Search for new breast cancer susceptibility genes

Oldenburg, R.A.

CHAPTER 4

MOLECULAR PROFILING

4.1. CHARACTERIZATION OF FAMILIAL NON-BRCA1/2 BREAST TUMORS BY LOSS OF HETEROZYGOSITY AND IMMUNOPHENOTYPING

R.A. Oldenburg[1,2], K. Kroeze-Jansema[1], H. Meijers-Heijboer[2], C.J. van Asperen[1], N. Hoogerbrugge[3], I. van Leeuwen[4], H.F.A. Vasen[4], A.M Cleton-Jansen[5], J. Kraan[1], J.J. Houwing-Duistermaat[6], H. Morreau[5], C.J. Cornelisse[5], P. Devilee[1,5]

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[1] *Center for Human and Clinical Genetics, Leiden University Medical Center, Leiden*
[2] *Dept. of Clinical Genetics, Erasmus Medical center, Rotterdam*
[3] *Dept. of Human Genetics, University Medical Center Nijmegen*
[4] *Netherlands Foundation for the Detection of Hereditary Tumors, Leiden*
[5] *Dept. of Pathology, Leiden University Medical Center, Leiden*
[6] *Dept. of Medical Statistics and Bioinformatics, Leiden University Medical Center, Leiden*

ABSTRACT

Purpose

Since the identification of *BRCA1* and *BRCA2*, there has been no major breast cancer susceptibility gene discovered by linkage analysis in breast cancer families. This has been attributed to the heterogeneous genetic basis for the families under study. Recent studies have indicated that breast tumors arising in women carrying a *BRCA1* mutation have distinct histopathological, immunophenotypic and genetic features. To a lesser extent, this is also true for breast tumors from *BRCA2* carriers. This indicates that it might be possible to decrease the genetic heterogeneity among families in which *BRCA1* and *BRCA2* have been excluded with high certainty (*BRCAX* families) if distinct subgroups of *BRCAX*-related breast tumors could be identified.

Experimental Design

Loss of heterozygosity analysis with at least one marker per chromosomal arm (65

markers) was used to characterize 100 breast tumors derived from 92 patients from 42 selected *BRCAX* families. In addition, the immunophenotype of 10 markers was compared to that of 31 *BRCA1*- and 21 *BRCA2*-related breast tumors.

Results and conclusions

The *BRCAX*-related tumors were characterized by more frequent LOH at 22q relative to sporadic breast cancer ($P<0.02$), and differed significantly from *BRCA1*- and *BRCA2*-related tumors in their positivity for Bcl2. However, cluster analyses of the combined data (LOH and immunohistochemistry) did not result in subgroups that would allow meaningful sub classification of the families. On chromosomes 2, 3, 6, 12, 13, 21 and 22 we found markers at which LOH occurred significantly more frequent among the tumors from patients belonging to a single family than expected on the basis of overall LOH-frequencies. Nonetheless, linkage analysis with markers for the corresponding regions on chromosomes 12, 21 and 22 did not reveal significant LOD's

INTRODUCTION

A positive family history remains one of the most important risk factors for breast cancer, with first-degree relatives of patients having an approximately 2-fold elevated risk. About 15% of all patients have a first-degree relative with breast cancer, and although germ-line mutations in *BRCA1* and *BRCA2* account for a substantial proportion of these cases,[1] these mutations explain only 20-25% of the overall excess familial risk.[2,3] Mutations in other genes such as *TP53* and *PTEN* are involved in rare multi-cancer syndromes and contribute very little to this risk. Mutations in *BRCA1* and *BRCA2* are strongly associated with families with at least 4 cases of breast cancer diagnosed before the age of 60 and one or more cases of ovarian cancer or male breast cancer.[1] However, in families with 4 or 5 cases of breast cancer, and no ovarian or male breast cancer cases, *BRCA1* and *BRCA2* mutations were significantly less frequent. Because such a familial clustering is unlikely to have occurred by chance, this has been taken as evidence that other breast cancer susceptibility genes must exist.[4] After the identification of *BRCA1* and *BRCA2*, several chromosomal regions have been implicated by linkage analysis to harbor a breast cancer susceptibility gene. In particular, linkage has been found with markers for 8p12-22 and 13q21,[5,6] but although mutations in *BRCA1* and *BRCA2* were excluded, these studies comprised either small or heterogeneous groups of families. Accordingly, these linkage results have proven difficult to replicate by others in independently collected sets of families.[7,8] It has

been argued that the inability to detect genetic linkage is largely due to a heterogeneous genetic basis for the families under study.[4]

It is now well established that breast tumors arising in women carrying a *BRCA1* mutation have distinct histopathological, immunophenotypic and genetic features.[9-14] This is also true for breast tumors from *BRCA2* carriers, although to a lesser extent. These findings indicate that it might be possible to subgroup the breast tumors derived from patients from families in which *BRCA1* and *BRCA2* have been excluded with high certainty (from now on called *BRCAX* families). This could possibly decrease the genetic heterogeneity within this group of families, and thereby increase the statistical power to detect linkage. Here, we used loss of heterozygosity and immunohistochemical analyses to characterize 100 breast tumors derived from *BRCAX* families. The *BRCAX*-related tumors were characterized by more frequent LOH at 22q relative to sporadic breast cancer, and differed significantly from *BRCA1*- and *BRCA2*-related tumors in their positivity for Bcl2. However, cluster analyses of the combined data (LOH and immunohistochemistry) did not result in subgroups that would allow useful sub classification of the families.

MATERIALS AND METHODS

Family selection

The families were ascertained through the Clinical Genetic Centers in Leiden, Rotterdam, and Nijmegen, as well as through the Netherlands Foundation for the Detection of Hereditary Tumors (STOET). Families were eligible if there were at least three cases of breast cancer diagnosed before the age of 60 from whom genotypes could be determined (N=216) or inferred (N=20). Families with cases of ovarian cancer or male breast cancer were excluded, and occurrences of other types of cancer were ignored. Pathological reports or medical reports were retrieved where available. Blood samples and paraffin-embedded tumor tissues were collected after obtaining written informed consent. The institutional ethical committees of all of the hospitals involved approved this study.

In total we collected 100 breast tumors derived from 92 patients from 42 selected *BRCAX* families. Nine of these 100 breast tumors belong to 8 *CHEK2**1100delC mutation carriers.[15] Although the families under study were not tested for mutations in other breast cancer susceptibility genes (such as p53, E-cadherin and PTEN), they did not show the phenotypic characteristic belonging to these cancer syndromes. We also collected 40 paraffin-embedded tumor samples from sporadic breast cancer

cases unselected for family history or age, and from 31 *BRCA1*-mutation carriers and 21 *BRCA2* mutation carriers.

BRCA1 and BRCA2 mutation Testing

In each family, the youngest breast cancer patient from whom a blood sample was available was tested for mutations in the *BRCA1* and *BRCA2* genes (and for many families the next youngest as well). The joint Clinical Genetic Centers applied a variety of methodologies. The largest central exons (exon 11 in *BRCA1* and *BRCA2*, exon 10 of *BRCA2*) were scanned by protein truncation tests.[16,17] The small exons were scanned for mutations by denaturating gradient gel electrophoreses (DGGE) or direct sequencing. All of the laboratories specifically assayed the presence of large founder deletions in *BRCA1* by deletion junction-PCR.[18] The entire coding sequences of *BRCA1* and *BRCA2* were investigated by conformation-sensitive gel electrophoresis (CSGE) in families that were incompletely scanned at the time of ascertainment.[19] Since 2002, each center offers full sequence analysis and DGGE covering the entire coding regions of both genes, and Multiple Ligation-dependent Probe Amplification (MLPA) to detect large deletions/duplications in *BRCA1*.[20]

Histology

Paraffin embedded tumor tissues were obtained and the breast tumors were histologically classified according to the WHO criteria.[21] An expert pathologist (H. Morreau, MD) assessed type of invasive cancer, histological grade, presence of *in situ* component and the presence of lymphocyte infiltrate. Age of the patient at time of diagnosis was available from pathological and medical reports.

LOH Analysis

On the respective H&E stained sections the areas of highest tumor density were selected. Four to six tissue cores (0.6 mm in diameter, Beecher Instruments, Silver Spring, MD) were punched from the designated area using a biopsy needle. DNA was isolated from these punches as described previously.[17] These punches generally contain >50% tumor cells. Normal DNA was isolated from the blood samples. For the LOH-analysis we used 65 fluorescence-labeled microsatellite markers selected from Weber Screening Set 6 and covering all chromosome arms.[22] Selection criteria were allele product-sizes below 250 bp (because PCR success rates with DNA isolated from paraffin-embedded material drops sharply with larger amplimers) and position in the telomeric half of a chromosome arm (because this will also detect mitotic recom-

bination events[23,24]). The PCR-products were visualized on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems) and analyzed with the Genotyper software version 3.7 NT (Applied Biosystems). The sporadic breast tumors were analyzed only for the six different markers on chromosome 22 and marker D11S15901 on chromosome 11, and the BRCA1-related breast tumors were analysed only for D4S1562 and D5S1471. Allelic imbalance was defined as the ratio of allele intensities in the normal versus the tumor DNA. An AIF (Allelic imbalance factor) of 1.70 or above was scored as 'LOH'.[25] A technical limitation in the interpretation of the allelic imbalance factor is the possible contamination of tumor DNA with non-malignant DNA. Although a biopsy needle to punch tissue cores does not prevent contamination with non-malignant cells, in 80% of the tumor DNA samples we detected at least one AIF >5.0, which is only achievable when relatively high proportions of tumor cells are present in the sample.[26]

Tissue-microarray (TMA)

Breast cancer tissue microarrays were prepared as described previously.[27] From each case three tissue cores were assembled in the TMA. In total 4 TMA blocks were constructed. Three blocks with *BRCAX* samples and one block with tumors samples from *BRCA1* and *BRCA2* mutation carriers.

Immunohistochemistry scoring

Immunohistochemical staining was performed by the labeled Streptoavidin biotin method (DAKO, Glostrup, Denmark) with a heat-induced antigen retrieval step.

One pathologist (HM) and one researcher (RAO) evaluated the immunohistochemical staining results. The percentage of stained nuclei, independent of the intensity, was scored for p53, ER, PR, and Cyclin D1. In the same way, the percentage of cells with cytoplasmic staining was scored for Bcl2. Her2/Neu was assessed in accordance with the DAKO HercepTest guidelines with a score of ≤ 1 considered negative. Cytokeratin 5/6, Cytokeratin 7 and Cytokeratin 19 were scored according to the presence or absence of membranous expression in the invasive component. The Chek2 staining pattern was scored as described earlier.[15] For p53 we used four different categories on the basis of any level of nuclear staining; 1) negative, 2) <25%, 3) 25-75% and 4) >75% positive nuclei. For ER and PR, a case was considered positive when ≥ 10 % of the nuclei stained above background. For Cyclin D1 the cut off limit was 30%. For bcl2 the cut off limit was 70%.

Statistics

Proportions were compared using chi-square statistics. Familial aggregation of LOH status at a marker was tested using a score statistic.[28] This statistic tests for the presence of an additive genetic effect. For this analysis AIF's between 1.3 and 1.7 were regarded as missing. Empirical p-values were computed by permutation of the LOH status among relatives of the same family.

Cluster Analysis

For the hierarchical cluster analysis we used the software programs Cluster and TreeView. The data was normalized, mean centered and average linkage clustering was applied. We renumbered the LOH data of 100 tumors as follows; AIF's>1.70 were scored as '1' (LOH), AIF's between 1.0 and 1.29 (retention of heterozygosity) as '–1', AIFs between 1.3 and 1.7 as '0', and homozygotes as missing. The immunohistochemical data for the different markers was scored as '1' when considered positive and '-1' when considered negative.

Linkage analysis

Genotypes were generated for 19 microsatellite markers on chromosome 12, 5 on chromosome 21, and 12 on chromosome 22. The markers were derived from Linkage Mapping Set version 2 (Applied Biosystems), and amplified from peripheral blood lymphocyte genomic DNA by standard PCR methods. DNA from CEPH 1347-02 was typed as reference to ensure consistency of allele sizing. Allele frequencies for parametric linkage analyses were calculated based on one randomly chosen individual from each family. Multipoint linkage analyses were carried out using the program GENEHUNTER version 2.1-B.[29] We used a model in which susceptibility to breast cancer is conferred by a dominant allele with a reduced penetrance and a population frequency of 0.003.[30,31] The risk of breast cancer by age 80 was assumed to be 0.85 in carriers and 0.096 in non-carriers. Risks are modeled in seven age categories (<30, 30-39, 40-49, 50-59, 60-69, 70-79, and 80+) as described.[31] We used the multipoint LOD-scores for each family to compute heterogeneity LOD scores, using the standard admixture model, and hence estimated the proportion of families (α) linked to the putative 'BRCAX' locus by maximizing the heterogeneity LOD score. Non-parametric linkage analyses were carried out by the program MERLIN version 0.9.12b 32.

TABLE 1

Histologic description of the different groups analyzed

%	BRCAx (n = 100)	Control (n = 40)	BRCA1 (n = 31)	BRCA2 (n = 21)
Ductal carcinoma in situ	1	0		4.8
Ductal carcinoma	81	85	100	95.2
Lobular carcinoma in situ	1	0		
Lobular carcinoma	10	5		
Colloid carcinoma	1	2.5		
Other	1	5		
Unknown	5	2.5		
Grade 1	20.7	29.4	0	5
Grade 2	50	41.2	12.9	45
Grade 3	29.3	29.4	87.1	50
	P < 0.0002*	P < 0.02**	P < .0002***	

DCIS, ductal carcinoma in situ; LCIS, lobular carcinoma in situ

*, grade of BRCA1 tumors versus grade of BRCAx tumors; **, BRCA1 versus BRCA2 tumors;

***, BRCA1 versus control tumors.

RESULTS

Histology

A total of 100 paraffin-embedded breast tumor samples could be retrieved from 92 patients from 42 early onset breast cancer families (*BRCAX*-families, defined as having at least 3 cases diagnosed before the age of 60, and no cases of ovarian or male breast cancer). We previously found 8 patients to carry the *CHEK2**1100delC mutation, representing 9 of these 100 breast tumors.[15]

The histological characteristics of this group of breast cancers, as compared to sporadic breast tumors (N=40) and the breast tumors from *BRCA1* (N=31) and *BRCA2* (N=21) mutation carriers are listed in Table 1. The most common histological type in all groups was infiltrating ductal carcinoma. Contrary to earlier suggestions,[33] lobular carcinoma was not significantly more often found in the *BRCAX* tumors relative to sporadic cancers. The *BRCA1* tumors were of higher grade than *BRCAX* tumors (P<0.001) and the *BRCA2* tumors (P= 0.01). Most of the *BRCAX* tumors were of grade II, and there was a trend towards grade being lower than that of *BRCA2* tumors (P=0.07), which is consistent with previously reported results.[9,11,33]

Immunohistochemistry

Three tissue microarray (TMA) blocks were constructed with 98 of the 100 *BRCAX* tumors, and one with 31 *BRCA1* and 21 *BRCA2*-related breast tumors. All tumors were stained with antibodies against ER, PR, P53, Bcl2, Her2/Neu, Cyclin D1, CHEK2, the basal cytokeratin 5/6 and the luminal cytokeratins 7 and 19, the immunohistochemical markers most commonly studied in *BRCA1*/2-associated breast carcinomas (Table 2). *BRCAX* tumors were significantly different from *BRCA1* tumors for ER ($p<0.001$), PR ($p=0.002$), Her2/Neu ($p=0.02$), Cyclin D1 ($p=0.02$), Bcl2 ($p<0.001$), and the basal CK5/6 ($p=0.0015$) staining. There were also significant differences between the *BRCA1* and *BRCA2* tumors for ER ($p=0.002$), Her2/Neu ($p=0.02$) and the basal CK5/6 ($p<0.001$) staining. *BRCAX* tumors differed significantly from both *BRCA1* and *BRCA2* tumors only for Bcl2 ($p<0.001$), while for CK5/6 this difference was borderline significant ($p=0.09$). As expected, the 9 tumors from *CHEK2**1100delC carriers were significantly more often negative for CHEK2 staining than *BRCA1*, *BRCA2*, and *BRCAX* tumors. Interestingly they are also significantly more often negative for luminal CK19 staining than *BRCAX* ($p=0.0008$) and *BRCA1* ($p=0.006$) tumors.

We combined the results of the luminal marker (CK19) together with the basal marker (CK5/6) expression to subdivide the *BRCAX* breast tumors into four different cellular phenotypes: 'luminal' (only expression of the luminal marker), 'basal' (expression of the basal marker and no expression of the luminal marker), 'mixed' (expression of the basal marker and expression of the luminal markers) and 'null' (no expression of basal and luminal markers).[34] In this subdivision CK7 was not included, because of the high percentage of tumors that stained positive in all groups. The results demonstrate that a high proportion of *BRCAX* breast carcinomas express the mixed phenotype or have a pure luminal phenotype (Table 3). The *BRCA1* tumors are more often of the mixed phenotype compared with *BRCAX* tumors ($p= 0.0017$) and with *BRCA2* tumors ($p=0.0007$). No significant difference was seen between the *BRCA2*- and *BRCAX*-tumors. The *CHEK2**1100delC related tumors showed a trend towards the null phenotype. Among the *BRCAX* tumors, the mixed tumors were more often positive for Her2/Neu relative to the luminal group ($p=0.02$), and the pure luminal tumors are more often grade III than the tumors with a null phenotype ($p=0.006$) (data not shown).

Genome-wide loss of heterozygosity (LOH)

The 100 BRCAX tumors were analyzed for LOH with 65 polymorphic markers representing all chromosomal arms. Of the potential 6,500 pair-wise normal/tumor com-

TABLE 2

Immunohistologic results

	BRCAx %	BRCA1 %	BRCA2 %	CHEK2 %	P
P53					
0	16.5	63	78.9	22.2	
<25%	62.6	3.7	10.5	77.8	
25-75%	9.9	7.4	5.3	0	
>75%	11	25.9	5.3	0	
ER					
Negative	33	85.7	41.2	44.4	0.0000009* 0.01†
Positive	67	14.3	58.8	55.6	0.002‡
PR					
Negative	43.5	78.6	61.1	33.3	0.002*
Positive	56.5	21.4	38.9	66.7	0.01†
Her2Neu					
Negative	74.5	96.1	72.2	77.8	0.02*
Positive	25.5	3.9	27.8	22.2	0.02‡
Cycline D1					
Negative	71.3	92.3	87.5	88.9	0.02*
Positive	28.7	7.7	12.5	11.1	
Bcl2					
Negative	39.1	88.9	94.1	33.3	0.000005* 0.0009†
Positive	60.9	11.1	5.9	66.7	0.00003⊙ 0.0009•
CHEK2					
Negative	13	22.3	16.7	66.7	0.04†
Positive	53.3	37	38.9	22.2	0.003#
Strong pos	33.7	40.7	44.4	11.1	0.02•
Cytokeratin 5/6					
Negative	54.3	19.2	76.5	55.6	0.0015* 0.04†
Positive	45.7	80.8	23.5	44.4	0.0002‡
Cytokeratin 7					
Negative	3.3	3.8	0	11.1	
Positive	96.7	96.2	100	88.9	
Cytokeratin 19					
Negative	27.9	11.1	33	66.7	0.0008†
Positive	72.1	88.9	67	33.3	0.006#

ER estrogen receptor, PR progesterone receptor,

* BRCA1 versus BRCAx tumors, † CHEK2 versus BRCA1 tumors, ‡ BRCA1 versus BRCA2 tumors,

⊙ BRCAx versus BRCA2 tumors, • CHEK2 versus BRCA2 tumors, # CHEK2 versus BRCAx tumors.

TABLE 3

Immunophenotype distribution based on the expression of the basal basal cytokeratin 5/6 and the luminal cytokeratin 19

%	Luminal	Basal	Mixed	Zero
BRCAx (n = 91)	35.2	8.8	36.3	19.8
BRCA1 (n = 27)	14.8	7.4	74.1	3.7
BRCA2 (n = 16)	43.8	6.3	18.8	31.3
CHEK2 (n = 9)	11.1	22.2	22.2	44.5
	P = 0.0017*		P = 0.00073**	

* BRCA1 versus BRCAx tumors

** BRCA1 versus BRCA2 tumors

parisons, 1,698 (26.1%) failed due to PCR problems of either the tumor DNA or normal DNA. Of the remaining 4,802, 1,220 (25.4%) were homozygous (not informative). Thus, in total 3,582 (55.1%) informative AIF's could be calculated. Using an AIF of 1.7 or greater as cut-off for LOH, the mean percentage of LOH among the markers was 30% (±6.3%), which is similar to the overall average LOH rate calculated from 151 published LOH studies of breast cancer.[35] LOH frequencies of 40% or greater were found at 1q41, 4p16, 11q22, 11q23.3, 16p13, 16q24, 17p12, 21q22, 22q11 and 22q13 (Figure 1), with the highest frequency found at D22S445 (59%). Whereas many of these chromosomal sites have also been highlighted in analyses of sporadic breast tumors, we did confirm that the percentage of LOH at D22S445 and D22S315 was significantly higher in BRCAX versus the sporadic breast tumors (respectively P<0.02 and P=0.035)(Figure 2). We also confirmed the high levels of allelic imbalance at 4q (7 of 12 informative cases) and 5q (4 of 9 informative cases) in *BRCA1*-related tumors.[36]

In 28 families we were able to assess LOH in at least 2 breast tumors from 2 patients. We tested whether there were loci at which LOH was found significantly more often within families than expected on the basis of overall LOH frequency at this locus in all our families. This was found for markers D2S125 (p=0.007), D3S2409 (p=0.045), D6S1552 (p=0.03), D12S2070 (p=0.02), D13S285 (p=0.02), D21S1255 (p<0.001) and D22S315(p=0.01). Of note, marker D22S445 did not show this familial clustering (p=0.35).

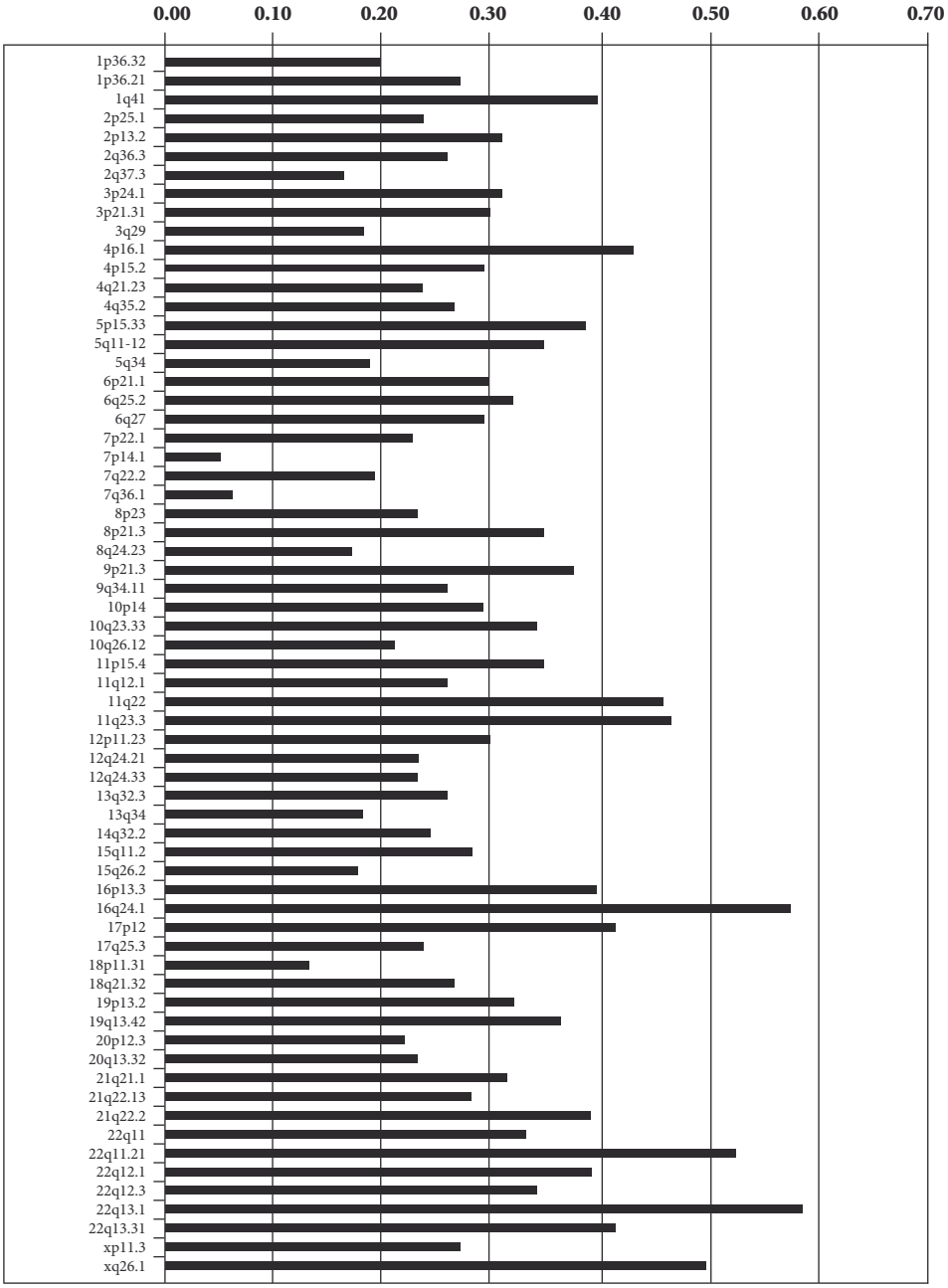

Figure 1. Percentages Loss of heterozygosity (LOH) for the different chromosomes.

The numbers above the graph represent the different chromosomes. A tumor was scored positive for LOH when having an AIF ≥ 1.70.

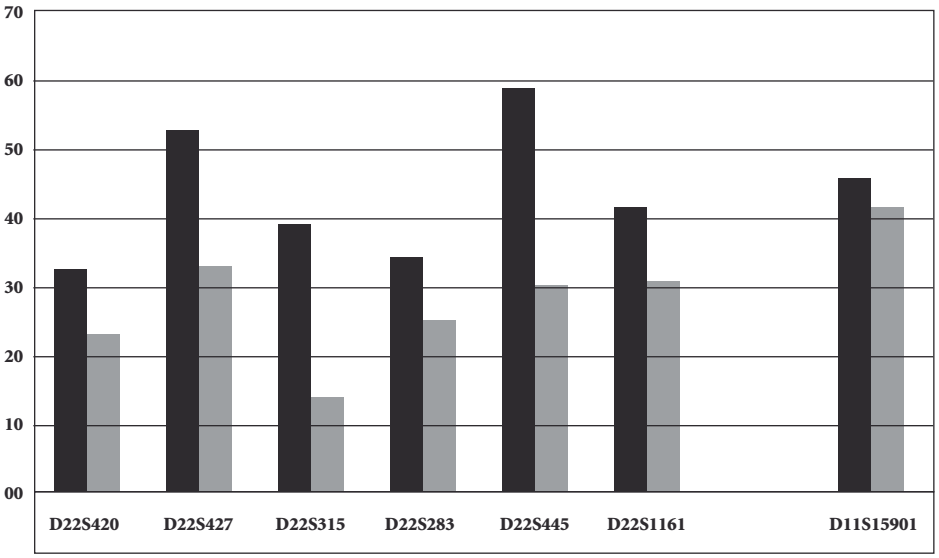

Figure 2

Percentages Loss of heterozygosity (LOH) found for the different microsatellite markers on chromosome 22 and 11. The black columns represent the BRCAx tumors and the grey columns the sporadic tumors.

Cluster analyses

We attempted to use the LOH data of 98 tumors in a hierarchical non-supervised clustering analysis by scoring AIF's>1.70 as '1', AIF's between 1.00 and 1.29 (retention of heterozygosity) as '–1', and AIF's between 1.30 and 1.70 and homozygotes as 'missing' in the software package 'Cluster'. Although the tumors were separated into two groups, these were not readily discernable on the basis of any single marker or combination of markers, nor did the tumors derived from the same family or the *CHEK2**1100delC carriers cluster together (data not shown). Adding the immunophenotyping and histological typing data did not resolve this.

Linkage analysis

We performed a linkage analysis in 55 families, complying with our selection criteria, for chromosomes with either a conspicuous LOH score (#22, at D22S445) or for which LOH showed significant familial clustering (#12, #21). For chromosomes 2, 3, 6 and 13 there were too few families for which linkage and LOH data could be combined to be statistically meaningful. The highest multipoint LOD score at chromosome 21 over all 55 families was -6.37 between markers D21S1256 and D21S1914. At the same locus, the non-parametric LOD (NPL) score was 1.72. Assuming hetero-

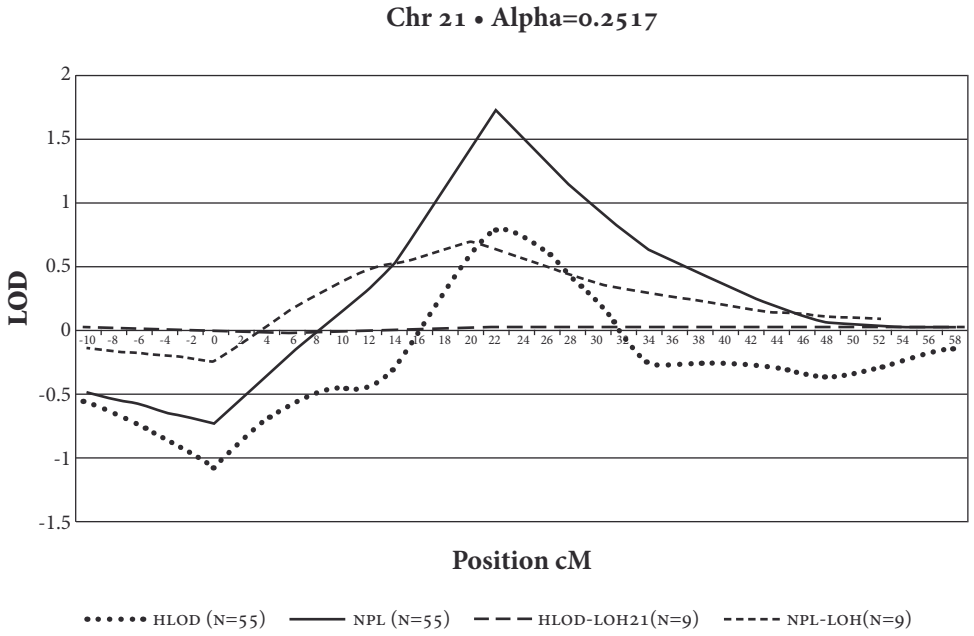

Figure 3

Heterogeneity logarithm of odds (HLOD) and nonparametric linkage scores (NPL) for chromosome 21; Alpha, the proportion of linked families calculated by the program GENEHUNTER; HLOD-LOH21, HLOD found for the 9 families in which the tumor of at least one patient showed LOH at marker D21S1255; NPL-LOH21, NPL-scores found for the 9 families in which the tumor of at least one patient showed LOH at marker D21S1255.

geneity, we found a non-significant heterogeneity LOD (HLOD) score of 0.80 (alpha=0.25). Selecting the 9 families in which the tumor of at least one patient showed LOH at marker D21S1255 decreased both the NPL and HLOD scores (Figure 3). Similar results were obtained for chromosome 12 (data not shown). In agreement with the absence of linkage, we were unable to detect consistent loss of the same parental allele on either #12 or #21 in the tumors from these families.

For chromosome 22, the highest multipoint LOD score was −11.34 between markers D22S303 and D22S315, and under the admixture model the estimated proportion of linked families was 0. When selecting the 12 families in which the tumor of at least one patient showed LOH at marker D22S445, the peak multipoint LOD score under heterogeneity was 0.06 (alpha=0.2) between marker D22S303 and D22S315 (27 cM proximal of D22S445).

DISCUSSION

We have analyzed 100 breast tumors from patients strongly selected for a particular familial background for loss of heterozygosity and immunophenotype analysis. To our knowledge, this is the first study analyzing LOH at all chromosome arms in such an extended and highly selected group of familial tumors. The main purpose of the study was to detect patterns of LOH and/or immunophenotype that would define distinct subgroups of tumors, on the basis of which we would then be able to stratify the families from which they derive. This is one approach to address the genetic heterogeneity problem, which is commonly believed to be the main reason for the inability to detect further moderate- to high-risk breast cancer susceptibility genes.[4,37] For this reason, we have selected cases from families with a high probability of segregating a breast cancer susceptibility gene, but with a minimal residual probability that this is *BRCA1* or *BRCA2*.

In many families we collected tumor tissues from two or more patients, allowing us to analyze whether certain genetic, immunohistochemical and morphological features were more prevalent within families than predicted by chance. We did indeed observe this for LOH with several markers, but not for any of the immunohistochemical markers. However, linkage analysis in the total group of 55 families did not produce significant LOD scores for any of these chromosomes, nor did linkage analysis in subgroups of families selected on basis of these LOH results. This suggests that LOH analysis of familial cases is unlikely to facilitate the detection of new breast cancer susceptibility loci by linkage analysis. It remains possible, however, that families in which multiple breast tumors show LOH at the same locus are caused by a shared genetic defect on another chromosome. A genome-wide linkage search in our families should address this. For example, it has been reported that breast tumors from families linked to *BRCA1* show more frequent LOH on 4q and 5q relative to sporadic breast cancer, which we have confirmed here.[36,38] Hence it might have been possible to detect linkage to BRCA1 among the families in which several tumors show LOH on 4q or 5q, rather than among families only selected on clinical phenotype. Although our LOH analysis covered all chromosome arms, certain LOH events may have escaped detection because of the limited number of markers we have used. LOH analysis with microarrays with 10,000 SNP's could indicate shared LOH regions with more accuracy, as was found for lung cancer.[39] LOH analysis with polymorphic markers detects any imbalance in parental chromosomes, including trisomy,[26] so that our 'LOH' scorings in fact reflect a wide range of different chromosomal aberrations. To distinguish between these, LOH data should be combined with (array-)CGH. This might be relevant

because we do not know at this stage whether other breast cancer susceptibility genes act according to Knudson's two-hit inactivation model.[26,40] It is conceivable, as was found for the *MET* oncogene in hereditary papillary renal carcinomas,[41] that trisomy (or copy-number gain) of the mutant allele contributes to susceptibility.

A better resolution for subgroup analysis of the tumors might be achieved by global gene expression analysis. Many different studies describe the possible classification of the heterogeneous group of sporadic breast cancers in distinct subtypes using microarray techniques.[42,43] Five different subtypes (one basal-like, one *ERBB2*-over-expressing, two luminal-like, and one normal breast tissue-like subgroup) have been recognized.[44] These tumor subtypes may represent different biological entities and might originate from different cell types. Four distinct phenotypes (pure luminal, mixed luminal/basal, pure basal and null) have been defined by immunostaining 1944 sporadic breast tumors with antibodies for both the luminal and basal phenotypes.[34] These subgroups were significantly different in their biological features and clinical course of the disease. In addition, another study[14] showed that the expression patterns from 15 fresh frozen tumor samples from 7 non-*BRCA1*/2 families clustered within their respective families, suggesting an underlying common genetic basis. The recently developed DASL-assay technique,[45] which makes gene expression analysis possible in archival paraffin-embedded tissues, may extend this observation to larger numbers of cases.

The hypothesis that genetic predisposition to breast cancer might preferentially give rise to certain subtypes is also supported by histopathological findings in *BRCA1* related tumors. These are generally of higher grade, show pushing margin growth patterns and high lymphocyte infiltration in comparison to sporadic cases.[33] They are also more often estrogen receptor (ER), progesterone receptor (PR) negative, Bcl2-negative, P53-mutated and negative for Her2/Neu amplification (our data, and refs[9,11,46]). In gene expression profiling, a basal-like gene expression pattern has been associated with *BRCA1* carriers.[13] We found most *BRCA1* tumors (81.5%) to belong to the pure basal or mixed phenotype category, based on cytokeratin 5/6 and cytokeratin 19 expression, as opposed to the *BRCA2* tumors which were mostly (75%) of the luminal or null phenotype. Intriguingly, *BRCAX* tumors were almost equally distributed over both categories. However, we noted that different tumors within the same family frequently belonged to different phenotype categories, indicating that it is unlikely that the basal/luminal phenotype is genetically determined in these cases.

The morphological and immunohistochemical results from *BRCAX* breast carcinomas and those arising in *BRCA1* and *BRCA2* mutation carriers are similar to those

recently reported by others.[9,11,47,48] Only Bcl2 displayed a significant difference between *BRCAX* tumors and *BRCA1*- or *BRCA2*-tumors (both p<0.0001), but the proportion of positive *BRCAX* tumors is not conspicuously different from what is observed in series of unselected sporadic breast tumors.[9] In general, the patterns of immunostaining and LOH in *BRCAX* tumors closely resemble those of sporadic breast tumors, with the possible exception of the 'mixed' phenotype (as defined by cytokeratins 5/6, 19) and LOH at chromosome 22. Two recent studies[49,50] have used classical CGH to analyze a small number of BRCAX-related breast tumors. Both these studies too found chromosomal aneuploidy patterns broadly resembling those of sporadic breast tumors, but did not identify chromosome 22 as a frequent target for aneuploidy. Conversely, regions on chromosome 8 and 19, identified by CGH,[50] were not observed by us. It should be noted, however, that a direct comparison of the *BRCAX* cases in these studies and ours is difficult due to differences in the applied selection criteria for *BRCAX* families. For example, the occurrence of ovarian cancer was not used to exclude families in the CGH studies,[49,50] increasing the probability that some are caused by undetected mutations in *BRCA1*.

Thus, in our families a clustering of sporadic, or sporadic-like breast cancer is seen. Yet, it has been argued that such familial clustering is unlikely to occur by chance but instead is more likely to have a genetic basis.[1] Therefore, if our families indeed have a genetic basis, our results suggest that this basis is the same as that for sporadic breast cancer. Analyses of genetic models to explain familial breast cancer have indicated that, after correction for *BRCA1* and *BRCA2*, the polygenic model incorporating multiple interacting low penetrance genes is the most likely explanation.[51-53] Such genes are also suspected to explain a substantial proportion of sporadic breast cancer. If more detailed analyses of this group of patients by high-resolution array-CGH or gene expression profiling confirms that these tumors resemble sporadic tumors very much, than this is in agreement with the idea that the remainder of familial risk to breast cancer is caused in a polygenic way. Finding these genes will be a challenge for years to come, but family studies will remain valuable in this regard because one is enriching for genetic susceptibility,[54] as was convincingly shown with the identification of the *CHEK2**1100delC variant.[15,55]

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