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Search for new breast cancer susceptibility genes

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CHAPTER 3.
PUTATIVE CANDIDATE GENES

3.1. EXTENDING THE P16-LEIDEN TUMOUR SPECTRUM BY RESPIRATORY TRACT TUMOURS

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KEY POINTS

- We studied eight different familial atypical multiple mole melanoma families with co-segregation of a p16-Leiden germline mutation.
- One family harbours an extraordinarily high number of tumours, comprising, breast, lung, and colon cancers, and oral squamous cell carcinomas (OSCC). In this family it seems that at least three of four lung cancer patients (one unknown), both OSCC patients, and only one of five individuals with breast cancer (two unknown) were carrying the p16-Leiden germline mutation. Immunohistochemical testing for p16 was performed and loss of heterozygosity (LOH) of the p16-Leiden wild type allele was analysed in different tumours. Additionally, four breast carcinomas and four lung tumours of eight p16-Leiden mutation positive patients from the seven remaining families were analysed.
- Immunohistochemistry of p16 was negative in all four analysed lung carcinomas.

LOH of the wild type p16 allele was present in one of three carcinomas tested. In both OSCC's, p16 immunohistochemistry was negative and LOH of the wild type allele was present in the one case analysed. Furthermore, immunohistochemistry of p16 was negative in one of five analysed breast tumours of mutation positive patients and only this tumour showed LOH of the wild type p16 allele.
- Our results suggest that the p16-Leiden germline mutation may be involved in susceptibility to lung cancer and OSCC development in some patients. There is no evidence for a dominant role of the p16-Leiden germline mutation in the development of breast cancer, although an interaction with as yet unidentified modifying factors cannot be ruled out.

INTRODUCTION

Familial atypical multiple mole melanoma (FAMMM; OMIM #155601) is characterised by the familial occurrence of melanoma of the skin in combination with multiple atypical precursor naevi.[1–4] The disease is inherited as an autosomal dominant trait, with germline mutations in the p16 (*CDKN2A*) gene having been reported in at least a quarter of FAMMM families. Previously, we reported an increased risk of pancreatic carcinoma in Dutch FAMMM families with a 19 bp deletion in exon 2 of the *CDKN2A/p16* gene (p16-Leiden; OMIM #600160.0003).[4]

Recently a patient with three carcinomas of the pharynx and oral cavity with a germline heterozygous *p16-Leiden mutation* was reported.[5] All three tumours showed inactivation of the retained wild type allele, with the somatic event being aberrant promoter methylation. Two other reports also described the occurrence of head and neck or oral squamous cell carcinomas (OSCC) in families with different *p16 germline* mutations.[6,7] A relationship between p16 germline mutations and breast cancer has also been suggested, although in the families studied, *BRCA1* and *BRCA2* mutations were not excluded.[8,9]

We studied a FAMMM family (EMC13769; Fig. 1) with co-segregation of the *p16-Leiden* germline mutation, with an extraordinary number of tumours comprising OSCC's, lung tumours, breast carcinomas, and colorectal carcinomas. We determined the mutation status in the various patients and investigated by loss of heterozygosity (LOH) analysis of the wild type allele in the tumours, in combination with immunohistochemistry, whether a causal relationship exists between the *p16-Leiden* mutation and the development of the different tumour type. Insufficient tissue was available for methylation studies. We additionally studied four breast tumours and four

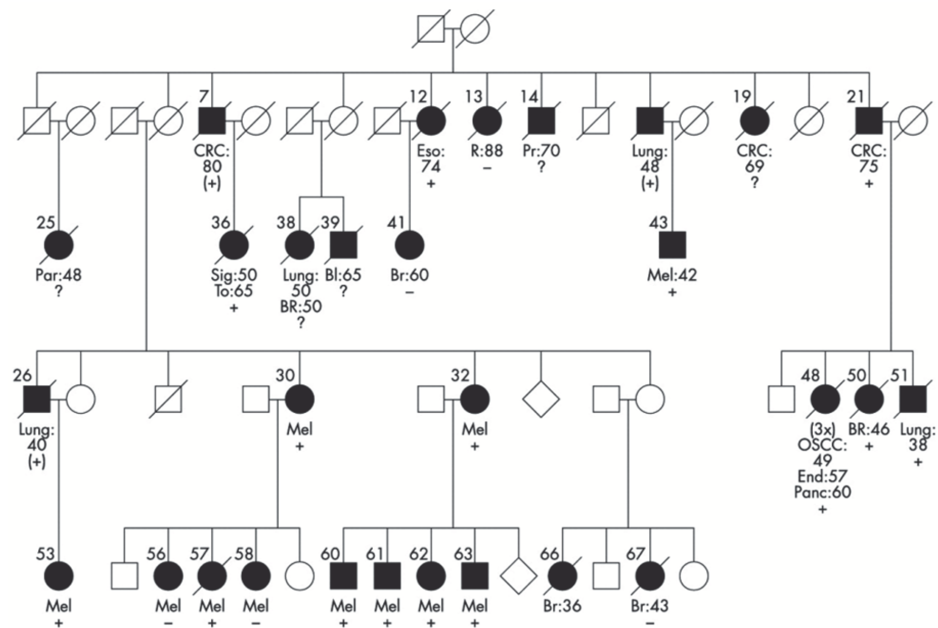

Figure 1; Pedigree of the family EMC13769.

Subject number appears above the symbol, age of diagnosis follows the diagnosis. Mel, melanoma; OSCC, oral squamous cell carcinoma. Cancer of the: Bl, bladder; Br, breast; CRC, colorectum; Eso, oesophagus; End, endometrium; Lung, lung; Panc, pancreas; Par, parotic gland; Pr, prostate; R, rectum; Sig, sigmoid; To, tongue. +, p16-Leiden positive; -, p16-Leiden negative; (+), obligate carrier; ?, p16-Leiden carrier status unknown.

lung tumours from eight other patients (from seven other families), all of whom carried a germline *p16-Leiden* mutation.

MATERIALS AND METHODS

Patients

Blood samples and/or paraffin embedded tumour samples were obtained for DNA solation from available subjects that had developed a carcinoma, to determine their *p16-Leiden* mutation status. Unavailable subjects with *p16-Leiden* positive offspring were classified as 'obligate carriers'. Informed consent was given by family members themselves or by their relatives, in case of deceased subjects. Tumours were pathologically verified whenever possible.

Tumour analysis

Paraffin embedded tumour tissues were obtained, and revision of histology was

performed. Areas of highest tumour density were selected for further molecular analysis. Serial sections were produced for immunohistochemical analysis.

DNA isolation

Genomic DNA of normal and tumour tissue was isolated from formalin fixed paraffin embedded material, resuspended in 96 µl of PK-1 lysis buffer (50 mmol/l KCL, 10 mmol/l Tris pH 8.3, 2.5 mmol/l $MgCl_2$, 0.45% NP40, 0.45% Tween 20, 0.1 mg/ml gelatine) containing 5% Chelex beads (Biorad, Hercules, CA, USA) and 5 µl proteinase K (10 mg/ml), and incubated for 12 h at 56°C. The suspension was incubated for 10 minutes at 100°C, centrifuged, and the supernatant carefully decanted.

Polymerase chain reaction amplification

The *p16-Leiden* deletion comprises 19 bp and removes nucleotides 225–243 of exon 2.[10] Genomic DNA from tumour and normal tissue was subjected to PCR amplification using labelled primers containing the 225–243 region; *p16*-forward-TET M1 (tumour) or FAM M1 (normal), sequence 5'-ATGATGGGCAGCGCCCGAGT-3' and *p16*-reverse A2, sequence 5'-ACCAGCGTGTCCAGGAAG-3' (Life Technologies). The total volume per reaction was 12 µl including 5 µmol of each primer (stock forward and reverse primer), a mix of 0.25 µl dNTP (10 mmol/l), 1.2 µl magnesium chloride (20 mmol/l), 1.2 µl bovine serum albumin (1 mg/ml), 1.2 µl AmpliTaq Gold buffer (without $MgCl_2$) and 0.25 µl AmpliTaq Gold DNA polymerase, 10 ng of normal or tumour DNA, and H_2O. The following conditions were used: 33 cycles of 1 minute at 96°C, 2 minutes at 55°C, 1 minute at 72°C, and a delayed extension step of 7 minutes at 72°C in a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Mixtures of 24 µl dionised formamide, 1 µl TAMRA 500 (Applied Biosystems) and 1.2 µl of PCR product were run on a ABI 310 Genetic analyser (Applied Biosystems) for 20 minutes with run profile GS STR POP4 (1.0 ml) C and analysed with GENESCAN 3.1 computer software (Perkin-Elmer Corp).

Loss of heterozygosity analysis

Owing to the 19 bp deletion, we could specifically analyse the fate of the wild type allele in terms of LOH. Analysis of LOH was possible when both normal and tumour tissue was available. LOH was scored when there was loss of intensity of one allele in the tumour sample with respect to the matched wild type allele from normal tissue. The quotient of the peak height ratios from normal and tumour DNA served as the allelic imbalance factor (AIF); that is, the ratio of the peak height at 101 bp of the

deleted allele and the peak height at 120 bp of the wild type allele. The threshold for allelic imbalance was defined as 40% reduction of one allele, agreeing with an AIF of <=0.59 or >1.3. The threshold for retention was defined to range from 0.76 to 1.3 as previously empirically determined.[11] AIF's of 0.60–0.75 and 1.3–1.69 were considered to belong to a so-called grey area, for which no definitive decision has been made.

Immunohistochemical testing for p16

Tissue sections (4 µm) were prepared on APES coated slides, and dried overnight in a 37°C oven. Sections were deparaffinised in xylene (3x5 minutes). Endogenous peroxidase was blocked by incubation in methanol/H_2O_2 0.3% for 20 minutes and sections were rehydrated with ethanol and distilled water. Antigen retrieval for p16 immunostaining was performed by microwaving in boiling 0.01 mol/l sodium citrate buffer (pH 6.0) for 10 minutes. After cooling for 2 hours and washing (2x5 minutes) in PBS, the sections were incubated overnight at room temperature with mouse anti-human p16 (1:500, clone JC8; Neomarkers Fremont, CA, USA) with tonsil tissue as positive control. Sections were subsequently washed (3x5 minutes in PBS) and incubated (30 minutes) with biotinylated secondary antibody in PBS/BSA 1%, washed (3x5 minutes in PBS) and incubated (30 minutes) with a horseradish peroxidase/streptavidin complex (SABC). Diaminobenzidine-tetrahydrochloride (DAB) was used as a chromogen, followed by counterstaining with haematoxylin. As a negative control, the primary antibody was omitted. Expression was scored by microscopic examination. Loss of p16 expression was scored when nuclei of tumour cells stained negative and nuclei of normal (stromal) cells stained positive (internal positive control).

BRCA1 and BRCA2 mutation screening

As described above, we were able to obtain tumour material of five *p16-Leiden* carriers with breast cancer. Three (NFDHT 1–3, table 1G0) had no first or second degree relative with breast cancer. The other two (EMC 13769 No 50 and LUMC 152, table 1G0) had several relatives with breast cancer diagnosed before the age of 60 years. Complete *BRCA1* and *BRCA2* mutation analysis was performed in the suspect families (EMC 13769 and LUMC 152) and found to be negative. We screened for germline mutations frequently detected in the Dutch population. Protein truncation tests[12] were also performed for PCR fragments of exon 11, and denaturating gradient gel electrophoresis was performed for the remaining exons and exon/intron junctions

of *BRCA1* and *BRCA2*. Additionally we screened for the deletions of exon 13 (3.8 kb) and exon 22 (510 bp) of *BRCA1*.[13]

Microsatellite instability

Microsatellite instability was analysed in a diagnostic setting as previously described using markers D2S123, D5S346, D17S250, BAT25, BAT26, and BAT40,[14] and immunohistochemical testing for MLH1, MSH2, and MSH6 was performed.[15]

RESULTS

Lung cancer

We analysed four different *p16-Leiden* families (Table 1, Fig. 1) with one or more cases of lung cancer. Family EMC13769 (Fig. 1) harbours four cases of lung cancer. One subject was a proven carrier of a germline *p16-Leiden* mutation (subject 51), two subjects are obligate carriers, and the *p16-Leiden* carrier status remains unknown for one (subject 38). The p16 immunohistochemistry analysis in the tumour of subject 51, a smoker, tested negative, and LOH of the wild type allele was found. The three other (NFDHT) families harbour 4 *p16-Leiden* mutation carriers with documented lung cancers. The immunohistochemistry analysis for p16 was negative in three analysed lung tumours. LOH of the wild type allele was ambiguous in one tumour, and in one tumour (carcinoid) retention was found (Table 1). In the other two tumours no normal tissue was available to perform the analysis.

Oral squamous cell carcinoma (OSCC)

Two subjects of family EMC13769 had a tumour originating in the oral cavity—that is, one tongue carcinoma (subject 36 at 65 years of age) and one subject with three primary OSCC's (subject 48 at 49 years). Immunohistochemical analysis of the tongue carcinoma was negative for p16 but lacked an internal positive control, and LOH analysis was not possible. Immunohistochemical analysis of the one of the three OSCC's from subject 48 (Fig. 1) tested negative for p16, and LOH of the wild type allele in this tumour was found (Table 1).

Breast cancer

We analysed five families with breast cancer. Family EMC 13769 shows five cases of breast cancer. Only one was carrying the *p16-Leiden* mutation (subject 50). Germline mutations in *BRCA1* & *BRCA2* were excluded for subjects no 41, 50 and 67. The p16 protein in the tumour from EMC13769 subject 50 stained positive and no LOH

TABLE 1

Results of LOH and immunohistochemical analysis in P16-Leiden mutation carriers.

Family	Subject no.	Anatomical site	Age at diagnosis	p16-IHC	Internal controle	Tumour (%)	LOH
Tumours originating in the lung and oral cavity							
EMC13769	36	OSCC (Tongue)	65	-	-		NA
	48	OSCC (1X)	49	-	+	>50	Yes
	51	Lung (adenocarcinoma)	38	-	+	>50	Yes
NFDHT4	1	Lung (SCC)	61	NA			A
NFDHT4	2	Lung (SCC)	48	-	NP		NA
NFDHT5		Lung (carcinoid)	46	-	+	70-80	R
NFDHT6		Lung (SCC)	56	-	+		NA
Tumours originating in the breast							
EMC13769	50	Breast*	46	+		>30	R
LUMC152		Breast*	41	-	+	50-60	Yes
NFDHT1		Breast	42	+		30	R
NFDHT2		Breast	47	+ †		30	R
NFDHT3		Breast	46	+ †			NA
Tumours orig**inating in the digestive tra**ct							
EMC13769	21	Colon‡	75	-	+	<30	R
	36	Sigmoid	52	+		>30	R

EMC - Erasmus MC; LUMC - Leiden University Medical Center;
NFDHT - Netherlands Foundation for the Detection of Hereditary Tumours;
OSCC - oral squamous cell carcinoma; SCC - squamous cell carcinoma;
’, No staining of tumour cells or internal control cells; NP - no internal control cells identified;
R - retention of the wild type allele; A - ambiguous;
NA - not analysed; **BRCA1* and *BRCA2* tested negative; † few positive tumour nuclei;
‡ microsatellite instability analysis: immunohistochemistry for MLH1, MSH2, and MSH6 positive.

was found (Table 1). Of the four additional typed breast carcinomas from *p16* mutation carriers from the families LUMC 152 and NFDHT 1–3 (Table 1), only one showed expression loss of the p16 protein with LOH of the wild type allele, although in two of four other analysed breast carcinomas only a few tumour nuclei stained positive (with the retention of the p16 wild type allele in one, the other not tested).

Digestive tract

Family EMC13769 harbours six cases of carcinomas of the digestive tract. Of the two tumours analysed (both patients had a germline *p16-Leiden* mutation), one tumour stained positive and one negative. Neither showed LOH (Table 1), nor microsatellite instability (microsatellite stable phenotype of the tumours with normal expression of MLH1, MSH2, and MSH6).

DISCUSSION

All lung and oral cavity tumours studied developed (most likely) in *p16-Leiden* mutation carriers. For two persons we cannot rule out the possibility that the *p16-Leiden* germline mutation in their offspring came from the non-bloodline spouses. However, as this family does not come from the 'Dutch region' where multiple *p16-Leiden* mutation carriers have been identified, we think that they are most probably obligate carriers of the same *p16-Leiden* mutation. The age of onset in most patients is unusually young and abrogation of p16 seems present in all analysed cases (4/4), a ratio that seems higher than that encountered in sporadic lung cancer (36–45%).[16] The *p16-Leiden* mutation might therefore indeed predispose carriers to an increased risk of lung and oral cavity carcinomas. With respect to lung cancer, this is supported by two other important observations. Firstly, an increased cumulative risk of developing lung cancer in male *p16-Leiden* mutation carriers was found compared with the general Dutch population (14.3% v 8.9%).[4] Secondly, *Cdkn2a* is the most likely candidate for the lung tumour susceptibility locus pulmonary adenoma progression gene 1 (*PAPG1*) in mice.[17,18] *PAPG1* has been mapped to a 1.5 cM segment on chromosome 4, which contains the *Cdkn2a* gene that encodes p16INK4a. *Cdkn2a* is polymorphic between the lung tumour resistant mouse strain BALB/cJ and the lung tumour susceptible A/J strain, and the resistant allele is preferentially lost in lung tumours of p16INK4a heterozygous mice. Additionally, germline deletion of the gene in mice leads to increased tumour size and notable histological signs of malignant progression.[17]

Sufficient information on the smoking habits of most subjects in our study was lacking. However, smoking may have contributed to the unusually early age of onset of three tumours, although one of the tumours is classified as an adenocarcinoma, a type not typically associated with smoking.

Our study does not provide evidence for a dominant role of *p16-Leiden* in the development of breast cancer. Breast cancer seems also statistically not increased in our cohort studied[4] However, in view of the early onset of breast cancer in our *p16-Lei-*

den positive cases, we cannot rule out a role for the gene in tumour progression, either due to haploinsufficiency or total abrogation of p16 as seen in one of our cases (LUMC152). Recently, it has been postulated for other genes that mutation or loss of a single allele may be sufficient to play an important role in progression towards cancer.[19] Furthermore, an interaction with as yet unidentified modifying factors (genetic and/or environmental) has yet to be elucidated.
Both analysed tumours from the digestive tract showed no LOH; however, one stained negative. In this case methylation might have inactivated the wild type allele, which is a frequent event in sporadic colon cancer.[20] The role of the *p16-Leiden* germline mutation in the development of colon cancer needs further research.
In conclusion, the *p16-Leiden* mutation not only seems to predispose to melanoma and pancreatic tumours but also to head and neck tumours[5–7], and tumours of the lung in some families. Promoter methylation[5] or loss of the wild type allele seems to be the mechanism for the 'second genetic hit'. Clinical criteria for *p16* germline mutation screening should be adapted accordingly.

ELECTRONIC-DATABASE INFORMATION

Online Mendelian Inheritance in Man (OMIM), www.ncbi.nlm.nih.gov/Omim/ (for FAMMM (OMIM 155601) and p16-Leiden (OMIM 600160.0003).

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3.2. THE CHEK2*1100delC VARIANT ACTS AS A BREAST CANCER RISK MODIFIER IN NON-BRCA1/BRCA2 MULTIPLE-CASE FAMILIES

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ABSTRACT

The frame-shifting mutation 1100delC in the cell-cycle-checkpoint kinase 2 gene (*CHEK2*) has been reported to be associated with familial breast cancer in families in which mutations in *BRCA1* and *BRCA2* were excluded. To investigate the role of this variant as a candidate breast cancer susceptibility allele, we determined its prevalence in 237 breast cancer patients and 331 healthy relatives derived from 71 non-*BRCA1*/*BRCA2* multiple-case early onset breast cancer families. Twenty-seven patients (11.4%) were carrying the *CHEK2**1100delC* variant. At least one carrier was found in 15 of the 71 families (21.1%). There was no evidence of cosegregation between the variant and breast cancer, but carrier patients developed breast cancer earlier than did noncarriers. We studied CHEK2 protein expression in 111, and loss of heterozygosity at *CHEK2* in 88 breast tumors from these patients. Twelve of 15 tumors from carriers showed absent protein expression as opposed to 3 of 76 tumors from noncarriers ($P < 0.001$). *CHEK2* loss of heterozygosity was associated with absence of protein expression but not with 1100delC carrier status. Thus, selecting for breast

cancer cases with a strong familial background not accounted for by *BRCA1* or *BRCA2* strongly enriches for carriers of *CHEK2**1100delC*. Our results support a model in which *CHEK2**1100delC* interacts with an as yet unknown gene (or genes) to increase breast cancer risk.

INTRODUCTION

First-degree female relatives of a breast cancer patient have an 2-fold increased risk to develop breast cancer.[1] Germ-line mutations in the *BRCA1* and *BRCA2* genes account for <5% of this familial risk.[2, 3] To explain the remainder of familial risk, various genetic models have been proposed. Models incorporating a single third hypothetical gene, *BRCA3*, or a number of common low penetrance genes with additive effect seem to fit equally well, although the latter fitted best when the known effects of parity on breast cancer risk were included.[3, 4] A mutation 1100delC in *CHEK2* has been proposed recently to be a low-penetrance breast cancer susceptibility allele.[5, 6] *CHEK2* is located on chromosome 22 and encodes the human orthologue of yeast *Cds1* and *Rad53*, which are G2 checkpoint kinases.[7] *CHEK2* is involved in cell cycle control and DNA repair through its ability to phosphorylate p53, Cdc25c, and *BRCA1*. The *CHEK2**1100delC* variant is a protein-truncating mutation that abrogates the kinase activity of the protein. It occurs in 0.3–1.4% of healthy control individuals,[5, 6, 8] but in about double that frequency among unselected cases of breast cancer. It is even further enriched among breast cancer cases with a positive family history in which *BRCA1* and *BRCA2* mutations have been excluded. Up to 5.5% of such cases may be carrying the *CHEK2**1100delC* variant, although it apparently incompletely segregates with breast cancer in the families of these cases.[5] Other variants in *CHEK2* seem to be very rare and are not enriched among familial breast cancer cases.[9-11] We have embarked recently on a genome-wide linkage search for new breast cancer susceptibility genes in a highly selected group of breast cancer families. Phenotypic and genotypic criteria[12] have minimized the probability that these families harbor mutations in *BRCA1* or *BRCA2*, but have selected for families that are caused by other high penetrant genes. Here, we investigate the role of the *CHEK2**1100delC* variant as a cause of breast cancer in these families.

MATERIALS AND METHODS

Families.

Families were ascertained through the Clinical Genetic Centres in Leiden, Rotterdam, and Nijmegen, as well as through the Netherlands Foundation for the Detec-

tion of Hereditary Tumors. 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 or inferred. DCIS or LCIS before the age of 60 as first primary cancer were also considered eligible diagnoses. Families with cases of ovarian cancer or male breast cancer were excluded, and occurrences of other cancer types were ignored. Seventeen of these 71 families were also part of the previous study identifying the *1100delC*CHEK2* variant as a low-penetrance breast cancer susceptibility gene.[5] The 71 families selected contained a total of 384 breast cancer patients, 297 of which diagnosed before the age of 60, 2 of which occurred in spouses (excluded from the statistical analysis), and 5 of which had in situ cancer (4 DCIS and 1 LCIS) only. There was one family where the third case diagnosed under 60 was an in situ cancer (combined DCIS/LCIS at age 53).

Pathology reports were retrieved for 260 patients (68%). For another 84 patients, diagnoses were confirmed by medical records, and retrieval of pathology reports was still in progress at the time when this study was finalized. For the remaining 40 cases, breast cancer diagnoses were ascertained by family interview only. 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.

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 different Clinical Genetic Centers applied a variety of methodologies. The large central exons (exon 11 in *BRCA1* and *BRCA2*, exon 10 of *BRCA2*) were scanned by protein truncation tests.[13] The small exons were scanned for mutations by denaturing gradient gel electrophoresis or direct sequencing. All of the laboratories specifically assayed the presence of large founder deletions in *BRCA1* by deletion junction-PCR.[14] For cases where scanning was still in progress at the time of sampling for the purpose of this research, we performed conformation-sensitive gel electrophoresis[15] covering all of the coding regions of both genes. This identified 10 different variants of uncertain clinical significance and 12 different polymorphisms. None of these were cosegregating with breast cancer or the *CHEK2*1100delC* variant.

Genotyping of the CHEK2*1100delC Variant.

The DNA sequence of exon 10 of *CHEK2*, where the 1100delC resides, is present in multiple homologous copies in the genome. For PCR, we used oligonucleotides 10F (5' TGT CTT CTT GGA CTG GCA GA; Fam-labeled) and 10R (5' ATC ACC TCC TAC CAG TCT GTG C), which specifically amplify the functional copy of *CHEK2*, relative to the nonfunctional pseudogenes.[16] The reaction volume of 10 µl contained 20 ng of genomic DNA, 1 µl 10' SuperTaq buffer (HT Biotechnology LTD.), 1 mM dNTPs, 300 mM of each primer, and 0.1 units of Silverstar DNA polymerase (Eurogentec). Annealing temperature was 65°C, and the PCR ran for 38 cycles. The resulting PCR-products were analyzed on an ABI3700, in fragment analysis mode. The wild-type allele runs as a 291-bp fragment and the mutant allele as a 290-bp fragment, which are readily separated into two peaks of about equal signal intensity in this assay. All of the positive samples were confirmed by sequencing as described previously.[5]

LOH Analysis.

LOH at the *CHEK2* locus was investigated by comparing the genotypes in normal and tumor DNA at four flanking markers, D22S420, D22S315, D22S280, and D22S283. CHEK2 maps between D22S315 and D22S280, which span an interval of 7 Mb. Four punches (5 mm long and 0.6 mm in diameter) were taken from paraffin-embedded tumor tissues, in the area where the tumor was located. These punches generally contain >50% tumor cells. DNA was isolated from these punches as described previously.[17] Allelic imbalance was defined as the ratio of allele intensities in the normal versus the tumor DNA. An AIF of 1.70 was scored positive.[18] LOH at the *CHEK2* locus was scored positive when the AIF- pattern was such that at least one proximal and one distal marker showed AIF 1.70 without interruption by a marker showing an AIF <1.70.

Tissue Array and Immunohistochemical Analyses.

All of the tumor samples were embedded in standard paraffin blocks. On the respective H&E-stained sections, a representative tumor area was selected. Two to four tissue cores (0.6 mm in diameter; Beecher Instruments, Silver Spring, MD) were punched from the designated area using a biopsy needle and arrayed into the recipient blocks. Using a tape-transfer system (Instrumedics, Hackensack, NJ), 4-µm sections were transferred to glass slides. For antigen retrieval, the deparaffinized sections were boiled in a microwave for 15 min in citrate buffer (pH 6.0) before incubation with a mouse monoclonal antibody, NCL-CHK2 (Novocastra Laboratories, LTD.,

Newcastle, United Kingdom), directed against the human CHEK2 protein. After this the slides were incubated with a second step antibody streptavidin-biotin labeled (Labvision) for 90 min. Two independent pathologists evaluated the staining results without prior knowledge of the mutation status of *CHEK2*. The tumors were scored as having an absent, weak, moderate, or high CHEK2 protein expression depending on the intensity of the staining regardless of the proportion of tumor cells falling in this category. When no staining was found, an absent protein expression was scored.

Statistical Analysis.
Prevalences, clinical characteristics of patients, and tumors were compared between groups by 2 tests. All of the tests of statistical significance were two-sided. A t-test was used to compare mean ages of onset between carriers and noncarriers. Additionally, Kaplan-Meier age of onset probability curves were estimated and differences were tested by the log-rank test. To obtain an impression of the size of the effect of a *CHEK2**1100delC* mutation on age of onset, a Cox-regression analysis was performed.

RESULTS

We investigated 71 families with a phenotype of early onset breast cancer, defined as having at least 3 cases diagnosed before the age of 60, and no cases of ovarian or male breast cancer. Mutations in *BRCA1* and *BRCA2* were excluded in at least the youngest breast cancer case from which a blood sample was available. These families contained a total of 384 breast cancer patients. We collected DNA samples from 237 patients, including all of those with in situ cancer, as well as from 331 family members without breast cancer and 54 spouses. Of the 622 individuals we were thus able to assay for the presence of the *CHEK2**1100delC* variant, we found 41 (6.6%) to be carriers (Table 1). The prevalence among breast cancer patients was 11.4% (27 of 237), which was significantly higher than the prevalence of the variant in healthy female family members (6 of 212; 2 = 12.047; df = 1; $P < 0.001$). Three carriers were known with in situ cancer (2 DCIS and 1 LCIS). Fifteen families (21.1%) had at least 1 positive individual for this variant. One of these was a family in which the only identifiable carrier was a woman with in situ cancer (DCIS; Fig. 1). The proportion of families in which at least 1 individual carried the *CHEK2* variant increased to 31.8% in families with >5 breast cancer patients diagnosed under 60 (Table 1). However, this trend was not statistically significant (2 = 2.6; df = 2; $P = 0.272$). In addition, *CHEK2*-posi-

TABLE 1

CHEK2*1100delC prevalences

Description	**Total**	**CHEK2+**	**%**
All sampled individuals	**622**	**41**	**6.6**
Male	154	8	5.2
Female	468	33	7
All sampled breast cancer cases	**237**	**27**	**11.4**
Cases diagnosed under 60	194	24	12.4
Cases diagnosed 60 or over	43	3	7.0
Cases with in situ cancer only	5	3	60.0
Healthy family members	**331**	**14**	**4.2**
Males	119	8	6.7
Females	212	6	2.8
Spouses [a]	**54**	**0**	**0**
Male	35	0	0
Female	19	0	0
All families	**71**	**15**	**21.1**
3 cases < 60	30	4	13.3
4 cases < 60	19	4	21.1
>= 5 cases < 60	22	7	31.8

[a] Two of these individuals were diagnosed with breast cancer.

TABLE 2

LOH at CHEK2

LOH at CHEK2	**Number of cases**	**CHEK2 carriers**	**%**
Positive[a]	11	3	27.3
Suspected[b]	29	5	17.2
Negative	29	3	10.3
Unknown[c]	20	3	15.0
Totals	89	14	14.0

[a] Cases in which at least one proximal and one distal marker showed AIF 1.70 without interruption by a marker showing an AIF < 1.70.

[b] Cases in which LOH was found only proximal or distal of CHEK2.

[c] Cases in which one of the reactions failed.

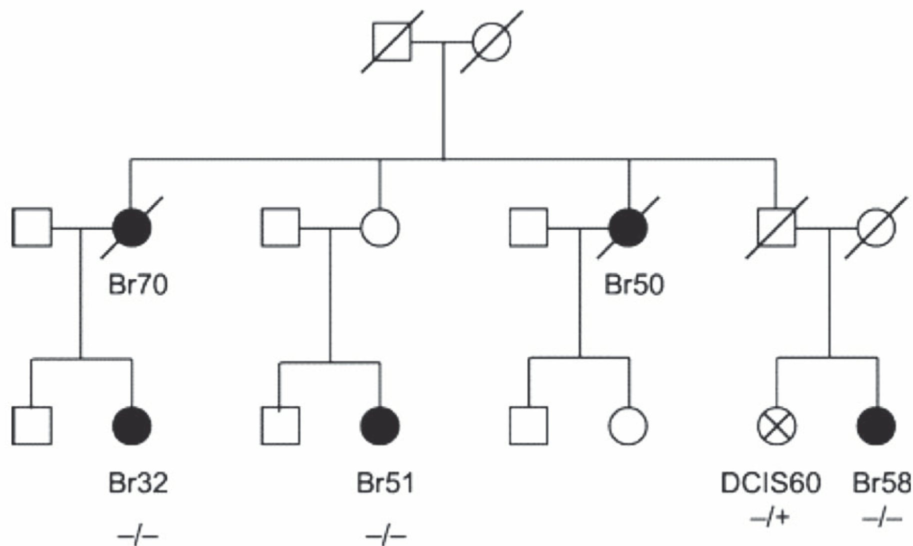

Fig. 1. Pedigree of family RUL154.
Filled symbols are individuals diagnosed with breast cancer, the age at diagnosis is given below the symbol. -/+ indicates that the individual carries the *CHEK2*1100delC* variant; -/- indicates the individual does not carry this variant.

tive families had on average slightly more blood-sampled cases than *CHEK2*-negative families (3.8 versus 3.2; data not shown). Although not a statistically significant difference, this indicates that the odds of detecting the variant is dependent on the number of blood-sampled breast cancer cases in a family.
In the 15 *CHEK2*1100delC*-positive families we defined the youngest carrier breast cancer case as the index patient. Under the null hypothesis of complete random Mendelian inheritance, we predicted that 12.9375 of the 54 affected relatives would be carrier of the variant. We observed 12 carriers, so that the null hypothesis could not be rejected. We performed LOH analysis in 89 archival breast tumor tissues from 88 breast cancer cases from these 71 families, at four markers mapping to either side of *CHEK2* (Table 2). LOH at *CHEK2* was found in 11 tumors, 3 of which derived from 2 *CHEK2*1100delC* carriers. In all 3 of the tumors, we could demonstrate that the lost allele was derived from the nontransmitting parent (data not shown). Although the 1100delC variant occurred 2.7 times more frequently among cases showing LOH at *CHEK2*, this difference was not statistically significant (2 = 1.239; df = 2; P = 0.538).
A tissue microarray with 111 tumors from 111 cases was stained with a mouse monoclonal antibody against the human CHEK2 protein. Examples of obtained staining

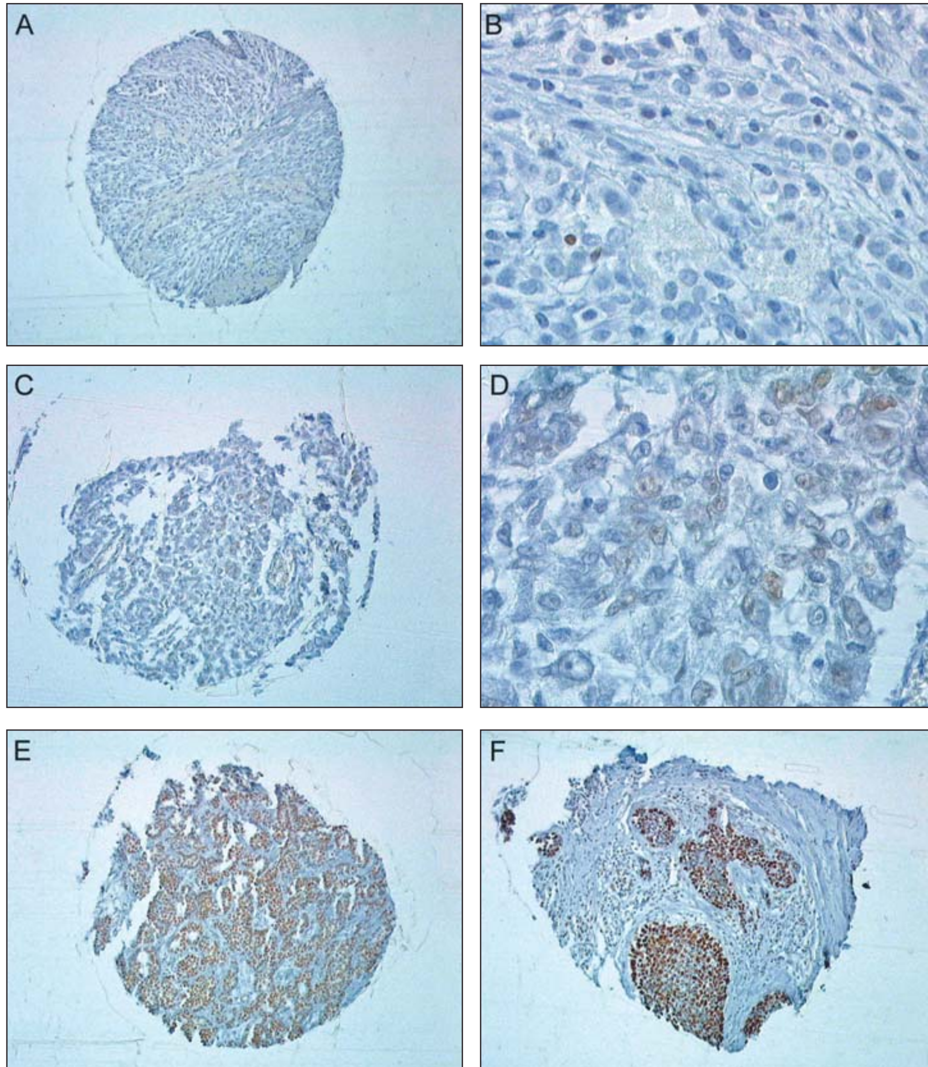

Fig. 2. Immunohistochemical staining of chek2 in human breast tumors on a tissue microarray.

The samples shown are from four different tumors and represent the four different scoring categories used here. A and B, absent protein expression in a tumor from a chek2*1100delC carrier. Note the scattered strongly staining normal epithelial cells as positive internal control (B). C–F, represent tumors from noncarriers. C and D, weak protein expression. E, moderate protein expression. F, high expression. Magnification x25 in A, C, E, and F. and x100 in B and D.

TABLE 3

Chek2 protein expression according to 1100delC carrier status and LOH

Variable	CHEK2 protein expression				Total
	Absent	Weak	Moderate	Strong	
CHEK2 +[a]	12	2	1	0	15
CHEK2 -[b]	3	41	27	5	76
LOH +[c]	3	7	0	0	10
LOH suspected[d]	7	12	8	1	28
LOH -[e]	0	11	14	2	27
LOH unknown[f]	4	9	5	1	19

[a] CHEK2 +, carriers of the 1100delC variant.
[b] CHEK2 -, noncarrier.
[c] LOH+, at least one proximal and one distal marker showed AIF 1.70 without interruption by a marker showing an AIF < 1.70.
[d] LOH suspected, one distal or proximal marker showed an AIF < 1.70 while the closest marker on the other side of CHEK2 was uninformative.
[e] LOH -, no LOH was found.
[f] LOH unknown, one of the reactions failed.

patterns are shown in Fig. 2. As noted in a previous study[19] there was considerable variability in the percentage of normal cells that were positive. CHEK2 protein expression was absent in 12 of 15 tumors from *CHEK2*1100delC* carriers (80.0%; Table 3). False-negative staining was considered unlikely, because in 6 of 12 tumors from *CHEK2*1100delC* carriers the stromal component stained normally.

Notably, the one tumor showing moderate protein expression was an in situ carcinoma (DCIS) from a patient from family RUL154 (Fig. 1). In comparison, only 3 of 76 tumors (3.9%) from noncarriers showed an absent CHEK2 protein expression (2= 52.709; df = 3; $P < 0.001$). For 37 tumors, protein expression and LOH data were available. CHEK2 protein expression was absent in 3 of 10 tumors with *CHEK2*-LOH, 2 of which were from *CHEK2*1100delC* carriers. The other 7 tumors with *CHEK2*-LOH all showed a weak CHEK2 protein expression. In comparison, all 27 of the tumors, which retained heterozygosity at *CHEK2*, showed some degree of protein expression (2 = 15.879; df = 6; $P = 0.014$). The mean age of diagnosis of the first primary tumor of *CHEK2*1100delC* carrier patients was not significantly different from that in noncarriers (48.3 versus 50.6 years; $P = 0.30$). However, any age difference may have been

masked by our selection for early onset breast cancer. Indeed, in a Kaplan-Meier analysis the age of onset distribution between the two groups was different ($P < 0.0001$). It is unlikely that this effect is confounded by differences in tumor grade because the percentage of grade III tumors was higher in noncarriers than in carriers (22 of 81 versus 1 of 9). A Cox-regression analysis revealed an odds ratio of 2.1 (95% confidence interval, 1.393–3.166; $P < 0.001$) for carriers to develop breast cancer relative to noncarriers (derived from *CHEK2*1100delC* positive and *CHEK2*1100delC* negative families). Among the 237 genotyped breast cancer patients in our cohort, 35 (14.8%) were known to have had a second primary
breast cancer. Five of these (14.3%) were positive for the *CHEK2* variant. Of the 202 patients with one primary breast cancer, 22 tested positive (10.9%). This difference was not statistically significant.

DISCUSSION

We found the *CHEK2*1100delC* variant in 11.4% of the breast cancer cases belonging to a highly selected group of families. This prevalence was substantially higher then reported previously by others. Two earlier studies[5, 6] selected familial breast cancer cases from families that were not linked to *BRCA1* or *BRCA2*, and found a prevalence of 5.1% and 5.5%, respectively. The families we studied are highly selected in several ways. First, they contain at least 3 breast cancer cases diagnosed before age 60 (the average number of breast cancer cases per family was 5.4). Second, they were selected against cases of ovarian and male breast cancer. Third, they all tested negative for mutations in *BRCA1* and *BRCA2*. On the basis of population incidence, the odds that 3 cases in a family occur under 60 by chance alone are very low, and, thus, they likely have a genetic basis. Hence, in this group of families we suspect an enrichment of a gene (or genes) other than *BRCA1* and *BRCA2* that may confer substantial breast cancer risks.[12] However, because we and others[5, 6] found no or weak evidence for cosegregation between *CHEK2*1100delC* and breast cancer, *CHEK2* is an unlikely candidate for such a gene. It is possible that other, more high-risk mutations in *CHEK2* exist that could account for these cases, but this has thus far not been substantiated by more comprehensive mutation scanning of the gene (9, 10, 20, 21). A more likely explanation for the data presented here is a model in which *CHEK2*1100delC* interacts with an as yet unknown rare gene (or genes) to confer breast cancer risks comparable with those conferred by *BRCA1* or *BRCA2*. Selecting for families caused by this rare gene would also enrich for *CHEK2*1100delC* carriers, which would act like a modifier of the breast cancer risk. The CHEK2 Consortium, studying families of

Dutch, German, United Kingdom, and North American origin, found the prevalence of the 1100delC variant to increase in families with 4 cases,[5] but the Finnish study found the highest prevalence among non-*BRCA1/2* cases with a moderate family history.[6] We also found weak evidence for increasing prevalence of *CHEK2**1100delC* among families with a more extensive family history of breast cancer. Even among populations with an apparently overall lower prevalence of the 1100delC variant,[8] this enrichment is observed. The higher allele frequency in Northern Europe as opposed to North America might be due to a founder effect of *CHEK2**1100delC*. The proposed risk modifying effect of *CHEK2**1100delC* is also supported by our finding that carriers in our families develop breast cancer systematically earlier than do noncarriers. Although this may be a peculiarity of this selected group of patients, a similar age-effect has been noted for genetic variants in *AR*, *HRAS1*, *RAD51*, and *AIB1* in carriers of *BRCA1* or *BRCA2* mutations.[22, 23, 24, 25] Alternatively, breast cancer in these families has a polygenic basis involving multiple interacting low-penetrance alleles,[26] one of which is the *CHEK2**1100delC* variant. The *CHEK2**1100delC* is approximately twice as prevalent among unselected breast cancer cases than among controls, suggesting it is a low-risk allele in its own right.[5, 6] In keeping with this, we found that *CHEK2**1100delC* is associated with breast cancer, but it was unable to explain the majority of breast cancer cases in these families. A role for *CHEK2* inactivation in breast tumor development is nonetheless supported by the highly significant association we found between *CHEK2**1100delC* carrier status and an absence of protein expression in the breast tumors. This confirms results obtained by others[6, 19] irrespective of minor differences in interpretation of immunohistochemical staining patterns among these studies. It would also explain the slightly earlier age of onset of breast cancer in 1100delC carriers, as these individuals only need to inactivate the wild-type allele whereas noncarriers would need to inactivate both copies of the gene. Paradoxically, the breast tumors of *CHEK2* carriers do not significantly more frequently show LOH at *CHEK2*. Hence, LOH may not be the only mechanism inactivating the wild-type allele, although the association between LOH and an absent protein expression we observed does indicate it is involved in some cases. Alternative mechanisms include promoter hypermethylation[27] and somatic mutations, but the roles of both appear to be marginal in breast cancer.[19, 28] Conceivably, other components of the pathway(s) regulating the expression and/or stability of CHEK2 protein are disturbed in these cases. An association with bilateral disease, but only a marginal trend toward earlier age of diagnosis was reported in one study.[6] In our cohort of cases we found an association between *CHEK2* carrier

status and earlier age of diagnosis but not between carrier status and multiple primary tumors. This could be a peculiarity of the selected families. Conceivably, many cases not carrying the *CHEK2* variant are carriers of another gene defect that predisposes them strongly to develop breast cancer. In combination with a long retrospective follow-up time, this may have masked the subtle effect of *CHEK2* on risk. In conclusion, we find a strong association between *CHEK2*1100delC* prevalence and breast cancer family history. Our results provide support for the hypothesis that this variant modifies the cancer risk conferred by an as yet unknown gene (or genes). Given the cancer occurrence in the families described here, this gene is expected to cause breast cancer risks comparable with those conferred by *BRCA1* and *BRCA2*. At this point it is in our opinion not appropriate to offer a predictive test for *CHEK2* in a clinical setting. The exact relative risk conferred by *CHEK2*1100delC* is not clear, but likely modest in comparison with *BRCA1* and *BRCA2*. In addition, estimates of breast cancer risk are difficult to make in these families, because the type of interaction (multiplicative or additive) and the role of other factors are presently unknown. Selecting for families with at least one carrier of the *CHEK2*1100delC* might reduce the genetic heterogeneity likely to exist among non-*BRCA1/BRCA2* families and facilitate the mapping of this breast cancer susceptibility gene by classical linkage analysis.

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3.3. EVALUATION OF LINKAGE OF BREAST CANCER TO THE PUTATIVE *BRCA3* LOCUS ON CHROMOSOME 13q21 IN 128 MULTIPLE CASE FAMILIES FROM THE BREAST CANCER LINKAGE CONSORTIUM

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ABSTRACT

The known susceptibility genes for breast cancer, including BRCA1 and BRCA2, only account for a minority of the familial aggregation of the disease. A recent study of 77 multiple case breast cancer families from Scandinavia found evidence of linkage between the disease and polymorphic markers on chromosome 13q21. We have evaluated the contribution of this candidate '*BRCA3*' locus to breast cancer susceptibility in 128 high-risk breast cancer families of Western European ancestry with no identified *BRCA1* or *BRCA2* mutations. No evidence of linkage was found. The estimated proportion (α) of families linked to a susceptibility locus at D13S1308, the location estimated by Kainu et al. [(2000) *Proc. Natl. Acad. Sci. USA* 97, 9603–9608], was 0 (upper 95% confidence limit 0.13). Adjustment for possible bias due to selection of families on the basis of linkage evidence at *BRCA2* did not materially alter this result (α = 0, upper 95% confidence limit 0.18). The proportion of linked families reported by Kainu et al. (0.65) is excluded with a high degree of confidence in our dataset [heterogeneity logarithm of odds (HLOD) at α = 0.65 was −11.0]. We conclude that, if a susceptibility gene does exist at this locus, it can only account for a

small proportion of non-BRCA1/2 families with multiple cases of early-onset breast cancer.

INTRODUCTION

Several genes are known to predispose to breast cancer. In the context of large multiple case families, the *BRCA1* and *BRCA2* genes are numerically the most important, accounting for most families segregating both early-onset breast cancer and ovarian cancer. However, as many as 60% of families with site-specific female breast cancer cannot be explained by *BRCA1* and *BRCA2*.[1,2] Moreover, population studies have demonstrated that these genes only account for 15% of the overall familial risk of breast cancer.[3,4] Even after allowing for other susceptibility genes that confer increased risk in the context of familial cancer syndromes, including *TP53* (Li Fraumeni), *PTEN* (Cowden), and *ATM* (ataxia telangiectasia), at least 80% of familial breast cancer risk is not explained by known genes, suggesting that other important susceptibility genes remain to be mapped. Outside the context of these specific syndromes, known genes other than *BRCA1*/*BRCA2* do not appear to account for a substantial proportion of high-risk breast cancer families. Linkage analysis in a set of 56 families with 3 or more cases of breast cancer yielded no evidence for a significant role of *PTEN*, although an attributable fraction of up to 35% could not be ruled out in a family set of this size.[5] However, direct mutation testing of the *PTEN* gene in a subset of these families has failed to identify any mutations, lending further support to the linkage results indicating that this locus is unlikely to account for a significant fraction of hereditary breast cancer.

To date, few additional candidate breast cancer susceptibility loci have been identified in families not attributable to any of the known genes. A potential susceptibility locus on chromosome 8p12–8p22 was identified through targeted linkage analysis of a region of frequent loss in breast tumors.[6,7] However, our analysis of a larger family series did not support the contribution of a putative gene at this locus to more than a small proportion [HLOD = 0.03, α= 0.03, upper 95% confidence limit (CL) 0.30] of high-risk families.[8]

These findings illustrate the difficulties inherent in efforts to identify additional susceptibility genes for a disease with high population prevalence. First, breast cancer is a genetically heterogeneous disease, and it is likely that there are multiple genes remaining to be identified among non-*BRCA1*/*BRCA2* families, with any one accounting only for a small proportion of such families. Second, in moderate-size families with a mixture of cases diagnosed at early and late ages, chance familial clustering of

cases may confound linkage-based approaches. Finally, penetrances of additional breast cancer susceptibility genes are likely to be lower than those associated with *BRCA1* and *BRCA2*.[9] Thus, analysis of a large family series with stringent selection criteria is required to achieve sufficient statistical power for unambiguous localization of novel susceptibility loci and meaningful evaluation of candidate genomic regions. To surmount these obstacles, our international collaborative group [Breast Cancer Linkage Consortium (BCLC)] has accrued, and continues to accrue, a collection of families appropriate to address the problem.

Recently, Kainu et al.[10] reported evidence for a novel breast cancer susceptibility locus on chromosome 13q21. They studied 77 families with multiple cases of breast cancer from Finland, Sweden, and Iceland in which no germline *BRCA1* or *BRCA2* mutations had been identified. Families were not specifically selected for early onset disease, nor were they excluded if one or more cases of ovarian cancer were present.

Initial analysis by comparative genomic hybridization (CGH) of tumors from 23 of these families and 14 others not analyzed further by linkage identified loss of 13q21–31 as a frequent and early event. Consistent loss of 13q21 in all five tumors from one family delineated a minimal region of haplotype sharing in these individuals as the target locus for a susceptibility gene. However, no evidence was presented for specific loss of the wild-type allele in these tumors, as would be expected for the underlying genetic model (inactivation of a tumor suppressor gene).

Genetic linkage analysis using 23 microsatellite markers from this region revealed supportive evidence of linkage to breast cancer. A maximum multipoint HLOD of 3.46 was found at marker D13S1308, with an estimated 65% of families linked. This marker lies ≈25 cM distal to *BRCA2* on chromosome 13q. Simulation studies to account for the possible confounding of linkage results by the proximity of these loci indicated that the linkage was unlikely to be the result of unidentified *BRCA2* mutations in a subset of families. However, the evidence for linkage was confined to a single pair of tightly linked markers (D13S1308/D13S1296) in this region, with linkage evidence dropping off quite rapidly surrounding this peak; indeed markers flanking a 2.1-cM region surrounding this peak yielded negative two-point LOD scores at recombination fractions up to 20%.

We present results from our attempt to confirm this linkage result through analysis of our series of 128 breast cancer families. In the remainder of this article, we refer to this locus as '*BRCA3*,' the quotation marks serving to emphasize the uncertainty regarding the existence and location of one or more such susceptibility loci.

TABLE 1

Summary of the families used in the 13q21 analysis

Age of diagnosis	Number of breast cancer cases in family				
	<3	3	4	5	>5
<50 years	51	48	19	5	5
<60 years	0	58	39	14	17
All cases:	0	26	36	25	41
Cases sampled/ genotyped	26	68	20	9	5

METHODS

Families.

Families were ascertained from cancer genetics or oncology centers in Europe (United Kingdom, Germany, Spain, Netherlands, France, and Israel), the United States, Australia, and Canada. One family was from Mexico. All families were Caucasian except the Mexican family that was of mixed European–Amerindian descent. Only families in which at least three women were diagnosed with breast cancer under age 60 years were eligible for the study. We excluded families in which cases of either ovarian cancer or male breast cancer were observed, because these phenotypes are strong predictors of *BRCA1* or *BRCA2* mutations.[1] Within these 128 families a total of 650 women were affected with breast cancer (median 5 per family); 56% of these cases were diagnosed under age 50. Samples from 409 affected individuals and 293 unaffected relatives were available for genotyping. Table 1 shows the characteristics of the families in more detail.
Entries are the number of families with the specified number of breast cancer cases of the indicated diagnostic criteria and sample availability.

Exclusion of *BRCA1* and *BRCA2*.

At least one breast cancer case from each family was screened for mutations in *BRCA1* and *BRCA2*, including all coding exons and splice junctions; in general, the sampled case with the youngest age at diagnosis was screened. This screening was performed using a variety of methods, including heteroduplex analysis (HDA), conformation sensitive gel electrophoresis (CSGE), and direct sequencing. Families from The Netherlands were also screened for the large genomic rearrangements that are known Dutch founder mutations, as these would not be detected by standard PCR-based screening methods. Other families were also tested for population-specific mutations, where

TABLE 2

Summary of markers used in the analysis

Marker	Map position, cM	Centers typed	Multipoint LOD score	
			Homogeneity	Heterogeneity (alpha = 0.65)
S1444	23.3	I	—	—
S1700	23.5	I	—	—
S260	23.7	S,L,I	−40.65	−14.25
S171	25.1	S	−36.07	−13.04
S1493	25.8	I	−33.83	−12.42
S267	26.9	S	−30.79	−11.51
S1293	26.9	I	−30.79	−11.51
S153	45.6	S	−32.54	−9.35
S788	45.6	I	−32.54	−9.35
S1317	51.0	L	−33.88	−10.35
S1262	51.0	I	−33.88	−10.35
S1308	52.6	S,L,I	−38.00	−11.03
S1296	52.6	I,L	−37.64	−10.93
S1291	53.2	L	−35.00	−10.06
S800	55.3	I	—	—
S166	55.3	S	—	—

I - IARC; S – ICR, Sutton; L – Leiden University. Based on published marker locations from Marshfield Medical Research Foundation (http://research.marshfieldclinic.org/genetics/). Note that BRCA2 is at position 24.8 on this map.

appropriate. Overall, we estimate that, taken together, these methods have an average sensitivity of 0.70.[1,11]

Genotyping.

Genotyping was carried out at the International Agency for Research on Cancer (62 families), Institute of Cancer Research (49 families), and University of Leiden (17 families). Genotypes were generated for 16 microsatellite markers within a 32-cM region of chromosome 13q21 spanning both BRCA2 and the putative 'BRCA3' locus (see Table 2). Not all centers genotyped all markers; Table 2 gives details on which

loci were genotyped at each center. Microsatellite repeats were amplified from peripheral blood lymphocyte genomic DNA by standard methods using published primer sequences (The Genome Database, http://gdbwww.gdb.org/). PCR conditions were specific to each genotyping center, as was fragment analysis. Internal consistency of allele sizing was achieved at each center by incorporating samples with known allele sizes on each gel. A common DNA sample (CEPH-1347-02) was typed to ensure consistency of allele sizing between centers. Allele frequencies were calculated separately for each center from the pedigree genotypes by using DOWNFREQ software, Version 1.1 (available through http://linkage.rockefeller.edu/soft/).

Statistical Analysis.

We performed standard parametric linkage analyses, essentially identical to our previous analyses of linkage in breast cancer families (e.g., refs. 1, 5, and 8) and to the analysis conducted by Kainu et al.[10] These analyses assume the model of susceptibility to breast cancer based on the segregation analysis of Claus et al.[9] Under this model, susceptibility to breast cancer is conferred by a dominant allele with population frequency of 0.003. The risk of breast cancer by age 80 is assumed to be 0.80 in carriers and 0.08 in noncarriers. Risks are modeled in seven age categories (<30, 30–39, 40–49, 50–59, 60–69, 70–79, and 80+) as described in Easton et al.[12]

Multipoint linkage analyses were carried out using the programs GENEHUNTER (V. 2.0-B; ref. 13), VITESSE,[14] and FASTLINK.[15] Genehunter was used where possible because it can analyze large numbers of polymorphic loci simultaneously and hence all of the markers we used could be incorporated into a single analysis. However, 33 families were too large to be accommodated by GENEHUNTER without discarding informative individuals. For these families we computed multipoint LOD scores by using either VITESSE (29 families) or FASTLINK (four families with multiple founders). The analyses assumed the intermarker distances as shown in Table 2.

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 '*BRCA3*' locus by maximizing the heterogeneity LOD score. A 95% confidence interval for αwas derived by computing the values of the heterogeneity LOD score that were within 0.83 (corresponding to a Z value of 1.96) of its maximum value. Ninety-nine percent confidence intervals were also computed.

Because the putative '*BRCA3*' locus on 13q21 is linked to *BRCA2*, we performed a

further analysis to allow for the possibility that preferential selection for families unlinked to *BRCA2* may have biased the results against linkage at '*BRCA3*.' In this analysis, we computed multipoint heterogeneity LOD scores at the candidate '*BRCA3*' locus, conditional on the LOD scores at *BRCA1* and *BRCA2*, according to the formula:

$$\text{LOD}(\theta_3) = \log_{10}\left[\frac{\alpha_1(1-\mu_1)10^{\text{LOD}_1(\theta_1)}+\alpha_2(1-\mu_2)10^{\text{LOD}_{2:3}(\theta_2)}+\alpha_3 10^{\text{LOD}_{2:3}(\theta_3)}+1-\alpha_1-\alpha_2-\alpha_3}{\alpha_1(1-\mu_1)10^{\text{LOD}_1(\theta_1)}+\alpha_2(1-\mu_2)10^{\text{LOD}_2(\theta_2)}+\alpha_3 10^{\text{LOD}_2(\theta_3)}+1-\alpha_1-\alpha_2-\alpha_3}\right]$$

In this formula α_1, α_2, and α_3 are the proportions of families meeting the eligibility criteria that are linked to *BRCA1*, *BRCA2* and '*BRCA3*,' respectively, and μ is the sensitivity of *BRCA1*/2 mutation screening. For the purposes of these analyses, α_1 and α_2 were set to 0.15 and μ to 0.7. $\text{LOD}_1(\theta_1)$ and $\text{LOD}_2(\theta_2)$ are the LOD scores at *BRCA1* and *BRCA2*, respectively, whereas $\text{LOD}_{2;3}(\theta_2)$ and $\text{LOD}_{2;3}(\theta_3)$ are the LOD scores at *BRCA2* and '*BRCA3*,' respectively, based on markers typed at both loci; $\text{LOD}_2(\theta_3)$ is the LOD score for '*BRCA3*' calculated using only markers at *BRCA2*. This calculated LOD score is the likelihood for the linkage data at '*BRCA3*' conditional on the existing linkage and mutation evidence at *BRCA1* and *BRCA2*, and hence corrects (albeit conservatively) for any bias in the '*BRCA3*' evidence produced by exclusion of families linked to *BRCA2*.

RESULTS

Total LOD scores were strongly negative throughout the 8-cM interval between D13S153 and D13S1291 (Table 2 and Fig. 1). At the location of '*BRCA3*' estimated by Kainu et al.,[10] D13S1308, the total LOD score was −38.00. Based on the admixture model, the estimated proportion of linked families (α) was 0, with an upper 95% confidence limit of 0.13. The estimated α was also zero for all possible positions in the interval D13S153-D13S1291. Of the 128 families, only four had a multipoint LOD score of greater than 0.5 at D13S1308, the highest of which was 0.67 (one additional family achieved a LOD score of 1.55 at a more distal marker, D13S800). Twelve families achieved LOD scores less than −1 at D13S1308.

We reanalyzed the data conditioning on the genotyping data at *BRCA1* and *BRCA2*. In

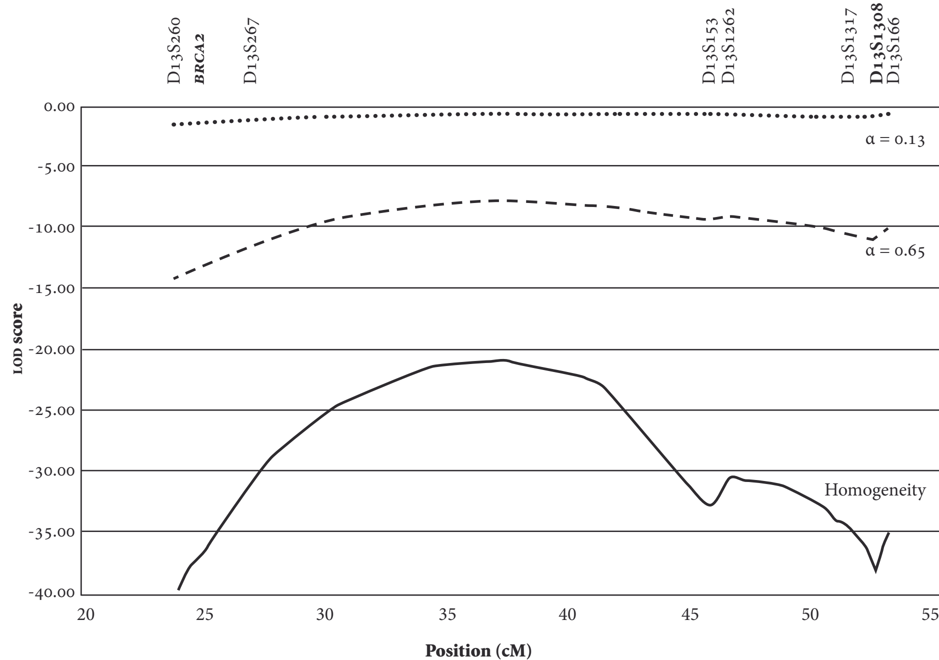

Figure 1. Multipoint LOD scores for the 128 families analyzed are shown graphically. The solid line represents scores obtained under the assumption of homogeneity; the dashed line assumes the proportion of linked families (α) to be 65%, as estimated by Kainu et al.[10]; and the dotted line represents the 95% upper confidence interval (α = 0.13).

this analysis the total LOD score was –25.08. In the heterogeneity analysis based on these conditional LOD scores, the estimated proportion of families linked to '*BRCA3*' was again 0, with an upper 95% confidence limit of 0.18. In the 95 families that could be analyzed with GENEHUNTER, we also analyzed the data by using the nonparametric method[13] to evaluate haplotype sharing among affected women. Again, no significant evidence of linkage was found (data not shown).

Discussion

Our results clearly conflict with those reported by Kainu et al.[10] Using a set of multiple case female site-specific breast cancer families analyzed for a similar set of markers within the candidate region and subjected to comparable statistical analysis, we found no evidence of linkage to 13q21. The proportion of linked families (65%) reported by Kainu et al.[10] is excluded with a high degree of statistical significance (the heterogeneity LOD score at α= 0.65 was –11.03 in our dataset). This is true even after

a conservative correction for possible bias due to potential exclusion of families linked at the *BRCA2* locus (conditional LOD at α= 0.65 was −7.64). In addition, under both unconditional and conditional analyses, the estimated proportion of linked families was 0, with upper 95% confidence intervals of 13% and 18%, respectively, indicating that if there is a susceptibility locus on 13q, it is likely to account for only a minority of breast cancer families. The paper of Kainu et al.[10] did not provide confidence limits on their estimated proportion of linked families. However, based on their LOD scores given under homogeneity and 65% heterogeneity, and assuming confidence intervals that are symmetrical about the best estimate, we have estimate a lower 95% confidence limit for α of 0.31. Thus the 95% confidence limits for the two studies do not overlap. Moreover, even when using a more stringent criteria of 99%, the upper confidence limit for our estimated proportion of linked families is 0.19 for the unconditional analysis and 0.26 for the analysis conditioning on *BRCA2* markers, further indicating a minor role, if any, for this locus.

There were some differences in selection criteria between the two studies. Our study was restricted to families in which at least three cases of breast cancer were diagnosed below age 60, whereas Kainu et al.[10] included families with three cases diagnosed at any age. Thus, our families may be more heavily selected for genes conferring high risk. It is perhaps noteworthy that the initial hypothesis-generating family analyzed by comparative genomic hybridization (CGH) in Kainu et al.[10] would not have qualified for our study because only two of the five cases were diagnosed under age 60. However, in the subset of 51 families with less than three cases diagnosed under age 50 (Table 1), there is also considerable evidence against linkage to this locus (multipoint LOD = −8.06; HLOD = 0; upper 95% CI for α = 24%; HLOD for α of 65% = −3.57). Thus it is unlikely that difference in age criteria can explain the differences in results between the two studies.

An additional difference in selection criteria was exclusion of families with any cases of ovarian cancer in our series, given the close association of this disease with *BRCA1* and *BRCA2*. Although no *BRCA2* mutations were identified in the family set of Kainu et al., the combination of detection methods applied to screening families have detection sensitivities of ≈0.70.[1,11] Thus, although simulated linkage results allowing for up to 25% of the families in the dataset of Kainu et al.[10] being due to undetected *BRCA2* mutations only exceeded the observed maximal lod score in 1 of 3,000 replicates, it is not known to what extent the seven families with ovarian cancer contributed to the observed overall LOD score.

The families in our study were drawn from Western Europe, or in descendent popu-

lations in North America and Australia, whereas the families studied by Kainu et al.[10] were from the Nordic countries. Although we have not specifically examined the ethnic origins of each family in our set, it is anticipated that the set of families from the United States and Canada ($n = 43$) are more ethnically heterogeneous, although most, if not all, are of Western European origin. Only a small minority of all of the families in our set are likely to be of Scandinavian origin, most notably the families ascertained in Minnesota, Seattle, and other parts of the Midwest, which have a high concentration of families descendent from emigrants of Sweden and Norway. One might speculate that the difference in the results observed is due to a population specific founder effect i.e., an excess of some specific mutation in '*BRCA3*' in the Nordic populations.

We believe this to be unlikely. The different Nordic populations have different population histories and do not originate from a single small founder population. Although closely related, the Swedish, Icelandic, and (to a lesser extent) Finnish populations are also genetically similar to English and Dutch populations.[17] If the observed linkage were due to a susceptibility allele that had reached a high frequency in the Swedish and Finnish populations, this allele would also be expected to occur at a detectable frequency in the British and Dutch families. On the other hand, if the linkage is the result of several different mutations in the candidate '*BRCA3*' gene, the expectation would be that (as in the case of *BRCA1* and *BRCA2*) mutations would also occur in the British, Dutch, and other populations, albeit the set of mutations might be different. Under either model, we would have expected to observe similar evidence of linkage in our families. Indeed, even when the prevalence of a population specific founder mutation has led to a specific susceptibility gene accounting for the majority of families of a hereditary cancer syndrome [e.g., *BRCA2* in the Icelandic population accounting for 61.4% of breast cancer families[18]; >50% of hereditary nonpolyposis colon cancer (HNPCC) families in the Finnish population attributable to two specific MLH1 mutations[19]], these same genes account for a substantial fraction of families with the same cancer syndrome in other populations (breast cancer reviewed in[20]; HNPCC[19]).

We conclude therefore that any contribution of a locus at chromosome 13q21 to familial breast cancer is likely to be small in breast cancer families of European origin. Further linkage studies in large series of multiple case families, or targeted association studies in large series of breast cancer cases and controls, will be needed to identify remaining genes underlying familial aggregation of the disease.

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