

# upQMPSF, a Method for the Detection of BRCA1 Exon Copy Number Variants

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**Abstract** Large insertions/deletions mutations are frequently found in genes associated with certain diseases such as hereditary cancers. These mutations are mostly overlooked by current classical screening techniques due to their certain limitations. This justifies the need to improve the existing techniques or design novel ones. A modified version of quantitative multiplex PCR short fluorescent fragment (QMPSF), termed universally primed QMPSF (upQMPSF), was developed. The modifications enhance multiplexing capacity, reduce cost, and improve the mutation detection spectrum. upQMPSF was used to screen germline mutations in 88 familial ovarian cancer patients negative for point mutations. upQMPSF successfully detected a 2.8 kb copy number gain spanning exon 15 of *BRCA1* gene mediated by Alu–Alu homologous-based recombination. upQMPSF is a cost-efficient, versatile method, and demonstrated efficiency in detecting structural variations as a potential method for genetic testing in clinical and research laboratories.

**Keywords** QMPSF · MLPA · *BRCA1/2* · *MLH1* · *RAD51*

## Background

Hereditary ovarian cancer accounts for only 5–10% caused by germline mutation in *BRCA1/2* genes (Wooster and Weber 2003). Close relatives carrying *BRCA1/2* gene mutation have elevated risks of developing cancers (Wooster and Stratton 1995; Pharoah and Ponder 2002). The focus of most mutation screening studies is on small size mutations such as missense, nonsense, and small insertions/deletions. Recently,

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other types of genetic changes are found to be responsible for the etiology of cancer predisposition. Large genomic rearrangements (LGRs) involve loss or gain of hundreds or thousands of DNA nucleotide bases that encompass one or more exons and sometimes the entire gene. This creates a shift in the reading frame and eventually produces a premature truncated protein. Different forms of repetitive DNA sequences in the intron gene region are believed to be the major mediator for genetic instability resulting in genomic deletions or duplications (Gu et al. 2008). LGRs are no exception. LGRs are believed to be regulated by stretches of DNA segments that are highly similar in sequences, such as short and long interspersed elements (van der Klift et al. 2005). Therefore, the formation of LGRs is due to DNA double-strand breaks (DSBs) followed by DNA repair through homologous recombination (HR) or non-homologous end joining (NHEJ) (Bishop and Schiestl 2000; Lupski 2007).

Higher frequency of LGRs is reported in *BRCA1* than *BRCA2* gene in hereditary breast and ovarian cancer patients, which is attributed to more Alu and L1 contents in *BRCA1* gene (Smith et al. 1996). Moreover, LGRs were reported in high frequency in British families with breast and ovarian cancer, which is likely due to a founder effect such as *BRCA1* exon13 duplications (Puget et al. 1999; Mazoyer 2000). In addition, LGRs are found to be frequently present in other cancer susceptibility genes such as *MLH1* and *MSH2* in hereditary non-polyposis colorectal cancer (HNPCC) (Charbonnier et al. 2000). Nevertheless, LGRs are mainly overlooked by most mutation detection methodologies due to their large size and heterozygous nature. Over the past years, multiplex ligation-dependent probe amplification (MLPA) and quantitative multiplex polymerase chain reaction of short fluorescent fragment (QMPSF) have been used to detect changes in exon copy number in multiple hereditary syndromes. Despite their advantages, both methods have certain limitations. Therefore, the aim of this study was to develop an improved method for the detection of LGRs and other mutation types.

## Materials and Methods

### HNPCC Patient Samples

Genomic DNA was extracted from blood of four patients diagnosed with HNPCC from McGill University to test upQMPSF efficiency in detecting LGRs. The anonymous samples are known to have LGRs in *MLH1* which was perilously detected and characterized using southern blot and DNA sequencing (Li et al. 2006). HNPCC samples contain single-exon 12 *JGH202-413*, *PFT1045*, and multi-exon 3–6 *AUS17392*, 6–8 *JGH200-82* deletions.

### Familial Ovarian Cancer Patient Samples

Genomic DNA extracted from the blood of 88 familial ovarian cancer patients was obtained from Gilda Rader Familial Ovarian Cancer Registry (GRFOCR) at Roswell Park Cancer Institute to establish upQMPSF and assess the contribution/

frequency of LGR in *BRCA1/2* and other susceptible genes. Patient samples were previously tested negative for point mutation and small insertions/deletions in *BRCA1/2* genes.

This study complies with the Helsinki Declaration, approval of ethics committees was obtained (protocol number I71705), and all patients were provided an informed consent.

### PCR Kit

AccuPrime™ *Taq* hot start DNA polymerase kit (Invitrogen, USA) with 10× PCR buffer I and II contain 200 mM Tris–HCl (pH 8.4), 500 mM KCl, 15 mM MgCl<sub>2</sub>, 2 mM dGTP, 2 mM dATP, 2 mM dTTP, 2 mM dCTP, thermostable AccuPrime™ protein, and 10% glycerol were used for PCR amplification in all three methods QMPSF, upQMPSF, and MM-PCR in equivocal amounts.

### QMPSF

The assay was performed using 120 ng genomic DNA from HNPCC patients in a total volume of 25 µl of PCR reagents (5 µl of primer mix, 2.5 µl AccuPrime™ 10X PCR buffer II, and 0.5 µl hot start *Taq* polymerase). Similar PCR conditions and primer sequences for *MLH1* were used as previously reported by (Charbonnier et al. 2000).

### MLPA

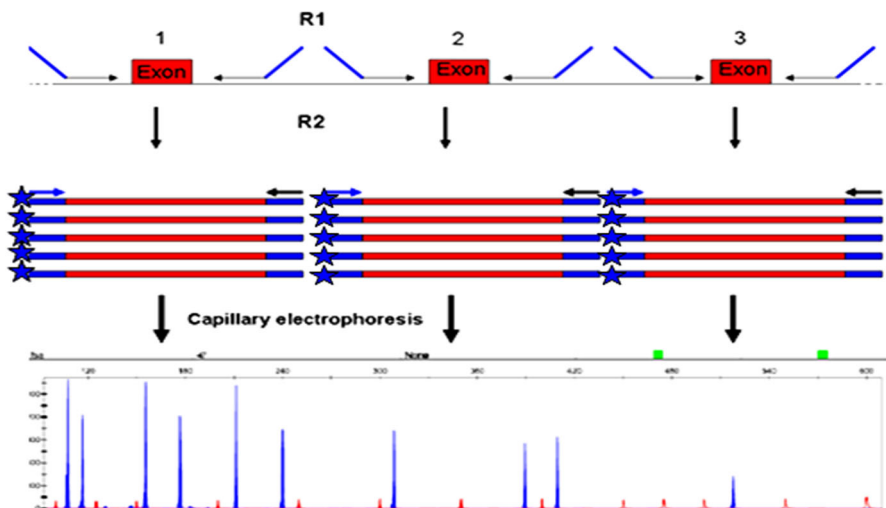
MLPA SALSA P003 *MLH1/MSH2* and P002 *BRCA1* kits were purchased from MRC-Holland (Amsterdam, Netherlands). MLPA was carried out according to the manufacturing protocol as following: 150–180 ng genomic DNA in a total volume of 5 µl was denatured at 95°C for 5 m. Samples were cooled to 25°C and SALSA probemix was added and heated to 95°C for 60 s, followed by probe hybridization for >16 h at 60°C. Ligation step performed by ligase-65 enzyme mix, incubation at 54°C for 15 m, and inactivation at 98°C for 2 m. PCR amplification of 35 cycles using a single fluorescent primer: 30 s at 95°C, 30 s at 60°C, 60 s at 72°C, and 20 m final elongation at 72°C.

### Mutation-specific Multiplex (MM-PCR)

MM-PCR was used to characterize the breakpoints and to confirm the upQMPSF detected exon15 duplication in *BRCA1* gene. A primer set was designed to be located at the 3' (F:ctattctgaagactcccagagc) and 5' (R3:gctcattgcccccaattgg) in intron (14 and 15) ends, along with *GAPDH* gene internal control primer pairs (F:gggtaaggagatgctgcatcg), (R:tgtagcactcaagacgtctgagc). 5 µl of primer mix (2.5 µl 10× PCR buffer II and 0.5 µl of *Taq* DNA polymerase) was added to 60–80 ng genomic DNA for a total volume of 25 µl. Samples were subjected to 38 cycles of PCR amplification under the following conditions: 95°C for 4 m, 94°C for 1 m, 63°C for 30 s, elongation at 68°C for 20 m, and final extension at 68°C for 20 m.

## upQMPSF

upQMPSF assay design relies on utilizing bioinformatics approaches of careful target sequence preparations and multiplex chimerical primer design steps. Exons and their flanking intron sequences were retrieved in a masked version using UCSC genome browser (Kent et al. 2002) in FASTA format with specific define. This included the following: gene name and location, exon position, sequence length, and acquired product size. Consecutive exons, separated with small introns, were combined into one target sequence. Multiplex chimerical primers design step was performed using FastPCR<sup>®</sup> primer design software in stringent primer properties criteria (Kalendar et al. 2009). Each chimerical forward and reverse primer sequences consist of 3' target-specific sequence and 5' unique universal sequences (Fig. 1). Primer sequences were examined for the absence of genetic variations at the binding sites and selected for each multiplex set. Two consecutive PCR amplification runs, R1 and R2, were carried out. In R1, a total volume of 25  $\mu$ l primer mix (1.5  $\mu$ l of low concentrated chimerical primers mix, 2.5  $\mu$ l 10 $\times$  PCR buffer II, and 0.5  $\mu$ l Taq polymerase) was added to 100–200 ng of genomic DNA. Two cycles of PCR amplification were performed under the following conditions: 95 $^{\circ}$ C for 4 m, 94 $^{\circ}$ C for 1 m, 58 $^{\circ}$ C for 1 m, and elongation at 68 $^{\circ}$ C for 1 m. Then, 8 cycles of amplification at 62 $^{\circ}$ C for 1 m and a final extension at 68 $^{\circ}$ C for 5 m were performed. In R2, 1.75  $\mu$ l aliquot of R1 products were added to 3.75  $\mu$ l of high concentrated universal primers (fluorescently labeled forward primer) for a total mixture volume of 25  $\mu$ l. The mixer was subjected to 20 amplification cycles, which is empirically chosen to achieve amplification within the exponential range. R2 conditions are set as following: 95 $^{\circ}$ C for 4 m, 94 $^{\circ}$ C for 1 m, 48 $^{\circ}$ C for 1 m, 68 $^{\circ}$ C for



**Fig. 1** Schematic representation of universal primer quantitative multiplex PCR short fluorescent fragments (upQMPSF) assay: two consecutive multiplex PCR amplifications (R1 and R2) using chimerical target-specific primers and universal primer pair with fluorescently labeled forward primer, respectively. Labeled PCR fragments are separated by capillary electrophoresis

1 m, and final extension at 68°C for 45 m to reduce the adenylation effect. All upQMPSF primer sequences were synthesized and purchased from Integrated DNA Technology (IDT), USA. Chimerical and universal primer concentrations were incrementally calculated based upon expected product sizes ranging between 0.3 and 1.8  $\mu\text{M}$  and were available upon request.

Flowchart summarizes the steps for the development of upQMPSF:

|   |   |
|---|---|
| Step 1: Target sequence preparation     | <ol style="list-style-type: none"> <li>1. Selection of target genomic regions (exon, intron, etc.)</li> <li>2. Sequence retrieval (masked version)</li> <li>3. Combine small size exons separated with small introns into one target sequence</li> <li>4. Formatting each target as separate in FASTA with define contains gene name, location, exon position, and length</li> </ol>                              |
| Step 2: Primer design                   | <ol style="list-style-type: none"> <li>1. Identify all possible forward and reverse primers for each target region using stringent criteria</li> <li>2. Primer check for self-annealing or hairpin structure using “OligoCal”</li> <li>3. Perform In-Silico PCR to avoid SNPs in the binding site, multiple matches in the genome. Also to confirm product size compatibility</li> </ol>                          |
| Step 3: Selection of multiplex sets     | <ol style="list-style-type: none"> <li>1. Select all possible multiplex sets for all regions based upon primer pair compatibility and product size</li> <li>2. Primers compatibility checking step is performed in term of primer dimerization imposed high melting temperature using (FastPCR)</li> </ol>  |
| Step 4: PCR amplification and detection | <ol style="list-style-type: none"> <li>1. Two consecutive PCR amplifications (R1 and R2) using chimerical and universal primers, respectively</li> <li>2. R2 chosen within an exponential amplification phase</li> <li>3. Fluorescently labeled products are detected by CE</li> <li>4. Multiple rounds of optimizations for comparable peak intensity, followed by assay reproducibility confirmation</li> </ol> |
| Step 5: Copy number analysis            | Calculation of normalized ratio “upQMPSF scores” (ratio test vs. normal samples of ratio of test peak vs. internal control) for all samples   |

The design of upQMPSF for *BRCA1* gene was used to detect exon copy number variants in two multiplex primer sets containing 27 amplicons for all 24 exons and the promoter region to enhance the cost efficiency (Table 1), including two products for the 3' and 5' ends of exon 11 (11a and 11b) in addition to internal control from different chromosomal region in each set for normalization.

upQMPSF was also developed for the detection of exon copy number variants in *BRCA2* gene. A total of 27 fragments representing partial exon regions, separated into two multiplex primer sets, including internal control from different chromosomal region in each set (Table 2).

Only two exon 11 fragments were designed spanning the 3' and 5' ends of (11a and 11b exons), respectively with their intron flanking regions for the detection of LGR mutations. Small size exons, such as 5, 6, 23, and 24 were combined into one amplicon in the multiplex set upQMPSF. *MLH1* and *RAD51* genes are designed in two multiplex primer sets for each gene.

**Table 1** upQMPSF multiplex primer sets for the detection of LGRs in *BRCA1*

| Name      | Primer sequence                              | Products size |
|-----------|--|---------------|
| SET1      |  |               |
| BCA1_pom* | <u>UPF</u> <sup>a</sup> -acctttaccagagcagagg | 140           |
| BCA1_pom* | <u>UPR</u> <sup>b</sup> -gctcgctgagacttctgga |               |
| BCA1_E8   | <u>UPF</u> -tttgaggtgtatccgctgc              | 161           |
| BCA1_E8   | <u>UPR</u> -ggccaacaattgctgactg              |               |
| BCA1_E12  | <u>UPF</u> -gcaaaggacaccacacac               | 182           |
| BCA1_E12  | <u>UPR</u> -taaggtgaagcagcatctgg             |               |
| BCA1_E7   | <u>UPF</u> -tttggcaggtttctgtagc              | 225           |
| BCA1_E7   | <u>UPR</u> -gggtttctctgtgttcttg              |               |
| BCA1_E2   | <u>UPF</u> -gaggcagagtggatggag               | 241           |
| BCA1_E2   | <u>UPR</u> -ccatctgtaagtgcagcac              |               |
| BCA1_E11a | <u>UPF</u> -acggctaattgtgctcactg             | 259           |
| BCA1_E11a | <u>UPR</u> -tctcagttcagaggcaacga             |               |
| BCA1_E6   | <u>UPF</u> -cctgtgtcaagctgaaaagc             | 289           |
| BCA1_E6   | <u>UPR</u> -cactgccatcacacggtt               |               |
| BCA1_E9   | <u>UPF</u> -ccatgccttaaccacttctctg           | 297           |
| BCA1_E9   | <u>UPR</u> -ccagcttcatagacaaaggttctc         |               |
| BCA1_E11b | <u>UPF</u> -tcaactcagaccaactcctg             | 315           |
| BCA1_E11b | <u>UPR</u> -accgttgctaccgagtgtct             |               |
| BCA1_E1   | <u>UPF</u> -tctccacaaggtccatcc               | 325           |
| BCA1_E1   | <u>UPR</u> -ggctggtcatgaggtcagga             |               |
| BCA1_E10  | <u>UPF</u> -tggtcagctttctgtaatcg             | 338           |
| BCA1_E10  | <u>UPR</u> -gattttgtgggtgtaaaaggtcc          |               |
| BCA1_E5   | <u>UPF</u> -cacggcgatacagccctact             | 356           |
| BCA1_E5   | <u>UPR</u> -gaaagggccttcacagtgtcc            |               |
| BCA1_E3   | <u>UPF</u> -caactccagactagcagggt             | 400           |
| BCA1_E3   | <u>UPR</u> -tgagatgtgcaccacagtg              |               |
| C1_mlh1   | <u>UPF</u> -tgctgggatctgagggtt               | 442           |
| C1_mlh1   | <u>UPR</u> -ggcagttgaggccctatg               |               |
| SET 2     |  |               |
| BCA1_E20  | <u>UPF</u> -tacagagtgtggggtgag               | 130           |
| BCA1_E20  | <u>UPR</u> -ccaaggtccaagcgagca               |               |
| C2_p53    | <u>UPR</u> -ctcacaacctccgtcatgtg             | 164           |
| C2_p53    | <u>UPR</u> -tgttttgccaactggccaag             |               |
| BCA1_E23  | <u>UPF</u> -actgtgctactcaagacca              | 183           |
| BCA1_E23  | <u>UPR</u> -acccaattgtggtgtgcag              |               |
| BCA1_E22  | <u>UPF</u> -ggcacaggtatgtgggcag              | 234           |
| BCA1_E22  | <u>UPR</u> -tggatggtacagctgtgtgg             |               |
| BCA1_E15  | <u>UPF</u> -cgtcaaatcgtgtggccca              | 245           |
| BCA1_E15  | <u>UPR</u> -tcacaattggtgctgatgtg             |               |
| BCA1_E19  | <u>UPF</u> -ggtatgagccacagtgcagg             | 254           |
| BCA1_E19  | <u>UPR</u> -tctttagggtgaccaccatc             |               |

**Table 1** continued

| Name   | Primer sequence                  | Products size                    |     |
|--|----------------------------------|----------------------------------|-----|
| BCA1_E16   | <u>UPF</u> -ttcttctggggtcaggcca  | 272                              |     |
| BCA1_E16   | <u>UPR</u> -cccagagtcagctcgtgttg |                                  |     |
| BCA1_E17   | <u>UPF</u> -tcgcctcatgtggtttatgc | 302                              |     |
| BCA1_E17   | <u>UPR</u> -tgtagaactgcaggattgc  |                                  |     |
| BCA1_E21   | <u>UPF</u> -tctgaggaaccccatcgtg  | 362                              |     |
| BCA1_E21   | <u>UPR</u> -gactgcttgccttgggaag  |                                  |     |
| BCA1_E14   | <u>UPF</u> -tgtcagcagaaggccttc   | 376                              |     |
| BCA1_E14   | <u>UPR</u> -cctgtctcagctgggtgc   |                                  |     |
| BCA1_E13   | <u>UPF</u> -tgaatgactgccttgggtcc | 405                              |     |
| BCA1_E13   | <u>UPR</u> -gcagagggataccatgcaac |                                  |     |
| <sup>a</sup> <u>UPF</u> : universal forward primer | BCA1_E18                         | <u>UPF</u> -ggctgtagcagcagtttcc  | 462 |
| <sup>b</sup> <u>UPR</u> : universal reverse primer | BCA1_E18                         | <u>UPR</u> -tgtgtgtgaacggacactg  |     |
| * Promoter region of BRCA1 gene                    | BCA1_E24                         | <u>UPF</u> -tcgatcccaagcactctcc  | 520 |
|  | BCA1_E24                         | <u>UPR</u> -acagccactactgactcgag |     |

## DNA Fragment Analysis

Fluorescently labeled PCR products from MLPA, QMPSF, and upQMPSF were analyzed on ABI PRISM 3130XL (Applied Biosystem, USA) as per fragment analysis protocol. 1  $\mu$ l of PCR product loaded with 10  $\mu$ l of formamide and 1  $\mu$ l size standard (Rox500, Applied Biosystem, USA) in a capillary plate. Fragment size, fluorescent intensity (peak height), and area size were collected and visualized using GeneMapper<sup>®</sup> software. Downstream quantitative data analysis was used for calculating upQMPSF score that represents the normalized ratio (NR) of Rt (R ratio for test peaks/internal control peak height in tumor samples)/Rn (R ratio for peak height/internal control in 80 normal samples). This score was analyzed and a cut off value for copy number variant was determined.

## DNA Sequencing Analysis

Breakpoint characterization of exon15 duplication was performed by amplifying a genomic region using MM-PCR. The PCR product was resolved on 1% agarose gel stained with Ethidium Bromide (EtBr) followed by DNA gel extraction via QIAquick gel extraction Kit (QIAGEN, USA).

## Results

### upQMPSF Assay Validation for LGRs Detection

The efficiency of upQMPSF in detecting LGRs was tested in comparison with QMPSF and MLPA using a set of four HNPCC patient samples with characterized

**Table 2** upQMPSF multiplex primer sets for the detection of LGRs in *BRCA2*

| Name         | Primer Sequence                     | Product Size |
|--------------|-------------------------------------|--------------|
| SET 1        |                                     |              |
| BRCA2_E4     | <u>UPF</u> -gcatatcaccacaccagc      | 133          |
| BRCA2_E4     | <u>UPR</u> -tgggaggattgcttgaggca    |              |
| BRCA2_E11b   | <u>UPF</u> -tgccaagtcagccacaca      | 186          |
| BRCA2_E11b   | <u>UPR</u> -agtgattggcaacacgaaagg   |              |
| BRCA2_E7     | <u>UPF</u> -ccttaatgatcagggcatttc   | 196          |
| BRCA2_E7     | <u>UPR</u> -tagaactaagggtgggtggtg   |              |
| BRCA2_E12    | <u>UPF</u> -ggagaacctcaatcaaaagaaac | 228          |
| BRCA2_E12    | <u>UPR</u> -actttgagaggcagtggtatca  |              |
| C1_p53F      | <u>UPF</u> -ctcacaacctccgcatgtg     | 302          |
| C1_p53R      | <u>UPR</u> -ctagtgggttcaggagggt     |              |
| BRCA2_E13    | <u>UPF</u> -tgctgatttctgttatgcttg   | 327          |
| BRCA2_E13    | <u>UPR</u> -agggtacacaggtaatcgctct  |              |
| BRCA2_E15    | <u>UPF</u> -acaaggaacgcgctcttcc     | 361          |
| BRCA2_E15    | <u>UPR</u> -actttagcaggatgaggcagg   |              |
| BRCA2_E2     | <u>UPF</u> -accgttcaggagatggga      | 371          |
| BRCA2_E2     | <u>UPR</u> -ggttctaagcaactgtgacg    |              |
| BRCA2_E1     | <u>UPF</u> -gcttctgaaactagggcgca    | 249          |
| BRCA2_E1R    | <u>UPR</u> -tcgtccaaccactaccac      |              |
| BRCA2_E11a   | <u>UPF</u> -gattctctgtcatgcctgcag   | 434          |
| BRCA2_E11a   | <u>UPR</u> -tctcagtgggcaacagctcaac  |              |
| BRCA2_E10    | <u>UPF</u> -gtctaaatggagcccagatgg   | 455          |
| BRCA2_E10    | <u>UPR</u> -agtccacttccagaggcttc    |              |
| BRCA2_E3     | <u>UPF</u> -tcaaagagcaagggtgactc    | 458          |
| BRCA2_E3     | <u>UPR</u> -tcctctgtgatagcttcgaga   |              |
| BRCA2_E14    | <u>UPF</u> -gttttggatggcaacctatgg   | 513          |
| BRCA2_E14    | <u>UPR</u> -ctatcatcagagccatgtcca   |              |
| BRCA2_E5-6   | <u>UPF</u> -tacacggtttccagcagctg    | 572          |
| BRCA2_E5-6   | <u>UPR</u> -tgccctgtatgaggcagaatg   |              |
| BRCA2_E8     | <u>UPF</u> -cacagatcatctgactttcca   | 604          |
| BRCA2_E8     | <u>UPR</u> -cccataaataggggactacgg   |              |
| SET 2        |                                     |              |
| BRCA2_E19    | <u>UPF</u> -ctgctaacagctactcggcct   | 143          |
| BRCA2_E19R   | <u>UPR</u> -cctccatcactgaaaaagcga   |              |
| BRCA2_E21    | <u>UPF</u> -aagacagcaagttcgtgctt    | 208          |
| BRCA2_E21    | <u>UPR</u> -ccttctgtgatggccagag     |              |
| BRCA2_E27    | <u>UPF</u> -aggaaagtctgtttccacacc   | 230          |
| BRCA2_E27    | <u>UPR</u> -gctgaaatgccttctgtgca    |              |
| BRCA2_E22    | <u>UPF</u> -tcaagggatgtcacaaccgt    | 241          |
| BRCA2_E22R   | <u>UPR</u> -tgggcattagtagtgattttgc  |              |
| BRCA2_E23-24 | <u>UPF</u> -cagatttaccagccacggga    | 253          |
| BRCA2_E23-24 | <u>UPR</u> -ttgccaactggtagctccaa    |              |



**Table 2** continued

| Name        | Primer Sequence                   | Product Size |
|-------------|-----------------------------------|--------------|
| BRCA2_E17   | <u>UPF</u> -ttcagggctctgtgtgacac  | 318          |
| BRCA2_E17   | <u>UPR</u> -acaactgcttgtgcaacat   |              |
| BRCA2_E18   | <u>UPF</u> -atggcagactgacagtgggt  | 327          |
| BRCA2_E18   | <u>UPR</u> -ggacctccaaaaactgcac   |              |
| BRCA2_E26   | <u>UPF</u> -tgcaaatgatccaagtgggt  | 342          |
| BRCA2_E26   | <u>UPR</u> -acctgtttgaaagtgtgcac  |              |
| BRCA2_E9    | <u>UPF</u> -gctgcaagtcatgtaagtctt | 362          |
| BRCA2_E9R   | <u>UPR</u> -gagcaatccttcaatgggtgc |              |
| C2_mlh1_E10 | <u>UPF</u> -tgagctaattggggcaaagg  | 384          |
| C2_mlh1_E10 | <u>UPR</u> -tgggcaaataggctgcatac  |              |
| BRCA2_E16   | <u>UPF</u> -gcagatttagcaggaggcgt  | 439          |
| BRCA2_E16   | <u>UPR</u> -gggtatgagccatccaccat  |              |
| BRCA2_E25   | <u>UPF</u> -ccagaatccaatcaggcct   | 467          |
| BRCA2_E25   | <u>UPR</u> -actgctgactgctgcaaacc  |              |
| BRCA2_E20   | <u>UPF</u> -tgtgcttgctgatacaat    | 561          |
| BRCA2_E20   | <u>UPR</u> -ggctctagttccaggcata   |              |

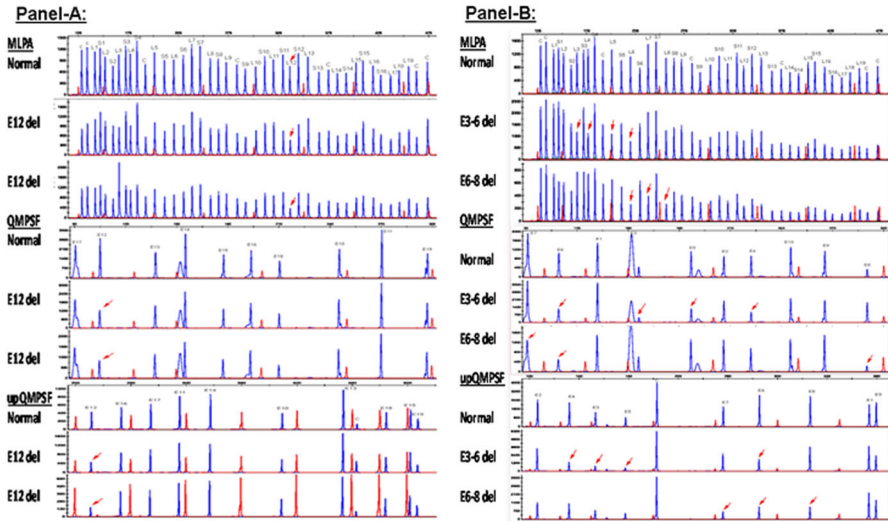
LGRs in *MLH1* gene. The result shows significant reduction in the exon deletion peak height when compared with its corresponding peak height in normal samples. This result was reproducible in all tested methods (Fig. 2a, b). upQMPSF score of *MLH1* set2 (exon 10–19) was calculated and displayed in a histogram with their corresponding frequencies.

Normal scores demonstrated a normal distribution pattern around a mean value of 1. Both JGH202-413 and PFT1045 patient with exon 12 deletions had upQMPSF scores of 0.5 and 0.55, respectively (Fig. 3a). These scores were located outside the region of normal variation in a range of 4 standard deviations. In another representation, the log<sub>2</sub> values of upQMPSF scores were plotted and the distribution revealed a copy number loss with value of  $\leq -0.5$  (Fig. 3b).

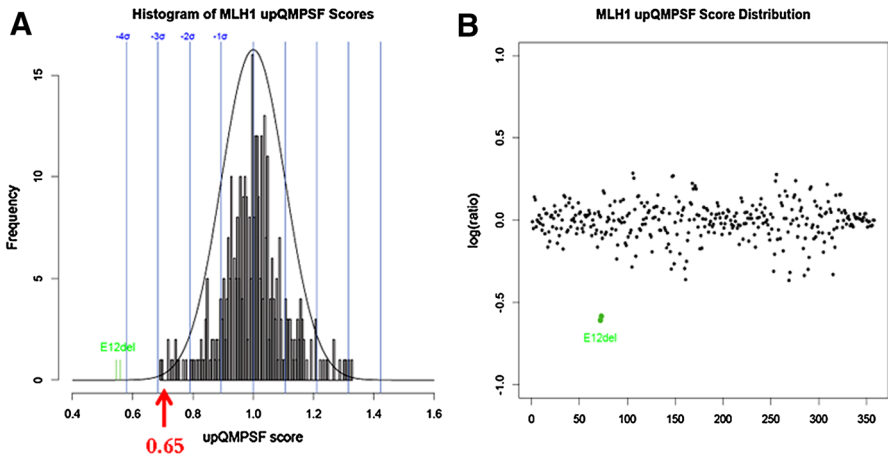
Other ovarian cancer susceptible genes, such as *BRCA2*, *MLH1*, and *RAD51* were screened by upQMPSF for the detection of LGRs; however, no exon copy number variant was found in tested ovarian cancer patients.

### Detection and Characterization of Exon 15 Duplication in *BRCA1* Gene

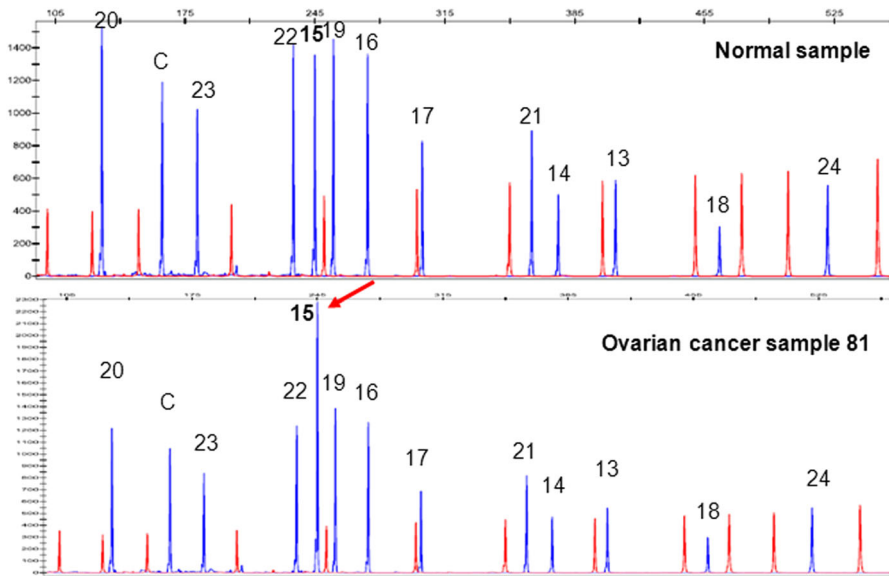
A case of exon 15 duplication was detected in patient 81 in *BRCA1* gene which was previously reported (Ramus et al. 2007). upQMPSF peak profile yielded an increase in exon 15 peak intensity when compared with neighboring normal peaks (Fig. 4). The intensity was visually distinguished from normal samples with upQMPSF score of 1.47, which is nearly 50% increase in comparison with overall normal samples, which averaged  $f = 1$ . This score is significantly separated from the normal variation range by a distance of 8 standard deviations (Fig. 5a). upQMPSF score  $\geq 1.35$  was



**Fig. 2** MLPA, QMPSF, and upQMPSF method comparison for the detection of MLH1 LGRs in HNPCC patients: **a** Lower peak height depicted by red arrow, represents the deleted copy of MLH1 exon12 in two different HNPCC patients (e12 in JGH202-413 and e12 in PFT1045) in comparison with normal peak height in control sample in three detection methods, including upQMPSF. **b** Similar representation with lower peak height for two HNPCC samples in MLH1 multi-exon deletions (e3-6 in AUS17392 and e6-8 in JGH200-82)



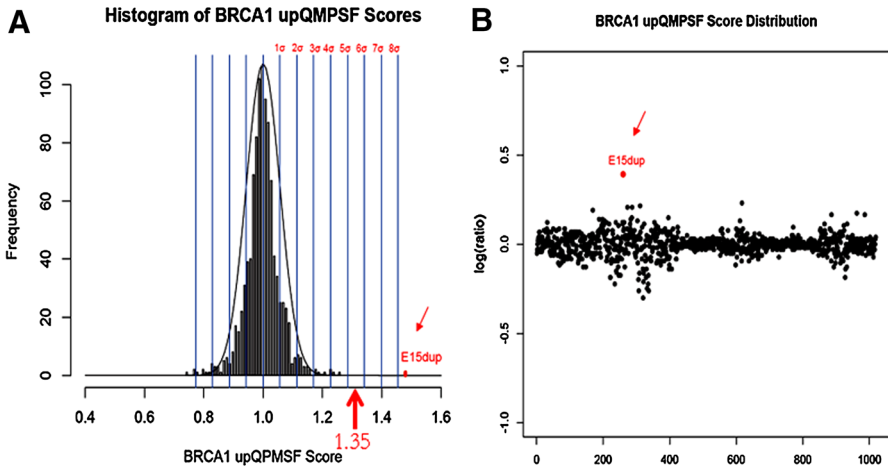
**Fig. 3** Statistical data analysis for MLH1 exon12 deletion in HNPCC patients: **a** Histogram shows upQMPSF scores for MLH1 set2 with their frequencies (black bars) in normal samples, upQMPSF score for MLH1 exon 12 deletions (green bars) were located outside the region of normal distribution  $\sim 4\times$  standard deviation from the mean of 1. upQMPSF score of 0.65 (red arrow) was chosen as a cut off value for quantitative detection of exon copy number loss. **b**  $\log_2$  values (black dots) of all upQMPSF scores distributed around the value of 0 in normal samples, upQMPSF score for MLH1 exon12 deletions (green lower dots) are shown to have a  $\log_2$  value of  $< -0.5$  demonstrated exon copy number loss



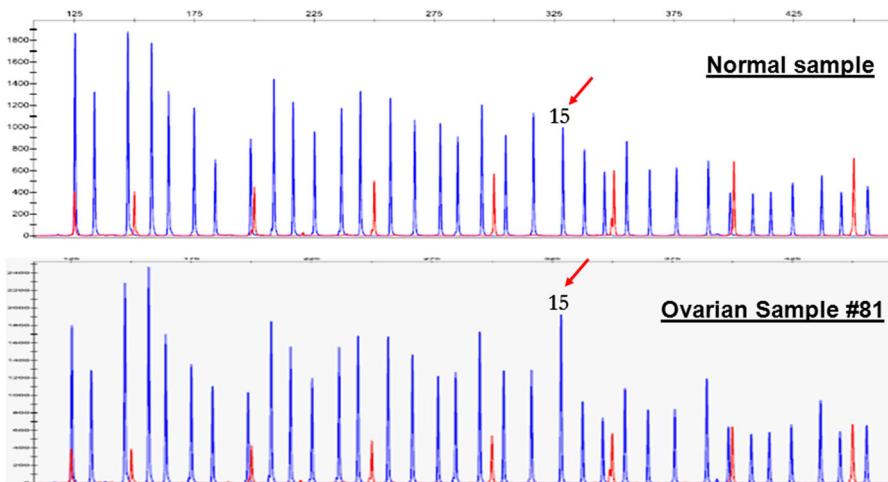
**Fig. 4** upQMPSF and the detection of BRCA1 exon 15 duplication in familial ovarian cancer patient 81: upQMPSF peak profile for BRCA1 set2 (exon 13–24), exon numbers are depicted on the top of each peak. Red arrow (lower panel) represents the detection of BRCA1 exon 15 duplication demonstrated by higher peak height in ovarian cancer patient 81 compared with the peak height in normal sample (upper panel) and other neighboring peaks

chosen as a cut off value for the detection of copy number gain. Log2 values of upQMPSF scores for *BRCA1* gene set2 scores revealed normal sample values around 0. Meanwhile, ovarian cancer samples with copy number gain had a value of  $\sim 0.48$  (Fig. 5b). upQMPSF replicates showed consistent pattern for patient 81 and the duplication was confirmed by MLPA with an increase in exon 15 peak height (Fig. 6). Additional multiplex primer subset was established to amplify two intron 15 fragments 15\_1 and 15\_2 in addition to exon 14, 15, 16 fragments. upQMPSF results showed copy number gain in amplicons 15 and 15\_1, but not 15\_2. The data confirmed and refined the duplicated region in *BRCA1* gene to  $\sim 3.8$  kb.

MM-PCR was used to characterize the breakpoints and to confirm the upQMPSF detected exon 15 duplication in *BRCA1* gene. A  $\sim 1.8$  kb band, smaller in size than the internal control, was detected in cancer patients but was not found in healthy family members (Fig. 7a). Sequence analysis of the detected product revealed a 2.8 kb genomic duplication that involved exon 15 of *BRCA1* gene, consisting of 1.15 of intron 14 and 1.65 kb of intron 15 (Fig. 7b). The 3' and 5' breakpoints contained Alu repeats sequences (Alu sg/x and Alu sp) with high sequence homology in similar orientation and sharing identical sequence of 20 bp “ATTCTCCTGCCTCAGCTCC”. This suggested that homologous-based recombination is the underlying mechanism for the duplication rearrangement (Fig. 7b). Three other family members of patient 81 in the pedigree (Fig. 8) were also



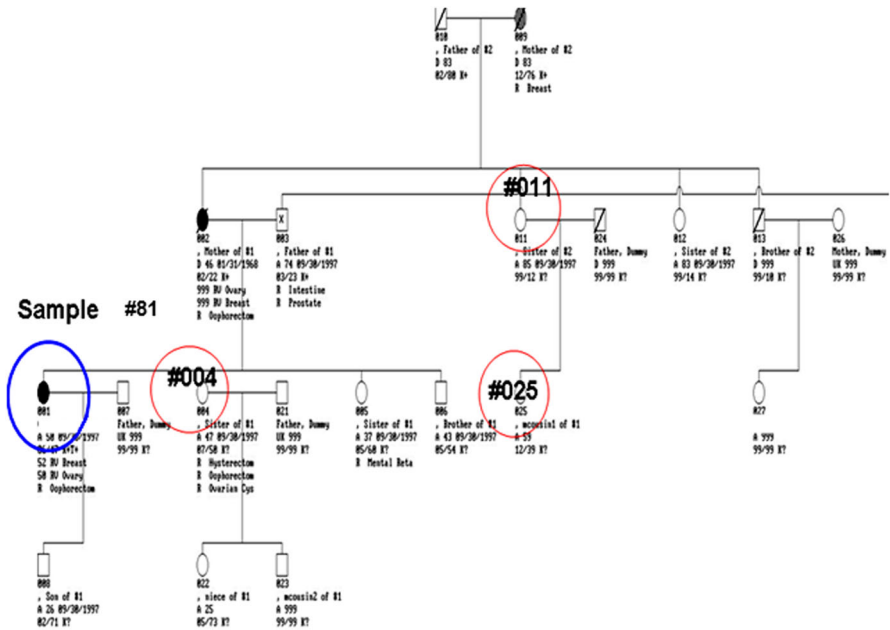
**Fig. 5** Statistical data analysis for BRCA1 exon15 duplication in familial ovarian cancer patient 81: **a** Histogram shows upQMPSF scores for BRCA1 set2 with their frequencies (*black bars*) in all normal samples. The score for BRCA1 exon15 duplication (E15dup, *red arrow*) was located outside the region of normal distribution  $\sim 8\times$  standard deviation from the mean of 1. upQMPSF score of 1.35 (*red arrow*) was chosen as a cut off value for the quantitative detection of exon copy number gain. **b**  $\log_2$  values (*black dots*) of all upQMPSF scores distributed around the value of 0 in normal samples; upQMPSF score for BRCA1 exon15 duplication (E15dup, *red arrow*) was shown to have a  $\log_2$  value of  $\sim 0.5$  demonstrating exon copy number gain



**Fig. 6** MLPA for BRCA1 exon15 duplication in familial ovarian cancer patient 81: MLPA result confirms the exon 15 duplication in ovarian cancer patient #81 detected by upQMPSF; the data shows increase in peak height in exon 15 in ovarian cancer sample in comparison with normal sample

screened to provide a prediction of cancer risk among family/relatives using upQMPSF, MM-PCR, and MLPA methods. All members were tested negative for exon 15 duplication mutation.





**Fig. 8** Family pedigree of ovarian cancer patient 81: Ovarian cancer patient 81 (blue, dark filled-in circle) and healthy family members (red circles 004, 025, and 011), which are found to be negative for BRCA1 exon 15 duplication

(Schouten et al. 2002; Casilli et al. 2002). Despite their advantages, both methods have certain limitations and disadvantages. MLPA is commonly used and commercially available. However, it is considered expensive, requires 16 h probe hybridization, and extra ligation step and lacks versatility in research due to its inability to be customized. QMPSF is simple, easy, and it relies on simultaneous amplification of different target sequences in multiplex fashion using region-specific primers. Nevertheless, QMPSF is considered costly, particularly in small-scale studies, due to the use of fluorescently labeled primers in each amplified region and lower multiplexing capacity compared with MLPA.

Due to these method limitations, there is a critical need for a simple and cost-effective technique for various types of mutation detections. The objective of the study is to develop an improved version of QMPSF with versatility, simplicity in the design, high efficiency, and reproducibility in detecting various mutation types. The newly created method was termed universal primer quantitative multiplex PCR short fluorescent fragments (upQMPSF). upQMPSF was established for quantitative detection of LGRs or exon/gene copy number changes. This is based upon simultaneous amplification of multiple genetic target regions using compatible multiplex region-specific chimerical primers, unique universal primers, and fluorescently labeled forward primer (Fig. 1). The improved concept replaces QMPSF individual fluorescently labeled primers with one universal primer to enhance robustness and improve assay cost efficiency by increasing multiplexing

capacity up to 15 products per set. Moreover, upQMPSF improves the detection resolution since the multiplex primers are designed in the intron regions. Thus, the entire exon region including the splicing sites (exon–intron boundary regions) will be included in the analysis.

In comparison with MLPA and QMPSF, results showed that upQMPSF is as sensitive as the other methods in detecting previously characterized LGRs in 4 HNPCC patient samples.

Moreover, upQMPSF was used to screen 88 ovarian cancer patients for LGRs in *BRCA1/2* genes. Exon 15 duplication in *BRCA1* gene spanning 2.8 kb was detected in patient 81. The duplication was confirmed, breakpoints were characterized and the mechanism of duplication was found to be mediated by Alu–Alu-based homologous recombination event. However, no LGRs were detected in *BRCA2* gene.

Germline mutations in *BRCA1* and *BRCA2* genes account for most but not all familial breast and ovarian cancer cases. Therefore, it is essential to examine LGRs in other ovarian cancer susceptibility genes. *MutL1 Homology (MLH1)* gene is a member of mismatch repair genes. Previous studies suggest that defects in *MLH1* gene also increase the risk of pancreatic, prostate, endometrial, gastric, and ovarian cancers (Lynch et al. 1998). In another report, *RAD51* gene was found to have a major role in initiating HR repair along with *BRCA1/2* genes at the site of DNA damage (Cousineau et al. 2005; Baumann and West 1998). Like *BRCA1* gene, *RAD51* gene sequence analysis showed high content of 89 Alu repeat compositions, which make it prone to HR events and LGRs. upQMPSF showed no LGRs was found in both *MLH1* and *RAD51* genes in tested samples.

## Conclusion

Various types of genetic abnormalities with different resolutions are found in cancer cells. They range in size from a point mutation/SNP to hundreds or thousands of bases of insertions and deletions such as large genomic rearrangements.

upQMPSF was developed for the detection of large size genomic rearrangements. A heterozygous genomic duplication spanning exon 15 was detected in *BRCA1* gene and mediated via Alu-based homologous recombination. No LGR was found in *BRCA2*, *MLH1*, and *RAD51* other hereditary ovarian cancer susceptible genes. The result concluded that LGRs are present in less than 1% in tested patient set. Nevertheless, LGRs detection in *BRCA1/2* should be included in the clinical genetic testing for the diagnosis of hereditary ovarian cancer.

When compared with currently available methods, upQMPSF is shown to be a sensitive, reproducible, cost-effective, easy to customize, and versatile method with improved detection resolution of LGRs and various types of mutations. As a mutation screening assay in genetic testing for hereditary diseases, upQMPSF allows for low cost and faster turn-around result.

Considering upQMPSF's capacity in detecting exon copy number variants, it should be considered for research and molecular diagnostic purposes with wider

spectrum for *BRCA1* gene mutation detection, as well as a safe alternative method, particularly for the confirmation or for first line of analysis.

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