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Development and applications of a real-time quantitative RT-PCR method (QRT-PCR) for BRCA1 mRNA

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Abstract

Objectives: To develop a real-time quantitative RT-PCR method for BRCA1 mRNA and then use it for the study of BRCA1 gene expression in human MCF-7 breast cancer cells after their exposure to antineoplastic agents and gamma irradiation.

Design and methods: The developed QRT-PCR method is based on the real-time monitoring of a fluorescein-labeled TaqMan probe, specific for BRCA1 mRNA, during PCR in the LightCycler. A BRCA1 PCR amplicon was purified, quantitated and used as a standard of known concentration for the development and analytical evaluation of the assay. The method was applied to study the alteration of BRCA1 gene expression after exposure to taxol, doxorubicin, 5-fluorouracil, etoposide or gamma irradiation in human MCF-7 breast cancer cells.

Results: The developed method is quantitative, highly specific for mRNA and highly sensitive (detection limit of 4 BRCA1 copies per μg of total RNA). We observed a reduction of BRCA1 expression for all antineoplastic agents used, while the gamma irradiated MCF-7 cells had an increase of expression with a peak at the 10 Gy dose.

Conclusions: The developed BRCA1 QRT-PCR method is quantitative, highly sensitive and specific. The proposed method is rapid, automated, and cost effective and can be used to study BRCA1 expression in a variety of clinical samples.

Keywords: BRCA1; Real-time QRT-PCR; Antineoplastic agents; Gamma irradiation

Introduction

The tumor suppressor gene BRCA1 was mapped and sequenced in 1994 due to its linkage to hereditary breast and ovarian cancer syndrome families [1]. Mutations in the gene are scattered throughout the entire coding region (approximately 5.5 kb) and confer, according to the most recent study, a lifetime susceptibility of 84% for breast cancer and 54% for ovarian cancer to female mutation carriers [2]. Our group, among many others worldwide, is involved in BRCA1 mutation screening and detection [3]. Among its many functions, the BRCA1 protein has a significant role both in the signaling of DNA damage as a member of the BASC complex and also in DNA repair by homologous recombination in double strand breaks or by nucleotide-excision during transcription-coupled repair [4]. Expression studies that are important for the elucidation of BRCA1 involvement in various cell mechanisms so far have been performed with semiquantitative techniques like RT-PCR, Northern blots or RNase Protection assays. The same applies to clinical studies where the measurement of BRCA1 mRNA levels in frozen tumor specimens has been recognized as a prognostic factor for future metastasis in sporadic breast cancer [5]. Modern real-time quantitative RT-PCR (QRT-PCR) methodology can provide significant and quantitative information about gene transcripts in an automated, rapid, versatile, and cost-effective way.

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In the present study, we report the development of a real-time QRT-PCR methodology for BRCA1 by using the LightCycler system technology [6,7] and its analytical validation for sensitivity and precision with calibration curves that used BRCA1 amplicons of known concentration as external standards. So far, analogous methods have been developed in other platforms and used BRCA1 and BRCA2 mRNA levels in tissue as a predictive factor for the effect of various antineoplastic agents in chemotherapy [8–10]. In cancer cell lines, studies of the effect of various agents, either chemotherapeutic or oncogenic or chemopreventive, have been performed with semiquantitative techniques [11–14]. In the present study, we have applied our methodology to quantitate the effect of four antineoplastic agents commonly used in chemotherapy, on BRCA1 expression in the human MCF-7 breast cancer cell line. We also report for the first time the effect of gamma irradiation on the expression of the BRCA1 gene in the human MCF-7 breast cancer cell line.

Materials and methods

Cell line and culture

The human mammary carcinoma cell line MCF-7 (obtained from ATCC, the American Type Culture Collection) was used for the preparation of the standard used for the calibration curve of the BRCA1 real-time PCR, as a positive control for the same reaction and also for the assessment of treatment with antineoplastic agents or ionizing radiation. MCF-7 is considered BRCA1-hemizygous since a large area of 2 Mb in chromosome 17, including the gene, is deleted and therefore weakly expresses the BRCA1 gene from the retained wild type BRCA1 allele [15].

MCF-7 cells were grown in a monolayer up to 80% confluency in DMEM/NUT.MIX F12 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS), 10 mg/L bovine insulin and 40 mg/L gentamicin sulphate [16]. MCF-7 cells, 2×10^6, were plated in T75 flasks and after a 24-h initial period the cells were either exposed to different doses of gamma irradiation (measured in Gy) from a ^{60}Co source (Theratron, Canada) or exposed for another 24 h in fresh medium containing the following antineoplastic agents: taxol (Bristol Myers Squib, USA), doxorubicin (Ebwe Arzteimitel, Austria), 5′FU (Hoffmann-La Roche, Switzerland) and etoposide (VP-16, Sigma-Aldrich, USA). All drugs were used at their IC_{50} concentrations, as determined previously by the Cell Titer 96 Aqueous cell proliferation kit (Promega, USA). After each treatment culture medium was replaced; cells were post-incubated for another 24 h in the fresh medium (without any agent), washed with PBS to remove the dead cells, trypsinized and harvested. During this entire period of cell culture, untreated cells (control) were still in the exponential phase of growth. Cell counts were performed by the trypan blue exclusion method using a hemocytometer as previously described [16]. Total RNA was extracted from the same number of living MCF-7 cells (1×10^6 cells) in all cultures, treated or untreated.

Total RNA isolation and cDNA synthesis

Total RNA isolation was performed by using the Trizol LS reagent (Invitrogen), according to the manufacturer's instructions. All preparation and handling steps of RNA took place in a laminar flow hood, under RNase-free conditions. The isolated RNA was stored in diethylpyrocarbonate (DEPC)-treated water at −80°C until further manipulations. RNA concentration was determined by absorbance readings at 260 nm with the UV-VIS (U-2000) spectrophotometer (Hitachi, Japan).

Reverse transcription of RNA was carried out with oligo $(dT)_{20}$ priming and the THERMOSCRIPT RT-PCR System (Invitrogen, USA). cDNA from 5 μg of total RNA isolated from MCF-7 cells was synthesized in 20 μL total volume, according to the manufacturer's instructions and stored then at −20°C. RNA integrity of the cDNA preparations was tested by PCR amplification of a 587-bp area of the β-actin housekeeping gene (primer sequences located in different exons are shown in Table 1). Checking for the proper size of any PCR reaction in this study was performed with electrophoresis of 10 μL PCR products on 2% agarose gels along with MW markers, staining with ethidium bromide and visualization under ultraviolet (UV) light.

Preparation of the BRCA1 standard

For the development and analytical evaluation of the BRCA1 real-time QRT-PCR, we prepared a BRCA1 gene

Table 1
Sequences of primers and TaqMan probe used in this study

Name	Oligonucleotide sequence (5′–3′)	Use	GenBank location
B1-F	GGCTATCCTCTCAGAGTGACA	Forward primer	4270–4292[a]
B4-R	CTGATGTGCTTTGTTCTGGA	Reverse primer	4451–4470[a]
β-actin-1	CCAAGGCCAACCGCGAGAAGATGAC	Forward primer	1624–1648[b]
β-actin-2	AGGGTACATGGTGGTGCCGCCAGAC	Reverse primer	2722–2746[b]
BRCA1 TM-2[c]	ACCACTCAGCAGAGGGATACCATGCAA-FL	TaqMan probe	4296–4322

[a] BRCA1 cDNA, GeneBank Accession Number U14680.
[b] β-actin DNA, GeneBank Accession Number E00829.
[c] Labeled with fluorescein (TIB MOLBIOL).

specific PCR amplicon as a standard. For this reason, in order to avoid contamination, extreme precautions were taken for the handling of the BRCA1 amplicon as in the case of a nested PCR methodology. cDNA was synthesized from total RNA extracted from MCF-7 cells and then amplified for BRCA1 by conventional PCR with primers and the cycling program described before [17] in a PTC-200 DNA Engine cycler (MJ Research, USA). The primers selected (B1-F and B-4R) are located in different exons (12 and 13, respectively) so they are targeting solely cDNA. All primers used are indicated in Table 1 along with their position in the GenBank sequence and were synthesized at the Lab of Microchemistry (FORTH, Greece). Appropriate blanks and negative control (genomic DNA) were run alongside. In the electrophoresis gel, no product was observed for the genomic DNA sample while for the MCF-7 sample the proper size for the BRCA1 mRNA amplicon was seen: 201 bp. Then, a significant amount of the amplicon, produced by many reactions of the same MCF-7 cDNA preparation, was purified by passing it through a column of the QIAquick Gel extraction kit (QIAGEN, Hilden, Germany). Purified BRCA1 PCR amplicon was quantitated by the PicoGreen DNA Quantification Kit (Molecular Probes, Eugene, OR, USA). This method was modified by our laboratory in the following way: 5 μL of a DNA solution of known concentration that is supplied with the kit (or its dilutions) was added along with 5 μL of the fluorophore Pico Green in LightCycler glass capillaries. A standard curve was created by using the fluorescence values measured with the Real Fluorimeter Mode in the LightCycler (range 5–500 ng/mL). Then, the fluorescence of the purified BRCA1 PCR amplicon was measured in triplicate as ng/mL and its corresponding concentration was converted in copies per μL by using the Avogadro constant and its molecular weight (number of bases of the PCR product multiplied by the average molecular weight of a pair of nucleic acids, which is 660, as stated in Ref [18], appendix C.1). Then, serial dilutions of the above-quantitated BRCA1 stock amplicon solution were kept in aliquots at −20°C and used throughout the study as external standards for the BRCA1 real-time PCR reaction (range of the standards: $3.3 \times 10^1 - 3.3 \times 10^6$ BRCA1 copies/μL).

Real-time QRT-PCR for BRCA1 mRNA

Real-time QRT-PCR for BRCA1 mRNA was performed using the LightCycler system (Roche Applied Science, Switzerland). The same primers, B1-F and B4-R, that were used in the conventional PCR were also used for the real-time QRT-PCR. Quantification is based on the real-time monitoring of the fluorescence emission of the fluorescein-labeled BRCA1 TM 2 TaqMan probe that is specific for the cDNA junction between exons 12 and 13 of the BRCA1 gene. This region, close to the 3′ end of the BRCA1 mRNA, is present in most of the splicing isoforms of BRCA1 mRNA detected so far [1,19,20]. The sequence of the TaqMan probe—designed and synthesized by TIB MOLBIOL (Berlin, Germany)—is also shown in Table 1, while the location of primers and probe of the proposed real-time RT-PCR assay for BRCA1 mRNA in relation to exons 12 and 13 is shown in Fig. 1.

The real-time QRT-PCR reaction was performed in the LightCycler glass capillaries in a total volume of 10 μL: 1 μL of the sample cDNA was added to 1 μL of the sense primer B-1F (final concentration: 0.5 μM), 1 μL of the antisense primer B-4R (final concentration: 0.5 μM), 1 μL of the probe BRCA-1 TM 2 (final concentration: 0.32 μM), 0.8 μL of 25 mM $MgCl_2$ (Roche Applied Science, final concentration: 3 mM), 1 μL of the LightCycler Fast Start DNA Master Hybridization Probes 10× reagent (Roche Applied Science) and DEPC-H_2O to the final volume. The reaction was initiated with a 10-min denaturation at 95°C and terminated with a 30 s cooling step at 40°C. The cycling protocol consisted of denaturation step at 95°C for 10 s, annealing at 60°C for 20 s and extension at 72°C for 20 s and repeated for 50 times. Ramp time for the third step was set to 2°C/s instead of the default 20°C/s Fluorescence detection was performed at the end of each extension step for 0 s at the F1 channel.

The point where the fluorescence rises above background noise (crossing point, Cp) is best calculated through the

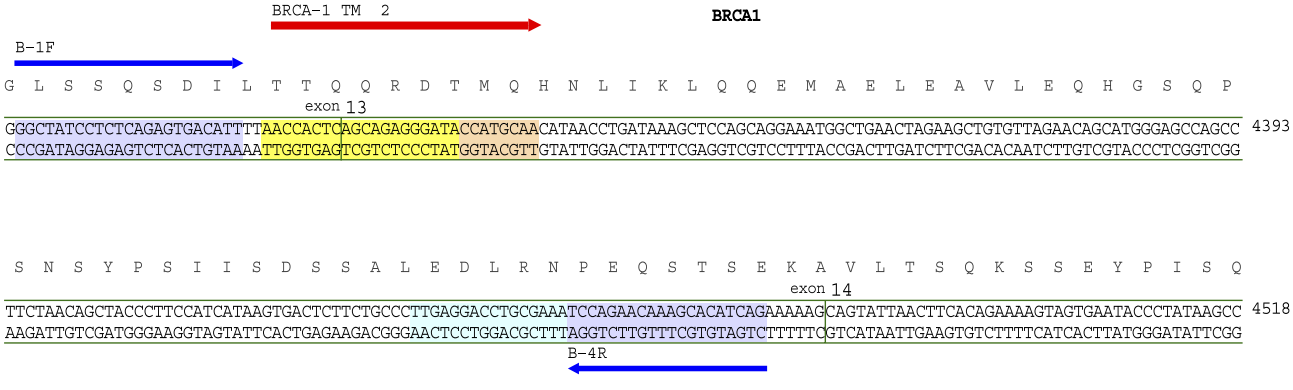


Fig. 1. Location of primers and TaqMan probe used in the real-time QRT-PCR assay for BRCA1 mRNA in relation to exons 12 and 13 (numbers correspond to the nucleotide position in BRCA1 cDNA GenBank accession no: U14680).

LightCycler software 3.1 as the second derivative maximum of the curve of the sample. For quantification, an external calibration curve was obtained by using the BRCA1 PCR amplicon standards (prepared as described above) as external standards of known concentration. The calibration curve was created by plotting the log number of BRCA1 copies corresponding to each standard versus the value of their corresponding crossing points (Cp). Sample concentration is expressed initially as BRCA1 copies per μL and then converted to copies per μg of total RNA. Standards, blanks, and positive control samples (cDNA samples of a known number of MCF-7 cells) were run alongside in the LightCycler.

Real-time QRT-PCR for HPRT-mRNA

Normalization of the samples is useful for adjustment in terms of cell numbers, quality of prepared RNA and presence of inhibitors. Most researchers use the expression levels of a housekeeping gene for normalization. We routinely employ the quantification of mRNA copies of the housekeeping hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene (LightCycler-h-HPRT gene set, Roche Applied Science). We used the standards provided with the kit and the manufacturer's instructions for the reaction mix but with a total volume of 10 μL and cycling conditions as those described above for the BRCA1 real-time PCR reaction.

Statistics

The Mann–Whitney *U* test for non-normally distributed groups was used to compare the levels of BRCA1 mRNA copies between the treated and untreated MCF cells. Data analysis was carried out with the SPSS 11.0 statistical package for Windows (SPSS Inc., USA).

Results

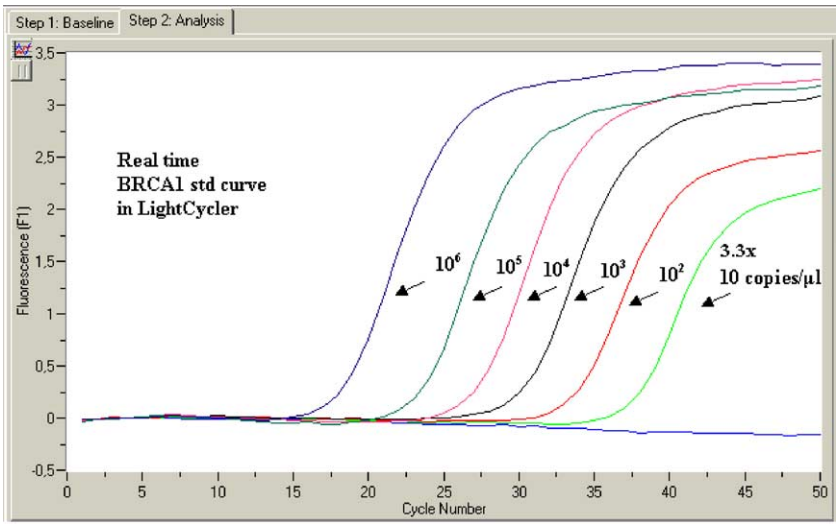
Development and analytical evaluation of real-time QRT-PCR for BRCA1 mRNA

In order to establish a specific, sensitive and reproducible real-time QRT-PCR methodology for BRCA1 mRNA, we firstly optimized the primer and probe concentrations, as well as reaction temperatures and times. Annealing temperature at 60°C, final Mg^{2+} concentration at 3 mM and ramp time 2°C/s for the extension step were found to be very critical since they increased both PCR efficiency by 5% and sensitivity by allowing the reliable detection of the less concentrated standard of 3.3×10^1 BRCA1 copies/μL. Using a ramp time of 2°C/s did not substantially increase the amplification time, while it allowed more stringent hybridization conditions of our TaqMan probe, which hybridizes very close to the forward primer, before Taq Polymerase starts polymerizing, hydrolyzing the probe and producing the fluorescent signal. When real-time PCR products obtained from the glass capillaries were also run in a gel, no other non-specific bands were observed besides the appropriate 201-bp band (data not shown). The analytical sensitivity and linearity of the proposed BRCA1 real-time RT-PCR assay was determined by using the BRCA1 external standards with known concentrations that were prepared as described above. Our calibration curves showed linearity over the entire quantification range (3.3×10^1–3.3×10^6 BRCA1 copies/μL) while the correlation coefficients were greater than 0.99 in all cases, indicating a precise log–linear relationship. The mean slope and intercept of the calibration curve was -3.57 ± 0.19 (CV = 5.2%, $n = 5$) and 42.63 ± 0.98 (CV = 2.3%, $n = 5$), respectively, while the PCR efficiency, expressed as $E = 10^{-1/\text{slope}}$, was 1.90 ± 0.07 (CV = 3.6%, $n = 5$), very close to the ideal value which is 2.00. The between-run CV's for the Cp values of the standards, analyzed in five different experiments over a period of 1 month, ranged from 0.6% to 3.9%. The within-run precision of the assay for the same standard (3.3×10^3 copies/μL) was calculated to be 0.4% (Cp ± SD: 31.87 ± 0,14, CV = 0.4%, $n = 5$). The overall reproducibility of the method was also very satisfactory, since the CV of the Cp's obtained for five different MCF-7 cell preparations (1×10^6 cells) subjected to the whole analytical procedure, which includes RNA extraction, cDNA synthesis and simultaneous quantitative real-time PCR in the LightCycler, was found to be 1.5% (Cp ± SD: 27.93 ± 0,42, CV = 1.5%, $n = 5$). The analytical detection limit of the method defined as 3.3 times the standard deviation of the Cp for the less concentrated standard divided by the mean slope of the calibration curve (D.L. = $3.3 \times SD_{std\ 1}$/slope) [21] was found to correspond to one BRCA1 copy/μL added cDNA and therefore 4 BRCA1 copies/μg of total RNA since we have 5 μg total RNA in a 20 μl cDNA solution. The quantitation limit (Q.L.) defined as three times the detection limit was therefore 12 BRCA1 copies/μg of total RNA. Real-time fluorescence monitoring of the PCR reaction in the LightCycler, as well as a typical calibration curve, displayed as a linear relationship between average Cp values and the logarithm of the number of BRCA1 copies for each standard, are shown in Figs. 2a and b, respectively.

Real-time quantification of BRCA mRNA copies in MCF-7 cells after exposure to various antineoplastic agents and gamma irradiation

The developed real-time QRT-PCR methodology was applied to study the expression of BRCA1 in the human breast cancer MCF-7 cells treated with doxorubicin, 5FU, taxol, and etoposide at their IC_{50} concentration. The same number of MCF-7 alive cells (1×10^6) was used for RNA extraction under the same conditions in order to appropri-

a



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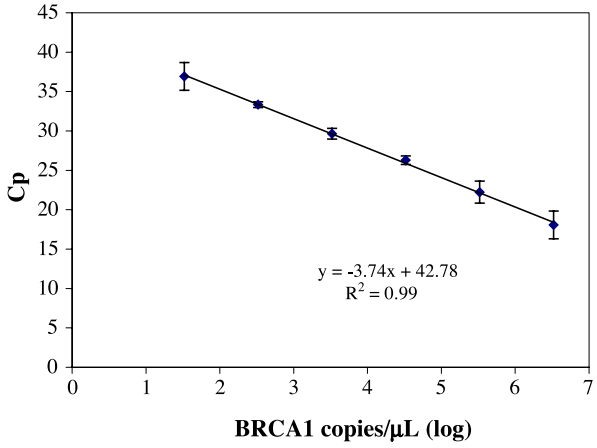

Fig. 2. a) Quantification of BRCA1 mRNA copies by real-time QRT-PCR in the LightCycler system: plot of fluorescence signal (F1) during amplification. Serial dilutions of a purified and quantitated BRCA1 cDNA amplicon were used as external standards. The curves from right to left correspond to 3.3×10^1, 3.3×10^2, 3.3×10^3, 3.3×10^4, 3.3×10^5, and 3.3×10^6 BRCA1 copies/μL of added cDNA, while the flat line represents the negative control. b) The graph shows the average crossing points (Cycle number) plotted versus the log of the number of BRCA1 copies/μL of the BRCA1 external standards ($n = 5$). Error bars represent ± 2SD of the Cp's of the BRCA1 external standards. Equation for the linear regression and corresponding correlation are shown in the graph.

ately compare BRCA1 copies between treated and untreated control MCF-7 cells. After ensuring that amplifiable material was present in all specimens by the qualitative amplification of the housekeeping gene β-actin, we performed the BRCA1 QRT-PCR assay. As recorded in Table 2, the three antineoplastic agents doxorubicin, 5′FU and taxol produced a significant decrease in BRCA1 expression to the MCF-7 cells ($P < 0.01$). However, for etoposide, a slight reduction of BRCA1 expression was observed ($P > 0.05$).

Table 2
Effect of antineoplastic agents on the expression of BRCA1 in 1×10^6 MCF-7 cells

Antineoplastic agent[a] (dose, μg/mL)	BRCA1 transcript (average copies/μg RNA ± SD)	HPRT transcript (average copies/μg RNA ± SD)	BRCA1/HPRT ratio	Normalized to control BRCA1/HPRT ratio (%)
Doxorubicin (4 μg/mL)	800 ± 280[b]	28 956 ± 2201	0.028	18[b]
5-Fluorouracil (10 μg/mL)	1224 ± 400 [b]	77 160 ± 3337	0.016	10[b]
Taxol (20 μg/mL)	1384 ± 112 [b]	48 920 ± 2950	0.028	18[b]
Etoposide (20 μg/mL)	7004 ± 1744	43 280 ± 4282	0.16	100
Control: 1×10^6 MCF-7 cells	10 236 ± 1604	63 400 ± 3964	0.16	100

[a] All experiments were performed in triplicate.
[b] $P < 0.01$.

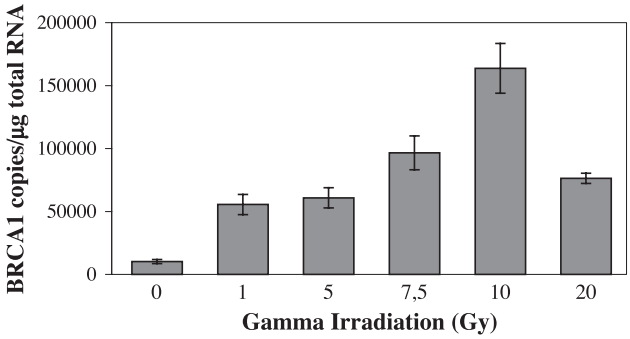

Fig. 3. Effect of gamma irradiation (Gy) on the levels of BRCA1 transcript in human breast cancer MCF-7 cells. Peak BRCA1 copies/μg of total RNA are obtained at 10 Gy. All experiments were performed in triplicate. Error bars represent ± 2SD of the BRCA1 copies obtained with the real-time methodology.

As graphically depicted in Fig. 3, ^{60}Co gamma rays produced a statistically significant increase in BRCA1 expression in the MCF-7 cells already at 1 and 5 Gy (5-fold increase compared to the MCF-7 untreated cells used as control), that peaks at 10 Gy (16-fold increase) and then decreases at 20 Gy (for all doses $P < 0.01$).

Real-time QRT-PCR for HPRT mRNA copies in MCF-7 cells after exposure to various antineoplastic agents and gamma irradiation

Real-time quantification of HPRT mRNA copies was performed for all samples in order to normalize the results. The real-time QRT-PCR reaction for the housekeeping HPRT gene was also a very efficient reaction under the conditions used: its efficiency (E) reached the value of 1.95 ± 0.03 (n = 3). Since its PCR product was of comparable size to that of BRCA1 (181 bp when run on a gel) and the difference between the values of the two efficiencies was within ± 0.05, the copies obtained from both reactions could be divided for accurate normalization.

HPRT mRNA copy results for the various treatments with the antineoplastic agents are shown in Table 2. Although there is some variation in HPRT gene expression, our conclusions concerning the expression of BRCA1 are not altered after normalizing our results according to the corresponding HPRT value of the control cells. However, normalization of our results for the gamma-irradiated MCF-7 cells according to the corresponding HPRT mRNA levels in the samples introduced an unpredictable variability for comparison among different doses of gamma irradiation. There was scattering in the HPRT values, especially for the high dose samples and this can be explained by the location of the above gene in a hypermutable region in the X chromosome. Many groups have described that gamma rays increase the mutation frequency at the HPRT locus in a dose-dependent exponential way and that the majority of mutations are large deletions of the gene [22,23]. Therefore, in this case, we normalized our results according to the μg of total RNA used for cDNA synthesis (Fig. 3). This approach for normalization of real-time PCR data is appropriate and has already been suggested by many researchers [21,24–26].

Discussion

The first goal of our study was to develop a rapid and sensitive real-time QRT-PCR methodology in the LightCycler platform in order to measure BRCA1 mRNA levels. This approach provides the most accurate results since quantitation is based on the exponential phase of the PCR product instead of using the endpoint accumulation of PCR product at the end of the plateau phase used in conventional PCR. The proposed method is suitable for clinical applications where many samples can be amplified and analyzed right afterwards with the proper software without the need for slab gels and complicated sample manipulation after PCR. The method is very rapid since the desired temperature in the LightCycler is reached with air heating/cooling, however, extra caution is needed when handling the glass capillaries since they can easily break.

The proposed method is targeting solely cDNA and not genomic DNA and is extremely sensitive with a detection limit of 4 copies per μg of total RNA and a low quantitation limit of 12 BRCA1 copies per μg of total RNA. The analytical performance of the assay was validated in detail through a series of experiments based on BRCA1 standards and in the MCF-7 breast cancer cell line. The BRCA1 external standards used were specifically designed, synthesized, and quantitated in a novel way in our laboratory. A similar approach for real-time external standard preparation has been also described very recently [27]. Detection of BRCA1 copies was linear over a range of 6 logs (3.3×10^1–3.3×10^6 BRCA1 copies/μL). By prolonging the ramp time of 2°C/s from the annealing to the elongation step, we observed an increase in PCR efficiency while the whole duration of the assay was increased only for 5 min. The absolute values of the slopes of the calibration curve as well the PCR efficiencies did not change significantly as evaluated in a set of five independent experiments performed in a period of 1 month. The assay showed a good performance over a 1-month period with an intra-assay coefficient of variation (between-run CV%) for Cps between 0.6% and 3.9%.

The first application of our methodology was the investigation of the effect of various antineoplastic agents commonly prescribed in chemotherapy on the expression of BRCA1 in the human MCF-7 breast cancer cell line. Doxorubicin and 5FU are cytostatic agents that arrest cells at S-phase; taxol is a microtubule-interfering agent while etoposide is a topoisomerase II inhibitor. All drugs were used at their IC_{50} concentration so that comparable cytotoxicity was attained. When we compared the number of BRCA1 transcripts found in cDNAs corresponding to the same number of treated and non-treated MCF-7 living

cells, a dramatic decrease was observed for most of the drugs, except for etoposide. Our results in antineoplastic agents are in accordance with previous studies where analogous decreases in BRCA1 expression were shown but in different agents (mostly adriamycin) in breast or ovarian cell lines [11,28,29]. In another study performed in prostate cancer cells, no effect was seen for BRCA1 expression after etoposide treatment, which is also in accordance with our results [12].

According to the results of the second application of our methodology, gamma irradiation led to an induction of BRCA1 expression in human MCF-7 breast cancer cells, in a dose-dependent manner. This can probably be explained as a response to DNA damage sensing. Analogous results that are consistent with the BRCA1 role in the DNA repair machinery have been obtained with the BRCA1 ortholog in maize after gamma irradiation [30]. In ovarian cancer cell lines, BRCA1 expression in response to ionizing radiation has been correlated to p53 functional status [31]. So far, other studies have not shown the effect of gamma rays on a breast cancer cell line but only on peripheral blood or lymphoblastoid cell lines [32,33]. Certainly, the proposed quantitative methodology could substantiate a significant amount of data performed by other researchers to the aforementioned questions.

When studying the effect of gamma irradiation on BRCA1 expression, normalization of our results with HPRT mRNA levels was found to be problematic. This was especially obvious for the high doses of gamma irradiation where partial or total deletions of the gene may be the reason for low levels of HPRT mRNA [22,23]. Lately, there is a considerable debate about the use of housekeeping genes for normalization of real-time PCR results. In a recent study, it was clearly shown that housekeeping gene expression should not be used to normalize real-time QRT-PCR results since a significant variation in the expression levels of 10 commonly used housekeeping genes was shown between individuals and tissues taken from the same individual [24]. On the contrary, it was shown by many groups that normalization to total RNA concentration is an acceptable alternative to the housekeeping gene approach. This approach was followed in our study of gamma irradiation [21,24–26].

The developed QRT-PCR method could also be used for the accurate measurement of BRCA1 mRNA levels in various tissues and mostly in the breast or ovarian cancerous tissues especially now with the advent of laser guided tissue microdissection. These QRT-PCR measurements could also assist in the elucidation of other genetic or epigenetic factors in the pathogenesis of breast or ovarian cancer. However, patients with loss of both chromosomes 17 or with a splicing BRCA1 mutation affecting the region detected or with extended methylation in the BRCA1 promoter resulting in the silencing of the gene [34,35] would test negative for the above assay.

Acknowledgments

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