

Rapid detection of most frequent Slovenian germ-line mutations in BRCA1 gene using real-time PCR and melting curve analysis

Srdjan Novaković and Vida Stegel

Unit of Molecular Biology, Institute of Oncology Ljubljana, Slovenia

Background. Detection of inherited mutations in cancer susceptibility genes is of great importance in some types of cancers including the colorectal cancer (mutations of APC gene in familial adenomatous polyposis - FAP, mutations in mismatch repair genes in hereditary nonpolyposis colorectal cancer - HNPCC), malignant melanoma (mutations in CDKN2A and CDK4 genes) and breast cancer (mutations in BRCA1 and BRCA2 genes).

Methods. This article presents the technical data for the detection of five mutations in BRCA1 gene in breast cancer patients and their relatives. The mutations - 1806C>T, 300T>G, 300T>A, 310G>A, 5382insC - were determined by the real-time PCR and the melting curve analysis.

Results and conclusion. In comparison to direct sequencing, this method proved to be sensitive and rapid enough for the routine daily determination of mutations in DNA isolated from the peripheral blood.

Key words: breast neoplasms – genetics; genes, BRCA1; mutation; polymerase chain reaction

Introduction

General screening for unknown mutations in the large genes such as BRCA1 and BRCA2 is time consuming and expensive. There is a whole range of procedures that should be performed prior to the final confirmation of the mutation. Habitually, the screening is

started by using the methods that provide information about the region of the gene where the mutation is positioned (e.g. protein truncation test - PTT, single-strand conformational polymorphism analysis - SSCP, denaturing gradient gel electrophoresis - DGGE).¹⁻³ Direct sequencing and determination of specific changes in the nucleotide sequence aims at the final confirmation and identification of mutation.

Yet, when the mutation is well determined and precisely described, then the detection can be performed in a less complicated and less expensive manner. One group of these methods comprises the analysis based on the determination of differences in the melting

Received 6 April 2005

Accepted 20 April 2005

Correspondence to: Srdjan Novaković, PhD, Unit of Molecular Biology, Institute of Oncology, Zaloška 2, 1000 Ljubljana, Slovenia. Tel. + 386 1 522 5118; Fax: +386 1 433 74 10; E-mail: snovakovic@onko-i.si

temperatures of perfectly matched base pairs and mutated variants.^{4,6} This article reports the determination of known *BRCA1* mutations - 1806C>T, 300T>G, 300T>A, 310G>A, 5382insC using the real time PCR on Light Cycler and melting curve analysis. The system is programmed to monitor the melting curve analysis of the allele specific fluorescent resonance energy transfer - FRET probes after the PCR, allowing direct typing of the sample without any further processing.

Methods

DNA was isolated from the peripheral blood using the DNA blood isolation kit (Quiagen, Hilden, Germany). The primers and probe sets for the detection of specific mutations were designed in our laboratory applying the Light Cycler Probe Design Software, Version 1.0, and synthesized by TIB Molbiol (Berlin, Germany).

The real-time PCR and melting curve analysis at the Light Cycler instrument (Roche Molecular Biochemicals, Mannheim, Ger-

many) was applied for the detection of mutations. PCR was performed according to the manufacturer's instructions (Light Cycler Fast Start master hybridization probes, Roche Molecular Biochemicals). Briefly, the DNA templates were selectively amplified in the PCR reaction (annealing temperature 52°C, 45 cycles) using specific primers described in Table 1. Beside specific primers, the specific hybridization probes conjugated with LC Red640 (Light Cycler Red 640 fluorescent dye) were added to the mastermix. At the end of PCR reaction, the melting curve analysis was performed through heating the mix to 95°C for 1 min followed by cooling in steps (58°C, 48°C, 40°C, 35°C) to 35°C and repeated gradual heating to 85°C. The data were collected during the gradual heating phase (35°C - 85°C).

Results and discussion

Several cancers appear to be related to *BRCA1* and *BRCA2* mutations including breast, ovarian, pancreatic, prostate, fallopian tube, la-

Table 1. The primers and probes used for the detection of mutations in *BRCA1* gene.

Mutation	Primer and probe names	Type and length of nucleotide sequence*
1806C>T	<i>BRCA1</i> F	primer - forward (23bp)
	<i>BRCA1</i> A	primer - reverse (24bp)
	Senzor	probe - senzor-FL (30bp)
	Anchor	probe - LC Red640-anchor-PH (34bp)
300T>G 300T>A	<i>BRCA1</i> in4 F	primer - forward (22bp)
	<i>BRCA1</i> in5 B	primer - reverse (20bp)
	C61G Sen G	probe - senzor-FL (23bp)
310G>A	C61G Anch	probe - LC Red640-anchor-PH (32bp)
	<i>BRCA1</i> in4 F	primer - forward (22bp)
	<i>BRCA1</i> in5 B	primer - reverse (20bp)
	C64Y Sen G	probe - senzor-FL (29bp)
5382insC	C64Y Anch	probe - LC Red640-anchor-PH (30bp)
	<i>BRCA1</i> in19 F	primer - forward (21bp)
	<i>BRCA1</i> in21 S	primer - reverse (22bp)
	5382insC Sen	probe - senzor-FL (22bp)
	Ex20 Anchor	probe - LC Red640-anchor-PH (28bp)

*the sequences of primers and probes are available through E-mail of corresponding author
LC - Light Cycler Red 640 fluorescent dye, FL - Fluorescein, PH - phosphate

ryngeal cancer, as well as adult leukemias and lymphomas.⁷ However, women with germ-line heterozygous mutations in *BRCA1* or *BRCA2* are primarily at increased risk of developing breast or/and ovarian cancer. The mutations in *BRCA1* and *BRCA2* predict the likelihood of breast cancer development by the age of 70 years of 45% to 87% and 26% to 84%, respectively. The odds of ovarian cancer for *BRCA1* and *BRCA2* mutation carriers are 16% to 63% and 10% to 27%, respectively.⁸ Even though the breast and ovarian cancers related to inherited mutations in cancer susceptibility genes represent a small proportion of all cancers (less than 10%), it is of great importance for the clinician to detect the patients who are carrying these mutations. Consequently, the determination of potential risk among the family members of the mutation carrier can be estimated and prevention measures can be undertaken. The criteria for the genetic testing of patients and relatives are based on the most common risk factors in hereditary cancer syndromes – early age cancer onset, presence of the same cancer in multiple relatives, occurrence of multiple primary cancers in one individual, development of an unusual type of cancer (e.g. breast cancer in male), being a member of a specific ethnicity.^{9,10} Considering the facts, that

Slovenian population is ethnically relatively closed and that the breast cancer in Slovenia is the most common cancer type affecting women, we started, at the Institute of Oncology Ljubljana in 2001, with genetic counseling and testing of individuals from families with multiple histories of breast and ovarian cancer.

Laboratory of Medical Genetics - Vrije University Brussels, provided general mutation screening of *BRCA1* and *BRCA2* genes for Slovenian patients. From these results, it was evident that the most frequent mutations in hereditary breast (and ovarian) cancer were the Slovenian founder mutations IVS162A>G in *BRCA2* and 1806C>T, 300T>G, 300T>A, 310G>A, 5382insC in *BRCA1* gene. Almost 80% (precisely 77%) of all determined mutations in Slovenian patients were covered by this mutation profile. With the purpose of simplifying the detection of mutations in *BRCA1* gene, we applied the real-time PCR followed by melting curve analysis using hybridization probes. The primers and probes were designed on the sequences of *BRCA1* gene covering the reported mutations - 1806C>T, 300T>G, 300T>A, 310G>A, 5382insC. For each type of mutation, the optimal conditions were settled and the positive inner control was used.

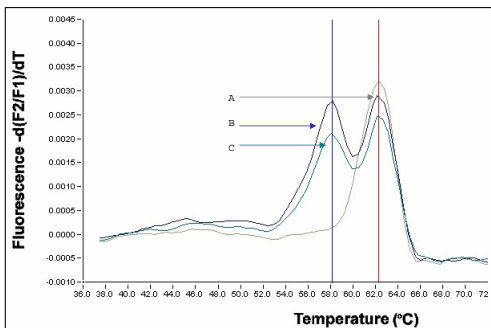


Figure 1. Detection of 1806C>T mutation in *BRCA1* gene by real-time PCR and melting curve analysis. DNA was isolated from the blood of a normal person and of a carrier of mutation. A - normal DNA (wild-type); B - positive control (mutant DNA); C - patient's DNA; wild type peak at 62°C; mutant peak at 58°C.

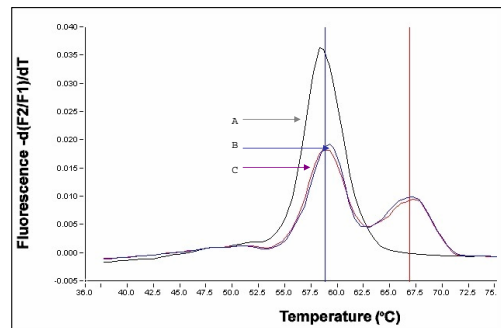


Figure 2. Detection of 300T>G mutation in *BRCA1* gene by real-time PCR and melting curve analysis. DNA was isolated from the blood of a normal person and of a carrier of mutation. A - normal DNA (wild-type); B - positive control (mutant DNA); C - patient's DNA; wild type peak at 58.5°C; mutant peak at 67°C.

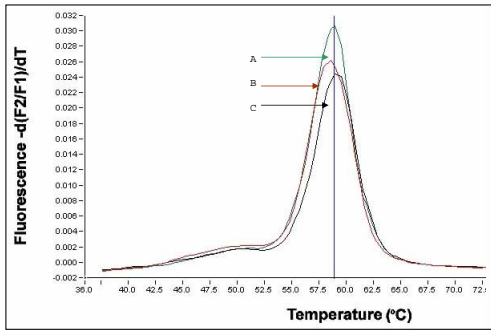


Figure 3. Detection of 300T>A mutation in *BRCA1* gene by real-time PCR and melting curve analysis. DNA was isolated from the blood of a normal person and of a carrier of mutation. A - normal DNA (wild-type); B - positive control (mutant DNA); C - patient's DNA; wild type peak at 58°C. Heterozygous mutant product for 300T>A mutation did not show any additional peaks.

The homozygous (wild-type) PCR product designed for the detection of 1806C>T on *BRCA1* gene showed a single peak at 62°C, whereas the heterozygous products (mutant) showed an additional peak at 58°C (Figure 1).

The homozygous (wild type) PCR product designed for the detection of 300T>G on *BRCA1* gene showed a single peak at 58.5°C, whereas the heterozygous products (mutant) showed an additional peak at 67°C (Figure 2). Unfortunately, the heterozygous product for 300T>A mutation did not show any addition-

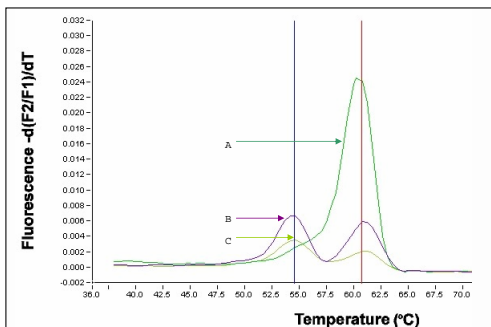


Figure 5. Detection of 5386insC on *BRCA1* gene by real-time PCR and melting curve analysis. DNA was isolated from the blood of a normal person and of a carrier of mutation. A - normal DNA (wild-type); B - positive control (mutant DNA); C - patient's DNA; wild type peak at 62.5°C; mutant peak at 67.5°C.

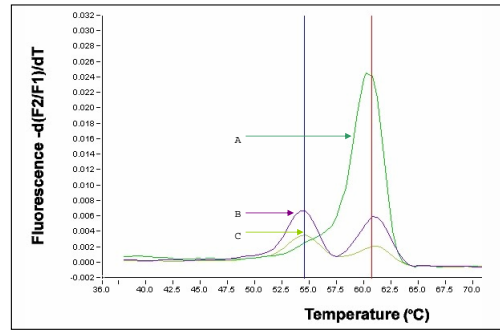


Figure 4. Detection of 310G>A on *BRCA1* gene by real-time PCR and melting curve analysis. DNA was isolated from the blood of a normal person and of a carrier of mutation. A - normal DNA (wild-type); B - positive control (mutant DNA); C - patient's DNA; wild type peak at 60.5°C; mutant peak at 54.5°C.

al peaks, thus making the mutation undetectable by this method (Figure 3). The reason for that was a too small difference (less than 2°C) between the melting temperatures of the wild type and mutated DNA sequence.

The homozygous (wild type) PCR product designed for the detection of 310G>A on *BRCA1* gene showed a single peak at 60.5°C, whereas the heterozygous products (mutant) showed an additional peak at 54.5°C (Figure 4).

The homozygous (wild type) PCR product designed for the detection of 5386insC on *BRCA1* gene showed a single peak at 62.5°C, whereas the heterozygous products (mutant) showed an additional peak at 67.5°C (Figure 5).

The majority of mutations detected with the real-time PCR and melting curve analysis were further subjected to direct sequencing. The number of tested individuals and of mutation positive individuals is listed in Table 2. An absolute correlation between the direct sequencing and the real-time PCR and melting curve analysis was obtained – the real-time PCR and melting curve analysis actually gave no false positive outcomes. The patient's DNA that was negative for mutations by real-time PCR and melting curve analysis was not subjected to sequencing in all cases, which precludes any conclusions concerning the false negative results of the method.

Table 2. Mutations in Slovenian patients detected by real-time PCR and melting curve analysis.

Type of mutation	Number of tested patients	Number of detected mutations	No. LC positive/No. direct sequencing positive*
1806C>T	87+90	12	10/10
300 T>G	5	2	2/2
300T>A	5	0	0/2
310G>A	2	1	1/1
5382insC	3	2	2/2

* number of positive samples detected by real-time PCR and melting curve analysis using Light Cycler (LC)/number of samples confirmed to be positive by direct sequencing

However, positive misleading results may be obtained because of unknown polymorphisms or a mutation within the target region affecting hybridization of probes and binding of primers, and consequently, the melting temperature of the product. It should also be mentioned that careful optimization of the reaction for each mutation is necessary before the analysis of the patients' DNA samples is performed in order to achieve an optimal sensitivity and specificity for the method. Especially important is to bear in mind that not only the physical features during the reaction, but also the nucleotide structure in the dimmer (e.g. higher melting temperature of the fragments that have higher percentage of GC) and the length of the dimmer fragment (the melting temperature of longer fragments is higher) affect the melting temperature. In this study, the PCR was optimized in respect to annealing temperature, concentration of Mg²⁺, and number of polymerization cycles; the reaction during the melting curve analysis was optimized in respect to the time and temperature differences in the intervals of cooling and heating steps.

In view of the present results, it could be concluded that the primers and probes for the detection of 1806C>T, 300T>G, 310G>A and 5382insC mutations in *BRCA1* gene were designed successfully. After the optimization, the real-time PCR and melting curve analysis using hybridization probes showed out to be an extremely sensitive method for the detection of known mutations. Even though the designed primers and probes were specific

for the mutation 300T>A, the method was not sensitive enough for this type of mutation. However, for the final conclusions about the sensitivity and specificity of the method, a larger number of samples should be included. Prior to this, possible false negative and positive results should be taken in consideration.

Acknowledgment

The study was supported by the Ministry of Education, Science and Sport of the Republic of Slovenia, grant number J3-3501 and J3-6363.

References

1. Roest PAM, Roberts RG, Sugino S, van Omen GJB, den Dunnen JT. Protein truncation test (PTT) for rapid detection of translation-terminating mutation. *Hum Mol Genet* 1993; **2**: 1719-21.
2. Hayashi K, Wenz HM, Inazuka M, Tahira T, Sasaki T, Atha DH. SSCP analysis of point mutations by multicolor capillary electrophoresis. *Methods Mol Biol* 2001; **163**: 109-26.
3. Myer RM, Lumelsky N, Lerman LS, Maniatis T. Detection of single base substitution in total genomic DNA. *Nature* 1985; **313**: 495-8.
4. Foy CA, Parkers HC. Emerging homogeneous DNA-based technologies in the clinical laboratory. *Clin Chem* 2001; **47**: 990-1000.
5. Wilhelm J, Pingout A. Real-time polymerase chain reaction. *Chembiochem* 2003; **4**: 1120-8

6. Gerard P, Pindolia MS, Worsham MJ. A rapid and sensitive approach to mutation detection using real-time polymerase chain reaction and melting curve analyses, using *BRCA1* as example. *Mol Diagn* 1999; **4**: 241-6.
7. DeVita TV, Hellman S, Rosenberg AS, editors. Principles and practice of Oncology 7th edition. Philadelphia: Lippincott Williams & Wilkins; 2005.
8. King MC, Marks JH, Mandell JB. Breast and ovarian cancer risks due to inherited mutations in *BRCA1* and *BRCA2*. *Science* 2003; **302**: 643-6.
9. Loman N, Johannsson O, Kristoffersson U, Olsson H, Borg A. Family history of breast and ovarian cancers and *BRCA1* and *BRCA2* mutations in a population-based series of early-onset breast cancer. *J Natl Cancer Inst* 2001; **93**: 1215-23.
10. Struwing JP, Hartge P, Wacholder S, Baker SM, Berlin M, McAdams M, Timmerman MM, Brody LC, Tucker MA. The risk of cancer associated with specific mutations of *BRCA1* and *BRCA2* among Ashkenazi Jews. *N Engl J Med* 1997; **336**: 1401-8.