

susceptible to certain infections than whites. For example, interindividual differences in susceptibility to infection with *C. pneumoniae* have been suggested to be determined by genetic variation in *MBL* (5, 8). Several lines of experimental evidence have implicated infection with *C. pneumoniae* as a factor that contributes to atherosclerosis (8, 12). If the *MBL* allele frequencies in the Inuit actually conferred resistance to *C. pneumoniae* infection, this might explain in part their apparent resistance to cardiovascular disease. Alternatively, the associations may have been related to linkage disequilibrium with other structural differences in *MBL* or at a linked locus, which would have provided the mechanistic basis for the associations.

The high prevalence of the *MBL A* allele and the low prevalence of the *MBL B, C*, and *D* alleles among the Inuit could have been the result of either a distant founder effect or selection. For example, the Inuit probably had been relatively disease free before contact with Europeans because the Bering Strait "cold-screen" eliminated many pathogens (13). However, after contact with Europeans, the introduction and spread of certain infectious diseases might have led to selection of the *MBL B, C*, and *D* alleles out of the Inuit population. A possible genetic association between *MBL* variation and the susceptibility to infections among the Inuit needs to be explored. Findings in Greenland Inuit are also consistent with these observations (14, 15).

Although the basis for the distinctive genetic architectural features of Canadian Inuit may never be determined, these people have significant differences in *MBL* allele frequencies compared with whites. Given the increasing interest in infectious causes for atherosclerosis and cardiovascular disease endpoints, it is reasonable to consider the possibility that interindividual differences in genetic susceptibility to infections may contribute to differences in the expression of cardiovascular endpoints. Our results, specifically the high frequencies of the resistant *MBL A* allele and *A/A* genotype and the low frequencies of the other *MBL* alleles in the Inuit, are thus consistent with the well-documented low prevalence of cardiovascular disease in these people. However, the findings are indirect and the relationship between these polymorphisms and atherosclerosis can only be validated in disease association studies.

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Simple and Rapid Detection of *BRCA1* and *BRCA2* Mutations by Multiplex Mutagenically Separated PCR, Pak Cheung R. Chan,^{1*} Betty Y.L. Wong,² Hilmi Ozcelik,^{1,3} and David E.C. Cole^{1,2,4†} (Departments of ¹Laboratory Medicine and Pathobiology and ⁴Medicine and Pediatrics (Genetics), University of Toronto, Toronto, Ontario, Canada M5G 1L5; ²Genetic Repository, Toronto General Hospital, Toronto, Ontario, Canada M5G 2C4; ³Department of Pathology and Laboratory Medicine and Centre for Cancer Genetics, Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada M5G 1X5; * Pak Cheung Chan is a recipient of a postdoctoral fellowship in Clinical Chemistry and Laboratory Medicine from the Ontario Ministry of Health, Canada; † address correspondence to this author at: Department of Laboratory Medicine and Pathobiology, Rm. 402, Banting Institute, 100 College St., University of Toronto, Toronto, Ontario, Canada M5G 1L5; fax 416-978-5650, e-mail davidec.cole@utoronto.ca)

BRCA1 and *BRCA2* are tumor suppressor genes that are inactivated during neoplastic development (1, 2). Germline mutations of the two genes are transmitted in the autosomal dominant fashion and predispose carriers to the development of ovarian and/or breast cancers (3, 4). Mutations in *BRCA1* are present in approximately one-half of the early-onset breast cancer families and 80% of the early-onset breast and ovarian cancer families (5), whereas *BRCA2* mutations are believed to account for a comparable percentage of inherited breast cancer cases (6). Women with germline mutations in *BRCA1* have a lifetime risk of 85% and up to 50% for breast and ovarian cancers, respectively.

Table 1. Nucleotide sequences of the primer sets.

Primer	Primer sequence ^a	Size of amplicon
<i>BRCA1</i> 185delAG		
Common forward (P1)	5'-ggttggcagcaatatgtgaa	
Wild-type reverse (P2)	5'-gctgacttaccagatgggactctc	335 bp
Mutant reverse (P3)	5'-cccaaattaatacactcttgc <u>g</u> tgcacttaccagatgggacagta	354 bp
<i>BRCA1</i> 5382insC		
Common reverse (P4)	5'-gacgggaatccaaattacacag	
Wild-type forward (P5)	5'-aaagcgagcaagagaatcgca	271 bp
Mutant forward (P6)	5'- aatcgaagaaaccaccaaa <u>g</u> tccttagcgagcaagagaatcacc	295 bp
<i>BRCA2</i> 6174delT		
Common reverse (P7)	5'-agctggtctgaatgttcttact	
Wild-type forward (P8)	5'-gtgggatttttagcacagctagt	151 bp
Mutant forward (P9)	5'-cagtctcatctgcaaatactcagggatttttagcacagcatgg	171 bp

^a Mismatched bases are underlined.

In individuals of Ashkenazi Jewish background, mutations such as 185delAG and 5382insC in *BRCA1*, and 6174delT in *BRCA2* are present in higher frequencies than other mutations because of founder effects (6–8). In one study, the three mutations accounted for 62% of Ashkenazi patients with ovarian and/or breast cancer (7). The high frequency of these mutations in the *BRCA* genes indicates their potential role in identifying individuals at risk.

Many methods have been reported for the study of *BRCA* mutations, including allele-specific oligonucleotide hybridization (8, 9), allele-specific PCR (10), PCR-mediated site-directed mutagenesis (11, 12), heteroduplex analysis (HDA) (13–15), single-strand conformation polymorphism (14, 16), and the protein truncation test (14, 15). Methods such as HDA, single-strand conformation polymorphism, and the protein truncation test, which are suitable for screening uncharacterized mutations, are

relatively more laborious, whereas allele-specific PCR, PCR-mediated site-directed mutagenesis, and allele-specific oligonucleotide hybridization are more efficient for screening of specific characterized mutations.

Here we describe a simple and rapid method for the simultaneous detection of three common mutations: 185delAG and 5382insC in *BRCA1*, and 6174delT in *BRCA2*. Blood samples were obtained with written informed consent. DNA was extracted from peripheral blood cells as described previously (17), coded, and analyzed in a blind fashion. Allele-specific oligonucleotide primers were designed on the basis of published *BRCA1* and *BRCA2* sequences (18, 19) and checked against the NIH database (<http://www.ncbi.nlm.nih.gov/Blast/>) for possible false priming. For each mutation, three primers (one common, one specific for the mutant, and one specific for the wild-type allele) were used. The competing mutant and wild-type primers were designed to differ by ~20 bp in size, allowing easy detection of the PCR products by routine electrophoresis and ultraviolet illumination after ethidium bromide staining. The mutant (long) and wild-type (short) primers both contain a mismatched base sequence near the 3' end. In the early cycles of amplification, the mismatched sequences generate mutagenized PCR products that are refractory to cross-amplification by the competing primer, thereby ensuring specificity of the reaction (20). The long (mutant) primer also incorporates two additional mismatched bases at two contiguous positions corresponding to the 5' end of the short (wild-type) primer. During the final cycles of the PCR reaction, heteroduplexes may be formed from the short and long products, but the contiguous mutagenized sequences in the long product prevent filling up of the short product by using the long strand as template. As a result, the mutant and wild-type products are separated mutagenically. The primer sequences and sizes of corresponding amplicons are shown in Table 1.

PCR amplification was carried out using the GeneAmp[®] PCR 2400 system from Perkin-Elmer. In each PCR reaction, 25 ng of genomic DNA was added to 10 μ L of reaction mixture consisting of 1 \times PCR reaction buffer (10

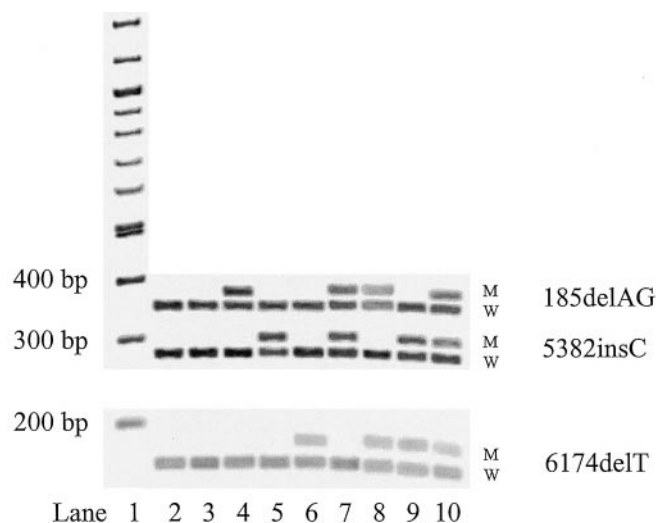


Fig. 1. Electrophoretogram of MS-PCR products.

Lane 1, 100-bp ladder; lanes 2 and 3, wild-type samples; lanes 4–6, patient samples with the 185delAG, 5382insC, and 6174delT mutations, respectively; lanes 7–9, equal volumes of 185delAG + 5382insC, 185delAG + 6174delT, and 5382insC + 6174delT, respectively; lane 10, equal volumes of each of the three mutations. M, mutant product; W, wild-type product.

mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 0.01 g/L gelatin), 3.25 mmol/L MgCl₂, 0.2 mmol/L dNTPs, and 50 kU/L AmpliTaq Gold™ (all from Perkin-Elmer). Allele-specific primers were added at 2.0 μmol/L for P1 and P3; 0.4 μmol/L for P2; 0.12 μmol/L for P4, P5, and P6; 0.31 μmol/L for P7 and P9; and 0.24 μmol/L for P8. Each PCR reaction consisted of an initial 12 min of AmpliTaq Gold activation at 95 °C, followed by 35 cycles of 15 s of denaturation at 94 °C, 15 s of annealing at 57 °C, and 30 s of extension (with an increment of 1 s for each subsequent cycle) at 72 °C, and a final extension step of 5 min at 72 °C. At the conclusion of the reaction, the PCR product was mixed with 2 μL of loading dye (200 g/L sucrose, 0.05 g/L bromophenol blue) and then separated on precast Clearose BG Mini (9 × 12 cm) Gels (Elchrom Scientific) by routine submerged electrophoresis (120 V, 80 min). The resolved amplicons were then stained with 0.5 mg/L ethidium bromide and viewed under ultraviolet illumination.

Like other PCR techniques, mutagenically separated PCR (MS-PCR) requires careful optimization of each reaction condition, including magnesium concentration, cosolvents (dimethyl sulfoxide or glycerol), and length and temperature of cycling stages. More importantly, the concentrations (relative and absolute) of individual primers must be determined empirically to give relatively equal amplification of the wild-type and mutant alleles. If a mutation is present in one of the alleles, two bands will be present. For the 185delAG mutation, the mutant and wild-type amplicons are 354 and 335 bp, whereas those of 5382insC are 295 and 271 bp, and those of 6174delT are 171 and 151 bp. Therefore, a minimum of three bands (absence of any mutant allele) and a maximum of six bands (all three mutations) can be detected. Fig. 1 shows the electrophoretogram with patterns for zero to three mutations present in a single sample. Because at least one of the two allelic PCR products is present, MS-PCR provides an intrinsic quality control against false negatives or PCR refractory conditions. The presence of wild-type and mutant allelic products also allows easy and objective interpretation of test results.

We evaluated this assay using a cohort of 66 Ashkenazi Jewish women at an increased risk for breast/ovarian cancer (all have a blood relative diagnosed with epithelial ovarian cancer). The genetic status of this cohort of patients had previously been ascertained by HDA (15) and DNA sequencing. Using the optimized procedure, we identified 7 individuals with the 185delAG mutation, 4 with the 5382insC mutation, 3 with the 6174delT mutation, and 52 without any of the three mutations. The results were identical to HDA and DNA sequencing, indicating the reliability of MS-PCR technique. Overall, the multiplex approach allows easy and simultaneous detection of the three mutations. This assay eliminates the need for radioisotopes, endonuclease digestion, and high-resolution electrophoresis. We conclude that this method is simple, reliable, and can be considered for routine use.

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