Available online at www.sciencedirect.com
ScienceDirect
Clinica Chimica Acta 390 (2008) 141 – 144
www.elsevier.com/locate/clinchim

Short communication

Asymmetric real-time PCR detection of *BRCA1* 5382insC mutation by melting curve analysis in the LightCycler

Christos Kroupis [a,b], Kostantinos Christopoulos [a], Myrto Devetzoglou [a], Ioannis Tsiagas [a], Evi S. Lianidou [a,*]

[a] *Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Athens 15771, Greece*
[b] *Laboratory of Clinical Biochemistry, Attikon University Hospital, Medical School, University of Athens, Greece*

Received 11 December 2007; received in revised form 23 December 2007; accepted 23 December 2007
Available online 17 January 2008

Abstract

Background: 5382insC *BRCA1* frameshift mutation is a common founder mutation for many populations worldwide and a high-risk allele for the development of hereditary breast and/or ovarian cancer. Our goal was to develop a novel, reliable and rapid method for its detection.
Methods: We developed an asymmetric real-time PCR method with hybridization probes in the LightCycler. Genotyping was performed by melting curve analysis.
Results and conclusions: The developed method was in concordance with reference methods when tested in 85 peripheral blood and 107 tumor DNA samples from Greek breast and/or ovarian cancer patients. The described method proved to be simple, cost-effective, easy to perform and rapid enough for routine use as a screening method in high-risk families and especially in the Greek, Slavic and Jewish populations where 5382insC mutation is the most common *BRCA1* mutation.

Keywords: *BRCA1*; 5382insC mutation; Real-time PCR; Melting curve analysis; LightCycler

1. Introduction

The tumor suppressor gene *BRCA1* is located on chromosome 17 and is linked to hereditary breast and ovarian cancer. BRCA1 protein has a significant role in the signalling of DNA damage and in DNA repair. Mutations are widely distributed throughout the whole gene and confer to mutation carriers a lifetime risk of 82% for breast cancer and 54% for ovarian cancer [1,2].

Our group is involved in *BRCA1* mutation screening in Greece [3,4]. We have found that the single-base insertion 5382insC in exon 20 of *BRCA1* resulting in frameshift and a truncated protein, is the most common mutation of this gene among Greek breast and ovarian cancer families since it was found in 7 out of the 85 families that we have studied [3]. In the same group of patients, another four scattered *BRCA1* and another three scattered *BRCA2* mutations were also detected; therefore 5382insC represents the majority of *BRCA1* mutations in Greece. This mutation is also present in high frequencies in Eastern–South Eastern Europe and Ashkenazi Jews [5–7]. Up to now, 5382insC is the second most common mutation in the *BRCA1* gene according to the Breast Information Core (BIC) database http://research.nhgri.nih.gov/projects/bic (1063 hits, Dec07). So far only heterozygotes have been detected for this mutation; the mutation is located in a critical area of the corresponding protein resulting to the inadequacy of mutant homozygotes to survive. Here, we describe a novel, rapid and reliable method that is based on real-time PCR and melting curve analysis that can easily distinguish between wild-type and mutant 5382insC heterozygotes samples.

2. Materials and methods

2.1. Patients

After obtaining informed consent, peripheral blood from 85 index cases of breast and/or ovarian cancer families were obtained from various hospitals in

* Corresponding author. Tel.: + 30 210 7274319; fax: + 30 210 7274750.
E-mail address: lianidou@chem.uoa.gr (E.S. Lianidou).

0009-8981/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2007.12.024

Greece. These samples were analyzed by PTT (Protein Truncation Test) and DNA sequencing for the whole *BRCA1* gene as previously described [3]. Seven of these patients were found to be positive heterozygotes for the *BRCA1* 5382insC mutation (8.2%, 7/85). This sample set was used for the initial development of the real-time PCR method in the LightCycler and the heterozygotes served as appropriate positive controls. Then, the optimized method was applied for *BRCA1* 5382insC mutation screening of 107 consecutive breast cancer tissue samples without any knowledge of the family history and whose histopathological characteristics were described elsewhere [8].

2.2. Methods

DNA was extracted from peripheral blood and tissue samples by using the QIAamp DNA Mini kit (Qiagen, Germany). DNA purity and quantity were determined by absorbance readings at 260 and 280 nm with a spectrophotometer (Hitachi U-2000, Japan).

Real-time PCR and melting curve analysis were performed in the LightCycler instrument (Roche Applied Science, Germany) [9]. The following primers embracing *BRCA1* exon 20 were designed *in silico* with the Primer Premier software, synthesized by FORTH (Greece) and used without any purification (positions are given according to *BRCA1* accession number L78833 in GeneBank):

Forward primer: 5′-ATA TGA CGT GTC TGC TCC AC-3′ (nt 71,518–71,537)
Reverse primer: 5′-CTG CAA AGG GGA GTG GAA TAC-3′ (nt 71,749–71,729)

The labeled hybridization probes were designed and synthesized by TIB MOLBIOL (Germany). Two different sensor hybridization probes were used: one specific for the wild-type allele (wt-probe) and one specific for the mutant 5382insC allele (mut-probe). Both were designed to work along with a common anchor probe in two separate reactions; one termed wt-rxn and the other termed mut-rxn. Their sequences are:

Sensor wt-probe: 5′-CGA GCA AGA GAA TCC CAG GAC (fluorescein)-3′ (nt 71,655–71,675),
Sensor mut-probe: 5′-CGA GCA AGA GAA TCC CCA GGA C (fluorescein)-3′ (nt 71,655–71,675),
Anchor probe: 5′-(LC Red640): AAG GTA AAG CTC CCT CCC TCA AGT TGA Cp-3′ (nt 71,679–71,706)

Real-time PCR was performed in glass capillary tubes (Roche Applied Science). The amplification mixture of a total volume of 10 μL included 100 ng of genomic DNA as template, 0.5 U of Taq DNA Polymerase (DyNAzyme TM II DNA Polymerase, FINNZYMES, Finland), and final concentrations of 1× supplied buffer, 4 mM $MgCl_2$, 200 μM dNTPs (Invitrogen, USA), 0.15 μg/μL bovine serum albumin (Sigma, USA), 0.15 μM of either sensor probe and 0.15 μM of anchor probe. Final primer concentrations for the asymmetric real-time PCR were 0.2 μM for the forward and 1 μM for the reverse primer. Sterile H_2O was used to supplement up to 10 μL.

The cycling protocol consists of pre-incubation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 20 s and extension at 72 °C for 20 s. The temperature ramp rate was 20 °C/s. Emitted fluorescence was measured at the end of each annealing step at the F_2/F_1 channel. Immediately after amplification, melting curve analysis was performed on the LightCycler. The melting curve protocol included raising the temperature at 95 °C for 30 s, cooling to 40 °C for 2 min and slow heating to 75 °C at a rate of 0.1 °C/s, during which time fluorescence measurements were continuously collected in the F_2/F_1 channel. The plot of the fluorescence *vs* temperature (F_2/F_1 *vs* T) of the LightCycler software (version 3.5) is useful for the monitoring of a successful amplification and then after the melting curve, its first derivative ($-\mathrm{d}(F_2/F_1)/\mathrm{d}T$ *vs* T) is useful for the identification of wild-type and mutant alleles by their different peaks. Also, by spinning upside down the capillaries in eppendorf tubes, their content could be obtained and agarose electrophoresis could be performed for the verification of the purity and size of the amplified PCR product.

In this set of 107 tumor samples, another established conventional PCR technique for the detection of the specific mutation was used (PSM, PCR mediated site directed mutagenesis), in order to compare results. The primers for the PSM method were the same as previously described [10] and the restriction enzyme *MvaI* (MBI Fermentas, Lithuania) was used to identify the specific single cut created in the amplified PCR product of the mutant allele.

3. Results

Real-time PCR amplification for the *BRCA1* 5382insC detection proved quite robust. The PCR product was free from other by-products or primer-dimers and at its correct size (232 bp) as judged by agarose gel electrophoresis. By using asymmetric PCR (forward/reverse primer ratio: 1/5), we have increased the template copies for the probes to anneal and therefore the emitted fluorescence signal. Therefore, the sensitivity of the method was increased by allowing even a 60 pg DNA sample to be efficiently amplified, detected and genotyped in either reactions (compared to 600 pg of a reaction with 1:1 ratio). The efficiency of both reactions was very good and at least 1.88 ($n=3$, close to the ideal 2.00, $r=0.99$) as calculated by the slope of a standard curve created by dilutions of a sample of known concentration (wild-type sample for the wt-rxn, mutant for the mut-rxn).

During melting curve analysis with reactions containing either of the sensor probes, wild-type patients produced one peak while mutant heterozygotes two peaks, one for the wild-type allele and one for the mutant *BRCA1* 5382insC. In the wt-rxn, the wild-type patients produced a single peak at 64.24 °C (mean) while the heterozygotes produced an additional peak for the mutant allele at a lower temperature (60.08 °C). In the mut-rxn, the wild-type patients produced a single peak at 60.50 °C (mean) while the heterozygotes produced an additional peak at a higher temperature (66.08 °C). In the mut-rxn, difference in melting temperatures (ΔTm) between wild-type and mutant alleles was more obvious (5.58 compared to 4.06 °C, Table 1, Fig. 1).

In the breast and ovarian cancer families group ($n=85$), our method was able to identify all seven heterozygotes without mistyping any other sample. Then we proceeded with the tumor DNA group ($n=107$), where we were able to identify an additional heterozygote. This result is in agreement with other studies that have looked at the percentage of BRCA carriers in unselected breast cancer patients [11]. The tumor heterozygote sample in both reactions deviated from the 1:1 allele ratio (Fig. 1) as expected since the majority of the cancerous cells in the specimen might have lost the wild-type allele by one of the mechanisms implicated

Table 1
Peak Tm values from *BRCA1* wild-type and 5382insC heterozygote patients in both real-time PCR reactions (total 192 samples: 85 peripheral blood and 107 tumor DNAs)

	Melting temperature, Tm (°C), Tm±SD (CV%)			
Reaction	Wild-type peak ($n=192$)	5382insC peak ($n=8$)	ΔTm (95% C.I.)	P*
Wt-rxn	64.24±0.29 (0.45)	60.08±0.32 (0.53)	4.16 (3.95–4.37)	<0.001
Mut-rxn	60.50±0.19 (0.32)	66.08±0.31 (0.47)	5.58 (5.44–5.72)	<0.001

*Student's *t*-test, C.I. = confidence intervals.

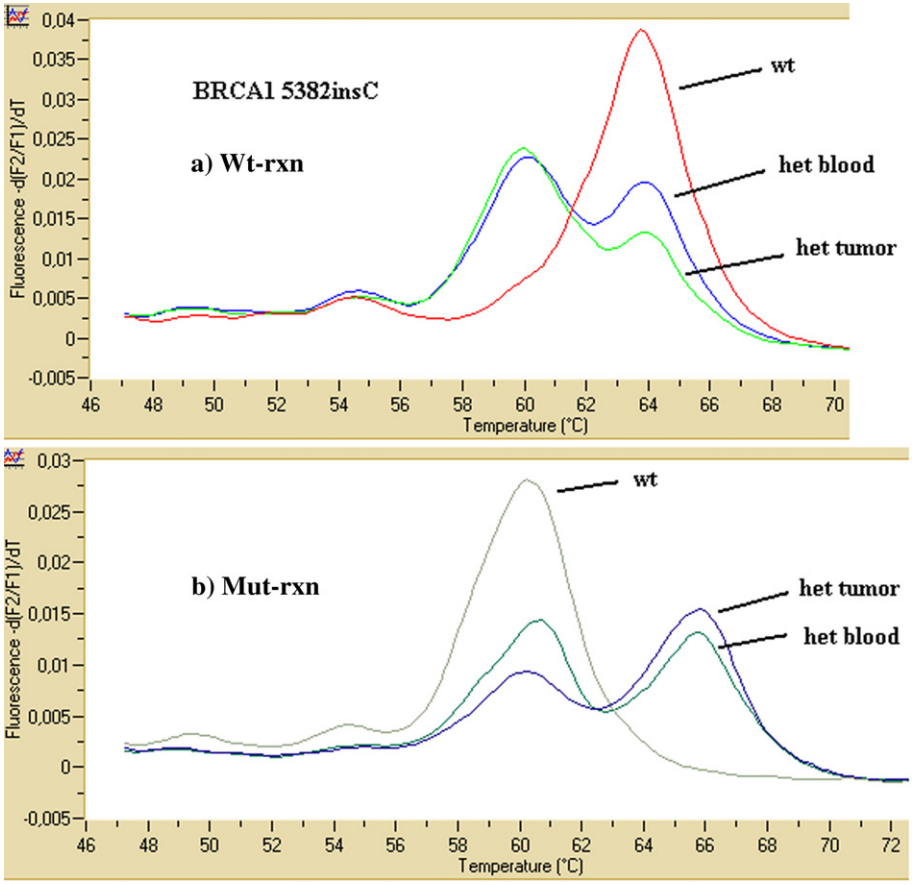

Fig. 1. Derivative melting curves for a *BRCA1* wild-type and two 5382insC heterozygote (het) samples (one peripheral blood and one tumor DNA). Results are shown for the (a) wt-rxn (wild-type sensor probe) and (b) mut-rxn (5382insC mutant sensor probe). *BRCA1* wt patients produce one high peak while heterozygotes produce two peaks of lower but almost-equal intensity. Tumor sample shows different than 1:1 allele ratio in both reactions as expected.

in carcinogenesis. There were no false positives or false negatives or contamination in any case. The comparison of results for all tumor DNA samples analyzed by real-time PCR and PSM-PCR revealed a 100% concordance. Statistics for Tm and ΔTm results for the totality of DNA samples ($n=192$) are presented in Table 1. Tm values of the peaks of wild-type and mutant alleles are statistically different in both reactions ($p<0.001$).

4. Discussion

Intense optimization of both asymmetric real-time PCR reactions for the detection of *BRCA1* 5382insC frameshift mutation led to very good amplification efficiencies and a significant Tm difference between the peaks of normal and mutant alleles so that easy identification of mutants of even low amount of DNA could be performed. To increase the cost-effectiveness of the detection, we propose the use of the wt-rxn for screening purposes in order to identify any sequence alterations underneath the area where the sensor probe hybridizes. Then the mut-rxn could be used for correct genotyping of the *BRCA1* 5382insC mutation.

The developed method is technically simple to perform and less prone to contamination since there is no need for post-amplification electrophoresis. Asymmetric real-time PCR followed by melting curve analysis is a rapid method (total time ~50 min) for the detection of *BRCA1* 5382insC mutation. It is also a high-throughput method since in the 32-position LightCycler carousel, thirty samples plus a blank and a control heterozygote, can be readily analyzed within 1 h after DNA extraction (and certainly could be further increased many-fold if a 384-well plate is used in the new LC480 platform). Finally and most important, it proved to be a reliable method in peripheral blood or tumor DNA specimens since it correctly genotyped 192 samples with 100% accuracy (comparison with other reference methods: DNA Sequencing and PSM). Analogous efforts have been performed by other groups in other platforms or for other *BRCA1* mutations [12,13].

Our method could be readily performed for diagnostic purposes in breast and/or ovarian cancer patients and for early identification of relatives who are carriers so that proper genetic counseling and medical monitoring could be initiated. Also it could be applied for the screening of high-risk families, in places where *BRCA1* 5382insC mutation is widespread: in any area with a substantial population of Greek, Slavic or Jewish ancestry. Pending an approved use for pre-implantation diagnosis, it could be also applied in the demanding setting of single-cell analysis due to its high sensitivity [14].

References

[1] King MC, Marks JH, Mandell JB. Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. Science 2003;302:643–6.

[2] Wooster R, Weber BL. Breast and ovarian cancer. N Engl J Med 2003;348:2339–47.
[3] Ladopoulou A, Kroupis C, Konstantopoulou I, et al. Germ line BRCA1 & BRCA2 mutations in Greek breast/ovarian cancer families: 5382insC is the most frequent mutation observed. Cancer Lett 2002;185:61–70.
[4] Kroupis C, Lianidou E, Goutas N, Vasilaros S, Yannoukakos D, Petersen MB. Genetic counseling of medullary breast cancer patients. Clin Genet 2004;65:343–4.
[5] Neuhausen SL. Founder populations and their uses for breast cancer genetics. Breast Cancer Res 2000;2:77–81.
[6] Gayther SA, Harrington P, Russell P, Kharkevich G, Garkavtseva RF, Ponder BA. Frequently occurring germ-line mutations of the BRCA1 gene in ovarian cancer families from Russia. Am J Hum Genet 1997;60: 1239–42.
[7] Csokay B, Tihomirova L, Stengrevics A, Sinicka O, Olah E. Strong founder effects in BRCA1 mutation carrier breast cancer patients from Latvia. Hum Mutat 1999;14:92.
[8] Kroupis C, Markou A, Vourlidis N, Dionyssiou-Asteriou A, Lianidou ES. Presence of high-risk human papillomavirus sequences in breast cancer tissues and association with histopathological characteristics. Clin Biochem 2006;39:727–31.
[9] Wittwer CT, Ririe KM, Andrew RV, David DA, Gundry RA, Balis UJ. The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. Biotechniques 1997;22:176–81.
[10] Rohlfs EM, Learning WG, Friedman KJ, Couch FJ, Weber BL, Silverman LM. Direct detection of mutations in the breast and ovarian cancer susceptibility gene BRCA1 by PCR-mediated site-directed mutagenesis. Clin Chem 1997;43:24–9.
[11] Syrjakoski K, Vahteristo P, Eerola H, et al. Population-based study of BRCA1 and BRCA2 mutations in 1035 unselected Finnish breast cancer patients. J Natl Cancer Inst 2000;92:1529–31.
[12] Pals G, Pindolia K, Worsham MJ. A rapid and sensitive approach to mutation detection using real-time polymerase chain reaction and melting curve analyses, using BRCA1 as an example. Mol Diagn 1999;4:241–6.
[13] Dufresne SD, Belloni DR, Wells WA, Tsongalis GJ. BRCA1 and BRCA2 mutation screening using SmartCycler II high-resolution melt curve analysis. Arch Pathol Lab Med 2006;130:185–7.
[14] Pals G, Young C, Mao HS, Worsham MJ. Detection of a single base substitution in a single cell using the LightCycler. J Biochem Biophys Methods 2001;47:121–9.