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SHORT REPORT

Mutations in the *RAD54* recombination gene in primary cancers

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Association of a recombinational repair protein RAD51 with tumor suppressors BRCA1 and BRCA2 suggests that defects in homologous recombination are responsible for tumor formation. Also recent findings that a protein associated with the MRE11/RAD50 repair complex is mutated in Nijmegen breakage syndrome characterized by increased cancer incidence and ionizing radiation sensitivity strongly support this idea. However, the direct roles of BRCA proteins and the protein responsible for NBS in recombinational repair are not clear though they are associated with the recombinational repair complexes. Since RAD51 forms a complex with other members of the *RAD52* epistasis group and with BRCA proteins, it is reasonable to ask if alterations of members of the *RAD52* epistasis group lead to tumor development. Here we describe missense mutations at functional regions of *RAD54* and the absence of the wild-type *RAD54* expression resulting from aberrant splicing in primary cancers. Since RAD54 is a recombinational protein associated with RAD51, this is the first genetic evidence that cancer arises from a defect in repair processes involving homologous recombination.

Keywords: RAD54; cancer; mutation

In yeast, double-strand break (DSB) repair occurs through a pathway of homologous recombination that involves the RAD52 epistasis group proteins. The structural and functional conservation of RAD51, RAD52 and RAD54 throughout evolution supports the idea that the recombinational repair pathway is retained in mammals (Essers *et al*., 1997; Bezzubova *et al*., 1997). RAD51 is associated with BRCA1 and BRCA2 tumor suppressor gene products, suggesting that a defect in recombination leads to tumor development (Scully *et al*., 1997; Sharan *et al*., 1997). Further evidence for this came from the identification of NBS1, responsible for Nijmegen breakage syndrome with increased cancer incidence, from the MRE11/RAD50 recombination complex (Varon *et al*., 1998; Carney *et al*., 1998; Matsuura *et al*., 1998). It is, therefore, probable that members of the *RAD52* epistasis group are altered in cancer.

To investigate whether *RAD54*, a member of the *RAD52* epistasis group, is mutated in human cancer, we performed SSCP analysis and direct sequencing of PCR products using mRNAs from 132 unselected primary tumors including 95 breast cancers, 13 colorectal cancers and 24 lymphomas. SSCP analysis exhibited two mutations (Figure 1a). Direct sequencing revealed a mutation, which did not show shifted bands of SSCP (Figure 1b). Histological analysis of a breast tumor Br7 (from a 63-year-old female patient with no obvious family history of cancer) showed an invasive ductal carcinoma. A G to A transition converted Gly to Arg at codon 325 within helicase motif III. The absence of the wild-type allele indicates that Br7 was homozygous for the mutation. The corresponding normal tissue shows the same transition, indicating that this is a germline mutation. As we could not detect this mutation by SSCP analysis, a restriction-based screen was performed. The mutation in Br7 abolishes an AccIII site, preventing the digestion of a PCR fragment. This assay revealed that this mutation was absent in 100 normal individuals (200 chromosomes).

A tumor KCo11 was derived from an adenocarcinoma of the colon. A C to A transversion converted Pro to His at codon 63 upstream of helicase domains (Figure 1c). The corresponding normal tissue shows the wild-type sequence, indicating that this is a somatic mutation. A tumor Ly18 was derived from a non-Hodgkin's lymphoma. A T to A transversion converted Val to Glu at codon 444 between helicase domains III and IV (Figure 1d). Since we could not obtain the corresponding normal tissue, we do not know whether this is a germline mutation. The presence of the wild-type alleles indicates that these tumors were heterozygous for the mutations. To exclude the possibility that base transitions in KCo11 and Ly18 represent polymorphisms, we examined mRNAs from 100 normal individuals (200 chromosomes) by SSCP analysis and confirmed that these transversions are absent in controls. Heterozygous mutations may be responsible for tumor formation if they act in a dominant-negative manner. In addition to these mutations (Table 1), a number of polymorphisms were identified (Table 2).

Based on sequence homology (Eisen *et al*., 1995), RAD54 belongs to the SNF2/SWI2 superfamily of

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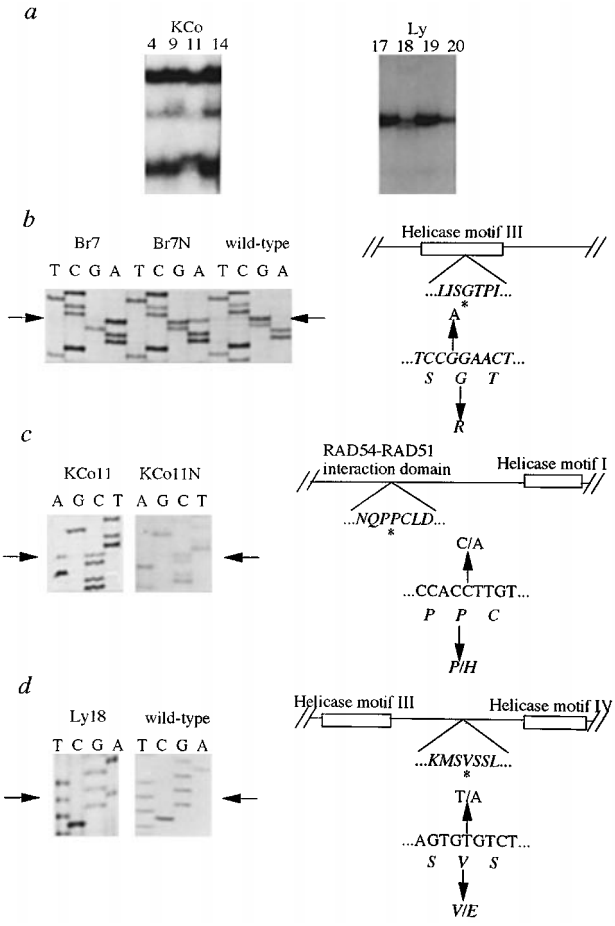

Figure 1 Point mutations of *RAD54* in primary cancer. (**a**) SSCP analysis of the *RAD54* cDNA amplified with primers N23 and N27 in colorectal cancer (left panel). SSCP analysis of the fragment amplified with primers N8 and N19 in lymphoma (right panel). (**b**) The *RAD54* homozygous mutation in Br7. Br7N is the corresponding normal tissue. A G to A substitution indicated by arrows results in a Gly to Arg conversion at codon 325 within helicase domain III. (**c**) The *RAD54* heterozygous mutation in KCo11. KCo11N is the corresponding normal tissue. A C to A substitution at codon 63 upstream of helicase motifs indicated by arrows results in a Pro to His mutation. (**d**) The *RAD54* heterozygous mutation in Ly18. A T to A transversion indicated by arrows converted Val to Glu at codon 444 between helicase motifs III and IV. RNAs were isolated from tumors and normal tissues by acid guanidium-isothianate method (Chomczynski and Sacchi, 1987). cDNAs were reverse transcribed, and nested PCR was performed (Sugimoto *et al*., 1991). The *RAD54* cDNA was divided into three segments to generate first PCR products suitable for the second PCR. The N-terminal one third of the cDNA was amplified with the first PCR primers N15 (5′-TAGGCCCAGGATGAGGAGGA-3′) and N18 (5′-AGGTTG-GATCCTC-CCTCCGA-3′). The region amplified by N15 and N18 was divided into three segments for the second PCR. The first segment was amplified with N23 (5′-ATGAGGAG-GAGCTTGGCT-3′) and N27 (5′-CTCCTTGTCAAGCTTCAG-3′). The second segment was amplified with N43 (5′-GCAGT-CAGCATGAAGCAT-3′) and N46 (5′-ACTGGTGACA-CACTCCCA-3′). The third segment was amplified with N45 (5′-AGAGGGAGTGAAATTCC-3′) and N18. The central region was generated with the first PCR primers N45 and N8 (5′-CTCCTCTTCCACACACTT-3′). This region was divided into four segments for the second PCR. The first segment was amplified with N5 (5′-ATGAGGTTGGGAAATGGC-3′) and N47 (5′-GAGGACTCCAACATGAAG-3′). The second segment was amplified with N41 (5′-ATCCAACCTCTGGCCAT-3′) and N42 (5′-ATTCAT-GGGCAGTCCCT-3′). The third segment was amplified with N50 (5′-TGGACAGCTTGAACACCA-3′) and N49 (5′-CAATTCTT-CTGCCGGTT-3′). The fourth segment was amplified with N19 (5′-AGGGACTGCCCATGAAT-3′) and N8. The C-terminal one-third was amplified with the first PCR primers N33 (5′-GGAGGACTTCTGATATCC-3′) and N14 (5′-CAGACCAG-CTGGTTATCA-3′). Then this region was divided into three segments for the second PCR. The first segment was amplified with N31 (5′-TTGCTTGAGGGCAAGATG-3′) and N36 (5′-TACAACCTTGGCTCGCTT-3′). The second segment was amplified with N37 (5′-GGATGGCACGATGTCCATT-3′) and N40 (5′-CTTCTTGTGGCTGAC-3′). The third segment was amplified with N39 (5′-TCTGGCGAGATGGTCAA-3′) and N24 (5′-TGGTTATCAGCGGAGGC-3′). Thirty cycles of amplification were performed with Taq polymerase (Amersham), each cycle consisting of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C. The PCR reactions were done in duplicates to exclude false positives possibly generated by Taq polymerase errors. Using first PCR products, the second round of PCR was performed with primers that were radiolabeled with T4 polynucleotide kinase and γ-^{32}P-ATP. Radiolabeled PCR products were denatured by boiling in 90% formamide and electrophoresed at 15°C on non-denaturing 5% polyacrylamide gel containing 10% glycerol, which was subjected to autoradiography. PCR products were directly sequenced in both directions using Sequenase PCR product sequencing kit (Amersham)

Table 1 Mutations in *RAD54*

Sample	*Tumor type*	*Codon*	*Nucleotide change*	*Amino acid change*	*Homozygosity*
Br7	Breast	325	gga→aga	Gly→Arg	Homozygous
KCo 11	Colon	63	cct→cat	Pro→His	Heterozygous
Ly18	Lymphoma	444	gtg→gag	Val→Glu	Heterozygous

We have analysed 132 primary tumors including 95 breast cancers, 13 colorectal cancers and 24 lymphomas for *RAD54* mutations

Table 2 Polymorphisms in *RAD54*

Codon	*Nucleotide change*	*Amino acid change*	*Frequency*
151	aag/gag	Lys/Glu	0.98/0.02
366	gac/gat	Asp/Asp	0.92/0.08
433	ccg/cca	Pro/Pro	0.98/0.02
730	gct/gcc	Ala/Ala	0.19/0.81

DNA and RNA helicases whose members play roles in genome integrity including transcriptional regulation (SNF2, MOT and BRM), chromosome stability (lodestar), nucleotide excision repair (ERCC6 and RAD16) and recombination (RAD54). Although helicase activities of members of the SNF2/SWI2 superfamily remain to be demonstrated, conservation of the helicase motifs throughout evolution strongly suggests their functional importance. Therefore, mutations that cause amino acid substitutions at helicase motifs can lead to their altered function. Seven helicase motifs characteristic of the SNF2/SWI2 superfamily are present in RAD54 (Kanaar *et al*., 1996; Rasio *et al*., 1997). Gly at codon 325, which was mutated in Br7, is at a conserved position in helicase motifs from yeast to human in members of the SNF2/SWI2 superfamily regardless of species and subfamily (Figure 2a). It is, therefore, highly likely that this mutation affects the function of RAD54 mediated by the helicase motif.

Although Pro at codon 63 and Val at codon 444 are outside helicase motifs, it is likely that these amino acid substitutions affect the function of RAD54. The interaction between RAD54 and RAD51 is mediated by the protein containing the N-terminal 142 amino acid sequence of human RAD54 (Golub *et al*., 1997). It is, therefore, likely that a missense mutation at this

a

SNF2	RLILTGTPLQNNL
BRM	RLLLTGTPLQNKL
LDS	RWALTGTPIQNKE
ERCC6	RIILSGSPMQNNL
RAD16	RTCLSGTPKQNRI
hRAD54	RVLISGTPIQNDL

*

b

GdRAD54	NVSSLSSITSLKKLCNHPALIYDKCVE
mRAD54	SVSSLSSITSLKKLCNHPALIYDKCVA
hRAD54	SVSSLSSITSLKKLCNHPALIYDKCVE

*

Figure 2 The conservation of the amino acids that were mutated in primary cancer. (**a**) The amino acid sequences of helicase domain III in the SNF2/SWI2 superfamily (Eisen *et al*., 1995). Identical amino acids conserved in the SNF2/SWI2 family are shown in bold. Glycine at codon 325 of RAD54, which was mutated in Br7, is indicated by an asterisk. SNF2 is a transcriptional regulator isolated from *S. cerevisiae* (Laurent *et al*., 1991). BRM is a regulator of *Drosophila* homeotic genes, brahma (Tamkun *et al*., 1992). LDS is the *Drosophila* lodestar protein whose mutations lead to excessive chromosome breakage and tangling (Girdham and Glover, 1991). ERCC6 is a human repair protein whose mutations lead to Cockayne's syndrome (Troelstra *et al*., 1992). RAD16 is a repair protein isolated from *S. cerevisiae* (Bang *et al*., 1992). hRAD54 represents human RAD54 (Kanaar *et al*., 1996). (**b**) The amino acid sequences of the region between helicase domains III and IV. Val at codon 444 of hRAD54 indicated by an asterisk is conserved from chicken to human. RAD54 of chicken and mouse are designated as GdRAD54 (Bezzubova *et al*., 1997) and mRAD54 (Kanaar *et al*., 1996), respectively

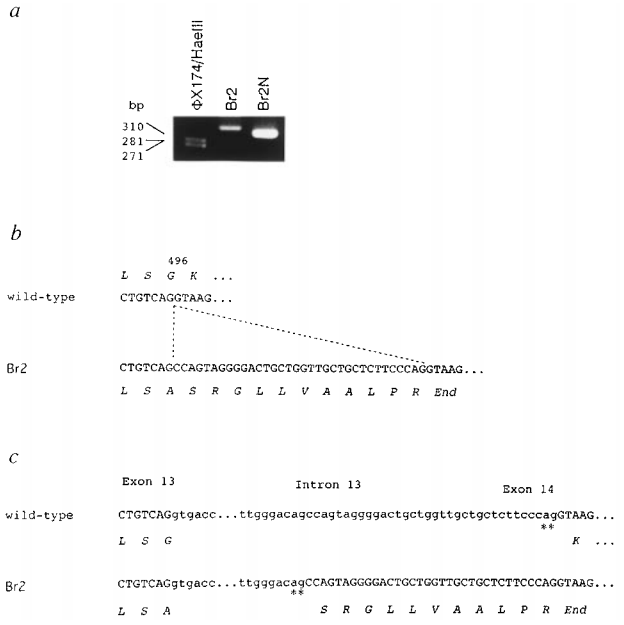

Figure 3 An aberrant transcript in breast cancer. (**a**) RT–PCR of *RAD54* amplified with primers N9 and N30 (5′-CCAATGAGATTGAGGCCA-3′). Br2N is the corresponding normal tissue. (**b**) Sequence comparison of the *RAD54* cDNA between wild-type (Br2N) and Br2. A 34 bp insertion at codon 496 caused a frameshift. (**c**) The sequence of exon-intron junctions from exon 13 to exon 14. Putative splice acceptor sites are indicated by asterisks. Br2 used the splice acceptor site not used in normal tissue, resulting in an addition of the 34 bp cDNA between exon 13 and exon 14. A genomic fragment corresponding to the aberrant transcript was amplified with primers N9 (5′-AAGTGTGTGGAAGAGGAG-3′) and N34 (5′-ACTTTGTCACTGCTACGG-3′) and cloned into the pCR2.1 vector (Invitrogen). Sequencing was performed using Sequenase DNA sequencing kit (Amersham)

region affects the interaction between RAD54 and RAD51. Val at codon 444 is conserved from chicken to human RAD54 throughout evolution (Figure 2b). ERCC6 contains several motifs of functional importance including an acidic part, nuclear location signals and casein kinase II phosphorylation sites outside helicase domains (Troelstra *et al*., 1992). These motifs are not well conserved in members of the SNF2/SWI2 superfamily. By analogy with the functional motifs outside helicase domains in ERCC6, it is possible that conserved regions outside helicase domains is important for RAD54 functions.

RAD54 maps to human chromosome 1p32 where loss of heterozygosity is observed in breast cancer (Rasio *et al*., 1997). It is possible that *RAD54* is the breast cancer susceptibility gene mapped to 1p32. However, the low mutation frequency of *RAD54* in breast cancer indicates that another tumor suppressor mapped to this region is frequently mutated in breast cancer.

Besides such mutations, RT–PCR demonstrated an aberrant transcript in a breast tumor Br2, an invasive ductal carcinoma from a 65-year-old female patient (Figure 3a). Br2 carried a 34 bp insertion at codon 496 upstream of helicase motif IV, resulting in a frameshift (Figure 3b). Since the site of the 34 bp insertion was the boundary between exons 13 and 14 (Rasio *et al*., 1997), we isolated the corresponding genomic clone and found that the insertion was derived from intron 13 (Figure 3c). It is possible that either a mutation at the splice acceptor site or an intronic mutation which creates a novel splice acceptor site is responsible for the aberrant transcript. Sequence analysis of the genomic fragments revealed no mutation around the splice acceptor site in the tumor and the presence of an alternative splice acceptor site in the wild-type allele, which was used as the sole splice acceptor site in Br2. It is still possible that a mutation at some distance from the splice acceptor site is leading to the abnormal splicing event. Even if the altered transcript is not caused by a mutation in *RAD54* itself, the resulting transcript could still lead to a truncated, presumably non-functional protein; adding weight to the evidence that alterations of RAD54 function can lead to cancer. The same aberrant transcript was identified in a breast tumor Br1, an invasive ductal carcinoma from a 73-year-old male patient. In contrast with Br2 in which no wild-type transcript was detected, Br1 expressed the wild-type mRNA.

This is the first genetic evidence that alterations of a gene directly involved in repair processes involving homologous recombination can contribute to carcinogenesis. *RAD54* was originally identified as a gene responsible for recombinational repair in *S. cerevisiae* (Emery *et al*., 1991). Null RAD54 cells in higher eukaryotes show reduced X-ray resistance and homologous recombination frequencies, suggesting that the RAD54 function is conserved throughout evolution (Essers *et al*., 1997; Bezzubova *et al*., 1997). Furthermore, human RAD54 has ATPase activity

that is absolutely dependent on double-stranded DNA (Swagemakers *et al*., 1998). Since biochemical studies indicate that yeast and human SWI/SNF complexes use the energy of ATP hydrolysis to disrupt nucleosome structure, it is suggested that RAD54 may play a role in chromatin remodeling (Peterson and Tamkun, 1995). Given that the RAD54 function is altered, we might expect that genetic instability is induced, which consequently leads to tumor formation. *RAD54* is altered in a small percentage of unselected primary tumors. In this respect, *RAD54* is similar to *BRCA1*, *BRCA2* and a BRCA1-associated RING domain gene (*BARD1*) (Thai *et al*., 1998). It is possible that other members of the *RAD52* epistasis group associated with the BRCA/RAD51 complex are mutated in cancer. This might account for the low mutation frequency of proteins in the BRCA/RAD51 complex in cancer. Although alterations of *RAD54* are found in a small fraction of primary tumors, these findings provide new insight into genetic instability underlying carcinogenesis.

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