-0819, 4-2018-0259), comprehensive multigene panel tests were performed for patients at high risk of hereditary breast and ovarian cancer syndrome in Yonsei Cancer Center[31]. Patients with at least one of the following risk factors were considered to be at high risk for hereditary breast cancer syndrome: at least one case of breast or ovarian cancer in first- or second-degree relatives, a first diagnosis of BC before the age of 40, or bilateral BC. This study aimed to evaluate the clinical implications of multigene panel testing of genes other than *BRCA* in Korean patients with *BRCA1/2* mutation-negative BC. Comprehensive multigene panel tests targeting 65 cancer predisposition genes were performed on genomic DNA extracted from the patient's peripheral blood samples. Genetic variants were classified in accordance with the American College of Medical Genetics and Genomics guidelines. Mutation carriers were referred to as patients with pathogenic or likely pathogenic variants.

A total of 1872 patients with high risk factors were enrolled in the PLEASANT study. In our study, patients with carcinomas other than BC, mutations detected in genes other than *RAD51D*, or missing clinical information were excluded. There were 14 patients with BC carrying germline pathogenic or likely pathogenic *RAD51D* mutations, and 1446 BC patients with wild-type *RAD51D* were included as the control group. A detailed flowchart of the study is presented in Figure 2.

The following clinicopathological characteristics of BC were obtained by reviewing the electronic medical records: age at diagnosis, next-generation sequencing results, molecular subtype, hormone receptor status, human epidermal growth factor receptor 2 (HER2) status, Ki67 expression level, tumor size, histologic grade, neoadjuvant chemotherapy (NAC), metastasis, and recurrence.

2.7. Definition of pathological complete response and molecular subtypes

Pathological complete response (pCR) was defined as the absence of residual invasive and in situ cancer in the breast and axillary lymph nodes, ypT0 ypN0, after NAC[32]. Patients with TNBC

were offered an anthracycline-, taxane-, nitrogen mustard-, or platinum-containing NAC. Patients with HER2-positive BC were offered a taxane-, or platinum-containing regimen in combination with trastuzumab or pertuzumab. Molecular subtypes of BC were determined based on hormone receptor status, HER2 status, and Ki67 expression level, which were assessed by immunohistochemistry of tumor tissues.

2.8. Statistical analysis and illustration

Statistical analyses were conducted to compare the clinicopathological features of germline *RAD51D* mutation carriers and non-carriers using R version 4.3.1 (Posit, Boston, MA, USA). Categorical variables were compared using the Fisher's exact test. Wilcoxon rank-sum tests were used to compare the continuous variables. The experimental data were statistically analyzed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). A *P*-value of <0.05 was considered statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$). The workflow used in this study was created using BioRender (Toronto, Canada).

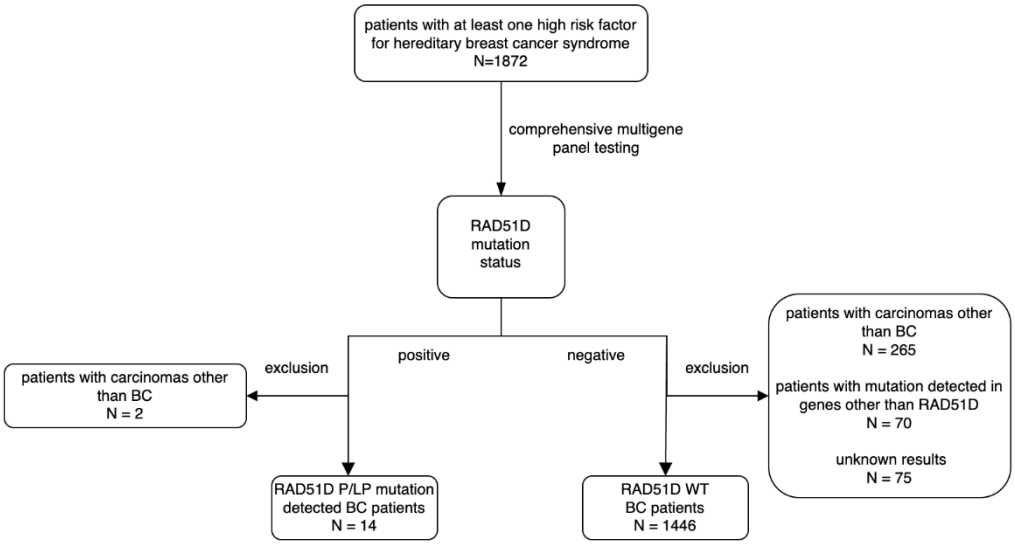

Figure 2. Flowchart of the study population. The flowchart shows the patient selection process.

3. RESULTS

3.1. Depletion of RAD51D reduces RAD51 expression, leading to HR deficiency

Defects in RAD51 paralogs cause impairment of RAD51 foci formation and HR deficiency[13,15,33]. To evaluate the effect of RAD51D-inactivation on RAD51 expression, we used siRNA to silence the *RAD51D* gene in MDA-MB-231, and MDA-MB-468 cells, which are TNBC cell lines known to have wild-type *BRCA1/2*. Proteins from siRNA-transfected TNBC cell lines were analyzed by western blotting. Successful *RAD51D* silencing was confirmed by RT-PCR and western blotting. Treatment with siRNA reduced *RAD51D* mRNA expression by approximately 80% (Figure 3), and downregulated protein expression by more than 70% (Figure 4). Decreased RAD51 expression was observed in TNBC cells transfected with siRAD51D compared to that in cells transfected with siControl (Figure 5). Because RAD51 mediates strand exchange to accurately repair DNA in HR, decreased RAD51 expression may be associated with HR deficiency.

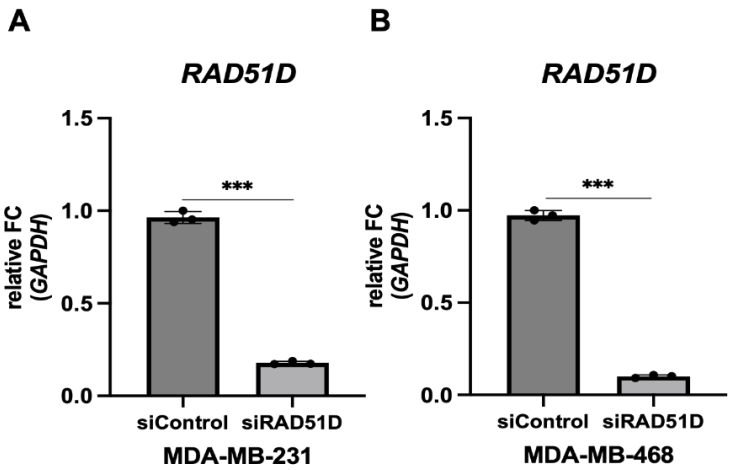

Figure 3. Relative *RAD51D* mRNA expression levels in TNBC cell lines treated with siControl or siRAD51D. (A) The relative *RAD51D* mRNA expression levels were significantly reduced in MDA-MB-231 cells, and (B) MDA-MB-468 cells after siRAD51D transfection. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$)

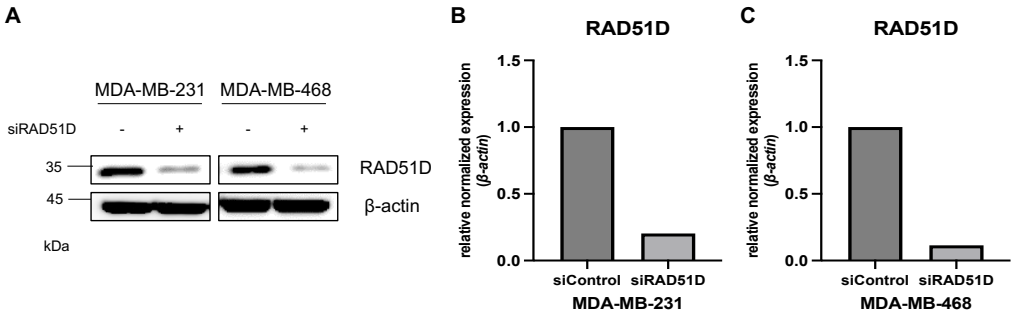

Figure 4. Relative RAD51D protein expression levels in TNBC cell lines treated with siControl or siRAD51D. (A) Western blot analysis was performed to confirm the knockdown of RAD51D in MDA-MB-231, and MDA-MB-468 cells. (B) Quantification of the RAD51D protein expression levels in MDA-MB-231 cells, and (C) MDA-MB-468 cells.

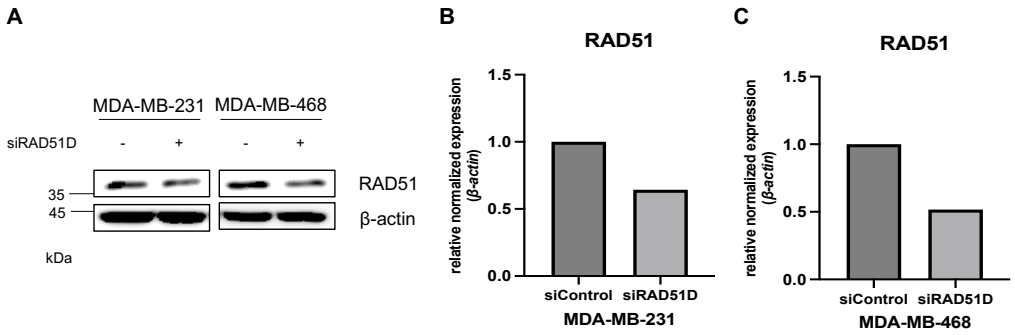

Figure 5. The impact of *RAD51D* silencing on RAD51 protein expression. (A) A decrease in RAD51 protein expression was observed in siRAD51D-treated MDA-MB-231 cells, and MDA-MB-468 cells. (B) Quantification of the RAD51 protein expression levels in MDA-MB-231 cell, and (C) MDA-MB-468 cells.

3.2. RAD51D-deficiency sensitizes triple-negative breast cancer cells to DNA-damaging agents

Previous studies have shown that patients with *BRCA*-mutated BC achieved a higher pCR rate than non-carriers after NAC[19,34]. Thus, we sought to determine the impact of RAD51D-deficiency on drug sensitivity to confirm whether RAD51D-deficient tumors can also benefit from DNA-damaging agents (Figure 6). The cell proliferation assay revealed that RAD51D-deficiency sensitized TNBC cells to cisplatin and olaparib, which induce genomic instability. Cisplatin was shown to be more effective than olaparib. Cisplatin was more toxic at low concentrations to BC cells treated with siRNA targeting *RAD51D* than to cells treated with siControl (Figure 7). No significant reduction in cell viability was observed following olaparib treatment (Figure 8). Olaparib treatment resulted in slightly greater differences in the viability of MDA-MB-231 cells compared to MDA-MB-468 cells.

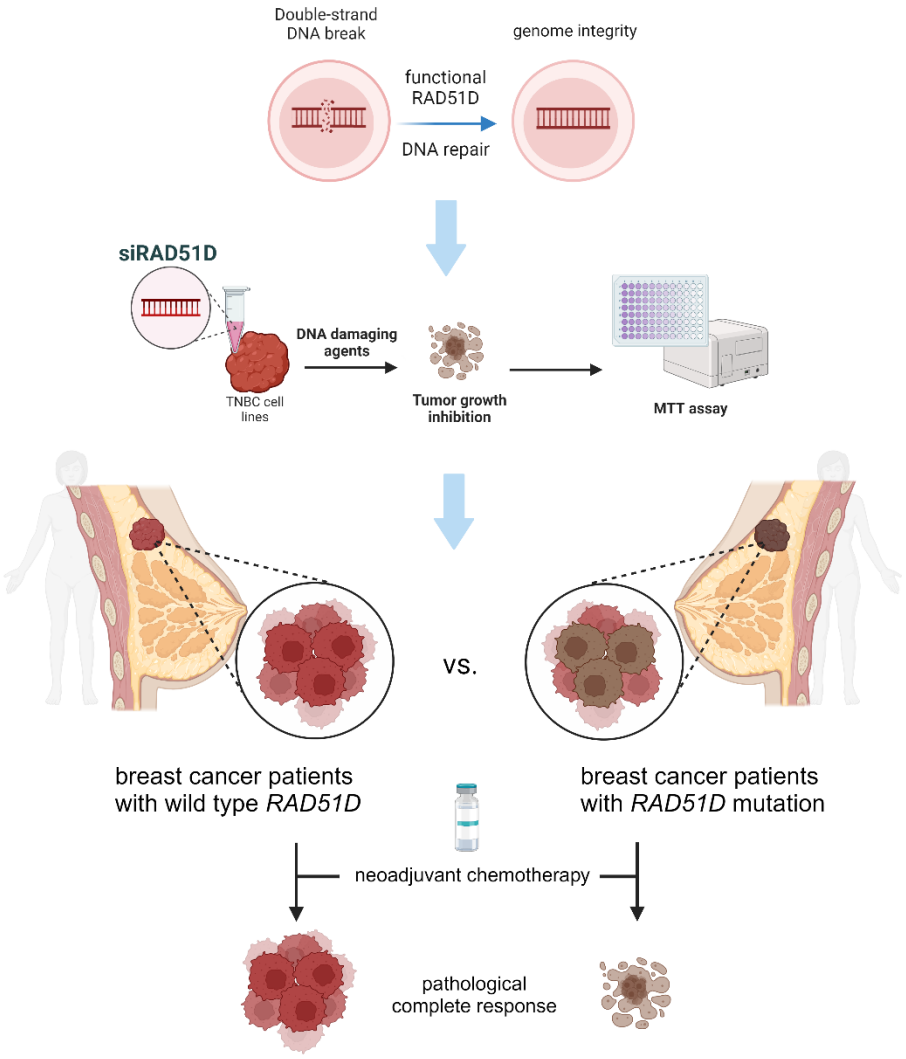

Figure 6. Study scheme showing nonfunctional RAD51D sensitizes cancer cells to DNA-damaging agents. The scheme of the hypothesis states that nonfunctional RAD51D confers sensitivity to DNA-damaging agents in experimental and clinical data.

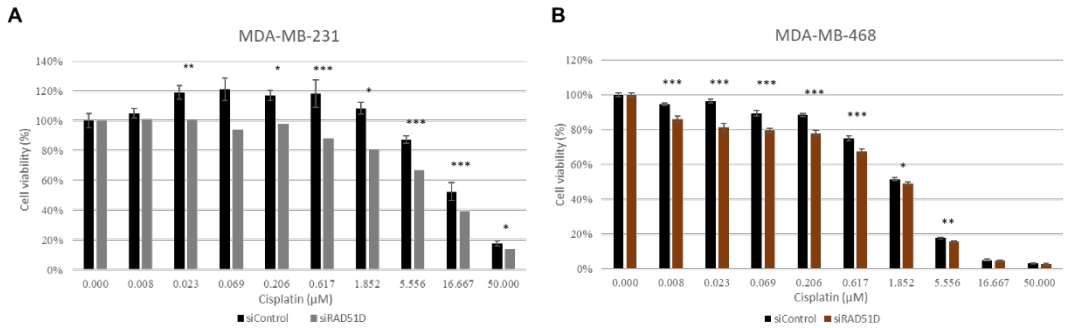

Figure 7. RAD51D-deficiency induces TNBC cells to be more sensitive to cisplatin. Cell proliferation assay revealed that siRAD51D-transfected (A) MDA-MB-231 cells, and (B) MDA-MB-468 cells were more sensitive to cisplatin, compared to siControl-transfected cells. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$)

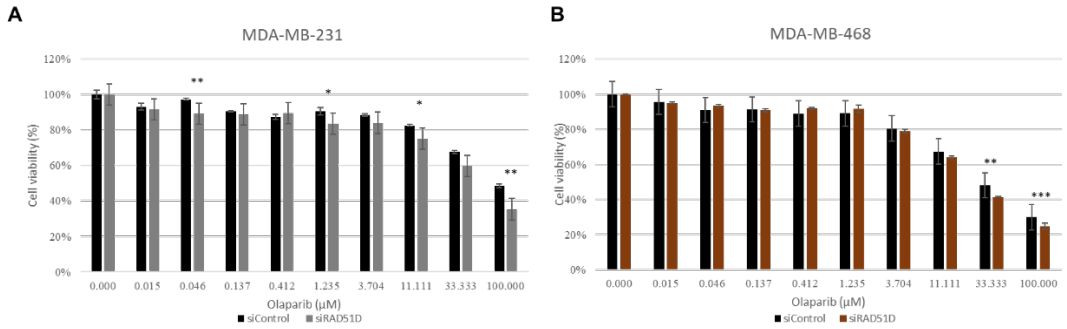

Figure 8. RAD51D-deficiency confers mild sensitivity to olaparib in TNBC cells. Cell proliferation assay was performed to evaluate olaparib sensitivity in (A) MDA-MB-231 cells, and (B) MDA-MB-468 cells. A slightly increased sensitivity was observed in siRAD51D-transfected cells. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$)

3.3. Differences in clinicopathological characteristics in germline *RAD51D* mutation carriers and non-carriers

RAD51D-deficient tumors were more susceptible to DNA-damaging agents *in vitro*, indicating that RAD51D contributes to the protection of genomic integrity. We also sought to investigate the clinicopathological features of *RAD51D*-mutated BC using clinical data (Figure 6). To characterize BC with *RAD51D* mutations, patients with germline *RAD51D* mutations were identified through comprehensive multigene panel tests. We identified 14 patients with BC harboring a germline *RAD51D* mutation in our cohort, with a prevalence of 0.97%. When comparing the clinical data, the *RAD51D* mutation carrier group showed more aggressive tumor phenotypes than the non-carrier group (Table 1). TNBC was more prevalent in the mutation carrier group (50%, n = 7) than in the non-carrier group (20.5%, n = 297) ($P = 0.014$). Mutation carriers were more likely to have estrogen receptor (ER)-negative ($P = 0.001$) and progesterone receptor (PR)-negative tumors ($P = 0.003$) than non-carriers. Cell proliferation was highly increased in tumors with *RAD51D* mutations, as confirmed by Ki-67 expression levels ($P = 0.048$). A tumor size ≥2 cm was more common in the carrier (64.3%, n = 9) than the non-carrier group (33.2%, n = 480) ($P = 0.003$). In the mutation carrier group, a higher proportion of patients received NAC ($P = 0.006$), and notably, they achieved better pCR than the non-carriers who received NAC ($P = 0.03$) (Figure 9). Although the proportion of TNBC in each group receiving NAC was not significantly different, the pCR rate was 66.7% (n = 6) in the mutation carrier group, whereas it was only 30.5% (n = 125) in the non-carrier group. These data were consistent with experimental data showing that RAD51D-deficient tumors were more sensitive to DNA-damaging agents. Most patients were diagnosed with BC before the age of 50 years, and there was no significant difference in age at diagnosis between the two groups because the patients enrolled in this study were at a high risk of hereditary BC.

Table 1. Clinicopathological characteristics between germline *RAD51D* mutation carriers and non-carriers

Status	WT (N=1446)	P/LP (N=14)	*P*-value
Age			
median	47.0 [39.0;56.0]	42.0 [38.0;52.0]	0.587
≤50	907 (62.7%)	10 (71.4%)	0.694
>50	539 (37.3%)	4 (28.6%)	
Subtype			0.003
Luminal A	610 (42.2%)	1 (7.1%)	
Luminal B	413 (28.6%)	3 (21.4%)	
HER2	126 (8.7%)	3 (21.4%)	
TNBC	297 (20.5%)	7 (50.0%)	
TNBC			0.014
no	1149 (79.5%)	7 (50.0%)	
yes	297 (20.5%)	7 (50.0%)	
ER			0.001
negative	424 (29.3%)	10 (71.4%)	
positive	1022 (70.7%)	4 (28.6%)	
PR			0.003
negative	610 (42.2%)	12 (85.7%)	
positive	836 (57.8%)	2 (14.3%)	
HER2			0.378
negative	1181 (81.7%)	10 (71.4%)	
positive	251 (17.4%)	4 (28.6%)	
unknown	14 (1.0%)	0 (0.0%)	
Ki67			
median	12.7 [5.0;32.0]	48.1 [6.3;72.1]	0.048
<14	752 (52.0%)	4 (28.6%)	0.196

≥14	683 (47.2%)	10 (71.4%)	
unknown	11 (0.8%)	0 (0.0%)	
Tumor Size			
median	1.5 [0.8; 2.3]	2.2 [1.5; 3.4]	0.023
<2 cm	960 (66.4%)	4 (28.6%)	0.003
≥2 cm	480 (33.2%)	9 (64.3%)	
unknown	6 (0.5%)	1 (7.1%)	
Histologic Grade			0.229
1	354 (24.5%)	1 (7.1%)	
2	561 (38.8%)	7 (50.0%)	
3	300 (20.7%)	5 (35.7%)	
unknown	231 (16.0%)	1 (7.1%)	
NAC			0.006
no	1036 (71.6%)	5 (35.7%)	
yes	410 (28.4%)	9 (64.3%)	
Metastasis			0.216
no	1117 (77.2%)	9 (64.3%)	
lymph node	298 (20.6%)	4 (28.6%)	
distant	31 (2.1%)	1 (7.1%)	
Recurrence			1
no	1432 (99.0%)	14 (100.0%)	
yes	14 (1.0%)	0 (0.0%)	

* WT; wild-type, P/LP; pathogenic/likely pathogenic

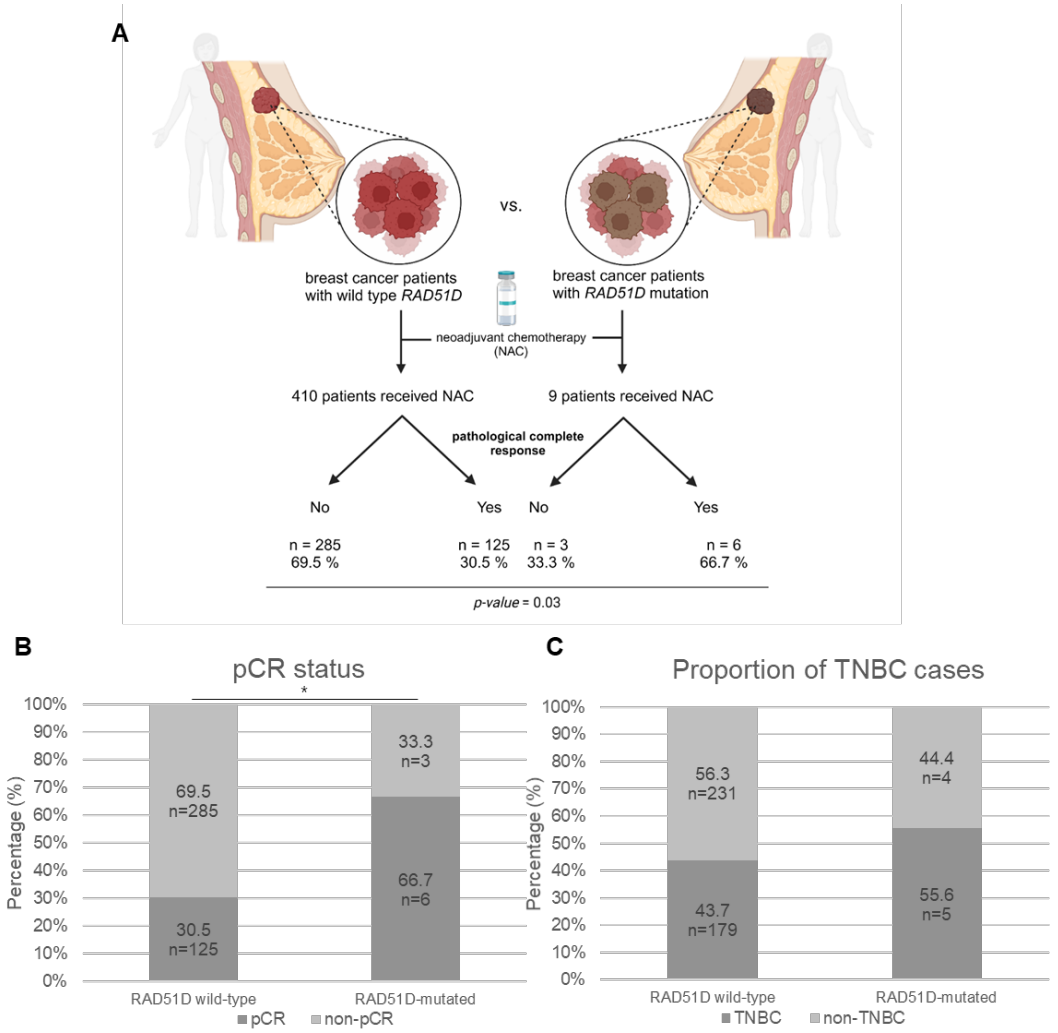

Figure 9. Pathological complete response according to *RAD51D* mutation status. (A) Mutation carriers achieved a better pCR than the non-carriers after receiving NAC (*P = 0.03*) (B) Bar graph showing the pCR status in the two groups (C) Bar graph showing the proportion of TNBC cases among patients receiving NAC.

3.4. Germline *RAD51D* variants identified in patients with BC

A total of 14 variants in *RAD51D* (NM_002878.3) were identified in the present study (Figure 10). The effects of the variants on the protein were frameshift (42.9%, n = 6), splicing (28.6%, n = 4), nonsense (21.4%, n = 3), or exon deletion (7.1%, n = 1). The most frequent mutation, p.Lys91IlefsTer13, was located in exon 4 and accounted for nearly 50% of cases. The second most frequent mutation in intron 9 disrupts RNA splicing. A novel deletion of exons 7–10 was observed in our cohort. All the variants are described in Table 2.

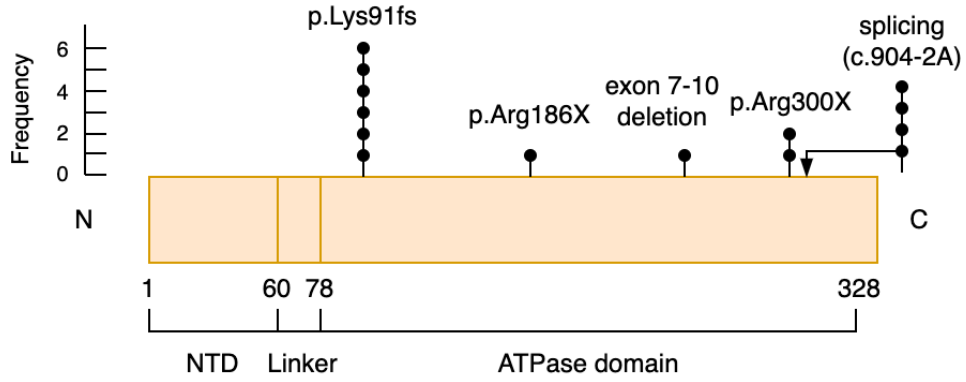

Figure 10. Distribution of deleterious mutations in *RAD51D* identified in this study. The diagram describes the distribution of the 14 variants in *RAD51D*. (* N; N-terminus, NTD; N-terminal domain, C; C-terminus)

Table 2. Pathogenic variants in *RAD51D* identified in this study

Location	Nucleotide	Protein	Molecular consequence	Genotype	Frequency	Reference
Exon 4	c.270_271dup	p.Lys91IlefsTer13	Frameshift	Hetero	6 (42.9%)	[35,36]
Exon 6	c.556C>T	p.Arg186Ter	Nonsense	Hetero	1 (7.1%)	[37,38]
Exon 7–10	exon 7–10 deletion	NA	Gross deletion	Hetero	1 (7.1%)	Novel, but exon deletion was observed in [39]
Exon 9	c.898C>T	p.Arg300Ter	Nonsense	Hetero	2 (14.3%)	[37,40]
Intron 9	c.904-2A>T	NA	Splicing	Hetero	4 (28.6%)	[41]

* NA: not applicable

4. DISCUSSION

In this study, we characterized *RAD51D*-mutated BCs and found that RAD51D-deficiency contributes to genomic instability. We determined whether *RAD51D* inactivation affects the expression of RAD51, which plays a crucial role in strand exchange with homologous DNA templates in HR[2,42]. RAD51 expression was downregulated as a result of *RAD51D* silencing. This result is consistent with a previous study showing that disruption of RAD51 paralogs leads to reduced HR activity, with disruption of RAD51C, and RAD51D causing the greatest reduction[13]. RAD51 nuclear foci formation, as assessed by RAD51 expression at DNA damage sites, was lower in RAD51 paralog-deficient cells than in wild-type cells under both spontaneous and irradiation-induced conditions[13]. As RAD51D is involved in HR-mediated DNA repair, we hypothesized that nonfunctional RAD51D induces HR deficiency, resulting in vulnerability to DNA-damaging agents such as platinum-based chemotherapy or PARP inhibitors.

Our study demonstrated that RAD51D-deficiency sensitizes BC cells to DNA-damaging agents *in vitro* and using clinical data. RAD51D-deficient TNBC cells were significantly more sensitive to cisplatin *in vitro*, and patients with *RAD51D*-mutated BC achieved a higher pCR rate than wild-type patients after receiving NAC. DNA-damaging agents significantly reduced tumor cell proliferation in RAD51D-deficient TNBC cells, which was similar to the trend observed in *BRCA*-mutated cancer cells, albeit to different degrees[43]. Cisplatin was shown to be more effective against these cells than olaparib in this study, whereas there was only a slight difference in sensitivity to olaparib between siRAD51D-transfected BC cells and siControl-transfected cells. Overall, our experimental data suggest that RAD51D contributes to protecting genomic integrity and conferring sensitivity to cisplatin but appears to be insufficient to induce significant synthetic lethality with PARP inhibitors alone. This study is noteworthy because there are few studies evaluating drug sensitivity in RAD51D-deficient TNBC cells and reporting on patients with *RAD51D*-mutated BC.

Remarkably, mutation carriers who received NAC achieved better pCR than non-carriers, consistent with the experimental results. Platinum-based NAC is associated with significantly increased pCR rates in patients with TNBC[44]; however, the proportion of TNBC in each group was not significantly different in our study. Higher pCR rates may be due to the intrinsic properties of RAD51D contributing to DNA repair rather than the effect of TNBC. There is supporting evidence

that breast cancers exhibiting BRCAness show enhanced sensitivity to chemotherapy[45,46]. BRCA status is associated with sensitivity to platinum-based chemotherapy and PARP inhibitors[47-49]. BRCA mutation carriers have higher pCR rates than non-carriers, and pCR is associated with a better prognosis, regardless of BRCA status[47]. In addition to BRCA1/2, RAD51-deficiency can be a predictive biomarker of response to platinum-based chemotherapy, and RAD51 overexpression is associated with resistance to chemotherapy[50,51]. Huang et al. revealed that regulation of RAD51 and RAD51D by microRNAs promotes chemosensitivity to DNA-damaging agents, mediated by HR defects[52]. The better efficacy of platinum-based chemotherapy and PARP inhibitors in tumors with BRCAness is related to defects in the DNA repair capacity[53,54]. These findings are consistent with those of the present study.

RAD51D-mutated BCs were shown to have phenotypes similar to *BRCA1*-mutated BCs. The mutation carrier group had a high incidence of TNBC, which is the most aggressive subtype of BC with a poor prognosis. *RAD51D*-mutated BCs were more likely to have ER-negative and PR-negative tumors than wild-type BCs. The cell proliferation rate was significantly higher in *RAD51D*-mutated BCs than in wild-type BCs. The median Ki67 expression level in the mutation carrier group was 48.1, whereas that in the non-carrier group was 12.7. These data indicated that *RAD51D*-mutated BCs were similar to *BRCA1*-mutated BCs rather than to *BRCA2*-mutated BCs[55]. While it has been reported that the mean age at diagnosis of patients with *RAD51D*-mutated BC is significantly younger than that of non-carriers, the age at diagnosis of the mutation carrier and non-carrier groups did not differ significantly in our study[56]. This is because the PLEASANT study group only included high-risk patients, and one of the criteria was a diagnosis of BC before the age of 40 years. This selection bias, targeting patients with a high risk for hereditary BC syndrome, may have resulted in a relatively high frequency of deleterious *RAD51D* mutations in our study compared to previous studies, which ranged from 0.07–0.38%[56-59].

Among the 14 cases of *RAD51D* variants, we identified six cases of the p.K91fs mutation. This mutation was previously found in UK and Chinese populations[35,36,56]. The frequency of the p.K91fs mutation in our study was 42.9%, which was higher than that in the UK population, but similar to the Chinese study by Chen et al. in that it accounted for the largest proportion of mutation cases. Although gross exon deletions in *RAD51D* have been reported previously, a novel deletion of exons 7–10 was observed in our cohort[39].

This study had some limitations. First, experiments were not performed to accurately assess HR deficiency in RAD51D-deficient tumor cells. A slight decrease in RAD51 expression was observed in the total protein of these cells, which is suggestive of HR deficiency. Therefore, experiments to demonstrate HR deficiency, such as RAD51 foci formation assays, are required to confirm the precise DNA repair capacity. The second limitation was the small number of patients with germline *RAD51D* mutations in the prospective cohort. Additional patient data are required to obtain more accurate results regarding *RAD51D* mutations. Further research should be performed to estimate HR deficiency in *RAD51D*-mutated patient tissues using homologous recombination deficiency testing to identify predictive biomarkers for platinum-based chemotherapy or targeted therapy with PARP inhibitors. Despite these limitations, experimental and clinical data confirmed that RAD51D is involved in maintaining genomic stability.

Our study underlines the association between germline *RAD51D* mutations and BC, and provides insights into the clinicopathological features of germline *RAD51D*-mutated BC. Notably, a significantly better response to DNA-damaging agents was observed in RAD51D-deficient BC cells in both *in vitro* and clinical data. Taken together, these results indicate that RAD51D contributes to the protection of genomic integrity.

5. CONCLUSION

As research on RAD51D is still insufficient, our study can help comprehensively understand the association between *RAD51D* mutations and BC. It was experimentally and clinically demonstrated that RAD51D-deficient BC cells are more sensitive to DNA-damaging agents than wild-type BC cells. A comparison of patient data revealed that *RAD51D*-mutated BC cases were similar to *BRCA1*-mutated BC cases. These findings can help to establish effective treatment strategies for patients with *RAD51D*-mutated BC.

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APPENDICES

BC : Breast Cancer
DDR : DNA Damage Response
DSBs : Double Strand Breaks
ER : Estrogen Receptor
HER2 : Human Epidermal Growth Factor Receptor 2
HR : Homologous Recombination
MMEJ : Microhomology-Mediated End Joining
NAC : Neoadjuvant Chemotherapy
NHEJ : Non-Homologous End Joining
PARP : Poly (ADP-Ribose) Polymerase
pCR : Pathological Complete Response
PR : Progesterone Receptor
RT-PCR : Real-Time Polymerase Chain Reaction
SDS: Sodium Dodecyl Sulfate
siRNA : Small Interfering Ribonucleic Acid
TNBC : Triple-Negative Breast Cancer

Abstract in Korean

RAD51D 배선 돌연변이가 유방암에 미치는 영향: DNA 손상 물질에 대한 감수성

BRCAness 란, 상동 재조합에 의해 매개되는 DNA 복구 과정에 손상이 생긴 것을 말한다. 상동 재조합 과정에 수많은 단백질이 관여하고, 관련 유전자에 배선 돌연변이가 있을 경우에 유방, 난소암 등 암에 걸릴 위험이 증가하기 때문에 BRCAness 의 개념을 확장하려는 연구가 이어지고 있다. 상동 재조합과 관련된 유전자의 변이를 이해하는 것은 유전성 유방암의 적절한 치료 전략을 제시하는 데에 도움이 될 것이다. 최근에는 RAD51D 가 유전체 보존에 중요한 역할을 한다고 보고되고 있다.

본 연구에서는 한국에서 배선 RAD51D 돌연변이와 유방암의 연관성을 조사하였다. 삼중 음성 유방암 세포주를 활용하여 RAD51D 의 발현을 억제한 유방암 세포에서 DNA 손상을 일으키는 항암제의 민감성을 확인하였다. RAD51D 기능이 불완전한 유방암 세포가 야생형 유방암 세포보다 cisplatin 에 더 민감하게 반응하고, olaparib 에 약간 더 민감하다는 것이 실험적으로 입증되었다. 더 나아가서 RAD51D 돌연변이를 갖는 유방암 환자의 임상 병리학적 특징을 분석하였다. 다중 유전자 패널 검사의 임상적 의의를 확인하고자 진행되었던 전향적 PLEASANT 연구에서 병원성, 또는 병원성 가능성이 있는 총 14 케이스의 RAD51D 변이가 확인되었다. 배선 RAD51D 돌연변이가 있는 유방암의 특징을 확인하고자, 돌연변이가 있는 유방암 환자 그룹과 야생형 유전자를 갖는 유방암 환자 그룹의 임상 데이터를 통계적으로 비교 분석하였다. 야생형 유전자를 갖는 유방암 1446 케이스와 비교했을 때, 야생형 유방암보다 더 공격적인 암의 특징을 보였다. 유방암 아형 중 삼중 음성 유방암이 가장 많이 차지하고 발병하였고, 종양 조직의 크기가 2 cm 보다 큰 케이스들이 유의미하게 많았다. 또한, RAD51D 돌연변이가 있는 유방암 환자 그룹이 야생형 유방암 환자 그룹보다 선행 항암 후 잔존 병변이 없는 병리학적 완전 관해가 더 잘 유도되는 것으로 나타났다. 이를 통해 RAD51D 기능이 불완전한 세포가 DNA 손상을 일으키는 항암제에 더 잘 반응하는 것을 실험적, 임상적으로 확인하였다.

RAD51D는 유전체 보존을 유지하는 데에 기여하며, 이 유전자의 불완전한 기능은 공격적인 유방암에 대해 감수성을 부여하는 것으로 나타났다. RAD51D 돌연변이가 있는 유방암은 BRCA1 유전자에 돌연변이가 있는 유방암과 유사한 특징을 보였고, 이는 PARP 저해제나 백금 기반 항암제로부터 효과를 볼 수 있다는 것을 알 수 있다.

핵심되는 말 : BRCAness, 상동재조합, RAD51D, 유전체 보존, 병리학적 완전 관해