Molecular and Cellular Pathobiology

The Retinoblastoma Gene Undergoes Rearrangements in BRCA1-Deficient Basal-like Breast Cancer

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Abstract

Breast tumors from BRCA1 germ line mutation carriers typically exhibit features of the basal-like molecular subtype. However, the specific genes recurrently mutated as a consequence of BRCA1 dysfunction have not been fully elucidated. In this study, we used gene expression profiling to molecularly subtype 577 breast tumors, including 73 breast tumors from BRCA1/2 mutation carriers. Focusing on the RB1 locus, we analyzed 33 BRCA1-mutated, 36 BRCA2-mutated, and 48 non-BRCA1/2–mutated breast tumors using a custom-designed high-density oligomicroarray covering the RB1 gene. We found a strong association between the basal-like subtype and BRCA1-mutated breast tumors and the luminal B subtype and BRCA2-mutated breast tumors. RB1 was identified as a major target for genomic disruption in tumors arising in BRCA1 mutation carriers and in sporadic tumors with BRCA1 promoter methylation but rarely in other breast cancers. Homozygous deletions, intragenic breaks, or microdeletions were found in 33% of BRCA1-mutant tumors, 36% of BRCA1 promoter–methylated basal-like tumors, 13% of non-BRCA1–deficient basal-like tumors, and 3% of BRCA2-mutated tumors. In conclusion, RB1 was frequently inactivated by gross gene disruption in BRCA1 hereditary breast cancer and BRCA1-methylated sporadic basal-like breast cancer but rarely in BRCA2 hereditary breast cancer and non-BRCA1–deficient sporadic breast cancers. Together, our findings show the existence of genetic heterogeneity within the basal-like breast cancer subtype that is based upon BRCA1 status. Cancer Res; 72(16); 4028–36. ©2012 AACR.

Introduction

Breast cancer is a vastly heterogeneous disease with respect to tumor biology and clinical course. Research over the past decades has identified numerous genetic alterations, but the driving events in breast tumor development are still not fully known. Global gene expression profiling has established molecular subtypes associated with characteristic pathologic and clinical traits (1–3). Subsequent studies have showed different DNA copy number aberrations and degrees of genomic instability in these subtypes (4–6). The basal-like subtype comprises 10% to 20% of all breast cancers and typically has negative estrogen receptor (ER), progesterone receptor (PR), and HER2 status, poor prognosis, and frequent genomic alterations (7). Breast tumors arising in patients with an inherited BRCA1 mutation are typically of basal-like subtype and share most features of sporadic basal-like breast cancer, whereas tumors from BRCA2 mutation carriers are generally ER- and PR-positive and of luminal phenotype (4, 8, 9). Diverse patterns of DNA copy number aberrations have been described in BRCA1- and BRCA2-mutated tumors supporting their evolution via distinct pathways (10). More recent data indicate that BRCA1-mutated and other basal-like tumors have almost indistinguishable DNA copy number profiles (4).

Mutational inactivation of both RB1 alleles is the primary molecular alteration causing the pediatric cancer retinoblastoma, but RB1 mutations have been found in a wide range of malignancies. However, the exact role of RB1 in breast cancer is unclear, and recent studies have found reduced RB1 protein expression in mainly basal-like and luminal B tumors (11). Interestingly, targeted deletion of both RB1 and p53 in mouse mammary progenitor cells induced triple-negative or basal-like tumors addicted to RB1 loss (12). These studies suggest

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that R11 inactivation is critical for a subset of breast cancers that possess a high proliferative capacity, although the underlying mechanism of R11 inactivation remains unclear.

To address these issues, we analyzed a comprehensive set of hereditary and sporadic breast cancers using genome-wide gene expression and genomic profiling. PAM50 stratification classified 85% of BRCA1-mutated as basal-like and 56% of BRCA2-mutated as luminal B. To identify genes that are specifically targeted in BRCA1-mutated tumors, we conducted genome-wide DNA copy number analysis of an extended set of BRCA1-mutated tumors. Genomic identification of significant targets in cancer (GISTIC) analysis highlighted chromosome 13q14.2 including the R11 gene as a common region of deletion. In addition, the finding was supported by an R11-specific homozygous deletion in a BRCA1-mutated case. Application of a R11-specific high-density oligonucleotide microarray identified intragenic rearrangements in 33% of BRCA1-mutated tumors, 36% of BRCA1-methylated cases, 13% of basal-like tumors, and none in non-basal-like tumors. By FISH analysis, a physical disruption of the R11 gene was observed that subsequently led to absence of protein expression. Overall, our results reveal that targeted loss of R11 is a recurrent event in basal-like breast cancers and specifically frequent in BRCA1-deficient breast cancer, which may have therapeutic implications.

Materials and Methods

Patients and tumor material
Freshly frozen breast tumor tissues (n = 577) were obtained from the Southern Sweden Breast Cancer Group tissue bank at the Department of Oncology, Skåne University Hospital (Lund, Sweden), The Helsinki University Central Hospital (Helsinki, Finland), and Landspítali University Hospital (Reykjavik, Iceland). In addition, DNA from 73 breast tumors with known BRCA1 or BRCA2 mutations was obtained from the same departments. Tumor and patient characteristics are summarized in Supplementary Table S1. The study was approved by the regional ethical committee in Lund (reg. no. LU240-01 and 2009/658), waiving the requirement for informed consent for the study, by the Icelandic Data Protection Committee and the National Bioethics Committee of Iceland and by the Helsinki University Central Hospital ethical committee (207/E9/07). For Icelandic and Finnish patients, written informed consent was obtained according to the National Guidelines.

All cell lines were obtained from American Type Culture Collection except for SUM-149, which was obtained from the originator S. Ethier (13), and L56Br-C1, which was established and characterized in our laboratory (14). All cell lines were used within 6 months of culturing after receipt. Cell line identities are further confirmed by the consistency of our global copy number profiles to those in the published literature. HCC1937, HCC1428, BT549, and L56Br-C1 were maintained in RPMI supplemented with 10% FBS, MDAMB-231 in Dulbecco’s Modified Eagle’s Media (DMEM) supplemented with 10% FBS, and MDA-MB-436 were cultured in McCoy’s 5A supplemented with 10% FBS. SUM-149 was maintained in Ham’s F12 supplemented with 5% FBS, 5 μg/mL insulin, and 1 μg/mL hydrocortisone and MCF-10A was cultured in DMEM/F12 supplemented with 5% horse serum, 20 ng/mL EGF, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, and 10 μg/mL insulin. All cell lines were cultured with penicillin/streptomycin.

Gene expression analysis

Global gene expression analysis of 577 breast tumors was conducted using oligonucleotide microarrays (Gene Expression Omnibus, GEO, platform GPL5345) produced at the SCIBLU Genomics Centre at Lund University (Lund, Sweden) as described (15). Normalization and data analysis were conducted as previously described (4). PAM50 classification was conducted as described (16), using gene expression centroids obtained from Parker and colleagues (3). Gene expression data for the complete cohort of 577 cases are available as GSE25307.

Bacterial artificial chromosome array–based comparative genomic hybridization analysis

Bacterial artificial chromosome (BAC) array–based comparative genomic hybridization (aCGH) data for 359 cases, all included in the 577-sample gene expression data set, were obtained from GEO (GSE22133) and analyzed as described (4). In addition, genomic profiles for 72 breast tumors comprising 38 BRCA1-mutated and 34 BRCA2-mutated cases were generated using the same BAC aCGH platform and analysis steps as for GSE22133, resulting in total 58 BRCA1-mutated and 66 BRCA2-mutated cases with BAC aCGH profiles when combined with samples in GSE22133. GISTIC (17) analysis was conducted individually for BRCA1- and BRCA2-mutated cases as previously described (4) using a q-value threshold of 0.25. BR1 gene copy number loss in sporadic and BRCA1/BRCA2-negative familial breast tumors was investigated using the 305 non-BRCA1- and BRCA2-mutated cases remaining in GSE22133 (4).

High-resolution aCGH analysis

Custom-designed 60-mer oligonucleotide high-resolution aCGH (HD-aCGH) arrays for interrogation of gene-specific alterations were designed using the Agilent eArray ver. 5.3 software (Agilent Technologies) as described (18). HD-aCGH analysis was conducted on (i) 4 BRCA1-mutant breast cancer cell lines (L56Br-C1, HCC1937, MDA-MB-436, and SUM-149), (ii) the basal-like BT549 cell line, (iii) the BRCA2-mutant HCC1428 cell line, (iv) 33 BRCA1- and 36 BRCA2-mutated breast tumors, (v) 15 non-BRCA1/2–mutated basal-like breast tumors, (vi) 11 nonhereditary basal-like breast tumors with BRCA1 promoter methylation, (vii) 6 nonhereditary luminal A breast tumors, (viii) 6 nonhereditary luminal B breast tumors, (ix) 5 nonhereditary HER2-amplified breast tumors, and (x) 5 nonhereditary normal-like breast tumors. Microarrays were processed as described (18). Breakpoint analysis was conducted using circular binary segmentation (α = 0.01; ref. 19). Agilent probes were mapped to the UCSC build 18. All tumors profiled by HD-aCGH were also profiled by BAC aCGH.

FISH and immunohistochemical analysis

FISH analysis was conducted as described (20) on L56Br-C1 and SUM-149 cells using BAC probes RP11-90M2, RP11-120G8, RP11-67M6, RP11-34B2, RP11-288P20, RP11-713F1, RP11-583K10, RP11-324O19, RP11-63B11, RP11-106G21, RP11-912M21, RP11-144N20, and RP11-946K7. Immunohistochemical analysis was conducted on paraffin-embedded breast tumors as previously described (20).
and RP11-639F5 positioned immediately centromeric of RB1 and RP11-108P5, RP11-165D7, and RP11-90K7 positioned immediately telomeric of RB1. Immunohistochemical (IHC) analysis of RB1 protein expression on different tissue microarray (TMA) was conducted according to manufacturer’s instructions (4H1, Cell Signaling).

**BRCA1 promoter methylation analysis**

BRCA1 promoter hypermethylation analysis was conducted using a PSQ HS 96 pyrosequencing system (Biotage) and included 2 CpG island regions as described (13). For each tumor sample to be determined as BRCA1 methylated, a methylation allele frequency ≥10% was required. In addition, a fully methylated as well as an unmethylated sample were included as controls. All electropherograms were manually checked.

**TP53 and RB1 sequencing**

Exon 4 to 10 of the TP53 gene and all coding exons of RB1 were analyzed by Sanger sequencing using an ABI3130XL.

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Figure 1. BRCA1-deficient breast cancer is associated with the basal-like molecular subtype. A, hierarchical clustering analysis of 577 breast tissue samples using the PAM50 gene set (3). In the heatmap, rows correspond to PAM50 genes and columns to samples. Red indicates elevated expression and green reduced expression. Tumors are broadly divided into the 5 major molecular subtypes, basal-like, HER2-enriched, luminal A and B, and normal-like as shown by corresponding PAM50 subtype classification. BRCA1-mutated and BRCA1-methylated cases are as indicated typically classified into the basal-like subtype, BRCA2-mutated cases are generally classified as luminal B, whereas sporadic and BRCA1/BRCA2-negative familial breast tumors (BRCAx) are distributed equally across all molecular subtypes. B, Fisher exact test for investigating an association between BRCA1 deficiency including BRCA1 mutation or gene promoter methylation and the basal-like subtype. C, Fisher exact test for investigating an association between BRCA2 mutation and the luminal B subtype. wt, wild-type.
sequence. Primers were designed using the Primer3 web software, and sequences were evaluated using Sequencher software (Genes Codes Corp.).

**Western blot analysis**
Whole-cell lysates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride. Primary antibodies directed against RB1 (4H1, Cell Signaling) and β-tubulin (Tu27, Covance) were used.

**Statistical analysis**
Kaplan–Meier analysis and Fisher exact test were conducted in R.

**Results**

**Comprehensive gene expression profiling of hereditary breast cancer**
Our analysis of global gene expression profiles from a cohort of 577 breast cancers (herein after referred to as the 577-set; Supplementary Table S1), including 34 tumors from BRCA1 germ line mutation carriers, confirmed the strong association between BRCA1 mutation and the basal-like subtype ($P = 2 \times 10^{-30}$, Fisher exact test; Fig. 1). In addition, we found that BRCA1 gene promoter methylation was strongly associated with basal-like classification (21 of 23 tumors; $P = 4 \times 10^{-13}$, Fisher exact test; Fig. 1A and B). Moreover, BRCA1 mutation and promoter methylation were mutually exclusive. Although BRCA2-mutated tumors were found in all subtypes, 56% of them were classified as luminal B ($P = 7 \times 10^{-8}$, Fisher exact test; Fig. 1C). In contrast, tumors from BRCA1/BRCA2-negative familial cases were distributed equally across all molecular subtypes (Fig. 1A).

**Molecular heterogeneity in BRCA1-mutated and basal-like breast cancer**
We were interested in examining the molecular heterogeneity of basal-like breast cancer, and in particular, focusing on tumors with and without BRCA1 dysfunction. Within the gene expression data, we identified 321 genes (false discovery rate = 20%) differentially expressed between BRCA1-mutant or BRCA1-methylated basal-like tumors compared with basal-like tumors with wild-type BRCA1. Interestingly, the expression of RB1, BRCA1, CDK6, and CCND1 were reduced in the BRCA1-deficient group. To further examine this heterogeneity, we used BAC-aCGH on 58 such tumors. GISTIC (17) analysis revealed 23 deleted regions of which many have previously been shown to characterize BRCA1-mutated breast cancer (4), including 3p21.31, 5q11.2, and the RB1 region at 13q14.2 where the latter was highlighted by an RB1 gene-specific homozgyous deletion and high frequency of heterozygous copy number loss (66%; Fig. 2).

**Identification of genetic rearrangement of the RB1 gene**
On the basis of our previous results (13) suggesting that tumors with double-strand break repair deficiency potentially have a higher frequency of intragenic rearrangements, we designed an HD-aCGH assay with 1,041 probes covering RB1 with an average spacing of 171 bp for identification of small intragenic RB1 lesions not detectable by BAC aCGH. Remarkably, HD-aCGH analysis of 33 BRCA1- and 36 BRCA2-mutated tumors revealed intragenic breakages and homozgyous multiexon deletions in RB1 in 9 (27%) and 2 (6%) cases, respectively, of the 33 BRCA1-mutated tumors, all being of the basal-like...
subtype. There was one BRCA2-mutated case with RB1 intragenic break; interestingly, this BRCA2 case was both ER-negative and classified as basal-like by gene expression profile. Furthermore, sequencing all RB1 exons in the 22 BRCA1-mutated tumors without intragenic RB1 breaks identified one additional case with a 28-bp frameshift deletion in exon 4 of RB1. Taken together, 36% of BRCA1-mutated tumors were found to harbor an RB1 gene alteration whereas only 3% of BRCA2-mutated tumors (P = 3.64 × 10^{-4}, Fisher exact test). To relate these findings to breast cancer without known BRCA1 or BRCA2 mutations, we used HD-aCGH to screen another 48 tumors representing all the 5 established molecular subtypes

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Number of cases</th>
<th>RB1 alteration, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA1-mutated</td>
<td>33</td>
<td>12 (36)*</td>
</tr>
<tr>
<td>BRCA2-mutated</td>
<td>36</td>
<td>1 (3)</td>
</tr>
<tr>
<td>BRCA1-methylated basal-like</td>
<td>11</td>
<td>4 (36)</td>
</tr>
<tr>
<td>Basal-like</td>
<td>15</td>
<td>2 (13)</td>
</tr>
<tr>
<td>HER2-enriched</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Normal-like</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Luminal A</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Luminal B</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
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*One case harbors a 28bp deletion detected by Sanger sequencing.

Consistently, RB1 intragenic breaks were observed only in basal-like tumors and predominantly in BRCA1 promoter–methylated basal-like tumors (36%) compared with BRCA1-nonmethylated basal-like tumors (13%; Table 1). Moreover, we screened the TP53 gene to investigate correlation between RB1 alterations and TP53 mutation. No difference in TP53 mutation frequency in RB1 wild-type or RB1 altered was found (P = 0.60, Fisher exact test).

Physical disruption of RB1 validated in BRCA1-deficient cell lines

Intragenic RB1 breaks were also found in 3 BRCA1-mutated breast cancer cell lines (L56Br-C1, HCC1937, MDA-MB-436) and 1 basal-like cell line (BT549), whereas a fourth BRCA1-mutated (SUM-149) and a BRCA2-mutated (HCC1428) cell line showed no alterations by HD-aCGH analysis (Fig. 3A). To corroborate the RB1 gene breakage, we conducted dual-color FISH analysis on metaphase spreads from L56Br-C1 and SUM-149 cells using 6 BAC probes mapping to either the 5' or 3' ends of the RB1 gene. A clear separation of fluorescent BAC probes was identified in L56Br-C1 cells indicating a physical disruption of the RB1 gene, whereas no such separation was observed in SUM-149 cells in line with the HD-aCGH results (Fig. 3B). Furthermore, confirming that the intragenic breaks result in loss of functional protein, no RB1 protein was detectable by Western blot analysis in the cell lines with intragenic breakage in RB1 (Fig. 3C).

RB1 protein expression in breast cancer

The correlation between BRCA1 deficiency, intragenic RB1 alterations, and RB1 protein loss was further substantiated by IHC analysis of a TMA comprising 21 BRCA1-mutated tumors of which 10 were also analyzed by HD-aCGH. Thirteen of 21...
cases (62%) displayed absent or low RB1 protein expression (0%-10% staining, Fig. 4A), and of the 10 HD-aCGH analyzed cases, 3 harbored an intragenic RB1 rearrangement and 1 an RB1 gene mutation resulting in reduced RB1 protein expression in all 4 cases (<10% positive cells; Fig. 4B and C). To extend the analysis of RB1 inactivation beyond BRCA1-mutated tumors, we first analyzed RB1 protein expression in a TMA comprising 84 samples for which we also had molecular subtyping data by gene expression profiling. These included 20 basal-like, 12 HER2-enriched, 14 luminal A, 13 luminal B, 14 normal-like, and 11 unclassified cases. Strikingly, only basal-like and luminal B tumors displayed absent or low RB1 protein expression (<10% positive cells) with basal-like tumors showing the highest frequency (P = 6.02 × 10⁻³, Fisher exact test; Fig. 5A). One BRCA1 promoter–methylated case with RB1 gene breakage was included in the 84-sample TMA; similar to BRCA1-mutated cases with abrogated RB1, this case had no RB1 protein expression (0% positive cells; Fig. 5B).

**Clinical impact of RB1 gene copy number loss in breast cancer**

In line with RB1 protein expression levels, the highest frequency of RB1 gene copy number loss was observed in basal-like and luminal B tumors when investigated in a set of 305 sporadic and BRCA1/BRCA2-negative familial breast cancers (4). RB1 deletion was not associated with poor clinical outcome in the 305 tumors from Jonsson and colleagues (4) overall (P = 0.47, log-rank test); however, in the subset of ER-positive tumors, RB1 deletion was associated with a worse outcome (P = 0.046, log-rank test) reflecting the high frequency of RB1 deletion in the clinically aggressive luminal B tumors.

**Discussion**

Early studies established the fact that breast tumors from BRCA1 and BRCA2 germ line mutation carriers have distinct genomic and phenotypic characteristics (9, 21–23). Subsequently, it was shown that the majority of BRCA1-mutated tumors belong to the basal-like subtype (2, 4), although it is still unclear whether there are molecular patterns that distinguish them from non-BRCA1–mutated basal-like breast cancer. Here, we report findings to support the hypothesis that BRCA1-deficient breast cancer in fact develops through distinct genetic pathways.

In our large 577-set of PAM50-classified breast cancers, we reinforce the strong correlation between BRCA1 mutations and the basal-like subtype and, additionally, show a strong association between BRCA1 methylation and the basal-like subtype as well as between BRCA2 mutations and the luminal B subtype. Genomic instability and frequent low-level copy number changes of typical patterns are hallmarks of BRCA1 and BRCA2 tumors (10); however, the identity of specifically targeted genes has been illusive. We recently showed that the PTEN tumor suppressor gene is disrupted by rearrangements in a fraction of BRCA1-deficient tumors (13), which encouraged us to search for additional similar hits. GISTIC analysis of genome-wide DNA copy number data from 58 BRCA1-mutated tumors revealed several affected regions, including 1q14.2, highlighted by a homozygous deletion at the RB1 locus.

Although loss of pRB expression in basal-like breast cancers has been reported (11), the mechanisms of RB1 inactivation have not been clearly shown. Thus, we designed a high-density
oligomicroarray covering RB1 at a resolution of 171bp and, interestingly, identified RB1 rearrangements in one third of BRCA1-mutated cases. Dual-color FISH analysis of metaphase chromosomes from BRCA1-mutated cancer cells confirmed the physical disruption of RB1. These aberrations are expected to result in absence of pRB protein expression, a feature commonly observed in basal-like tumors, but also in luminal B tumors (11). We found that only 1 of 36 BRCA2-mutated tumors harbored an intragenic RB1 breakage. Interestingly, this particular case was one of the few BRCA2 tumors classified as basal-like. Furthermore, by analysis of 48 non-BRCA1/2-mutated tumors, representing all molecular subtypes, RB1 breakages were only observed in basal-like cases. The fact that these preferentially were cases with BRCA1 methylation further substantiates the strong selection for dual BRCA1 and RB1 inactivation and identifies RB1 disruption as a rather specific feature of BRCA1 dysfunctional breast cancer whether it is by germ line mutation or somatic promoter hypermethylation. It is interesting to note that 1 of 2 tumors with RB1 breakage and unconfirmed BRCA1 inactivation was classified as basal-like and obtained from a patient with family history of breast and ovarian cancer, suggesting that an unrevealed BRCA1 mutation may exist also for this deviating case. Accordingly, none of the luminal B classified tumor harbored RB1 breakage, despite frequent loss of pRB expression, suggesting that other genetic, epigenetic, or posttranslational mechanisms are operational in the luminal B subtype of breast cancer.

An intriguing observation was the similarly high frequency of RB1 breakages in BRCA1-mutated and BRCA1-methylated cases, which may reflect that BRCA1 methylation is an early event that drives or influences tumor progression in a manner similar to a germ line BRCA1 mutation. The genetic association of BRCA1 and RB1 was further supported by the demonstration of a physical interaction between these 2 major tumor suppressors (24). Thus, the highly proliferative capacity of basal-like BRCA1-mutated breast cancers could potentially be due to a combination of both BRCA1 and RB1 abrogation. It has also been hypothesized that combined inactivation of RB1, TP53, and BRCA1 confers a basal-like phenotype. However, no difference in the frequency of somatic TP53 mutations was observed between BRCA1-mutated tumors with and without RB1 gene breakage (46% mutated in both groups). Our findings are contradictory to results from a recent study of ovarian cancer (25), where BRCA1/2 inactivation was found to be mutually exclusive to RB1 inactivation (and CCNE1 activation), which may suggest the role of tumor type-specific oncogenic networks.

In summary, our study reveals that targeted loss of RB1 is a recurrent event in basal-like breast cancers, a subgroup characterized by high genomic instability. RB1 intragenic breaks
are only observed in basal-like tumors and predominantly in BRCA1-mutated or BRCA1 promoter-methylated cases, resulting in significantly reduced or absent pRB expression. These novel results define a genetic heterogeneity within the basal-like subtype, based on BRCA1 status. This may have implications for understanding differential response to chemotherapy of triple-negative or basal-like breast cancer to drugs that target DNA repair deficiency or for development of new drugs that compensate for RBl dysfunction.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Vallon-Christersson, S.K. Gruberger-Saal, C. Heagrét, E. Johnson, P. Heikkilä, B.A. Agnarsson, O.T. Johannsson, P. Malmström, M. Fernö, H. Olsson, N. Loman, H. Nevanlinna, Å. Borg
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Jonsson, J. Staaf, J. Vallon-Christersson, M. Ringnér, K. Holm, C. Persson, D. Graubau, P. Heikkilä, O.T. Johannsson, Å. Borg

References


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