**J Med Genet** 2001;38:361–368

**Abstract**

Introduction—A small fraction of breast cancer is the result of germline mutations in the *BRCA1* and *BRCA2* cancer susceptibility genes. Mutation carriers frequently have a positive family history of breast and ovarian cancer, are often diagnosed at a young age, and may have a higher incidence of double or multiple primary breast tumours than breast cancer patients in general.

**Objectives**—To estimate the prevalence and spectrum of *BRCA1* and *BRCA2* mutations in young Danish patients affected with bilateral or multifocal breast cancer and to determine the relationship of mutation status to family history of cancer.

**Subjects**—From the files of the Danish Breast Cancer Cooperative Group (DBCG), we selected 119 breast cancer patients diagnosed before the age of 46 years with either bilateral (n=59) or multifocal (n=61) disease.

**Methods**—DNA from the subjects was screened for *BRCA1* and *BRCA2* mutations using single strand conformation analysis (SSCA) and the protein truncation test (PTT). Observed and expected cancer incidence in first degree relatives of the patients was estimated using data from the Danish Cancer Registry.

**Results**—Twenty four mutation carriers were identified (20%), of whom 13 had a *BRCA1* mutation and 11 carried a *BRCA2* mutation. Two mutations in *BRCA1* were found repeatedly in the material and accounted for seven of the 24 (29%) mutation carriers. The mutation frequency was about equal in patients with bilateral (22%) and multifocal breast cancer (18%).

The incidence of breast and ovarian cancer was greatly increased in first degree relatives of *BRCA1* and *BRCA2* mutation carriers, but to a much lesser degree in relatives of non-carriers. An increased risk of cancer was also noted in brothers of non-carriers.

**Conclusions**—A relatively broad spectrum of germline mutations was observed in *BRCA1* and *BRCA2* and most of the mutations are present in other populations. Our results indicate that a diagnosis of bilateral and multifocal breast cancer is predictive of *BRCA1* and *BRCA2* mutation status, particularly when combined with information on the patients’ age at diagnosis and family history of breast/ovarian cancer.

Keywords: breast cancer; mutations; *BRCA1*; *BRCA2*

**BRCA1 and BRCA2 mutation status and cancer family history of Danish women affected with multifocal or bilateral breast cancer at a young age**


Hereditary breast and ovarian cancer (HBOC) syndrome is characterised by familial clustering of breast cancer diagnosed at an early age and increased incidence of ovarian cancer. Mutations in two cancer susceptibility genes, *BRCA1* and *BRCA2*, seem to be responsible for the majority of breast cancers associated with the HBOC syndrome and these genes may also account for a small (~5%) but significant fraction of the total breast cancer incidence in western countries.

Since the cloning of *BRCA1* and *BRCA2* in 1995 and 1996, several functional studies have been published. The *BRCA1* and *BRCA2* proteins are involved in regulation of gene transcription and DNA repair that is based on homologous recombination. Disruption of their normal function probably results in inability to repair chromosome breakage, which may lead to cancer when accumulation of such aberrations occurs in genes that control proliferation and/or differentiation. Although the basic function of the proteins may overlap and both genes interact with the RAD51 protein, which is essential for recombination based DNA repair, mutations in the two genes have to some degree been associated with a distinct phenotype. For example, the profiles of somatic chromosome changes appear to be distinct in *BRCA1* and *BRCA2* defective tumours. The cancer spectrum is also slightly different in *BRCA1* and *BRCA2* linked families; for example, ovarian cancer is reported to be more common in *BRCA1* mutation carriers while *BRCA2* mutations are associated with increased risk of male breast cancer and cancer of the pancreas. The pathology associated with mutations in the two genes also appears to differ. There is also some evidence for heterogeneity in the expression of both *BRCA1* and *BRCA2* mutations, which may be caused by additional genetic or environmental factors and allelic variation. Studies of high risk families indicate that the penetrance associated with positive *BRCA1* and *BRCA2* mutation status is high, or close to 90% in terms of lifetime risk. Lower estimates of the penetrance have been obtained from studies of cancer incidence in first degree relatives of mutation carriers who have been identified without previous consideration of their family history.
Multiple primary cancer is well established as a consequence of inherited cancer syndromes. In HBOC families, a high incidence of bilateral breast cancer and the occurrence of breast and ovarian cancer in the same subject have been noted. The clinical and biological significance of multiple ipsilateral tumour lesions remains an unsolved puzzle. Bilateral and ipsilateral multiplicity have features in common and are associated with one another. Macroscopically distinct tumour lesions may result from intramammary tumour spread as well as growth of independent primary tumours. Bilateral breast cancers are frequently noted in BRCA1 and BRCA2 families, but the relationship of ipsilateral multiplicity to these 119 patients was obtained from patients diagnosed with breast cancer before the age of 46 years and a median age at onset of breast cancer for these 119 patients was 39 years (range 24–45 years). Bilateral breast cancer was defined as synchronous if bilaterality was diagnosed simultaneously or less than 12 months apart (n=12), and as metachronous if bilaterality occurred 12 or more months apart (n=46).

All patients are identified in the DBCG registry by their full name and their unique personal identification number. This number, assigned to all citizens in Denmark alive on 1 April 1968, incorporates sex and date of birth and permits accurate linkage of information between registries. For patients born after that date, the personal identification number was assigned at birth.

A total of 180 control DNA samples from healthy subjects that we used to compare allele frequencies were kindly provided by Cand Scient Hans Eiberg (Copenhagen family bank).

DNA AND RNA EXTRACTION
Most of the tumour samples were derived from tissue processed for hormone receptor analysis (frozen nuclear pellets or tissue homogenate) and some were obtained as fresh frozen tissue. DNA was extracted using a salting out procedure with reagents provided with the Wiz kit (Promega). Total RNA was isolated using QuickPrep Total RNA extraction kit (Pharmacia Biotech) and the manufacturer’s protocol.

MUTATION ANALYSIS
All samples were subjected to mutation analysis by SSCA and PTT. In addition, the control samples were screened by SSCA for variants encoding missense changes that were suspected to be pathogenic.

SSCA was done for all coding BRCA1 exons with primers originally obtained from Dr Nigel Spurr (ICRF, Clare Hall Laboratories, UK). In the case of BRCA2, SSCA was done for all exons except 10 and 11, which were analysed with PTT. The primers were as previously described. Thermal cycling was performed in 15 µl volumes with 0.2 mmol/l dTTP, dGTP, and dATP, 0.01 mmol/l “cold” dCTP, and 0.25 µCi α-32P dCTP (Amersham), 3 pmol of each primer; 30 ng sample DNA, 0.2 units Dynazyme (Finnzyme Oy), and the buffer provided with the enzyme. Thermal cycling was done in five steps with annealing temperatures of 62°C (three cycles), 60°C (five cycles), 58°C (eight cycles), 55°C (12 cycles), and 53°C (17 cycles), respectively. The amplified products were diluted 5× in a formamide loading buffer, denatured at 95°C for five minutes, and cooled on ice for 5–10 minutes before loading onto the gel. Then 3 µl of the sample was electrophoresed in a 0.5 × MDE gel (FMC Bioproducts) using 0.6 × TBE running buffer under three different conditions: (1) 18–24 hours at 4–6 W in a cooling room (4°C), (2) 6–8 hours at 30 W in a cooling room, and (3) 18–24 hours at room temperature at 4–5 W and a gel containing 5% glycerol. The gels were exposed to x-ray film (Hyperfilm MP, Amersham) in a −80°C freezer for 12–24 hours.

Primers used in PTT for amplification of genomic DNA from BRCA1 (exon 11) and BRCA2 (exons 10 and 11) have been described elsewhere. Exon 11 of the BRCA1 gene was amplified in three overlapping segments. For BRCA2, a single reaction was needed for exon 10 while exon 11 was screened using four different sets of primers. Thermal cycling was done with the Expand High Fidelity PCR system (Boehringer Mannheim) using the manufacturer’s protocol. The PCR product was transcribed and translated using the TnT Quick Coupled Transcription/Translation System (Promega). Typically, a 10 µl reaction contained 8 µl TnT T7 Quick mastermix, 1.5 µl...
BRCA1 and BRCA2 mutation status

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municipalities.

committees of Copenhagen and Frederiksberg

Classification of Diseases, Seventh Revision
cancers were counted and categorised accord-

date of death, or 1 December 1999. Observed

with breast cancer to the date of emigration,

extended from the date of birth of the proband

in Denmark. The period of follow up for the

which has been in operation since 1942,

date of death, and name. The Cancer Registry,
died before 1 April 1968, their date of birth,

personal identification numbers or, if they had

sisters and 84 brothers) were found.

mothers and 95 fathers) and 162 sibs (78

local parish registers. A total of 192 parents (97

number of the mother, or occasionally from the

Register by using the personal identification

located in the files of the Central Population

of the 119 breast cancer patients were also

patient, and the date of death obtained from

the families had lived at the date of birth of the

population registers of the localities in which

before 1936 or who had died before 1 April

identification number and on date of death or

was linked to the files of the Central Population

RELATIVES IDENTIFICATION OF CANCER IN FIRST DEGREE

IDENTIFICATION OF CANCER IN FIRST DEGREE

RELATIVES

By use of the personal identification number,
each of the breast cancer patients under study

was linked to the files of the Central Population

Register. For parents born after 1936 and alive

at the date of the start of the Central

Population Register on 1 April 1968, infor-

mation was obtained on full name and personal

identification number and on date of death or

emigration, if applicable. For parents born

before 1936 or who had died before 1 April

1968, such information was searched manually

in the files of the “parish registers”, that is, the

population registers of the localities in which

the families had lived at the date of birth of the

patient, and the date of death obtained from

the Danish mortality files. Sisters and brothers

of the 119 breast cancer patients were also

located in the files of the Central Population

Register by using the personal identification

number of the mother, or occasionally from the

local parish registers. A total of 192 parents (97

mothers and 95 fathers) and 162 sibs (78

sisters and 84 brothers) were found.

Data on the patients’ relatives were linked

with the Danish Cancer Registry, using the

personal identification numbers or, if they had

died before 1 April Brca1, their date of birth,

date of death, and name. The Cancer Registry,

which has been in operation since 1942,
collects information on all patients with cancer

in Denmark. The period of follow up for the

occurrence of cancer among parents and sibs

extended from the date of birth of the proband

with breast cancer to the date of emigration,
date of death, or 1 December 1999. Observed

cancers were counted and categorised accord-
ing to a modified version of the International

Classification of Diseases, Seventh Revision

(ICD-7).

The protocol was approved by the ethical

committees of Copenhagen and Frederiksberg

municipalities.

PCR product, and 0.5 µl S

labelled methio-
nine (in vivo cell labelling grade, Amersham).

After incubation at 32°C for one hour the pro-
tein products were diluted 5 x in protein load-
ing buffer, denatured, and electrophoresed on a
12.5% SDS-PAGE gel. The gels were dried

exposed to an x ray film for 8-24 hours.

For RT-PCR analysis, cDNA was synthe-
sised from total RNA using ExpandTM reverse

transcriptase and amplified with the Expand

High Fidelity PCR system using protocol

suggested by the manufacturer (Boehringer

Mannheim).

All samples displaying aberrant mobility in

the SSCA or PTT gels were reamplified and 5
µl of the amplified product was treated with a
mixture of exonuclease I and shrimp alkaline

phosphatase. One µl of this mixture was

sequenced using the Thermo Sequenase

radiolabelled terminator cycle sequencing kit

(Amersham). The products were electro-

phoresed on a 6% urea sequencing gel using

0.8 x glycerol tolerant gel (GTG) buffer. The

gels were fixed and dried before exposure to the

x ray film.

Identification of cancer in first degree

relatives

Results

Among the 119 patients, a total of 49 germline
sequence variants were identified in the two
genomes, including pathogenic mutations (n=19),
rare sequence variants (n=15), and common
polymorphisms (n=15). The 19 pathogenic
mutations were found in 20% (24/119) of the
patients. The majority of these mutations lead
to premature termination of the protein trans-
lation. Twelve are frameshift mutations, two are
missense, and one of the mutations affects a
splice site. Only four of the mutations are

identified in the two genes, including pathogenic
mutations (n=19), rare sequence variants (n=15),
and common polymorphisms (n=15). The 19
pathogenic mutations were found in 20% (24/119)
of the patients. The majority of these mutations
lead to premature termination of the protein
translation. Twelve are frameshift mutations, two
are nonsense, and one of the mutations affects a
splice site. Only four of the mutations are

amino acid changes (missense). Six of the

pathogenic mutations had not been reported in

the Breast Cancer Information Core database

(BIC, http://www.nhgri.nih.gov/Intramural_re-

search/Lab_transfer/Bic) by May 2000 and

are considered novel.

BRCA1 mutations

Thirteen BRCA1 mutation carriers and eight
distinct mutations were detected in our assays

(table 1A). Five of the mutations result in a

truncated protein and three are missense
mutations. All these mutations have been

submitted previously to the BIC database by oth-

ers. Two mutations were recurrently found in

our material. A frameshift mutation in exon 11,

2594delC, was found in four of the patients

and three patients had a nucleotide substitu-
tion 5208T>C leading to an amino acid
change (C1697R).

The other two missense mutations found in

BRCA1 were C61G and A1752P. Position

1697 is in a conserved stretch of 23 amino

acids that is identical in the human, canine, and
dog in this position is replaced by a

mouse Brca1.19 The arg1697 allele was tested in

a yeast based assay for loss of C-terminal

transactivation activity (Vallon-Christersson et

al, submitted). Basically, the assay measures

the ability of BRCA1 C-terminal sequences

that are fused with a GAL4 DNA binding
domain to activate transcription of a reporter
gene.20 The BRCA1 construct containing

arg1697 was not able to activate transcription

in this system, further supporting the C1697R

as a pathogenic mutation. Neither the C1697R

variant nor A1752P were found in the control
material. The region around position 1752 is

quite well conserved, although alanine found in

human and dog in this position is replaced by a

serine in the mouse. In the yeast assay, the

The expected numbers of cancers among first
degree relatives of the breast cancer study

group were calculated by multiplying the

number of person years at risk for each

subgroup of relatives, respectively, by the sex

specific cancer incidence rates for the Danish

population in five year age groups and calendar

periods of observation. Standardised incidence

ratios (SIRs), taken as the ratios of the

observed to the expected number of cancers,

and associated 95% confidence intervals (CIs)

were calculated assuming a Poisson distribu-
tion of the observed numbers of cancers.18 The

Fisher exact test was used to compare the

prevalence of positive mutation status between
disease classes.

Epidemiological analysis

EPIDEMIOLOGICAL ANALYSIS

The expected numbers of cancers among first
degree relatives of the breast cancer study

group were calculated by multiplying the

number of person years at risk for each
Table 1: Information for the BRCA1 and BRCA2 mutation carriers identified in this study. Position of mutation by exon; nucleotide change; type of mutation, ie nonsense, frameshift, splice site, or missense; effect of mutation on the protein sequence; patient’s age at diagnosis; type of cancer, ie unilateral multifocal disease or bilateral; presence of cancer in first degree relatives of the probands and types of cancer.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Mutation</th>
<th>Type</th>
<th>Effect</th>
<th>Age</th>
<th>Characteristics</th>
<th>Family history of cancer (site)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proband 1st degree relatives</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| 5 | 300T>G | Missense | C61G | 36 | Bilateral | Yes (breast) |
| 11 | 2594delC | Frameshift | Stop 845 | 36 | Bilateral | Yes (ovary) |
| 11 | 2594delC | Frameshift | Stop 845 | 41 | Multifocal | No |
| 11 | 2594delC | Frameshift | Stop 845 | 28 | Multifocal | Yes (breast) |
| 11 | 2594delC | Frameshift | Stop 845 | 34 | Bilateral | No |
| 11 | 351G>T | Non-sense | Stop 1134 | 28 | Bilateral | No |
| 11 | 3819del | Frameshift | Stop 1242 | 34 | Bilateral | No |
| 11 | 3829delT | Frameshift | Stop 1263 | 39 | Bilateral | Yes (ovary) |
| 18 | 5208T>C | Missense | C1697R | 35 | Multifocal | Yes (breast*, cervix) |
| 18 | 5208T>C | Missense | C1697R | 41 | Bilateral | Yes (breast, nasopharynx, skin) |
| 18 | 5208T>C | Missense | C1697R | 44 | Bilateral | Yes (pancreas) |
| 20 | 7375G>C | Missense | A1752P | 41 | Multifocal | Yes (breast) |
| 20 | 5382insC | Frameshift | Stop 1829 | 32 | Bilateral | Yes (ovary*, rectum) |

*Bilateral mutation, †Novel mutations.

Table 2: Unknown and rare sequence variants identified in the patient group. Name of variant; gene name; effect of variant on the protein level; number of carriers and their age at first diagnosis of cancer; family history and types of cancers diagnosed in first degree relatives.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Gene</th>
<th>Amino acid change</th>
<th>No of patients (age)</th>
<th>1st degree relatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>710C&gt;T</td>
<td>BRCA1</td>
<td>None</td>
<td>1 (42)</td>
<td>Yes (bladder, eye)</td>
</tr>
<tr>
<td>3238G&gt;A</td>
<td>BRCA1</td>
<td>S1040N</td>
<td>3 (43,44,27)</td>
<td>Yes (prostate)</td>
</tr>
<tr>
<td>4158A&gt;G</td>
<td>BRCA1</td>
<td>R1347G</td>
<td>3 (44,44,45)</td>
<td>Yes (prostate, melanoma, rectum)</td>
</tr>
<tr>
<td>4654G&gt;T</td>
<td>BRCA1</td>
<td>S1512I</td>
<td>1 (39)</td>
<td>No</td>
</tr>
<tr>
<td>4931A&gt;G</td>
<td>BRCA1</td>
<td>None</td>
<td>1 (38)</td>
<td>Yes (connective tissue)</td>
</tr>
<tr>
<td>5396+47ins12</td>
<td>BRCA2</td>
<td>Y24C</td>
<td>3 (40,37,39)</td>
<td>No</td>
</tr>
<tr>
<td>1029+56G&gt;T</td>
<td>BRCA1</td>
<td>None</td>
<td>1 (39,33)</td>
<td>Yes (oesophagus)</td>
</tr>
<tr>
<td>7236+62A&gt;G</td>
<td>BRCA2</td>
<td>None</td>
<td>2 (42,42)</td>
<td>No</td>
</tr>
<tr>
<td>7663+53G&gt;T</td>
<td>BRCA2</td>
<td>None</td>
<td>2 (42,35)</td>
<td>Yes (prostate)</td>
</tr>
<tr>
<td>9079G&gt;A</td>
<td>BRCA2</td>
<td>A2951T</td>
<td>1 (34)</td>
<td>Yes (lung, lip)</td>
</tr>
<tr>
<td>9485+26T&gt;C</td>
<td>BRCA2</td>
<td>None</td>
<td>1 (42)</td>
<td>No</td>
</tr>
<tr>
<td>10204A&gt;T</td>
<td>BRCA2</td>
<td>K320X</td>
<td>1 (42)</td>
<td>No</td>
</tr>
<tr>
<td>10338G&gt;A</td>
<td>BRCA2</td>
<td>None</td>
<td>3 (40,37,39)</td>
<td>No</td>
</tr>
<tr>
<td>10462A&gt;G</td>
<td>BRCA2</td>
<td>J3412V</td>
<td>1 (41)</td>
<td>No</td>
</tr>
</tbody>
</table>

For BRCA2 mutations, eleven mutations were detected of which six have not been reported before (table 1B). None of the mutations was identified in more than one patient. Ten resulted in a truncated protein including one splice site and one nonsense mutation. Only one missense mutation was considered pathogenic.

The splice site mutation occurs in the invariant position of the donor site in intron 15 (7845+1G>A). Tumour RNA was available from the patient so the effect of this mutation could be assessed. RT-PCR of a segment containing exons 14-18 gave a band of 900 bp in this sample and the normal sized band of 1050 bp was missing. Sequencing of the aberrant band indicated the absence of exon 15. The only missense change in BRCA2 pro1752 allele was able to activate transactivation, although in non-pathogenic (polymorphic) amino acid changes tested this ability was reduced. Two other missense changes have been reported near this position (R1751Q and P1749R) and the site may therefore represent a mutation hotspot. The third missense mutation found in BRCA1, C61G, has been seen previously in many breast cancer families throughout Europe. This variant affects the critical ring finger motif in BRCA1, which is required for binding to the BAP1 and BARD1 proteins. Five BRCA1 variants of unknown significance were found in this study (table 2). The three amino acid changes belonging to this category are all located in non-conserved positions of the BRCA1 protein. One of them, S1040N, has previously been found in healthy controls. The carrier of another variant, S1512I, also had a mutation in the BRCA2 gene suggesting that this variant may also be a neutral polymorphism. A 12 bp insertion/duplication in intron 20 (IVS20+47ins12) was found in two patients in our group but was not present in the healthy control population. This variant has been found previously in Polish breast cancer families and at low frequency in normal Polish controls. More work is therefore needed to elucidate the connection of this variant to familial predisposition.

Of the 13 BRCA1 carriers identified in this study, nine had bilateral breast cancer and four had multifocal disease (table 3). The mean time interval between surgery for the first primary cancer and the second occurrence in the contralateral breast was 53 months, about the same as for non-carriers (46 months). The mean age at first diagnosis was 37 years for subjects positive for BRCA1 mutation, which is two years lower than for non-carriers.
Table 3: Patient classification according to mutation carrier status and type of breast cancer

<table>
<thead>
<tr>
<th>Mutation carrier status</th>
<th>Multifocal unilateral</th>
<th>Synchronous bilateral</th>
<th>Metachronous bilateral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-carriers</td>
<td>70 (53%)</td>
<td>8 (6%)</td>
<td>37 (41%)</td>
</tr>
<tr>
<td><strong>BRCA1 mutation positive</strong></td>
<td>4 (31%)</td>
<td>1 (8%)</td>
<td>8 (61%)</td>
</tr>
<tr>
<td><strong>BRCA2 mutation positive</strong></td>
<td>7 (64%)</td>
<td>3 (27%)</td>
<td>1 (9%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>81 (61%)</td>
<td>12 (10%)</td>
<td>46 (39%)</td>
</tr>
</tbody>
</table>

Table 4: Observed number of malignant neoplasms in first degree relatives of the patients (Obs) and standardised incidence ratios (SIR) classified by cancer type and mutation status

<table>
<thead>
<tr>
<th>Mutation status</th>
<th>All cancers</th>
<th>Breast or ovary cancer</th>
<th>Other cancers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No of relatives</td>
<td>Obs</td>
<td>SIR</td>
</tr>
<tr>
<td>All categories</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mothers</td>
<td>97</td>
<td>35</td>
<td>1.4</td>
</tr>
<tr>
<td>Fathers</td>
<td>95</td>
<td>26</td>
<td>1.0</td>
</tr>
<tr>
<td>Sisters</td>
<td>78</td>
<td>11</td>
<td>2.3*</td>
</tr>
<tr>
<td>Brothers</td>
<td>84</td>
<td>11</td>
<td>3.3*</td>
</tr>
<tr>
<td><strong>All relatives</strong></td>
<td>566</td>
<td>85†</td>
<td>1.4*</td>
</tr>
</tbody>
</table>

| BRCA1 carriers  |             |     |     |     |     |     |     |
| Mothers         | 12          | 10  | 3.9*| 7   | 10.3*| 3   | 1.6 |
| Fathers         | 12          | 3   | 1.0 | 0   | 0.0  | 3   | 1.0 |
| Sisters         | 11          | 4   | 8.4*| 3   | 18.1*| 3   | 3.2 |
| Brothers        | 14          | 0   | 0.0 | 0   | 0.0  | 0   | 0.0 |
| **All relatives** | 75          | 17  | 2.5*| 10  | 11.6*| 7   | 1.2 |

| BRCA2 carriers  |             |     |     |     |     |     |     |
| Mothers         | 10          | 3   | 1.4 | 3   | 4.9* | 0   | 0.0 |
| Fathers         | 10          | 2   | 0.8 | 0   | 0.0  | 2   | 0.8 |
| Sisters         | 8           | 1   | 3.1 | 1   | 9.7  | 1   | 0.0 |
| Brothers        | 5           | 1   | 0.0 | 0   | 0.0  | 1   | 4.7 |
| **All relatives** | 51          | 7   | 1.3 | 4   | 5.5* | 3   | 0.7 |

| Unknown variants |             |     |     |     |     |     |     |
| Mothers         | 10          | 2   | 0.6 | 0   | 0.0  | 2   | 0.8 |
| Fathers         | 10          | 3   | 0.9 | 0   | 0.0  | 3   | 0.9 |
| Sisters         | 2           | 0   | 0.0 | 0   | 0.0  | 0   | 0.0 |
| Brothers        | 9           | 3   | 9.5*| 0   | 0.0  | 3   | 9.5*|
| **All relatives** | 60          | 8   | 1.1 | 0   | 0.0  | 8   | 1.2 |

| Wild type genotype |             |     |     |     |     |     |     |
| Mothers           | 65          | 20  | 1.1 | 10  | 2.1* | 10  | 0.8 |
| Fathers           | 63          | 18  | 1.1 | 0   | 0.0  | 18  | 1.1 |
| Sisters           | 57          | 6   | 1.6 | 3   | 2.3  | 3   | 1.2 |
| Brothers          | 56          | 7   | 3.0*| 0   | 0.0  | 7   | 3.0*|
| **All relatives** | 380         | 53† | 1.3 | 13  | 2.1* | 40  | 1.1 |

*Statistically significant by 95% confidence limits.
†Including two affected children.

(Table 3 continued)

(S2704F) was found in one patient but not in healthy controls. This is a relatively well conserved region and both the mouse and rat protein have a serine in this position. Another potential missense mutation affecting the highly conserved N-terminal Y42C was detected in three patients. This variant has previously been found in familial breast cancer patients who do not carry any apparent protein truncating mutation in BRCA1 or BRCA2 (T Frank, personal communication). In addition, studies of BRCA2 in a yeast based system have localized transactivation activity in the region encoded by exon 3 that appears to be destroyed by the introduction of this amino acid change.27

However, screening of the control material showed one subject with this change whose family history of cancer was unknown. We therefore treat Y42C here as an unknown variant.

Other rare polymorphisms and variants of unknown significance were found at four positions in the BRCA2 coding region and as many intronic positions. Of these, there were two missense changes, A2951T and I3412V. The A2951T variant has been referred to in the BIC database as a polymorphism. Residue 3412 is absent in the rat and mouse BRCA2 sequence and appears to be located in a trivial region, as it is upstream of the known polymorphic nonsense change K3326X, which was also found in one patient in this study.28

Of the 11 BRCA2 mutation carriers identified in the study, seven had multifocal breast cancer (table 3). The remaining four bilateral cases had a short relapse free time interval (mean=11 months) and two of them had synchronous disease. The average age at diagnosis was about the same as for the BRCA1 carriers (36 years).

FAMILY HISTORY OF CANCER

The cancer incidence in first degree relatives of the breast cancer patients included in the study is shown in table 4. The incidence of all cancers was significantly higher than expected for the combined group and the estimated standardized incidence ratio (SIR) was 1.4. Increased cancer incidence was observed in sisters of the patients (SIR 2.3). This is largely because of excess of breast and ovarian cancer (SIR 4.3).

As expected, the occurrence of these cancer types in both sisters and mothers is particularly associated with a positive BRCA1 and BRCA2 mutation status. The SIRs for breast/ovarian cancer in the relatives of BRCA1 and BRCA2 mutation carriers were 11.6 and 5.5, respectively. In addition, breast cancer occurred at younger ages in relatives of mutation carriers and the median age at diagnosis was 43 years (range 33-64) but was 54.5 years for relatives of non-carriers (range 39-81). The corresponding figures for ovarian cancer were 46 (range 41-47) and 51 years (41, 51, and 70 years), respectively. Relatives of patients with the wild type genotype also had a significantly increased risk of breast and ovarian cancer but the incidence was only twofold greater than that expected. Other cancer types make up about 42% (10 of 24) of occurrences in first degree relatives of the BRCA1 and BRCA2 mutation carriers, but the same type of cancer never appeared more than once. There is no incidence of breast or ovarian cancer in relatives of patients with a nucleotide variant of unknown significance, implying that in most cases these variants are benign polymorphisms.

Interestingly, the number of cancers observed in brothers of the patients was higher than expected. This effect appears to be mainly associated with brothers of probands that are negative for BRCA1 and BRCA2 mutations, that is, those carrying unknown variants (SIR 10) or do not have a mutation in either gene (SIR 3.0). Various cancer types were observed in the brothers, including cancer of the lung, urinary bladder, skin, testes, and lymphoma.

Discussion

Of the 19 distinct BRCA1 and BRCA2 gene mutations identified, six are novel changes and these were all in BRCA2. The majority of mutations have previously been detected outside Denmark, including four mutations dispersed over Europe and North America, seven previously detected in North America, and two previously detected in Europe. Two mutations were found recurrently and account for 29% of all the carriers identified in the study group.

(Family History of Cancer)
The most common mutation, 2594delC in BRCA1, has been found previously in Danish breast cancer families and is one of the most common mutations found in breast cancer families from southern Sweden.\textsuperscript{17} Besides being frequently observed in families from the Nordic countries, 2594delC has also been found recurrently in Wales,\textsuperscript{23} which may reflect migration in the Viking age. The second most common mutation, 5208T>C, which was observed in three patients may have a more recent origin since it has not been detected in breast cancer families outside Denmark. In spite of these two recurrent mutations, it appears that the mutation spectrum in Denmark is quite broad, particularly with respect to BRCA2, which argues against a major influence of genetic isolation in the past. The majority of the subjects in this study come from Seeland, which is the most populated area in Denmark and harbours the capital, Copenhagen. Patients from the north of Jutland are under-represented in this material and, consequently, founder mutations that are important in that area may have been missed.

Twenty four BRCA1 or BRCA2 mutation carriers were identified among 119 breast cancer patients. The observed prevalence of 20% must, however, be viewed in the context of the screening methods and patient selection criteria used. Since the screening methods used in this study are not fully sensitive, some mutations may have been missed. We used three different conditions for SSCA to maximise the sensitivity, which proved to be important since six of the 49 changes found were detected only by one of the conditions used. Mutations in non-coding regions are beyond the scope of this study, that is, changes in introns or regulatory sequences and large genomic deletions. These may nevertheless constitute a significant proportion of the mutations, for example, many Dutch breast cancer families are known to segregate large genomic deletions in BRCA1.\textsuperscript{30} It is therefore apparent that the mutation frequency given by most screening studies, including our own, are conservative estimates.

The mutation frequency observed here is higher than in most previous studies of consecutive patient series. However, direct comparison with the results of others is rarely meaningful since different patient selection criteria have been used and populations may vary in the frequency of BRCA1 and BRCA2 mutations. Some examples can nevertheless be given; for instance, in one BRCA1 mutation screening study of young American breast cancer patients (<45 years) with an affected first degree relative, 15 carriers were identified in a group of 208 (7.2%).\textsuperscript{31} In our study, 36% of the patients with an affected first degree relative had a mutation in BRCA1. In a British population based study of both BRCA1 and BRCA2, Peto \textit{et al}\textsuperscript{2} found a mutation frequency of 5.9% in patients diagnosed with breast cancer before the age of 36 and 4.1% in the age range 36-45 years. The corresponding figures were much higher in this study, 33% and 16%, respectively. In another study of Ashkenazi Jewish breast cancer patients with bilateral disease, the frequency of BRCA1 and BRCA2 mutations was found to be 82% in cases with age at onset before 42 years\textsuperscript{33} as compared to 29% in our material under the same criteria. The broad mutation spectrum observed in this study and the small number of country specific mutations suggests that the mutation frequency does not significantly differ from that observed in other western populations. We therefore suspect that the 4-6 times higher mutation frequency observed in our study as compared with the above mentioned British and American studies is mainly because of the inclusion of multiple cancer in the selection criteria.

As expected, a positive family history of breast or ovarian cancer is more frequent in carriers of BRCA1 or BRCA2 mutations (50%) than in those not carrying any mutation (14%). Nine of 19 first degree relatives with breast cancer and five of eight first degree relatives with ovarian cancer come from a BRCA1 or BRCA2 mutation positive family. In addition, the age at diagnosis of breast and ovarian cancer was approximately 10 years lower in relatives of mutation carriers. Moreover, three of the five pedigrees that have four or more cancer patients include a proband of positive mutation status (fig 1). It is therefore apparent that BRCA1 and BRCA2 explain a large portion of the hereditary component associated with this group of patients. This may either indicate that mutations in other genes that predispose to hereditary breast cancer are rare in the Danish population or that such mutations are less likely to lead to multiple breast cancer. The high prevalence of cancer in brothers of probands who do not carry a mutation in BRCA1 or BRCA2 could signify an additional genetic risk factor (table 4). However, the spectrum of cancers observed in the

\begin{figure}
\centering
\includegraphics{ pedigree.png}
\caption{Pedigrees of patients with more than two first degree relatives affected with cancer. The probands are indicated with an arrow. Only affected sibs are shown in the pedigree. Age at diagnosis, site of cancer (bil=bilateral), and mutation status of the probands is given below the symbols.
}\end{figure}
The mutation status of BRCA1 and BRCA2 in patients with familial breast cancer.

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