MULTIPLEX-PCR GENERATES FALSE POSITIVES IN DETECTION OF THE BRCA1 185delAG RECURRENT MUTATION

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Abstract : Breast and ovarian cancer are common pathologies in women, with increasing incidences worldwide. In hereditary breast and ovarian cancer (HBOC) families, a large percentage of cases are attributable to hereditary factors compatibles with dominant autosomal transmission of a major tumour suppressor gene with incomplete penetrance. Screening for BRCA1 mutations is now standard practice for HBOC cases in western world, and permits medical follow-up and genetic counselling. Over 300 BRCA1 germinal mutations are stored in the Breast Cancer Information Core (BIC) mutation database. 185delAG, an Ashkenazi founder mutation, is a recurrent BRCA1 mutation in eastern European populations. Several screening methods were proposed for detection of 185delAG. We demonstrate that one screening methods generates false positives by unspecific amplifications, therefore being inappropriate for molecular diagnosis.

INTRODUCTION

Breast and ovarian cancer are very common pathologies in women, with increasing incidences worldwide including Romania [8,12,16]. In hereditary breast and ovarian cancer (HBOC) families, a large percentage of cases are attributable to hereditary factors compatibles with dominant autosomal transmission of a major tumour suppressor gene with incomplete penetrance [6]. Germinal mutations in BRCA1 gene are responsible for about 45% of families with increased incidence of breast cancer and of over 80% of families with significantly increased incidence of both early-onset breast and ovarian cancer [8]. Screening for mutations in these two genes is now standard practice for HBOC cases in Western Europe and North America, and permits medical follow-up and genetic counselling adapted to the needs of individuals in such families. The consequences of germ-line mutation of either of the BRCA genes are serious: by 50 years of age, 45% of BRCA1 carriers and 20% of BRCA2 carriers will have already developed breast cancer, compared to about 3% of non-carriers [15]. Over 300 BRCA1 and 100 in BRCA2 germinal inactivating mutations were found, most of them causing the lost of function by premature protein synthesis termination [14]. BRCA1 recurrent founder mutations were found in cancer cases and healthy patients from HBOC and non-HBOC families [11], especially three known founder mutations (300T>G, 185delAG and 5382insC). These three mutations were previously described as Ashkenazi Jewish founder mutations, also found frequently in general population [3]. Eastern European studies revealed a high frequency of the 185delAG mutation [2,4,7,13]. Several rapid and cheap pre-screening methods were developed for the detection of 185delAG. Here we describe the dangerous diagnosis of false positives by one multiplex-PCR method previously described [3].

MATERIAL AND METHODS

HBOC families were recruited when three or more breast or ovarian cancer cases were diagnosed within the same family line. Situations with less then three cases were considered for cancer cases were diagnosed before age 40, for breast and ovarian cases in the same family, for breast cancers in men, for bilateral breast cancer cases and for any medullar breast cancer. All patients agreed by written informed consent. Familial anamneses were drawn and recruited patients were included on a BRCA study list.

Genomic DNA was extracted from 10 ml peripheral blood by optimization of the Wizard™ Genomic DNA purification kit (Promega Inc, Madison, WI, USA). Two samples of 5 ml were processed in parallel, and DNA was eluted in 1 ml TE Buffer. After appropriate dilutions in 50 µl, DNA amount was estimated by spectrophotometry, using the DU800 spectrophotometer (Beckman Coulter Inc, Fullerton, CA, USA). Multiplex-PCR was optimized and performed for detection of the recurrent 185delAG BRCA1 mutation, using as reference method the 4-primers multiplex-PCR [Fitzgerald et al., 1996]. PCR was performed in a final volume of 50 µl, using variable quantities of reagents following optimization steps. Amplifying program was also optimized alternatively on thermocyclers as Mastercycler™ Gradient (Eppendorf AG, Hamburg, Germany) or PalmCycler™ (Corbett LifeSciences / Qiagen, Germantown, MD, USA). Amplification aliquots were migrated, using the Sub-Cell™ System for Submerged Horizontal Electrophoresis (Bio-Rad Laboratories Inc, Hercules, CA, USA) at 5V/cm on 2% or 3% agarose gels containing 0,5 µg ethidium bromide for 1 ml gel solution. Following electrophoresis, gels were visualised under UV in a G:BOX Chemi™ Gel Documentation System (Syngene, Cambridge, UK) and interpreted with GeneSnap™ and GeneTools™ software.

RESULTS AND DISCUSSION

Several recurrent BRCA1 mutations (185delAG, 5382insC, or 300T>G) are proved to be responsible for a majority of HBOC families in eastern European populations. The185delAG founder Ashkenazi mutation represents a deletion of two successive A and G nucleotides within BRCA1 exon 2. In the HUGO approved systematic nomenclature [5], where nucleotide numbering starts with A of initiator ATG in position 1, the mutation is generating a frameshift in codon 23 and a premature termination 17 amino acids downstream. Hence, the correct event happening is in fact the c.68_69delAG. The obsolete nomenclature system, actually widely used in ancient literature and by mutation databases as the BIC [1], uses to start numbering 119 nucleotides 5'UTR upstream the initiation ATG codon, therefore considering there is a 185delAG alteration. As a convention, we will systematically refer to HUGO nomenclature, using NCBI Genbank reference sequences U14680 for coding sequence, NC_000017.9 for genomic sequence and NP_009227.1 for protein sequence. Though, we'll keep the 185delAG appellation as overwhelming literature data is using it.

In order to quickly screen for 185delAG heterozygous presence in our HBOC patients, we chose a 4 primers multiplex-PCR method previously described [3]. In the BRCA1 sequence below (issued from NC_000017.9 genomic sequence), the bold letters represent exon2, containing the underlined initiator ATG. Nucleotides A and G, deleted when 185 delAG (c.68_69delAG) mutation is present, are caps underlined.

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1261 aaaatgataa aatgaagttg tcatttttata aacctttaa aaagatatat atatatgttt
1321 ttctaatgtg ttaaagttca ttggaacaga aagaaatgga tttatctgct cttcgcgttg
1381 aagaagtaca aaatgtcatt aatgctatgc agaaaatctt agAGtgtccc atctggtaag
1441 tcagcacaag agtgtattaa tttgggattc ctatgattat ctcctatgca aatgaacaga
1501 attgacctta catactaggg aagaaaagac atgtctagta agattaggct attgtaattg
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The multiplex-PCR method uses 4 primers in the same reaction in order to discriminate between wild-type and mutated allele, in virtue of generating several amplicons of different sizes depending of the normal or heterozygous status of the matrix DNA. As highlighted in figure 1, two distal control primers (forward P1 and reverse P2) generate a 242 (wild-type) or 240 (mutated) base pairs fragment. Two other primers compete for matrix allele in order to amplify differentiable fragments. P3 is a forward wild-type specific primer, which amplify, together with reverse P4 a 118 bp fragment, corresponding to the wild-type allele. P2 is a reverse mutation-specific primer which will generate, together with forward P1, a 168 bp fragment corresponding to the 185delAG allele. If P2 and P3 primer competition is hold in, when putting together all 4 primers we should discriminate between a normal wild-type individual (242 + 118 bp fragments profile) and a 185delAG heterozygous patient (240 + 168 + 118 bp fragments profile).

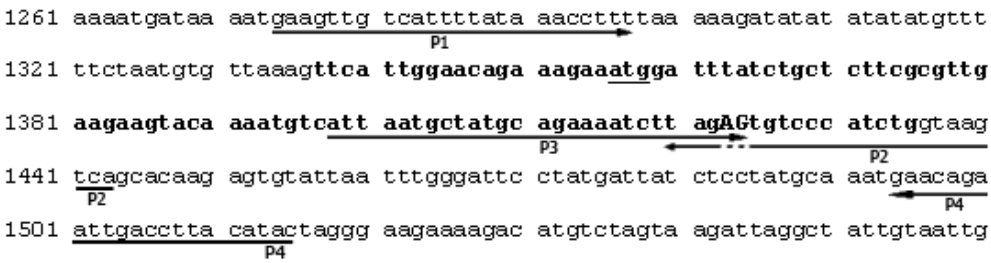

Figure 1. Distribution of 4 primers along a genomic sequence sparing BRCA1 exon 2.

The optimization steps comprised all experiences requisite in order to prepare an efficient final protocol, adapted to local equipment and working conditions. Each time a new technique is implemented or when working background changes, each research step requires a deepened and recurring analysis in order to establish an optimal efficiency of all reaction parameters within the new conditions. Each PCR reaction has its own optimal parameters, but times and often these parameters are comprised within functional intervals. Inside such interval, each parameter may slightly vary without perturbing the global efficiency of the reaction; therewith, modifying simultaneously several parameters within the limits of the functional interval can shift the whole reaction to failure. Time and again, researchers who do not manage to make a functional PCR reaction locate at a borderline of the functional interval, where one only parameter should be changed; modifying any other parameter pushes the reaction far away from the optimal conditions. This problem is even more complex when using multiplex-PCR, with different functional intervals for each amplification, and with global parameters which have to comply with all involved reaction parameters. During the optimization of our 4-primers multiplex-PCR reaction, we managed to find out the optimal conditions for each of the following parameters.

Genomic DNA quality and purity was checked following extraction by spectrophotometry. Different amounts (50-500 ng) of DNA were used for amplification. We identified a threshold of 100 ng genomic DNA to use for all amplifications to take place simultaneously. With less then 100 ng, only the control amplification (P1+P4) generates a detectable band signal. The quantity of each primer was also optimized, taking into account the possible competition between P2 and P3, as explained further. Also, in order to avoid the primer dimmer apparition, quantities of each primer, depending on magnesium concentrations, were finally situated between 50 and 100 pmoles (1-2 µM in the reaction volume). We established the final dNTP concentration at 0,2 mM each, PCR reaction buffer at 1X, we chose to use 1,25 TaqPolymerase units for each reaction.

Important parameters involved in PCR efficiency concern cycling conditions, including annealing temperature, duration of each amplification step and number of cycles. To optimize a complete PCR program, numbers of options are available on modern thermocyclers, like gradient and increment possibilities, touch-down annealing or hot-start enzymatic activity. We managed to conceive a PCR program adapted to all primer combinations within our multiplex assay, comprising an initial denaturation step of 10 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C denaturation, 30 seconds on gradient temperature annealing, and 60 seconds at 72°C extension. A final extension step of 5 minutes at 72°C was completed by quick cooling and storage at 4°C. Optimal annealing temperature, generally depending on each primer Tm and on salt concentration, was identified by gradient temperature amplifications. Given primers length and base composition, a preliminary amplification program was generated for each primer pair separately, using the Optimase™ ProtocolWriter from Transgenomic™. Subsequently, a general recommended annealing temperature, respecting relative Tms of each primer, was established to be at 60°C. In order to test this temperature, a gradient amplification was conceived, using ± 4°C annealing conditions simultaneously for the same 4-primers multiplex PCR. This option is available on several thermocyclers, by unequal heat distribution along the thermo-block during only the annealing step. Hereby, samples annealed at different temperatures (Table I) can be migrated simultaneously in gel in order to find out the optimal temperature. Figure 2 reveals electrophoretic profiles of the samples annealed in Table I thermal conditions (60°C ± 4°C).

Table I. Effective temperatures of thermo-block loci, when using a 60°C ± 4°C temperature gradient.

Block	1	2	3	4	5	6	7	8	9	10	11	12
Annealing temperature (°C)	56	56,7	57,4	58,1	58,8	59,5	60,2	60,9	61,6	62,3	63	63,7

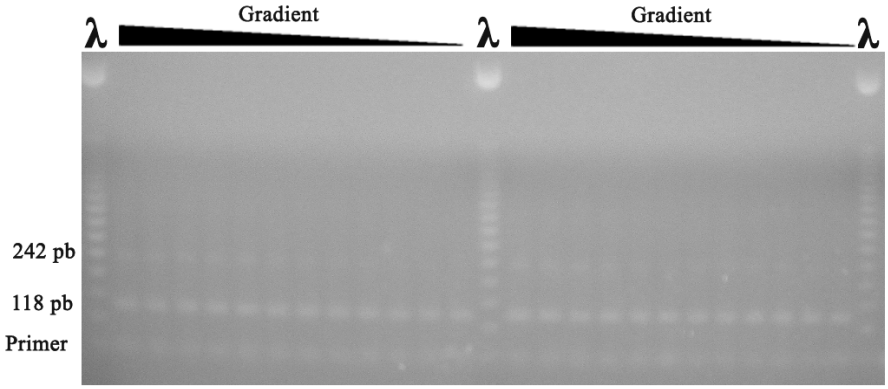

Figure 2. Electrophoretic profiles of the samples annealed in Table I thermal conditions (60°C ± 4°C). (λ = 50bp step ladder)

In figure 1 above, one can remark that the 118 bp fragment (P3+P4) is well amplified at any of temperatures ranged between 56 and 64°C, whereas the 242 bp fragment is amplified only at annealing temperatures lesser then 58°C. Both fragments are co-amplified at low temperatures, however we chose not to lower temperature more then necessary, and to continue the reactions at 58°C, which allows enough amplification conditions without nonspecific products apparition.

$MgCl_2$ concentration was varied between 1,5-3 mM, with considerable effects on reaction specificity. In fact, annealing specificity depends equally on primer sequence and primer Tm, value depending of salt concentration of the reaction medium. While magnesium is a necessary co-factor of DNA thermostable polymerase, its ions stabilize double stranded DNA, arising the relative Tm values. Little Mg^{2+} concentration generates more stringent DNA base pairing conditions during the annealing step, so that too little $MgCl_2$ may cause partial or total failure of amplification reactions. On the other side, high Mg^{2+} or Mn^{2+} concentration reduces DNA polymerase fidelity; too many $MgCl_2$ can generate unwanted nonspecific amplification products. Because free dNTP seize Mg^{2+} ions, their concentration should also be adapted to dNTP concentration. In our case, optimization showed that optimal $MgCl_2$ concentration has to be 1,5 μM, as explained below.

A controversial result is presented in figure 3. Lanes 1-3 correspond to awaited results for 4-primer multiplexing at 58°C, with obvious 118 bp and less intense 242 bp fragment band. In order to intensify the 242 bp signal, we tried (lanes 4 and 5) the same amplification using only primers P1 and P4, but adding a double quantity of Taq polymerase (2,5 units per reaction) and a maximum $MgCl_2$ concentration of 3 mM. Both modifications explain the high intensity of the obtained 242 bands. On the other side, an unexpected additional band appears upstream, corresponding to a DNA amplicon longer than 242 bp. Taking account that only 2 primers were used, the additional band obviously corresponds to a nonspecific unwanted product (highlighted with ? in figure 3), appearing intensively lane 4 (when 100 ng matrix DNA and 50 pmoles P1 and

P4 were used), whilst less intensive lane 5 (when 500 ng matrix DNA and 100 pmoles P1 and P4 were used). This would mean that lesser DNA and more Mg^{2+} are propitious factors for the apparition of nonspecific products.

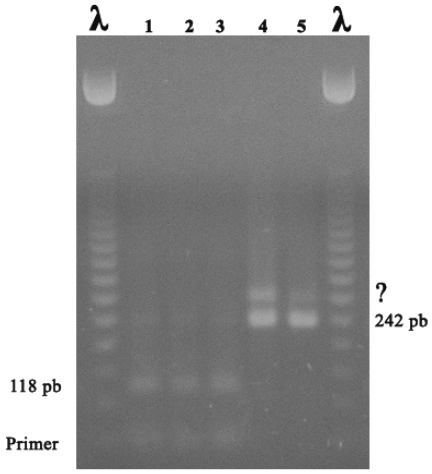

Figure 3. Multiplex-PCR with 4 primers (lanes 1-3) and 2 primers (lanes 4-5), by varying different parameters of the reaction. (λ = 50bp step ladder)

The final establishment of optimal conditions for multiplex 4-primers amplification were supposed to be determined by the following experiment, whose results are synthesized in figure 4. A non-template control lane 14 shows no contamination within our experiment. All reactions were performed at 58°C annealing temperature, value which permits the amplification of both expected fragments. The different reaction conditions for lanes 1-13 are presented in table II. We used a relative constant amount of genomic DNA around 100 ng, constant amounts (50 pmoles of P1 and P4), and variable amounts of Taq polymerase. Two reactions (lanes 2 and 4) were performed with higher magnesium concentrations, whereas two other reactions (lanes 11 and 13) were performed by adding primers P2 (50 pmoles) and P3 (100 pmoles). This strategy should allow amplification of all combinations available, putting serious chances in advantage of the P1-P2 mutant specific amplification. As we can see figure 4, a primer competition is confirmed lanes 11 and 13, where adding P2 and P3 pushed the reaction towards a 118 bp fragment amplification (P3-P4) instead of the previous 242 amplified lanes 1-10 and 12. Still, in lanes 11 and 13 both fragments are equally amplified, which confirmed the optimal conditions for a good multiplex-PCR.

Table II. Reaction conditions and reagent amounts for the reactions migrated in figure 4 (lanes 1-14)

Lane	ADN (patient)	Genomic DNA (ng)	Primers (pmoles)	Taq (UE/reaction)	$MgCl_2$ (mM)
1	00101AN	100	P1(50) + P4(50)	2,5	1,5
2	00101AN	100	P1(50) + P4(50)	2,5	3
3	00101AN	100	P1(50) + P4(50)	1,25	1,5
4	00101AN	100	P1(50) + P4(50)	1,25	3
5	00102ML	120	P1(50) + P4(50)	2,5	1,5
6	00201CN	100	P1(50) + P4(50)	2,5	1,5
7	00301NM	120	P1(50) + P4(50)	2,5	1,5
8	00601EF	100	P1(50) + P4(50)	2,5	1,5
9	00701AC	130	P1(50) + P4(50)	2,5	1,5
10	00801ET	120	P1(50) + P4(50)	2,5	1,5
11	00101AN	120	P1(50) + P4(50) + P2(50) + P3(100)	2,5	1,5
12	00101AN	80	P1(50) + P4(50)	2,5	1,5
13	00101AN	110	P1(50) + P4(50) + P2(50) + P3(100)	2,5	1,5
14	No template control	0	P1(50) + P4(50)	2,5	1,5

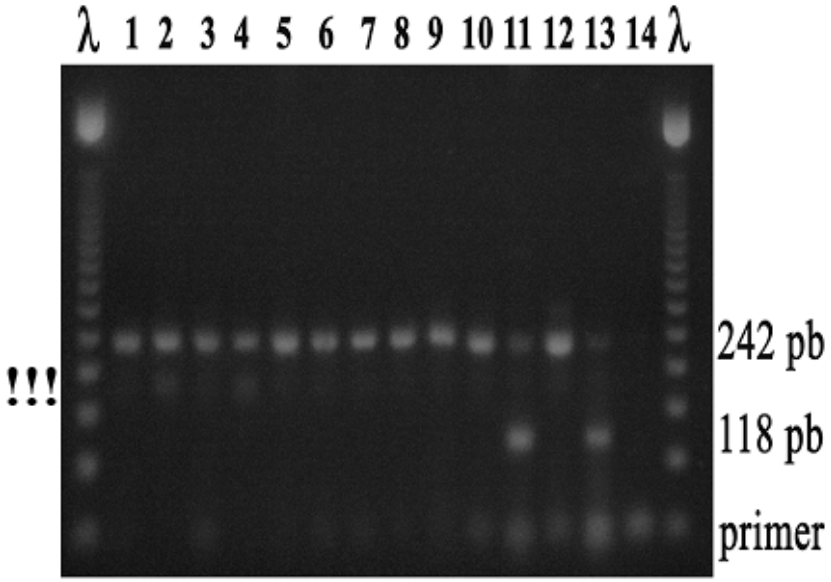

Figure 4. Multiplex-PCR using reagents and conditions synthesized in table II. (λ = 50bp step ladder)

One also can see in figure 4 that a little more magnesium concentration lead to the apparition (lanes 2 and 4) of another unexpected nonspecific product (highlighted with !!!). Although few intense, the bands corresponding to this product migrate somewhere between 150

and 200 bp, which is unfortunately exactly the size domain expected for the mutation specific amplicon (P1+P2) to migrate (168 bp). This nonspecific band appears when only P1 and P4 are used, in patients wild-type for the 185delAG mutation. What could happen in the case of mutated patients? The next optimization step should be testing of the optimal conditions for P1-P2 amplification, using a positive control patient. Still, if any product appears within the 150-200 bp domain, is this product the mutated 168 bp fragment, or is it merely another unspecific product due to temperature or magnesium concentration? Are we allowed to perform a risky diagnosis in those conditions?

CONCLUSIONS

After a long, meticulous, nerve-racking and time-consuming optimization, we managed to find out the optimal conditions for a simultaneous amplification of the 242 control and the 118 wild-type specific products, generating in this way a functional multiplex-PCR. Unfortunately, the event observed in figure 4 (lanes 2 and 4) is in fact a good example of false-positive. Often encountered in routine molecular analysis, this situation is blurring the whole multiplex technique, due to its dangerous false positive diagnosis. For this reason, the 4 primers multiplex-PCR detection of the BRCA1 185delAG mutation [3], was abandoned and should not be recommended as a good diagnosis method.

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