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

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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 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.

**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 an 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 evolutionarily 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.

**Abbreviations:** AI, allelic imbalance; BRCT, BRCA1 carboxy-terminal; DSB, double-strand break; NBS, Nijmegen breakage syndrome.
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 lacking a strong family history of 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 chemotherapy 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. 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-04126, BR-01163) 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 unselected 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).

**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).

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**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).

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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 NBS1 Leu150Phe carriers and one previously observed carrier (17) was verified by microsatellite marker analysis (data not shown).

**RAD50 IVS3-1G>A leads to frameshift**

The effect of RAD50 IVS3-1G>A on mRNA splicing was evaluated by cDNA-specific amplification of 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 RAD50 214delG transcript was present at lower levels than the wild-type, which indicates that it is unstable (data not shown).

**Loss of the wild-type allele is not implicated in the tumorigenesis of RAD50 and NBS1 mutation carriers**

The AI analysis was performed in the carriers of three potentially pathogenic alterations (Table I). Of the breast tumors of eight RAD50 687delT, one RAD50 IVS3-1G>A and two NBS1 Leu150Phe carriers, only two showed AI: one positive for RAD50 687delT and one for NBS1 Leu150Phe. However, both tumors showed decrease in the intensity of the mutant allele (data not shown), which was confirmed by sequencing.

### Table I. Pathogenic* RAD50 and NBS1 germ line changes observed in unselected Northern Finnish breast cancer cases

<table>
<thead>
<tr>
<th>Allele</th>
<th>Effect on protein</th>
<th>Carrier frequency</th>
<th>P-value OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAD50 687delT</td>
<td>Frameshift → stop codon at 234</td>
<td>2.5% (8 out of 317)</td>
<td>0.6% (6 out of 1000)</td>
</tr>
<tr>
<td>RAD50 IVS3-1G&gt;A</td>
<td>Frameshift → stop codon at 78</td>
<td>0.3% (1 out of 317)</td>
<td>(0 out of 1000)</td>
</tr>
<tr>
<td>NBS1 448C&gt;T</td>
<td>Leu150Phe in the BRCT domain</td>
<td>0.6% (2 out of 317)</td>
<td>(0 out of 1000)</td>
</tr>
<tr>
<td>ALTOGETHER</td>
<td></td>
<td>3.5% (11 out of 317)</td>
<td>0.6% (6 out of 1000)</td>
</tr>
</tbody>
</table>

*Based on the effect on the protein and allele prevalence in patients versus controls.

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Table II. Chromosomal aberrations in heterozygous \textit{RAD50} 687delT, \textit{RAD50} IVS3-1G>A and \textit{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$^a$</th>
<th>Complex chromosomal rearrangements$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{RAD50} 687delT ($n=4$)$^c$</td>
<td>0.4 SD = 0.9$^d$</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>\textit{RAD50} IVS3-1G&gt;A</td>
<td>BR-0516</td>
<td>–</td>
<td>11.1</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>BR-0490</td>
<td>–</td>
<td>2.7</td>
<td>4.1</td>
</tr>
<tr>
<td>\textit{NBS1} Leu150Phe</td>
<td>BR-0483</td>
<td>–</td>
<td>2.8</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>BR-0480</td>
<td>1.4</td>
<td>2.7</td>
<td>1.4</td>
</tr>
<tr>
<td>Controls ($n=6$)$^c$</td>
<td>–</td>
<td>1.5 SD = 1.3</td>
<td>0.3 SD = 0.8$^e$</td>
<td>–</td>
</tr>
</tbody>
</table>

$-$ = not observed.

$^a$Inversions, ring chromosomes, translocations ($\leq$3 break rearrangements).

$^b$Translocations ($\geq$4 break rearrangements), marker chromosomes.

$^c$For \textit{RAD50} 687delIT 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.

$^d$Observed only in one carrier with 1.7 per 100 metaphases showing telomere fusions.

$^e$Observed only in one control with 2.0 per 100 metaphases showing simple chromosomal rearrangements.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Increase in spontaneous chromosomal aberrations in T-lymphocytes of heterozygous \textit{RAD50} 687delIT, \textit{RAD50} IVS3-1G>A and \textit{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 \textit{RAD50} IVS3-1G>A carrier showing complex chromosomal rearrangements csb(4)(p14), der(13)del(13)(q11q14)t(13;16)(q14q10), der(16)t(13;16)(q14q10), der(21;22)(q10q10). Csb = chromosome break, der = derivative chromosome, del = deletion, t = translocation.}
\end{figure}
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 $RAD50\ 687\text{de}T$ and in one $NBS1\ \text{Leu150Phe}$ carrier. All observed chromosomal aberrations were considered random, as no preference for specific break site or evidence for clonality was observed.

**Discussion**

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

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 $RAD50\ 687\text{de}T$ and $NBS1\ \text{Leu150Phe}$ alleles, in addition to a novel $RAD50\ IVS3-1\text{G}>\text{A}$ splicing mutation, appeared to associate with predisposition to cancer.

The current results suggest that $RAD50\ 687\text{de}T$ 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 $RAD50$ carriers in the current cohort (0.6%, 2 out of 317) is higher than expected from the population frequency ($0.008$). No difference in allele frequency between cases with (2 out of 70) or without (6 out of 247) family history of breast and/or ovarian cancer was observed, which is at least partly due to the low number of cases considered as familial. Nevertheless, in the two families displaying both $RAD50\ 687\text{de}T$ and breast cancer, clustering of the disease cannot be explained by this mutation alone. The incomplete segregation suggests that $RAD50\ 687\text{de}T$ is a low-penetrance susceptibility allele, which possibly multiplies the cancer risk in cooperation with susceptibility alleles in other genes and environmental factors, as suggested by the polygenic model for breast cancer susceptibility. According to this model the genetic susceptibility to breast cancer is due to several loci, each conferring a modest independent risk ($22$). Consequently, these low-penetrance alleles might account for a substantial fraction of breast cancer incidence without necessarily causing evident familial clustering. This also suggests the possibility that a significant portion of the seemingly sporadic cancer cases could in fact be due to genetic predisposition ($23$).

It appears that $RAD50\ 687\text{de}T$ does not produce a stable protein product (C. Attwooll and J. Petrini, unpublished data). This dosage variation may have direct influence on DNA damage recognition and response pathway activities ($24$), which is supported by the increase of genomic instability in $RAD50\ 687\text{de}T$ carriers. The identification of another $RAD50$ mutation, $IVS3-1\text{G}>\text{A}$, associated with genomic instability further suggests the existence of additional cancer susceptibility alleles in this evolutionarily conserved gene. These may, however, not only be very rare, affecting perhaps only one or two families, but also population specific. Consequently, this suggests that screening of the $RAD50$ gene for additional germline mutations in other populations might be beneficial.

The association of $NBS1\ \text{Leu150Phe}$ with breast cancer susceptibility is supported by the identification of two more carriers in the current cohort ($0.6\%$, 2 out of 317). Yet, the families displaying $NBS1\ \text{Leu150Phe}$ do not show exclusively breast, but also other malignancies, including gynecological and stomach cancer. This feature was also evident in the previously reported mutation-positive family ($17$). Although $NBS1\ \text{Leu150Phe}$ has been so far observed only in affected individuals, its frequency in the studied cohort is too low to reach statistical significance when compared with controls ($P = 0.058$). However, its pathogenicity is supported by the increase in chromosomal instability in mutation carriers. It is possible that substitution of the evolutionarily conserved Leu150 residue is critical for BRCT domain function. This domain seems essential for the interaction between $NBS1$ and histone $\gamma$-H2AX after irradiation ($25$), suggesting that it plays an important role in DNA damage recognition. Indeed, other changes in conserved residues of the BRCT domain, GlyGly136–137GluGlu and Tyr176Ala, have been shown to disrupt the Mre11 complex nuclear focus formation and to block NBS1 phosphorylation after irradiation ($26$). Additionally, the $NBS1\ \text{Ile171Val}$ allele that is pathogenic for acute lymphoblastic leukemia and aplastic anemia ($12,27$) has been associated with an increase in numerical and structural chromosomal aberrations ($27$), suggesting a defect in the DNA damage response.

The tested heterozygous carriers of $RAD50\ 687\text{de}T$, $RAD50\ IVS3-1\text{G}>\text{A}$ and $NBS1\ \text{Leu150Phe}$ 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 5q23-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-penetration 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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