RAD50 and NBS1 are breast cancer susceptibility genes associated with genomic instability

Katri Heikkonen1, Katrin Rapakko1, Sanna-Maria Karpinnen1, Hannele Erkko1, Sakari Knuttila2, Tuia Lundan1,2, Arto Mannermaa1, Anne-Lise Borresen-Dale3, Åke Borg4, Rosa B.Barkardottir5, John Petrini6 and Robert Winqvist1,6

1Department of Clinical Genetics, University of Oulu/Oulu University Hospital, Oulu, Finland, 2Department of Pathology, Haartman Institute, University of Helsinki and HUSLAB Helsinki University Central Hospital, Finland, 3Department of Genetics, Norwegian Radium Hospital, Oslo, Norway, 4Department of Oncology, Lund University Hospital, Lund, Sweden, 5Department of Pathology, University Hospital of Iceland, Reykjavik, Iceland and 6Department of Molecular Biology, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

*To whom correspondence should be addressed at: Department of Clinical Genetics, Oulu University Hospital, P.O. Box 24, FIN-90029 OYS, Finland. Tel: +358 8 3153228; Fax: +358 8 3153243; Email: robert.winqvist@oulu.fi

The Mre11 complex, composed of RAD50, NBS1 and MRE11, has an essential role in the maintenance of genomic integrity and preventing cells from malignancy. Here we report the association of three Mre11 complex mutations with hereditary breast cancer susceptibility, studied by using a case–control design with 317 consecutive, newly diagnosed Northern Finnish breast cancer patients and 1000 geographically matched healthy controls (P = 0.0004). RAD50 687delT displayed significantly elevated frequency in the studied patients (8 out of 317, OR 4.3, 95% CI 1.5–12.5, P = 0.008), which indicates that it is a relatively common low-penetration risk allele in this cohort. Haplotype analysis and the screening of altogether 512 additional breast cancer cases from Sweden, Norway and Iceland suggest that RAD50 687delT is a Finnish founder mutation, not present in the other Nordic cohorts. The RAD50 IVS3-1G→A splicing mutation leading to translational frameshift was observed in one patient, and the NBS1 Leu150Phe missense mutation affecting a conserved residue in the functionally important BRCA1 carboxy-terminal (BRCT) domain in two patients, both being absent from 1000 controls. Microsatellite marker analysis showed that loss of the wild-type allele was not involved in the tumorigenesis in any of the studied mutation carriers, but they all showed increased genomic instability assessed by cytogenetic analysis of peripheral blood T-lymphocytes (P = 0.006). In particular, the total number of chromosomal rearrangements was significantly increased (P = 0.002). These findings suggest an effect for RAD50 and NBS1 haploinsufficiency on genomic integrity and susceptibility to cancer.

Introduction

RAD50 is a highly conserved DNA double-strand break (DSB) repair factor. Together with NBS1 and MRE11 it composes the Mre11 complex with functions in sensing and early processing of DSB, cell cycle checkpoints, DNA recombination and maintenance of telomeres (1–3). This protein complex integrates DNA repair with checkpoint signaling through the ATM kinase, acting both upstream and downstream of ATM in the DNA damage response pathway (4–7). Even minor disturbances of Mre11 complex activity have profound effects on the genomic integrity, and all three components have been implicated in recessive genetic instability disorders. Hypomorphic mutations in MRE11 and NBS1 result in ataxia-telangiectasia like disorder (ATLD) and Nijmegen breakage syndrome (NBS), respectively (8,9), and recently mutations in RAD50 have also been described in a patient with NBS phenotype (T. Dörk, personal communication). While no cancer predisposition has been reported in ATLD (10,11), NBS patients are cancer-prone, particularly to lymphomas. In addition, heterozygous NBS carriers have been suggested to have increased cancer risk, and germline alterations in NBS1 have been shown to associate with a variety of malignancies, including leukemia, malignant melanoma, prostate, breast and ovarian cancer (12–16).

On the basis of the central role of the Mre11 complex in the maintenance of genomic integrity, 151 breast and/or ovarian cancer families were previously screened for germline mutations in the entire coding regions of the RAD50, NBS1 and MRE11 genes (17). Two novel, potentially breast-cancer-associated mutations were identified: RAD50 687delT leading to frameshift with premature translation stop at codon 234 was observed in two families, and NBS1 Leu150Phe affecting an evolutionary conserved residue in the middle of the functionally important BRCA1 carboxy-terminal (BRCT) domain was observed in one family. In addition, two other novel RAD50 alterations, Ile94Leu and Arg224His classified as unknown variants, were observed. This prompted us to evaluate the overall significance of these alterations in breast cancer susceptibility by studying a second breast cancer cohort. Here we report the prevalence of these alleles, and a novel RAD50 truncation allele, in an unselected cohort of 317 consecutive, newly diagnosed breast cancer cases and 1000 healthy population controls. For the further evaluation of the geographical occurrence of RAD50 687delT, altogether 512 additional breast cancer patients from Sweden, Norway and Iceland were screened for this mutation. We also provide evidence for a pathogenic effect of the three Mre11 complex alterations on genomic instability assessed by cytogenetic analysis of peripheral blood lymphocytes.

Material and methods

Subjects

An unselected cohort of 317 breast cancer patients were screened for RAD50 687delT, RAD50 Ile94Leu, RAD50 Arg224His and NBS1 Leu150Phe alterations. DNA samples were isolated from blood lymphocytes of patients operated at the Oulu University Hospital from April 2000 through July 2004.
The diagnoses were pathologically confirmed. The mean age at cancer diagnosis was 58 years (ages varied between 29 and 95). A written informed consent was obtained from all patients, who also filled out a questionnaire concerning breast, ovarian and other cancer prevalence in their family. Only patients with breast and/or ovarian cancer in first- or second-degree relatives were considered to be familial cases. The healthy control group (N = 1000) used as reference was collected from May through July 2002 and constituted of anonymous cancer-free Finnish Red Cross blood donors originating from the same geographical region as the studied patients (17). The mean age of the controls was 41 years (ages varied between 18 and 66).

The blood samples of the patients identified in Oulu selected for chromosomal analysis were collected at least 2 years after the initial breast cancer diagnosis. These patients filled out a questionnaire concerning cancer treatment received and other possible chronic diseases, to exclude those where recent or ongoing radio- and/or chemotherapy, or other medication might have influence on peripheral lymphocyte chromosomal stability. Seven of the eight cases had received radiotherapy and the time from last treatment to sample collection for chromosomal analysis was for one patient 9 months, and for the rest 2–4 years. Three patients had also received chemotherapy; the time from last treatment was for two cases 2 years and for one case 1 year. The controls used in chromosomal analysis were healthy, age-matched female individuals.

To evaluate the geographical prevalence of RAD50 687delT, additional breast cancer cohorts from Sweden (Lund, 130 cases), Norway (Oslo, 136 cases) and Iceland (Reykjavik, 246 cases) were screened for this mutation. Approval for the study was obtained from the Ethical Boards of the involved University Hospital health care districts.

Mutation screening

Conformation sensitive gel electrophoresis (18) was used for mutation detection. All findings were confirmed by sequencing with the Li-Cor IR2 4200-S DNA Analysis system (Li-Cor, Lincoln, NE). The following primers were used: for RAD50 687delT and Arg224His in exon 5, forward 5'-GTGACAGCATAATATGCCAGTTCCACGA-3', reverse 5'-TGATTAGCAGGTACACAGGATC-3'; for RAD50 Ile4Leu in exon 3, forward 5'-TGGCTTTTTCCTAGAACCACAC-3', reverse 5'-GAAACACACCATACTCAGACCAA-3'; for NBS1 Leu150Phe in exon 4, forward 5'-AGTITTAAAAAGTCTAAAATTGGCACTC-3', reverse 5'-ACAAGGGATGGGATGTTGTA-3'.

Statistical analysis

Fisher's exact test was used to evaluate the differences in the allelle frequencies between cases and controls. Independent samples t-test for normally distributed variables and Mann-Whitney U-test for non-normally distributed variables was used to determine the statistical difference in the number of different chromosomal aberrations between the two groups categorized according to their genetic status. Comparisons were done with SPSS (version 12.0 for Windows, SPSS, Chicago, IL), and all P values were two-sided.

Evaluation of RAD50 IVS3-1G>A effect on mRNA splicing

Lymphoblast cell lines were established from two carriers (BR-0416, BR-0490) of a novel RAD50 IVS3-1G>A mutation identified during screening for the BRCA1 and BRCA2 non-carriers. Cells were grown in RPMI 1640 medium containing 20% fetal bovine serum, 1% L-glutamine and gentamycin (10 μg/ml) at 37°C in a 5% CO2 atmosphere. cDNA was isolated with the FastTrack<sup>®</sup> 2.0 Kit (Invitrogen, Carlsbad, CA) and cDNA was synthesized using the RevertAid<sup>™</sup> First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD). The effect of RAD50 IVS3-1G>A on splicing was estimated by cDNA-specific amplification of a 488 bp fragment with following primers: forward 5'-ATAATTTGGTGTTGACCAATGG-3' (exon 1), reverse 5'-CTCCTGGCAGAAGTTTTCTAAGG-3' (exon 5). The presence of incorrectly spliced transcripts was evaluated by electrophoresis in 1% agarose gel and sequencing.

Microsatellite marker analysis

DNA markers DSS2057, DSS1984 and DSS2002 were used for haplotype analysis of RAD50 687delT carriers, and to analyze allelic imbalance (AI) in the available tumors of RAD50 687delT and RAD50 IVS3-1G>A carriers. Markers DSS1800, DSS888, DSS1811 and DSS1724 were used for the corresponding analyses of NBS1 Leu150Phe carriers. Normal and tumor DNA were extracted from paraffin-embedded tissues (19). The PCR products were analyzed with the Li-Cor using an IRD800-labelled forward primer, and allele intensity ratios were quantified with the Gene Profiler 4.05 analysis program (Scanalytics, Fairfax, VA). AI was calculated from the formula AI = (T2 × N1)/(T1 × N2), where T1/2 represents tumor and N1/2 the corresponding normal alleles. A value > 1.67 or < 0.60 was considered to indicate AI, meaning that the intensity of one allele had decreased >40% (20).

Cytogenetic analyses

The cytogenetic analysis of peripheral blood T-lymphocytes of RAD50 687delT, RAD50 IVS3-1G>A, NBS1 Leu150Phe carriers, and mutation-negative, age-matched healthy controls was carried out in short-term cultures (21). A minimum of 50 Giemsa-banded metaphases were evaluated for spontaneous chromosomal abnormalities by light microscopy and photographed with an automatic chromosome analyser (Cytovision version 3.1, Applied Imaging, Santa Clara, CA).

Results

RAD50 687delT is a founder mutation with incomplete penetrance

A cohort of 317 consecutive, newly diagnosed breast cancer patients operated at the Oulu University Hospital from April 2000 through July 2004 were screened for the 4 Mre11 complex alterations. The frequency of the observed alleles was compared with those of 1000 population controls. Altogether eight RAD50 687delT carriers (8 out of 317) were identified among the studied breast cancer cases, the frequency being significantly higher than in controls (6 out of 1000) (P = 0.008, OR 4.3, 95% CI 1.5–12.5). The mean age at diagnosis did not differ between patients with RAD50 687delT mutation (57.9 years) and non-carriers (58.0 years).

Of the studied 317 cases 70 were considered familial, two of which (BR-0492, BR-0279) were found positive for RAD50 687delT. The study of additional affected family members, however, indicated incomplete segregation of the mutation (Figure 1A). Two RAD50 687delT carriers (BR-0225, BR-0234) had family history of other cancers (at least two cases in first- or second-degree relatives), whereas the rest (BR-0412, BR-018, BR-0077 and BR-0116) showed no evidence for clustering of cancer in the family.

Owing to the relatively high frequency of RAD50 687delT in the Finnish cohort, altogether 512 breast cancer cases from Sweden, Norway and Iceland were screened to obtain more information about the geographical occurrence of this allele. However, RAD50 687delT was not observed, which suggests that it is a Finnish founder mutation. The common origin of the mutation was confirmed by microsatellite marker analysis of the 14 carriers, which all shared the same haplotype not present in the 8 analyzed non-carriers (data not shown).

Other alterations observed in the Northern Finnish unscreened breast cancer cohort

Of the other studied RAD50 alterations Arg224His was observed twice (2 out of 317, 0.6%), which did not differ from the controls (9 out of 1000, 0.9%), whereas Ile94Leu was not detected (0 out of 317). Consequently, the current results do not support the association of these two changes with breast cancer susceptibility. However, when screening for the Ile94Leu alteration a novel RAD50 splicing mutation, RAD50 IVS3-1G>A, was observed in one patient (BR-0490) with breast cancer at the age of 56. There were several breast cancer cases in the family (Figure 1B), but only two additional ones were available for mutation testing: the sister of the index case was identified as carrier, whereas the maternal cousin tested negative. RAD50 IVS3-1G>A was not present in 1000 population controls (Table I).

The NBS1 Leu150Phe substitution was observed in two patients (BR-0483, BR-02114), but not among controls (P = 0.058, Table I). Patient BR-0483 had breast cancer at the age of 58, and was considered to be a familial case as her daughter had had ovarian cancer at age 17 (Figure 1C).
In addition, the daughter tested positive for the Leu150Phe allele. Case BR-02114 had breast cancer at the age of 54. She had family history of stomach and thyroid cancer (Figure 1C), but no additional DNA samples were available for mutation testing. The common origin of the two currently identified \( \text{NBS1} \) Leu150Phe carriers and one previously observed carrier (17) was verified by microsatellite marker analysis (data not shown).

\[ \text{RAD50 IVS3-1G} > \text{A} \]

leads to frameshift

The effect of \( \text{RAD50 IVS3-1G} > \text{A} \) on mRNA splicing was evaluated by cDNA-specific amplification of \( \text{RAD50} \) exon 3. Only one PCR product corresponding to the predicted size was observed in 1% agarose gel. However, sequencing revealed that IVS3-1G>A leads to one base pair deletion between exon 2 and 3 in the mRNA sequence (214delG), thus resulting in a premature translation stop at codon 78. This observation was supported by the Splice site prediction program (http://www.fruitfly.org/seq_tools/splice.html). Even though IVS3-1G>A abolishes the existing acceptor site (score 0.97), it creates a new one right next to the previous site (score 0.11). The \( \text{RAD50} \) 214delG transcript was present at lower levels than the wild-type, which indicates that it is unstable (data not shown).

\[ \text{RAD50 687delT} \]

, \( \text{RAD50 IVS3-1G} > \text{A} \) and \( \text{NBS1} \) Leu150Phe carriers display genomic instability

To investigate whether these three heterozygous mutations lead to genomic instability, cytogenetic analysis of peripheral blood T-lymphocytes from four affected \( \text{RAD50 687delT} \) (BR-018, BR-0492, BR-04126, BR-01163), two \( \text{RAD50 IVS3-1G} > \text{A} \) (BR-0516, BR-0490) and two \( \text{NBS1} \) Leu150Phe (BR-0483, BR-0480) carriers was performed. Six mutation-negative age-matched healthy controls were used as reference. The carriers showed a significant increase in the number of chromosomal aberrations (Table II and Figure 2) \((P = 0.006)\). Particularly, both simple and complex chromosomal rearrangements were found more frequently in carriers than...
Table II. Chromosomal aberrations in heterozygous RAD50 687delT, RAD50 IVS3-1G>A and NBS1 Leu150Phe mutation carriers, and healthy controls

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Telomeric associations</th>
<th>Chromatid/chromosome breaks, deletions</th>
<th>Simple chromosomal rearrangements&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Complex chromosomal rearrangements&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAD50 687delT&lt;sup&gt;c&lt;/sup&gt; (n = 4)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.4 SD = 0.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.5 SD = 3.2</td>
<td>4.3 SD = 0.8</td>
<td>3.5 SD = 3.2</td>
</tr>
<tr>
<td>RAD50 IVS3-1G&gt;A</td>
<td>BR-0516 –</td>
<td>11.1</td>
<td>6.3</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>BR-0490 –</td>
<td>2.7</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>NBS1 Leu150Phe</td>
<td>BR-0483 –</td>
<td>2.8</td>
<td>8.3</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>BR-0480 –</td>
<td>1.4</td>
<td>1.4</td>
<td>–</td>
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<tr>
<td>Controls (n = 6)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>–</td>
<td>1.5 SD = 1.3</td>
<td>0.3 SD = 0.8&lt;sup&gt;e&lt;/sup&gt;</td>
<td>–</td>
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</table>

<sup>–</sup> = not observed.
<sup>a</sup>Inversions, ring chromosomes, translocations (≤3 break rearrangements).
<sup>b</sup>Translocations (>4 break rearrangements), marker chromosomes.
<sup>c</sup>For RAD50 687delT and controls containing more than two individuals analysed, the number of aberrations per 100 metaphases shown is the mean for pooled data. SD = standard deviation.
<sup>d</sup>Observed only in one carrier with 1.7 per 100 metaphases showing telomere fusions.
<sup>e</sup>Observed only in one control with 2.0 per 100 metaphases showing simple chromosomal rearrangements.

Fig. 2. Increase in spontaneous chromosomal aberrations in T-lymphocytes of heterozygous RAD50 687delT, RAD50 IVS3-1G>A and NBS1 Leu150Phe carriers. Comparison of (A) the frequency of cells with chromosomal aberrations and (B) the frequency of aberration type per 100 metaphases. Bars represent the mean ± SD of pooled data for mutation carriers (n = 8) and healthy controls (n = 6). A minimum of 50 Giemsa-banded metaphases were analysed for each individual. (C) Metaphase of RAD50 IVS3-1G>A carrier showing complex chromosomal rearrangements csb(4)(p14), der(13)del(13)(q11q14)(13:16)(q14q10), der(16)(13;16)(q14q10), der(21;22)(q10q10). Csb = chromosome break, der = derivative chromosome, del = deletion, t = translocation.
in healthy controls ($P = 0.002$ and $P = 0.010$, respectively), whereas for chromosome/chromatid breaks and deletions the difference did not reach statistical significance ($P = 0.204$). In contrast, telomeric associations were rare as they were seen only once in one RAD50 687delT and in one NBS1 Leu150Phe carrier. All observed chromosomal aberrations were considered random, as no preference for specific break site or evidence for clonality was observed.

**Discussion**

Given the importance of the Mre11 complex in the maintenance of genomic integrity and in preventing cells from malignancy, we have evaluated the significance of four Mre11 complex germline alterations in breast cancer susceptibility. Of these RAD50 687delT and NBS1 Leu150Phe alleles, in addition to a novel RAD50 IVS3-1G>A splicing mutation, appeared to associate with predisposition to cancer.

The current results suggest that RAD50 687delT is a Finnish founder mutation, which confers increased risk of breast cancer. The risk is apparent in women unselected for family history: the frequency of breast and/or ovarian cancer was observed, which is at least partially due to the low number of cases with family history of breast and/or ovarian cancer. The risk is apparent in women unselected for family history: the frequency of breast and/or ovarian cancer was observed, which is at least partially due to the low number of cases with family history of breast and/or ovarian cancer. clonal analysis of genomic instability might be beneficial.

**Effect of RAD50 and NBS1 on genomic integrity**

The tested heterozygous carriers of RAD50 687delT, RAD50 IVS3-1G>A and NBS1 Leu150Phe all showed an increase in genomic instability compared with healthy controls. Although based on a small number of individuals analysed, the total number of chromosome aberrations observed in mutation carrier T-lymphocytes (mean 13.6, SD 8.4) was increased 7-fold compared with non-carrier healthy controls (mean 1.9, SD 1.9) ($P = 0.006$). In particular, the number of chromosomal rearrangements was significantly increased ($P = 0.002$). As the Mre11 complex has been implicated in multiple cellular responses to DNA damage (3), the elevated frequency of spontaneous translocations in mutation carriers could reflect a deficiency in DNA repair. In fact, experimental analyses have shown that DSBs are the principal lesions involved in the process of chromosomal aberration formation. Defects in the DSB repair pathways may lead to broken chromosomes and chromosome rearrangements, and this genomic instability could be connected to increased mutation rates of cancer-related genes, ultimately contributing to tumorigenesis (28). Interestingly, epidemiological studies have shown that people with elevated frequency of chromosomal aberrations in their peripheral blood lymphocytes have a significantly elevated risk of developing cancer. Consequently, it has been suggested that the occurrence of chromosomal aberrations could be a relevant biomarker for cancer risk, reflecting either early biological effects of genotoxic carcinogens or individual cancer susceptibility (29).

The elevated spontaneous chromosomal instability of heterozygous mutation carriers suggests an effect for RAD50 and NBS1 haploinsufficiency on genomic integrity. Indeed, it has previously been indicated that in the case of tumor suppressor genes participating in the maintenance of the genomic stability the gene-dosage effect rather than biallelic inactivation is sufficient to exert a cellular phenotype that leads to tumorigenesis. However, these genes may show differences in tissue-specific protein-dosage thresholds, below which they fail to operate normally (24). For NBS1 heterozygosity and cancer predisposition there is supporting evidence also from mouse models. In addition to lymphomas, heterozygous NBS1 knockout mice developed a wide array of tumors affecting the liver, mammary gland, prostate and lung. Additionally, it was demonstrated that haploinsufficiency,
not loss of heterozygosity, was the mechanism underlying tumor development. Moreover, this study provided evidence that NBS1 heterozygosity predisposes cells to malignancy most likely because of chromosomal instability and defects in DNA repair (30).

As the current results suggest that Mre11 complex mutations act in a haploinsufficient manner, it raises the question about the importance of inactivation of these genes also in sporadic cancer cases. The first suggestion of reduced dosage of RAD50 at the somatic level already comes from acute myeloid leukemia (AML), since a strong correlation between AML and chromosomal aberrations involving the RAD50 containing 9q23-q31 chromosomal region has been observed (31). However, no direct implication of RAD50 defects in AML has yet emerged. Nevertheless, it is rather interesting that the currently identified RAD50 IVS3-1G>A positive family also shows one case of leukemia.

In conclusion, current results support the contribution of RAD50 687delT, RAD50 IVS3-1G>A and NBS1 Leu150Phe in hereditary susceptibility to breast cancer, and probably also other cancers. In particular, RAD50 687delT seems to be a relatively common low-penetrance breast cancer susceptibility allele in Northern Finland, and the risk associated with it is apparent in women unselected for family history. The elevated spontaneous chromosomal instability in peripheral blood lymphocytes of heterozygous mutation carriers suggests an effect for RAD50 and NBS1 haploinsufficiency on genomic integrity and predisposition to cancer.

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References


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