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ORIGINAL ARTICLE

A novel BRCA2 mutation in an Indonesian family found with a new, rapid, and sensitive mutation detection method based on pooled denaturing gradient gel electrophoresis and targeted sequencing

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Background: Breast cancer is increasing in Indonesia and other developing countries. Germline mutations in the BRCA1/2 genes are most strongly associated with a high risk for breast cancer development. There have been no reports on BRCA1/2 gene mutations in the Indonesian population. Genetic research yielding insight into mutations affecting the Indonesian population can help in risk assessment of individual patients.
Aims: To screen the BRCA1/2 genes for mutations in early onset Indonesian breast cancer patients and their families with a new, simple, and sensitive BRCA1/2 mutation screening strategy based on denaturing gradient gel electrophoresis (DGGE) and targeted sequencing.
Methods: DNA was isolated from the blood of four Indonesian breast cancer patients from high risk families and seven family members, and the polymerase chain reaction was performed with specially designed primers throughout the BRCA1/2 coding sequences to produce fragments suitable for pooled DGGE analysis. The aberrantly migrating samples were reamplified and sequenced.
Results: Two mutations were found in exons 13 and 16 of BRCA1 and two mutations in exons 2 and 14 of BRCA2, which turned out to be established polymorphisms according to the Breast Cancer Information Core. In addition, a novel 6 bp deletion in exon 11, leading to a premature stop, was found in BRCA2.
Conclusion: Pooled DGGE and targeted sequencing revealed four BRCA1/2 polymorphisms and one novel BRCA2 mutation in a group of Indonesian patients at high risk of hereditary breast cancer. This illustrates that the proposed method is sensitive and particularly suited for screening unknown populations.

Breast cancer is the most common malignancy in women in the Western world, accounting for 32% of all female cancers, and is responsible for 18% of cancer deaths in women. The hereditary form of breast cancer constitutes about 5% of breast cancer cases overall.[1] The first major gene responsible for hereditary breast cancer susceptibility, BRCA1, was mapped to 17q21 in 1990,[2] and was cloned in 1994.[3] A second gene involved in the hereditary breast cancer syndrome, BRCA2, was identified several months later.[4] Both genes have long coding sequences and complex genomic structures: BRCA1 comprises 5592 bp, composed of 22 coding exons that encode 1863 amino acids,[3] and BRCA2 comprises 10 443 bp, with 26 coding exons that encode 3418 amino acids.[4] In addition to breast cancer, ovarian cancer,[5] fallopian tube cancer,[6] and primary peritoneal cancer are part of the BRCA1/2 cancer spectrum in women.

> "By identifying the endemic Indonesian mutations, we hope to offer better risk assessment for women in the Indonesian population who are susceptible to the BRCA1/2 related hereditary cancers"

Over 80% of families with two or more cases of premenopausal breast cancer and two or more cases of ovarian cancer are believed to carry a germline BRCA1 or BRCA2 mutation.[7 8] Comprehensive evaluation of breast cancer susceptibility genes can be used to provide women with information concerning their risk of developing cancer, and guide the decision on the necessity of preventive surgical measures.

It has been shown that ethnically different populations exhibit different germline mutation spectra in the BRCA1 and BRCA2 genes.[9–11] There are no previous publications on BRCA1/2 mutation detection in the Indonesian population. By identifying the endemic Indonesian mutations, we hope to offer better risk assessment for women in the Indonesian population who are susceptible to the BRCA1/2 related hereditary cancers. There are no exact data on breast cancer incidence in Indonesia, but a study by Soeripto *et al* in 1982 (unpublished results) showed that the age standardised incidence rate in the Jogjakarta region was 6.17, ranking second after cervical cancer (7.69). One of the central themes in cancer risk assessment is the efficiency and accuracy of genetic screening methods. Currently, the ability to conduct large scale, population based studies is constrained by the lack of an accurate and inexpensive method for mutation detection. The large size of the BRCA1 and BRCA2 genes, and the scattered distribution of mutations throughout the genes, complicate the task of mutation detection and make rapid screening for mutations a major technical challenge. A technique that scans stretches of DNA for unknown mutations should be optimised to detect as close to 100% of the sequence alteration as possible. Here, we describe the results of the application of a recently developed rapid and sensitive method for the detection of BRCA1/2 mutations[12] based on denaturing gradient gel electrophoresis (DGGE)[13] and targeted sequencing on an Indonesian group of high risk

Abbreviations: DGGE, denaturing gradient gel electrophoresis; PCR, polymerase chain reaction; PTT, protein truncation test; SCCP, single strand conformational polymorphism

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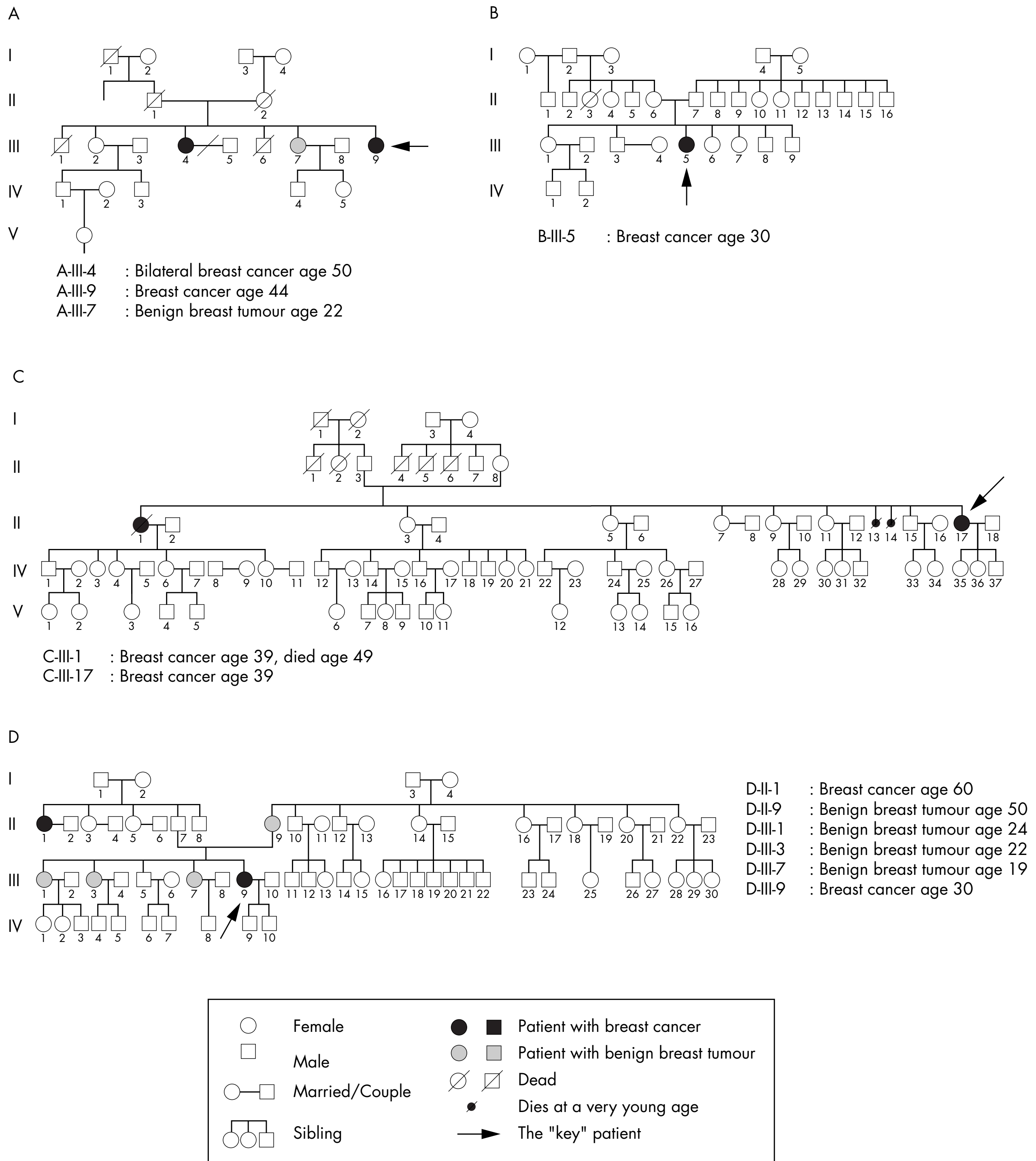

Figure 1 Pedigrees of four Indonesian families screened for BRCA1/2 mutations with a novel, rapid, and sensitive mutation detection method based on pooled denaturing gradient electrophoresis and targeted sequencing.

patients. This method appears to be particularly suited for the analysis of unknown populations.

MATERIALS AND METHODS

Patients

From 1996 to 1998, four Indonesian families were selected from patient pools at three hospitals in Jogjakarta, Indonesia; three with hereditary breast cancer based on Moller's criteria,[14] and one with one early onset patient without a family history. Figure 1 shows the pedigrees of these four families. Information regarding potential risk factors for breast cancer was obtained through a structured face to face interview. Information on family history (up to the date of diagnosis of the patient's breast cancer) was elicited by asking each patient to identify all first and second degree female blood relatives. For each identified relative, the interviewer then asked the year of birth, vital status, year of death (if applicable), history, and type of cancer (if any).

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Table 1 Primer sequences used to amplify BRCA1 exon by exon for denaturing gradient gel electrophoresis[12]

Fragment	Forward primer	Reverse primer	Size (bp)
Exon 2-1	ATGATAAAATGAAGTTGTC*	ACACTCTAAGATTTTCTGC	204
Exon 2-2	TTATCTGCTCTTCGCGTTG	CTTCCCTAGTATGTAAGGTC*	202
Exon 3	GCGCGTTGAGCCTCATTTATTTTC	ACAAAAGCTAATAATGGAGC*	185
Exon 5	GTATTCTTTCTACAAAAGG*	TCCAACCTAGCATGATTAC	208
Exon 6	GGTTGATAATCACTTGCTG*	CACTTGAGTTGCATTCTTG	223
Exon 7	ATACATAGGGTTTCTCTTG*	AGAAGAAAACAAATGGTTT	293
Exon 8	TTGCTTGACTGTTCTTTACC*	ACTTAAAAAAACCTGAGACC	209
Exon 9	CCCTTTTAATTAAGAAAAC*	ACTAAATAGGAAAATACCAG	191
Exon 10	CATTTGACAGTTCTGCATAC*	TTCAGTGCCTGTTAAGTTG	217
Exon 11-1	ATGACAATTCAGTTTTTGAG*	TATTACTGGGTTGATGATG	147
Exon 11-2	AGCTGCTTGTGAATTTTCTG*	ATAAACTGCTGTTCTCATGC	243
Exon 11-3	TTTTTACAAATACTCATGCCAGCTC*	TAGGATTCTCTGAGCATGGC	313
Exon 11-4	TTTTTTTTTTGTGTGAGAGAAAAGAATGG*	CATCTACCTCATTTAGAACG	272
Exon 11-5	GAATCAAATGCCAAAGTAGC*	GGACGCTCTTGTATTATCTG	329
Exon 11-6	ATTATAGGAGCATTTGTTAC	TTTTCGAGTGATTCTATTGG*	324
Exon 11-7	CAAAAGGTGATTCTATTCAG*	ATAAGGTGGGCTTAGATTTC	275
Exon 11-8	CAGGCATATTCATGCGCTTG*	GAAAGTATCGCTGTCATGTC	281
Exon 11-9	GAGTAACAAGCCAAATGAAC	GGGGTCTTCAGCATTATTAG*	242
Exon 11-10	TTGTCAATCCTAGCCTTCC*	ATTAGTCCCTTGGGGTTTTC	324
Exon 11-11	AATAAATGTGTGAGTCAGTG*	ACATTCCTCTTCTGCATTTC	300
Exon 11-12	ATTCAAGGTTTCAAAGCGCC	GTATATTAACTGTCTGTAC*	238
Exon 11-13a	AGGAAGAAAATCAAGGAAAG*	TAATGAGTCCAGTTTCGTTG	223
Exon 11-13b	GCCAAATGTAGTATCAAAGG	CAGGTGACATTGAATGTTCC*	248
Exon 11-13c	AAAATCTGCTAGAGGAAAAC	TCATCACTGGAACCTATTTC*	259
Exon 11-13d	TAAAGAAGCCAGCTCAAGC	CTGAAATCAGATATGGAGAG*	325
Exon 11-13e	GCAAGAATGTCAAGAAGTAG*	CCATCATCTAACAGGTCATC	181
Exon 11-13f	AGTCATGCATCTCAGGTTTG*	ATAAGTTCTCTTCTGAGGAC	280
Exon 11-14	CTTTCACCCATACACATTTG	TGCAGTCATTTAAGCTATTC*	277
Exon 11-15	GAGTGTCTGTCTAAGAACAC*	TATTTGCAGTCAAGTCTTCC	221
Exon 11-16	GTTTCTTCACAGTGCAGTG	AAATAGACTGGGGCAAACAC*	296
Exon 12	GTCTGCTTTTACATCTGAACC*	AATGCAAAGGACACCACACAC	221
Exon 13	GCGCGATTTCATTTTCTTGGTGCC	GGGAAGGAAAGAATTTTGC*	305
Exon 14	TCAGAACAAAGCAGTAAAG*	AAGATGTCAGATACCACAG	257
Exon 15-1	ATTGGTGGCGATGGTTTTC*	CTCCTCCACATCAACAACCT	204
Exon 15-2	ACTACCCATCTCAAGAGGAG	AAATCAAAGTGTTTGTTCC*	195
Exon 16-1	GACCAGAACTTTGTAATTC*	CCAGCAGTATCAGTAGTAT	299
Exon 16-2	AAAGTTGCAGAATCTGCCC	TAAGTCTTAGTCATTAGGG*	252
Exon 17	GTGCTAGAGGTAACTCATG*	CAGCAGATGCAAGGTATTC	213
Exon 18	ACAGCACTTCCTGATTTG	TCTGAGGTGTTAAAGGGAG*	222
Exon 19	TCTATCTCCGTGAAAAGAG	CTGGTTAGTTTGTAACATC*	176
Exon 20	TGCTCCACTTCCATTGAAG*	TTTGTCAACTTGAGGGAGG	220
Exon 21	CCTCTCTCCATTCCCCTG*	AAGGCTGGTGCTGGAACTC	182
Exon 22	GCCTGGGTTAAGTATGCAG*	ATTGTGTCCTCCCTCTCTG	210

*GC clamp sequence (CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCG) is attached to this site.

The patients were interviewed for family history using standardised questionnaires and blood was taken after informed consent.

DNA amplification

DNA was isolated from peripheral blood using simple and rapid genomic DNA extraction. Two types of buffer—cell membrane lysis buffer, containing sucrose and triton X-100, and nuclear membrane lysis buffer, containing guanidine thiocyanate, sodium *N*-lauroyl sarcocinate, and β mercaptoethanol—were used. By using this method, only a small amount of peripheral blood (0.5 ml) was needed. The DNA was stored at 4℃.

Polymerase chain reaction (PCR) fragments suitable for DGGE analysis were designed based on the theoretical melting profile of each exon, as described previously.[12] A 40 mer GC clamp was attached to the short oligonucleotide to obtain optimal melting profiles. Tables 1 and 2 provide the PCR primer sequences for BRCA1 and BRCA2, respectively.

Genomic DNA was amplified using 100–200 ng of template DNA, 10 pmol of the mixture of 60 mer primers, 1mM MgCl, and 1 U Platinum Taq in 50 µl PCR reactions. The amplification conditions in a Robocyler (Stratagene, Canada) were five minutes at 94℃ (hot start), followed by five cycles of one minute at 94℃, one minute at 52℃, and one minute at 72℃; five cycles of one minute at 94℃, one minute at 50℃, and one minute at 72℃; and 30 cycles of one minute at 94℃, one minute at 48℃, and one minute at 72℃; with five minutes extra at 72℃. The final incubation was followed by five minutes at 94℃ and 15 minutes at 50℃ to enhance the formation of heteroduplex molecules.

Denaturing gradient gel electrophoresis

Primers for DGGE were obtained from Ingeny (Goes, the Netherlands). Aliquots (4–6 µl) of the PCR products were mixed with 2 µl of standard dye loading buffer and electrophoresed through a 20 cm 9% polyacrylamide gel (acrylamide/bisacrylamide, 37.5/1) containing a linear 20–65% denaturing gradient (100% UF: 7M urea/40% deionised formamide). A 12% polyacrylamide stacking gel was poured to create solid slots for efficient loading of the PCR products, which prevented difficulties caused by the high urea concentration. Electrophoresis was performed in 0.5 Tris acetate EDTA buffer at 58℃ for 16 hours.[15] Gels were stained with ethidium bromide and photographed under an ultraviolet transilluminator.

To increase the throughput of the DGGE procedure, three to four different amplicons with relatively large melting temperature differences were pooled. The fragment pool was designed using a computer program that was based around melting profiles and sequences (tables 3 and 4). For each different exon, seven samples from four different families were loaded side by side to compare their band patterns. The aberrantly migrating samples were reamplified using

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Table 2 Primer sequences used to amplify BRCA2 exon by exon for denaturing gradient gel electrophoresis[12]

Fragment	Forward primer	Reverse primer	Size (bp)
Exon 2	TCCCTGTGTAAGTGCATT	CACTTTCTCGGTGTAATT*	229
Exon 3	ACTAAGGTGGGATTTTTT*	CGCCCCCCCAGTCTACCATATTG	350
Exon 4	CACTGAATTATTGTACTG	ATATGTAGGAAAATGTTTC*	226
Exon 5	AAATAACCTAAGGGATTT	CATTTCTAGTATTCTAAG*	170
Exon 6	CTTAACAATTTTCCCCTT	GCTATTGTCAAATTCTCA*	169
Exon 7	GATCAGGGCATTTCTATA*	CGCCCCTCATCTGCTCTTTCTTG	248
Exon 8	GTTTTTGCATTCTAGTGAT*	GTTAGCAATTTCAACAGTCT	184
Exon 9	GAGAGTTTTTATACTAGTGA*	ACAGAGCAAGACTCCACCTC	278
Exon 10-0	ATGTGCTTCTGTTTTATACT*	CGCCCGCCAAACTATCTTCTTCAGAGGT	205
Exon 10-1	TTTTTTTTTTAGTATATGAAACAGTTGTAG*	CTTCTGATTTGCTACATTTG	291
Exon 10-2	CTCATTTGTATCTGAAGTGG*	CGCCCTTTTGGTCACATGAAGAAAT	255
Exon 10-3	GGAGCCCAGATGGCGAAAA*	CTGTTTCCTCATTTAATGGC	234
Exon 10-4	GCGCCACGTATTTCTAGCCTACC	TTAAAGTTTGGATCAGTCAT*	294
Exon 10-5	GAGAATCACCTAAAGAGACT*	GGGGGGCCAGCTTCCATTATCAAT	210
Exon 10-6	GTTTGCTCACAGAAGGAGGA*	CCTGCATTCTTCAAAGCTAC	256
Exon 10-7	AGCCACCACCACACAGAATT	CTTTCGGTATTTTTTTTCCT*	171
Exon 10-8	GCTATACATGATGAAACATC*	GTACCTGAATCAGCATTTGC	177
Exon 10-9	GCTTTTGAAGCACCACTTAC	GGAATCGTCATCTATAAAAC*	138
Exon 11-1	CGCGTGAATGTGATTGATGGTAC	CTGTAGTTTTTCCTTATTAC*	244
Exon 11-2	ATCTTGATTATAAAGAAGCA*	TGAATGTTGTACTGGGTGAC	219
Exon 11-3	AAAAGAAGAGGTCTTGGCTG	GACTAGGTTTGACAGAACA*	214
Exon 11-4	AGCACTCTTATTTTAACTCC	TTTTCAGGTGGCAACAGCTC*	255
Exon 11-5	AATGTCAGACAAGCTCAAAG*	TTGGATTACTCTTCGATTTG	245
Exon 11-6	TAGCATCACCTTCAAGAAAG*	CTGTGTCTCCATATAAAACC	316
Exon 11-7	TGACTTGTGTAAACGAACCC	GTCCTGCCCATTTGTTCATG*	286
Exon 11-8	GGACATCTCCTTGAATATAG*	TTCAACACAAGCTAAACTAG	265
Exon 11-9	GAGCAAAATGTTCTTCAAAG*	AATTTCTGCCTTTTGGCTAG	301
Exon 11-10	CCCCTCAGATGTTATTTTCC*	GATCAGCATCTCTGCATTCC	271
Exon 11-11	AAGTGCCTGAAAACCAGATG	ATGAGCAGAATAAAAGCCCC*	282
Exon 11-12	AGTGCTTCTGGTTATTTAAC	CTTACAGTTTTATCATTATG*	285
Exon 11-13	GCAGAGGTACATCCAATAAG*	TTACTTGAATCACTGCCATC	312
Exon 11-14	GAAGATAACAAAATATACTGC*	TTACCATGACATGCTTCTTG	288
Exon 11-15a	GAAGATTTGTCAGATTTAAC	TATGTCAGAATCTAATTCAG*	310
Exon 11-15B	ATCAGAAACCAGAAGAATTG*	TACCAACTGGGACACTTTCT	184
Exon 11-15C	CAGACATAGTTAAACACAAA	ATTTCACTAGTACCTTGCTC*	265
Exon 11-15D	AGGAATCTTTGGACAAAGTG	GTTTTGAGATTTTCAGTTTG*	325
Exon 11-15E	TTCTATTGAGACTGTGGTGC*	TGACCATCAAATATTCCTTC	327
Exon 11-15F	TGTGAGTCAGACTTCATTAC	GTTAGACATGCTACTGTTAC*	250
Exon 11-15G	GTACTATAGCTGAAAATGAC*	TCCTCAACGCAAATATCTTC	319
Exon 11-15H	AAAAGATGCAAATGCATACC	CCCTACCTCAAAATTATTAC*	179
Exon 11-15I	ATGCAGCCATTAAATTGTCC	TACTTTACTGAAACTGTCTG*	197
Exon 11-15J	CAGTGGTAAAATCGTTTGTG*	TCTTCACTCTGAATGTCAGC	283
Exon 11-15K	TTATGATGAATGTAGCACGC	CTTCCCTATACTACATTTAC*	206
Exon 11-15L	CACCTTGTGATGTTAGTTTGG	ACCACATTATATGAAAAGCC*	362
Exon 11-15M	GAAGAAAATACTGCTATACG*	ATCAAATTCCTCTAACACTC	211
Exon 11-15N	TACAGCAAGTGGAAAGCAAG	TTATTTTCTGAAGAACCACC*	301
Exon 11-16	CTTCCTCGTGTTGATAAGAG*	GGCATGACTTGGCAGTTTAG	472
Exon 11-17	GGAAGATGATGAACTGACAG	GTGATTGGCAACACGAAAGG*	214
Exon 12	GACTTTTGAGAAATAAAACTG*	GATCCACCTGAGGTCAGAAT	278
Exon 13	CGCCCGTAATATAAAATAATGGTTTCC*	AAACGAGACTTTTCTCATAC	196
Exon 14-1	CGCCCGCCGTGTACTAGTCAATAAACTT	AGCAGAAACTTGATAAAATG*	244
Exon 14-2	ATCTTCAAGCAATTTAGCAG*	TACTATCATCAGAGCCATGT	277
Exon 14-3	AAAACAGACAAAAGCAAAAC*	CATCACACAATTGTCATAC	225
Exon 15-1	GGTGTGCTTTTTAAATTC*	CTTCTTAATTCGCATATCCTG	151
Exon 15-2	ATTACAAGTCTTCAGAATGCC*	GCGGGAAAAGCCATCAGTATTGTAG	254
Exon 16	GTGTGATACATGTTTACTT*	GGTAGAGGGAATACATAAA	358
Exon 17	CAGTATCATCCTATGTGGTT*	CTGCCGTATATGATTACGTA	303
Exon 18-1	CTCAGTTATTCAGTGACTTG*	GATCTAACTGGGCCTTAACA	354
Exon 18-2	TTGAACTTACAGATGGGTGG	CTGATTTTTACCAAGAGTGC*	248
Exon 19	CGCCCGCCATATTTATTAATTTGTCC	TATGGTAAGTTTCAAGAATAC*	252
Exon 20	TATGTGACTTTTTTGGTGTG	CTCTAAGACTTTGTTCTCAT*	281
Exon 21-1	GTGAATTAATAATCCTTTTG*	GCACGAACTTGCTGTCTTGT	127
Exon 21-2	CATATTTACCATCACGTGCA	GCCTCATTATATGTCCTCTT*	177
Exon 22-1	CTAGTTACAATAGATGGAAC*	TGTGACATCCCTTGATAAAC	254
Exon 22-2	TGGAATCTGCTGAACAAAAG	CTGATAAAAACAAAGCATTTAC*	169
Exon 23-1	GATAATCACTTCTTCCATTTG	GTTAGCTCTTTCAGATTTAC*	205
Exon 23-2	GAAGGAAAGAGATACAGAAT*	CCCGCCCGTTCCATAAACTAACAAGCAC	219
Exon 24	GTGCTTGTTAGTTTATGGAA*	CGCCCAAAAATTAACTATATTGTGC	265
Exon 25-1	CTTGCATCTTAAAATTCATC*	GGTTGCTTGCAGCAATTAAC	221
Exon 25-2	CCCGCCCTGGCAATAAAGTTTTGGAT	CAAAATGTGTGGTGATGCTG*	283
Exon 26	GGAAATACTTTTGGAAACAT*	TTCCTTGAGTTTACATTAAC	282
Exon 27-1	ATGATAGGCTACGTTTTCAT*	TTGCAGTTCTTTTGGTCATC	220
Exon 27-2	GTCTTGTAAAGGGGAGAAAG*	TGAGGAGAATTCAGTTCTT	256
Exon 27-3	CTGCACAGAAGGCATTTCAG*	CAGAGATGTAGTACAACGTC	333
Exon 27-4	ACCAGTTCAGAAGATTATCT	GTCAATAATTTATTGTCGCC*	256

*GC clamp sequence (CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCG) is attached to this site.

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Table 3 Amplicon pools created for BRCA1 to increase the throughput of denaturing gradient gel electrophoresis

Gene	Pool	Exons in amplicon pool
	A	7,18, 10, 20
	B	9, 6, 8, 23
	C	5, 2B, 11-3, 16B
	D	14, 11-4, 22, 24
BRCA1	E	2A, 11-1, 16A
	F	19, 13, 21
	G	17, 11-14, 3
	H	11-15, 15A, 15B
	I	11-16, 11-2, 12
BRCA1 exon 11	A	11-5, 11-8, 11-10
	B	11-13b, 11-13e, 11-13f
	C	11-7, 11-12, 11-13c
	D	11-9, 11-11, 11-13a
	E	11-6, 11-13d

Each amplicon pool contained three to five amplicons with relatively large melting temperature differences.

sequencing primers and sequencing was performed using the Big Dye cycle sequencing kit according to the manufacturer's instructions. The reaction products were analysed using an ABI DNA sequencer 310 and sequence files were edited using the Sequence Navigator program.

RESULTS

In the 11 patients, aberrantly migrating bands (fig 2) were found in exons 13 and 16 of the BRCA1 gene, and in exons 2, 11, and 14 of the BRCA2 gene (table 5). We then sequenced the exons with aberrantly migrating bands (fig 3). The aberrantly migrating bands found in exons 13 and 16 of BRCA1 and exons 2 and 14 of BRCA2 appeared to be caused by single nucleotide substitutions producing the same amino acid. Consultation with the Breast Cancer Information Core (last accessed 1 February 2005; http://www.research.nhgri.nih.gov/projects/bic) revealed that these substitutions were indeed established polymorphisms.

We detected one deleterious BRCA2 mutation in patient B-III-5 (fig 4). This mutation was a deletion of six nucleotides in exon 11 (c.2472delTAAATG) at codon 824. The mutation did not cause a shift of the reading frame, but affected three codons and resulted in a premature stop codon. There is no such mutation registered in the Breast Cancer Information Core (http://www.research.nhgri.nih.gov/projects/bic), so this is a novel mutation. This patient had early onset breast cancer and there was no family history of cancer. Table 5 summarises the genetic aberrations found, and table 6 shows the clinicopathological data of the patients with breast cancer.

Table 4 Amplicon pools created for BRCA2 to increase the throughput of denaturing gradient gel electrophoresis

Gene	Pool	Exons in amplicon pool
BRCA2	A	11-1, 27-1, 19
	B	23-1, 18-1, 10-4, 11-3
	C	8, 20, 27-3, 10-5, 21-1
	D	15-1, 23-2, 26, 10-6
	E	12, 10-7, 2, 24, 27-2
	F	5, 6, 11-17, 15-2
	G	11-16, 9, 27-4, 18-2
	H	10-1, 10-3, 10-10, 10-8
	I	25-1, 14-2, 22-1, 25-2
	J	14-3, 10-9, 22-2
	K	4, 3, 7
	L	16, 11-2, 21-2
	M	13, 17, 14-1, 10-2
BRCA2 exon 11	A	11-4, 11-8, 11-15h
	B	11-5,11-10,11-13, 11-15f
	C	11-6, 11-12, 11-15d
	D	11-7, 11-14, 11-15c
	E	11-9, 11-15g, 11-15m
	F	11-15e, 11-15L, 11-15n
	G	11-15a, 11-15i, 11-15j
	H	11-11, 11-15b, 11-15k

Each amplicon pool contained three to five amplicons with relatively large melting temperature differences.

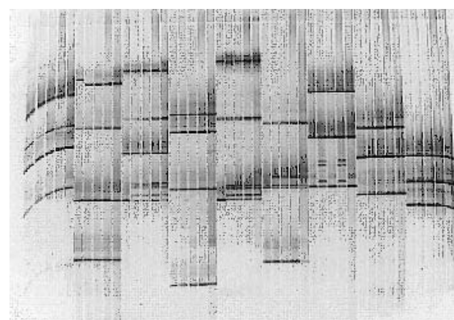

Figure 2 Pooling of five samples of 24 exons in the BRCA1 gene for denaturing gradient gel electrophoresis. Each pool consists of three to four amplicons selected on the basis of their melting behaviour. The fragments for each pool can be seen in table 3. The aberrant bands appeared in fragment 9 (pool B), fragment 16B (pool C), fragments 2A and 16A (pool E), fragment 13 (pool F), fragment 3 (pool G), and fragment 11-15 (pool H); these fragments were further analysed by sequencing.

DISCUSSION

The increasing demand for mutation detection in disease genes, either known or presumed, can be solved by automated sequencing using fluorescent dyes.[16] However, only a few laboratories are equipped for the broad application of this costly and labour intensive strategy. As alternatives to sequencing methods, which determine the exact nature and location of each base along a DNA fragment, various mutation scanning procedures have been developed. These methods, which rely on the recognition of a sequence variation between mutant and wild-type DNA on the basis of an altered electrophoretic migration pattern, provide a simple means for determining whether a given DNA sample harbours a mutation in a particular gene.

The most well established scanning procedures are single strand conformational polymorphism (SCCP) analysis, DGGE, chemical cleavage of mismatch, RNase cleavage, the protein truncation test (PTT), and heteroduplex analysis. Among these methods, SCCP, DGGE, PTT, and heteroduplex analysis are the most widely used because of their accuracy, simplicity, lack of toxicity, and/or relative affordability.

We focused on DGGE because this method, when optimised, has the highest mutation detection rate (close to

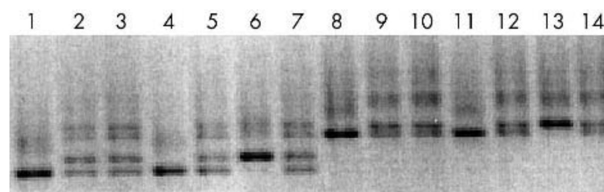

Figure 3 Results of denaturing gradient gel electrophoresis (DGGE) analysis of BRCA1. DGGE of Indonesian patients at high risk for hereditary breast cancer. Exon 16: lanes 1, 4, and 6 displayed a darker single band as opposed to samples in lanes 2, 3, 5, and 7, which showed lighter double bands. The dark band was in the same position as one of the double bands. Exon 13 was similar: lanes 8, 11, and 13 displayed a dark single band, whereas lanes 9, 10, 12, and 14 showed light double bands. These aberrant bands appeared to represent single nucleotide substitutions, with no consequences for the amino acid sequence of the protein (polymorphisms).

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Table 5 Mutations and polymorphisms found in the BRCA1 and BRCA2 genes using the proposed method

Patient	Exon, gene	NT	Codon	Base change	AA change	Designation	Mutation type
B-III-5	11, BRCA2	2699	824	Del TAAATG	Leu to stop	2699 del TAAATG	Nonsense
A-III-9	13, BRCA1	4427	1436	T to C	Ser to Ser	4427T/C	Polymorphism
B-III-5							
D-III-9							
A-III-9	16, BRCA1	4956	1613	A to G	Ser to Gly	S1613G	Polymorphism
B-III-5							
D-III-9							
D-III-9	2, BRCA2	203	–	G to A	5′UTR	203 >A	Polymorphism
D-III-9	14, BRCA2	7470	2414	A to G	Ser to Ser	7470A/G	Polymorphism

AA, amino acid; NT, nucleotide; UTR, untranslated region.

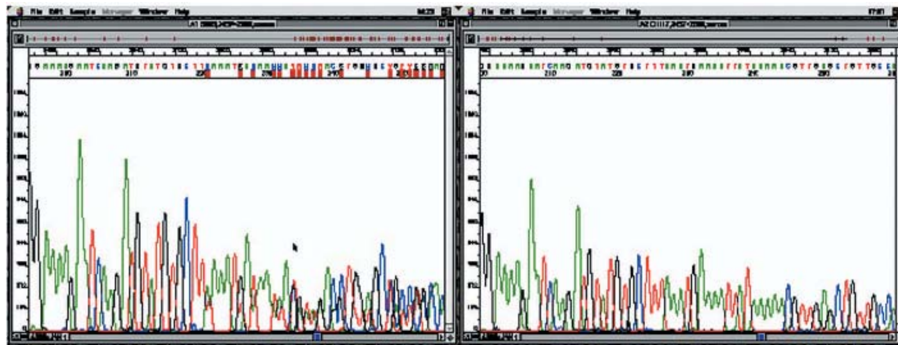

Figure 4 Sequencing traces of BRCA2 exon 11 of a 30 year old patient with early onset breast cancer, showing the 2699delTAAATG mutation on the left, with the normal control on the right.

100%[17]) compared with SCCP and heteroduplex analysis. Additional advantages of this methodology are the possibility of optimising the analysis by computer simulation and the non-radioactive approach. In DGGE, during electrophoresis, double stranded DNA amplified by PCR migrates through a gel containing an increasing concentration of denaturant (urea and formamide). As double stranded DNA is electrophoresed through the denaturing gradient, it will melt and change its conformation in such a way that the mobility of the molecule is dramatically reduced. To prevent complete strand dissociation and to facilitate the detection of mutations in the higher melting domains, a GC rich fragment (GC clamp) is introduced during fragment amplification. The GC clamp increases the percentage of single base changes detectable by DGGE, theoretically to 100%.[18 19] In principle, four bands are detectable in a heterozygous state after denaturation and renaturation, corresponding to the two homodimers and two heterodimers. Although DGGE has been applied to BRCA mutation screening before,[20–22] we used a new technique based on DGGE after exon by exon PCR amplification of the complete BRCA1 and BRCA2 sequences,[12] and sequenced aberrantly moving bands. This yielded four polymorphisms consisting of single nucleotide substitutions, underlining the sensitivity of the method. Intelligent pooling of the amplicons before electrophoresis greatly improved the throughput of the method.

Double gradient DGGE is based on the combination of two linear gradients, a primary denaturing gradient (urea and formamide) and a collinear secondary porosity gradient (polyacrylamide).[23] This secondary gradient suppresses band broadening during electrophoresis and thus improves the resolution of the DGGE banding pattern. Hayes *et al* compared double gradient DGGE gels with various porosity gradients to a standard 9% polyacrylamide gel, and showed that mutations with different melting profiles cannot be appropriately detected using a single DGGE condition.[15]

PTT is a widely applied screening technique,[5] but is especially helpful for known mutations in particular populations, and less suitable as a primary screening approach for new mutations in unknown populations. Furthermore, PTT only detects mutations that result in stop codons and lead to premature termination of translation, thereby producing truncated proteins. A possible advantage of PTT is that it conveniently misses harmless polymorphisms. PTT is usually only applied for detecting mutations in exon 11 of the BRCA1 gene and exons 10 and 11 of the BRCA2 gene, which account for more than 60% of the coding sequence. However, mutations are distributed throughout the entire coding sequence, with no apparent clustering or hot spots.[24]

"Intelligent pooling of the amplicons before electrophoresis greatly improved the throughput of the method"

More than 1893 distinct germline BRCA2 mutations have been found to date (http://www.research.nhgri.nih.gov/projects/bic), and the number is expected to increase further. The pattern of mutations is similar to that seen in BRCA1 and in many other tumour suppressor genes. At

Table 6 Clinicopathological data of the patients with breast cancer showing BRAC1/2 mutations and/or polymorphisms

Patient	Age	Tumour type	Tumour size	TNM	Stage	Lymph nodes positive	Pregnancies	Children	Contraceptives
A	44	Ductal	2	T2N0M0	II A	0/11	0	0	None
B	30	Ductal	1	T1N0M0	I	0/10	0	0	None
C	39	Ductal	3	T2N0M0	II A	0/10	5	3	IUD
D	30	Ductal	2.5	T2N0M0	II A	0/10	2	2	Oral contraceptives for 4 years, IUD

IUD, intrauterine device.

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Take home messages

- Four BRCA1/2 polymorphisms and one novel BRCA2 mutation were found in a group of Indonesian patients at high risk of hereditary breast cancer using pooled denaturing gradient gel electrophoresis and targeted sequencing
- This new and high throughput method is sensitive and particularly suited for screening unknown populations

present, all mutations that clearly cause disease result in premature termination of translation or absence of a transcript. Approximately 75% of truncating mutations are small deletions, 15% are small insertions, and 10% are base substitutions leading directly to termination codons (http://www.research.nhgri.nih.gov/projects/bic).[25–27] In addition, the novel 2699delTAAATG mutation described in our present paper leads to a premature stop codon. The patient with this mutation had early onset breast cancer and no family history of cancer. It has been suggested that the probability of harbouring a BRCA2 mutation among patients with early onset breast cancer is relatively independent of a positive family history for the disease.[25]

Mutations in the BRCA2 gene have been found mainly in families with a high incidence of female and male breast cancer,[4 26 28 29] whereas the risk of ovarian cancer is lower than in BRCA1 families.[4 26 30 31] Loss of heterozygosity has been found in 30–45% of sporadic breast tumours,[32 33] indicating a role for BRCA2 also in sporadic breast tumours, although very few somatic mutations have been reported.[24 34 35] Other tumour types seen repeatedly in BRCA2 families include those of the prostate, larynx, pancreas, and colon.[36 37]

In conclusion, we describe a novel BRCA2 mutation and four polymorphisms in the BRCA1/2 genes found in the Indonesian population using a new high throughput and highly sensitive method to screen BRCA1 and BRCA2 for mutations based on exon by exon PCR amplification followed by pooled DGGE and sequencing of aberrant bands. This is an ideal approach for screening populations for unknown mutations, and has the ability to detect single base differences using non-toxic and relatively simple and inexpensive methods.

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The patients gave full consent for their details to be published.

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