Altered DNA Binding by the Human Rad51–R150Q Mutant Found in Breast Cancer Patients

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The human Rad51 protein (HsRad51) catalyzes homologous pairing and strand exchange between single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) during recombinational repair of double-stranded DNA breaks. An *HsRad51* mutation that results in the substitution of Gln for Arg150 (R150Q) was found in bilateral breast cancer patients; however, the consequences of this R150Q mutation have not been elucidated. To determine how this HsRad51(R150Q) mutation affects HsRad51 function, in the present study, we purified the HsRad51(R150Q) mutant. The purified HsRad51(R150Q) was completely proficient in the ATP-hydrolyzing activity. A gel filtration analysis revealed that HsRad51(R150Q) also retained the polymer formation ability. In contrast, the ssDNA- and dsDNA-binding abilities of HsRad51(R150Q) were clearly reduced, as compared to those of HsRad51. These differences in the DNA-binding properties between HsRad51(R150Q) and HsRad51 may be important to account for the tumorigenesis in breast cancer patients with the HsRad51(R150Q) mutation.

Key words Rad51; homologous recombination; DNA repair; tumorigenesis; breast cancer; DNA binding

The Rad51 protein is the eukaryotic ortholog of the bacterial RecA protein,[1] which functions in homologous recombination. In mice, the *Rad51*-gene knockout results in early embryonic lethality,[2,3] indicating that the Rad51 protein is essential for mammalian development. In chicken DT40 cells, the *Rad51*-gene knockout causes cell death, with the accumulation of unrepaired double-stranded DNA breaks (DSBs).[4] This fact indicated that the Rad51 protein is required for DSB repair in higher eukaryotes.

When DSBs occur, single-stranded DNA (ssDNA) tails are produced at the DSB sites.[5,6] The Rad51 protein assembles onto the ssDNA tails, and forms a helical filamentous polymer.[7—10] The BRCA2 protein, a tumor suppressor, and replication protein A (RPA) play important roles in this Rad51 assembly step onto the ssDNA tail.[11—16] The Rad51–ssDNA filament then binds to intact double-stranded DNA (dsDNA). A homologous sequence between the ssDNA and dsDNA molecules is aligned within the Rad51 filament, and the ssDNA forms a heteroduplex with the complementary strand of dsDNA (homologous pairing). The heteroduplex region is then extended by the Rad51-mediated strand exchange. These homologous-pairing and strand-exchange reactions catalyzed by Rad51 are the key steps in DSB repair through homologous recombination.[17—20]

In humans, mutations in the *Rad51* gene have been identified in several tumors,[21—27] suggesting the involvement of the human Rad51 protein (HsRad51) in tumor suppression mechanisms. Most of the *HsRad51* mutations in tumor cells were found in its non-coding region, but a missense HsRad51 mutation, in which Arg150 is replaced by Gln (R150Q), was identified in patients with bilateral breast cancer.[21] However, the effect of this R150Q mutation on the biochemical activities of HsRad51 has not been reported yet. Therefore, in the present study, we purified the HsRad51(R150Q) mutant, and tested its biochemical activities *in vitro*.

MATERIALS AND METHODS

Preparation of HsRad51 and HsRad51(R150Q) The *HsRad51(R150Q)* mutant gene, inserted at the *Nde*I site of the pET15b expression vector (Novagen, Darmstadt, Germany), was constructed using a Quik Change kit (Stratagene, La Jolla, CA, U.S.A.). The hexahistidine-tagged HsRad51 and HsRad51(R150Q) proteins were expressed in the *E. coli* JM109(DE3) strain, which also carries an expression vector for the minor tRNAs (Codon(+)RIL, Novagen, Darmstadt, Germany). The proteins were purified on nickel–nitrilotriacetic acid (Ni–NTA) agarose (Qiagen, Hilden, Germany), as previously described.[28,29] The hexahistidine tag was then removed from the HsRad51 portion with thrombin protease (GE Healthcare Bio-Sciencess, Piscataway, NJ, U.S.A.). HsRad51 and HsRad51(R150Q) without the hexahistidine tag were then dialyzed against 100 mM Tris–acetate buffer (pH 7.5), containing 7 mM spermidine and 5% glycerol. During this dialysis step, HsRad51 and HsRad51(R150Q) were precipitated (spermidine precipitation),[30] and then the proteins were dissolved in 100 mM potassium phosphate buffer (pH 7.0), containing 150 mM NaCl, 1 mM EDTA, 2 mM 2-mercaptoethanol, and 10% glycerol. HsRad51 and HsRad51(R150Q) were further purified by chromatography on a MonoQ column (GE Healthcare Bio-Sciences, Piscataway, NJ, U.S.A.). The purified HsRad51 and HsRad51(R150Q) were dialyzed against 20 mM HEPES–NaOH buffer (pH 7.5), containing 150 mM NaCl, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, and 10% glycerol. Protein concentrations were determined by the Bradford method[31] with bovine serum albumin as the standard protein.

DNAs The ϕX174 phage ssDNA and dsDNA used in the DNA-binding and ATPase assays were purchased from New England Biolabs (Ipswich, MA, U.S.A.). All of the DNA concentrations are expressed in moles of nucleotides.

ATPase Activity HsRad51 (2.5 μM) or HsRad51(R150Q) (2.5 μM) was incubated with 1 mM ATP (Roche Applied Sci-

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ence, Basel, Switzerland) in 22 mM HEPES buffer (pH 7.5), containing 15 mM NaCl, 1 mM $MgCl_2$, 1% glycerol, 0.01 mM EDTA, 0.2 mM 2-mercaptoethanol, 1 mM dithiothreitol, and 0.1 mg/ml bovine serum albumin, in the presence or absence of ssDNA. In the ssDNA-dependent reaction, the ϕX174 circular ssDNA (40 μM) was used as the substrate. In the high salt conditions, the reaction mixture contained 1.52 M NaCl. The reaction was performed at 37 °C. After a 10 min pre-incubation in the absence of ATP, the reaction was initiated by adding 1 mM ATP. At each indicated time, a 20 μl aliquot of the reaction mixture was removed and mixed with 30 μl of 100 mM EDTA, to quench the reaction. The amount of inorganic phosphate released was determined by a colorimetric assay.[32,33] Briefly, 500 μl of a malachite green solution [0.034% (w/v) malachite green oxalate, 1.05% (w/v) hexaammonium heptamolybdate tetrahydrate, and 0.1% (w/v) polyvinyl alcohol in 1 M HCl] was mixed with 50 μl of sample solution (*i.e.* the reaction mixture quenched with EDTA). After 1 min, 50 μl of 34% (w/v) sodium citrate dihydrate was added to stop further color development. The absorbance at 655 nm was measured with a 96-well micro plate reader (Bio-Rad, Hercules, CA, U.S.A.). A 1 mg/ml phosphate ion standard solution (Wako Pure Chemicals, Osaka, Japan) was used to prepare phosphate standards.

Assays for DNA Binding For the ssDNA binding, the ϕX174 circular ssDNA (20 μM) was mixed with HsRad51 or HsRad51(R150Q) in 10 μl of 26 mM HEPES buffer (pH 7.5), containing 45 mM NaCl, 1 mM $MgCl_2$, 3% glycerol, 0.03 mM EDTA, 0.6 mM 2-mercaptoethanol, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and 1 mM ATP. For the dsDNA binding, the supercoiled ϕX174 dsDNA (10 μM) was mixed with HsRad51 or Rad51–R150Q in 10 μl of 24 mM HEPES buffer (pH 7.5), containing 30 mM NaCl, 1 mM $MgCl_2$, 2% glycerol, 0.02 mM EDTA, 0.4 mM 2-mercaptoethanol, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and 1 mM ATP. For the competitive ssDNA and dsDNA binding, the ϕX174 circular ssDNA (20 μM) and the linear ϕX174 dsDNA (10 μM) were mixed with HsRad51 or HsRad51(R150Q) in 10 μl of 26 mM HEPES buffer (pH 7.5), containing 1 mM $MgCl_2$, 3% glycerol, 0.03 mM EDTA, 0.6 mM 2-mercaptoethanol, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 1 mM ATP, and 45 mM or 200 mM NaCl. The reaction mixtures were incubated at 37 °C for 10 min, and were then analyzed by 0.8% agarose gel electrophoresis in 1×TAE buffer (40 mM Tris–acetate and 1 mM EDTA) at 3.3 V/cm for 2 h. The bands were visualized by ethidium bromide staining.

Gel Filtration HsRad51 (150 μg) and HsRad51(R150Q) (150 μg) were analyzed by Superdex 200 HR 10/30 (GE Healthcare Bio-Sciences, Piscataway, NJ, U.S.A.) gel filtration chromatography. The elution buffer contained 20 mM HEPES–NaOH (pH 7.5), 200 mM NaCl, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, and 10% glycerol, and the flow rate was 0.3 ml/min.

RESULTS

ATPase Activity of HsRad51(R150Q) To study the effect of the HsRad51(R150Q) mutation *in vitro*, we purified HsRad51(R150Q) to near homogeneity by a four-step purification method,[34] including nickel–nitrilotriacetic acid (Ni–NTA) agarose column chromatography, removal of the hexahistidine tag from HsRad51 with thrombin protease, spermidine precipitation, and MonoQ column chromatography. The HsRad51 used in the present study was also purified by this four-step purification method. The concentrations of HsRad51(R150Q) and HsRad51 used in this study were determined by the Bradford method.[31] The purities and concentrations of HsRad51(R150Q) and HsRad51 were confirmed by an SDS-PAGE analysis with Coomassie Brilliant Blue staining (Fig. 1A).

All members of the Rad51 family contain the Walker-type ATPase motif, and HsRad51 reportedly hydrolyzes ATP in the presence of ssDNA or under high salt conditions.[35] Therefore, we tested the ATP hydrolyzing ability of HsRad51(R150Q). Consistent with the previous report, ATP hydrolysis by HsRad51 is stimulated in the presence of ssDNA or NaCl (1.52 M) (Figs. 1B, C). The purified HsRad51(R150Q) exhibited exactly the same ATPase activity as HsRad51 in the presence of the ϕX174 circular ssDNA (Fig. 1B). HsRad51(R150Q) also exhibited salt-induced ATPase activity very similar to that of HsRad51 in the absence of DNA (Fig. 1C). These results indicated that the R150Q mutation affected neither the ssDNA-dependent nor DNA-independent ATP hydrolysis by HsRad51.

Polymer Formation Ability of HsRad51(R150Q) The

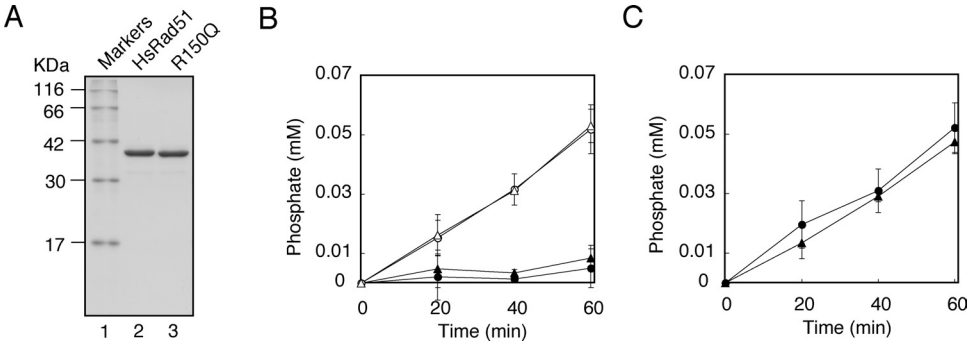

Fig. 1. Purification and ATPase Activities of HsRad51 and HsRad51(R150Q)

(A) Purified HsRad51 (1.6 μg, lane 2) and HsRad51(R150Q) (1.6 μg, lane 3) were analyzed by 15% SDS-PAGE with Coomassie Brilliant Blue staining. Lane 1 indicates the molecular mass markers. (B) The ssDNA-dependent ATPase activity. Time course experiments are shown. Circles and triangles indicate the experiments with HsRad51 and HsRad51(R150Q), respectively. Open symbols indicate experiments with ssDNA (40 μM), and closed symbols indicate experiments without ssDNA. (C) Salt-induced ATPase activity. Circles and triangles indicate the experiments with HsRad51 and HsRad51(R150Q), respectively. Closed symbols indicate experiments in the presence of 1.52 M NaCl, respectively.

Rad51 protein forms a self-associated polymer, which is considered to be an active form of the protein. Therefore, we tested the polymer formation ability of HsRad51(R150Q). Gel filtration chromatography revealed that HsRad51(R150Q) formed polymers by self-association, like HsRad51: the protein eluted in the void volume from the Superdex 200 gel filtration column (Fig. 2). Therefore, the R150Q mutation did not appear to affect the subunit–subunit contact in the HsRad51 polymer. To eliminate non-specific interactions between the protein and the Superdex 200 gel matrix, the experiments were performed in the presence of 200 mM NaCl.

DNA Binding Activities of HsRad51(R150Q) We next tested the DNA-binding activity of HsRad51(R150Q). To do so, we employed agarose gel shift assays with ϕX174 circular ssDNA and ϕX174 supercoiled dsDNA as substrates. As shown in Fig. 3A, HsRad51(R150Q) bound to ssDNA and dsDNA. HsRad51 cooperatively binds to DNA, and forms a helical filamentous complex.[8] Thus, increasing the number of HsRad51 molecules bound to the DNA molecule retards the migration of the HsRad51–DNA complexes in the agarose gel.

A careful comparison of the migration distances between HsRad51(R150Q) and HsRad51 revealed that the HsRad51(R150Q)–ssDNA complexes migrated faster than the HsRad51–ssDNA complexes in the presence of the same amount of the protein (Fig. 3B). These results indicated that the number of HsRad51(R150Q) molecules bound to ssDNA is less than that of HsRad51. Similarly, the HsRad51(R150Q)–dsDNA complexes also migrated faster than the HsRad51–dsDNA complexes in the presence of the same amount of protein (Fig. 3C). Therefore, these results indicated that HsRad51(R150Q) binds to ssDNA and dsDNA, but its DNA-binding ability is reduced as compared to that of HsRad51. This mild DNA-binding defect of HsRad51(R150Q) could not be detected in the ssDNA-dependent ATPase assay (Fig. 1B), because the assay was per-

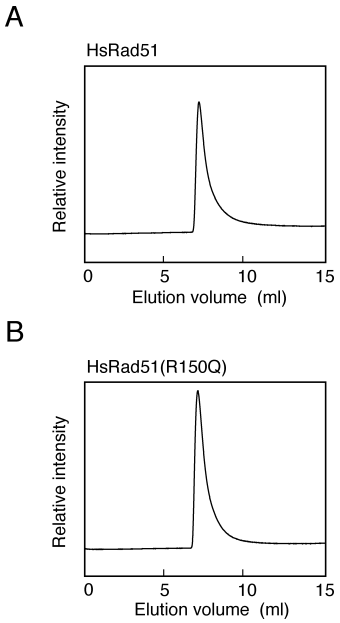

Fig. 2. Gel Filtration Analysis of HsRad51(R150Q)

Elution profiles of HsRad51 and HsRad51(R150Q) in Superdex 200HR chromatography. HsRad51 (A); HsRad51(150Q) (B).

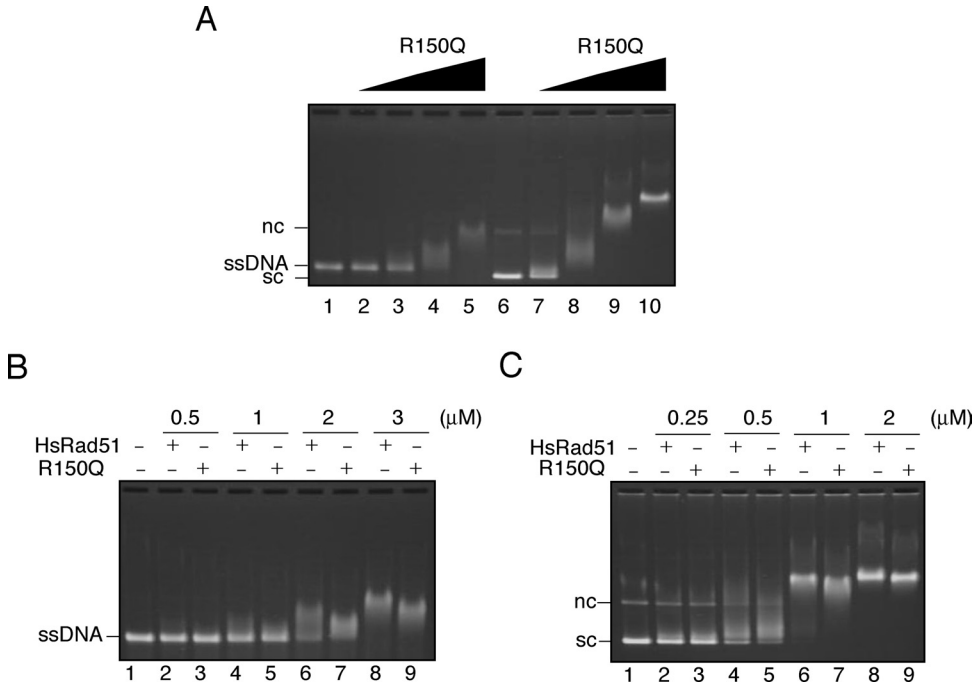

Fig. 3. The DNA-Binding Activities of HsRad51(R150Q)

(A) The ssDNA-binding and dsDNA-binding experiments. The ϕX174 circular ssDNA (20 μM, lanes 2—5) or the supercoiled ϕX174 dsDNA (10 μM, lanes 7—10) was incubated with HsRad51(R150Q) at 37 °C for 10 min. Protein concentrations were 0.25 μM (lane 7), 0.5 μM (lanes 2, 8), 1 μM (lanes 3, 9), 2 μM (lanes 4, 10), and 3 μM (lane 5). Lanes 1 and 6 are control experiments without HsRad51(R150Q). "nc" and "sc" indicate nicked circular dsDNA and supercoiled dsDNA, respectively. (B) Comparison of ssDNA binding between HsRad51(R150Q) and HsRad51. The ϕX174 circular ssDNA (20 μM) was incubated with HsRad51(R150Q) or HsRad51 at 37 °C for 10 min. Lane 1 is a control experiment without HsRad51. Lanes 2, 4, 6, and 8 indicate the experiments with HsRad51, and lanes 3, 5, 7, and 9 indicate the experiments with HsRad51(R150Q). Protein concentrations were 0.5 μM (lanes 2, 3), 1 μM (lanes 4, 5), 2 μM (lanes 6, 7), and 3 μM (lanes 8, 9). (C) Comparison of dsDNA binding between HsRad51(R150Q) and HsRad51. The ϕX174 supercoiled dsDNA (10 μM) was incubated with HsRad51(R150Q) or HsRad51 at 37 °C for 10 min. Lane 1 is a control experiment without HsRad51. Lanes 2, 4, 6, and 8 indicate the experiments with HsRad51, and lanes 3, 5, 7, and 9 indicate the experiments with HsRad51(R150Q). Protein concentrations were 0.25 μM (lanes 2, 3), 0.5 μM (lanes 4, 5), 1 μM (lanes 6, 7), and 2 μM (lanes 8, 9). The samples were analyzed by 0.8% agarose gel electrophoresis in 1×TAE buffer.

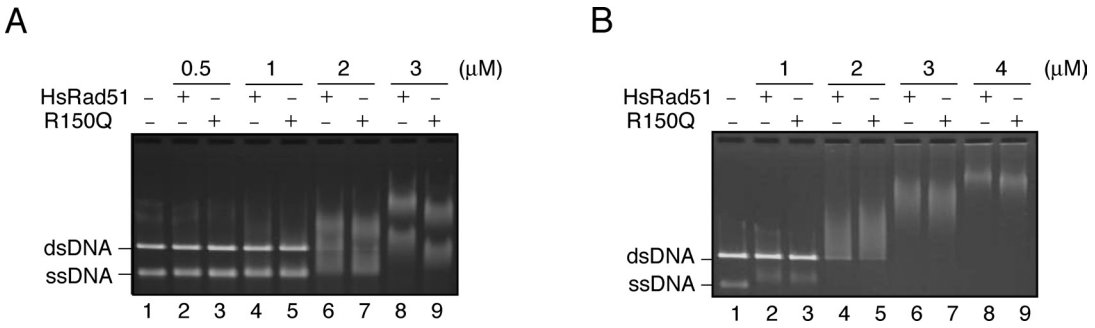

Fig. 4. Competitive ssDNA and dsDNA Binding

The ϕX174 circular ssDNA (20 μM) and the linear ϕX174 dsDNA (10 μM) were incubated with HsRad51 or HsRad51(R150Q) in the presence of 45 mM NaCl (low salt conditions; A) or 200 mM NaCl (high salt conditions; B). (A) Lane 1 is a control experiment without HsRad51. Lanes 2, 4, 6, and 8 indicate the experiments with HsRad51, and lanes 3, 5, 7, and 9 indicate the experiments with HsRad51(R150Q). Protein concentrations were 0.5 μM (lanes 2, 3), 1 μM (lanes 4, 5), 2 μM (lanes 6, 7), and 3 μM (lanes 8, 9). (B) Lane 1 is a control experiment without HsRad51. Lanes 2, 4, 6, and 8 indicate the experiments with HsRad51, and lanes 3, 5, 7, and 9 indicate the experiments with HsRad51(R150Q). Protein concentrations were 1 μM (lanes 2, 3), 2 μM (lanes 4, 5), 3 μM (lanes 6, 7), and 4 μM (lanes 8, 9). The reaction mixtures were incubated at 37 °C for 10 min, and were then analyzed by 0.8% agarose gel electrophoresis in 1×TAE buffer.

formed in the presence of an excess amount of ssDNA (40 μM ssDNA for 2.5 μM protein).

HsRad51 Preferentially Binds to ssDNA over dsDNA in the Presence of 200 mM NaCl When the DNA-binding experiments were performed under the low salt conditions (45 mM NaCl), HsRad51 bound to ssDNA and dsDNA with no obvious preference (Fig. 4A). Interestingly, we found that HsRad51 preferentially bound to ssDNA over dsDNA in the presence of 200 mM NaCl. As shown in Fig. 4B (lane 2), under the high salt conditions (200 mM NaCl), HsRad51 preferentially formed the complexes with ssDNA, and the free ssDNA specifically disappeared. HsRad51(R150Q) also preferentially bound to ssDNA in the presence of 200 mM NaCl (Fig. 4B, lane 3), and the ssDNA-binding preference was not observed under the low salt conditions (Fig. 4A). Under both the high salt and low salt conditions, HsRad51(R150Q) exhibited reduced DNA-binding activity as compared to HsRad51 (Fig. 4), confirming that HsRad51(R150Q) is moderately defective in its DNA-binding activity.

DISCUSSION

Rad51 is an essential enzyme for DSB repair by homologous recombination in eukaryotes. Consistent with its importance in DNA repair, the *Rad51*-knockout in chicken DT40 cells causes cell death, with the accumulation of spontaneous chromosome breaks.[4] In addition, the *Rad51*-knockout in mice results in early embryonic lethality.[2,3] These facts suggest that a severe Rad51 defect in higher eukaryotes causes cell death, rather than tumorigenesis.

The HsRad51(R150Q) mutation was found in patients with bilateral breast cancer.[21] In order to assess whether the R150Q mutation directly affects the HsRad51 function, in the present study, we purified HsRad51(R150Q) and tested its biochemical activities. Purified HsRad51(R150Q) possesses the ATPase, polymer formation, and DNA-binding activities; however, the ssDNA- and dsDNA-binding abilities of HsRad51(150Q) were moderately, but clearly, decreased as compared to those of HsRad51. If the HsRad51(R150Q) mutation caused a severe defect in the HsRad51 function, then the mutation may induce cell death rather than tumorigenesis, as in the case of the *Rad51*-knockout cells.[2—4] Therefore, the mild defects exhibited by the HsRad51(R150Q) protein may account for the breast cancer tumorigenesis; however, we cannot exclude the possibility that HsRad51(R150Q) may have defective interaction(s) with other tumor suppressors, such as BRCA2.

According to the structure of the ATPase domain of HsRad51,[36] the side chain of Arg150 is located close to the main chain CO atom of Tyr178. The distance between the Nη atom of Arg150 and the main chain O atom of Tyr178 is about 2.9 Å, which is suitable for the formation of a hydrogen bond between them. Therefore, the R150Q mutation of HsRad51 may disrupt this interaction between Arg150 and Tyr178, and may affect the tertiary structure of HsRad51. Further biochemical and structural analyses with HsRad51(R150Q) and tumor suppressors will be required to understand how this HsRad51 mutation affects tumorigenesis.

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