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ORIGINAL ARTICLE

Capillary electrophoresis as alternative method to detect tumor genetic mutations: the model built on the founder *BRCA1 c.4964_4982del19* variant

Maria De Bonis[1] · Angelo Minucci[1] · Giovanni Luca Scaglione[2] · Elisa De Paolis[1] · Gianfranco Zannoni[3] · Giovanni Scambia[2,3] · Ettore Capoluongo[1,4]

Abstract

Despite some populations show a wide spectrum of different BRCA pathogenic variants (PVs), particular ethnic groups carry at high frequency a single or a few recurrent PVs, usually due to a founder effect. The identification of these founder PVs, with simple molecular methods, improves *BRCA1/2* testing and cancer risk assessment. In this study, we developed a rapid and reliable PCR method, coupled with capillary electrophoresis (CE) for genotyping the Italian founder *BRCA1 c.4964_4982del19* (rs80359876) variant. In addition, we compared the performance of two CE platforms: (Agilent 2100 Bioanalyzer and the Experion Automated Electrophoresis system) to identify this variant. Our findings suggest that CE represents a simple and standardized diagnostic strategy for the unambiguously identification of the *BRCA1 c.4964_4982del19* variant, on both germline and somatic DNA samples. The results and performance obtained by two platforms are absolutely superimposable in terms of specificity and sensitivity, as well as for their feasibility, time of analysis and costs.

Keywords *BRCA1/2* genes · Capillary electrophoresis · Italian founder *BRCA1/2* mutations

Abbreviations

HBOCS	Hereditary breast and ovarian carcinoma syndrome
PVs	Pathogenic variants
iPARP-1	Poly(ADP-ribose) polymerase 1-inhibitors
HGSC	High-grade serous carcinoma
NGS	Next generation sequencing
CE	Capillary electrophoresis
FFPE	Formalin fixed paraffin embedded
LOH	Loss of heterozygosis
MPS	Massively parallel sequencing

✉ Ettore Capoluongo
e.capoluongo@idi.it

[1] Università Cattolica del Sacro Cuore, Fondazione Policlinico Universitario Agostino Gemelli, Polo Scienze delle Immagini, di Laboratorio ed Infettivologiche, Largo Agostino Gemelli 1, 00168 Roma, Italy

[2] Fondazione di Ricerca e Cura Giovanni Paolo II, Laboratorio di Oncologia Molecolare, Largo Agostino Gemelli 1, 86100 Molise, Campobasso, Italy

[3] Università Cattolica del Sacro Cuore, Fondazione Policlinico Universitario Agostino Gemelli, Polo Scienze della Salute della Donna e del Bambino, Largo Agostino Gemelli 1, 00168 Roma, Italy

[4] Istituto Dermopatico dell'Immacolata Istituto di Ricovero e Cura a Carattere Scientifico, Dipartimento di Diagnostica di Laboratorio e Biologia Molecolare Clinica, Via dei Monti di Creta 104, 00167 Roma, Italy

Introduction

Hereditary breast and ovarian cancers syndrome (HBOCS) is associated with germline PVs within *BRCA* susceptibility genes [1–3]. However, investigating *BRCA* mutational status plays a key role not only for the identification of familial cancer predisposition but also to address therapeutic choices. In fact, iPARP-1 were cleared by FDA and EMEA being effective for targeted treatment of high-grade serous carcinoma (HGSC) patients harboring germline and/or somatic *BRCA* PVs [4].

Conventional methods used to identify *BRCA* germline PVs are time-consuming and expensive, due to the large size of the genes. The recent introduction of next generation sequencing (NGS) bench-top platforms is a great promise, which is rapidly revolutionizing genetic screening in diagnostic and clinical applications [5].

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Furthermore, NGS allows to detect somatic mutations, that may be present in a low proportion of the total DNA at limit of detection not achievable by Sanger sequencing [6]. Since NGS technique still remains expensive for cost of machines and infrastructure, a stepwise mutational analysis screening for the most common *BRCA1/2* variants could allow for cheaper affordable first-line molecular testing strategy [7, 8].

BRCA mutational landscape widely varies among different populations [9]. In fact, while some populations present a large spectrum of different PVs, particular ethnic groups show high frequency of a single or a few recurrent *BRCA* PVs, usually due to a founder effect [7].

In the last years, several founder PVs were well-studied, as the (a) *BRCA1 c.68_69delAG* and *c.5266dupC*, (b) *BRCA2 c.5946delT* in the descendants of Ashkenazi Jews (c) the *BRCA2 c.771del5* that is identifiable in approximately 8% of both Icelandic breast and ovarian cancer cases [10].

In Italy, some recurrent founder PVs have already been reported, each one confined within a limited regional geographic area. The most significant examples are: *BRCA1 c.1378dupA* and *c.3228_3229delAG* in Tuscany [11, 12], *BRCA1 c.4964_4982del19* in Calabria and Sicily [13], *BRCA1 c.5181_5183delGTT* in Veneto [14], *BRCA2 c.8537delAG* and *c.3723del3insAT* in Sardinia [15, 16], and finally the more recently *BRCA1 c.190T>C* in the Lombardy [17].

In these case, a preliminary screening of founder PVs can improve *BRCA* testing and cancer risk assessment, reducing the cost and turn-around time of molecular testing and also to define patient's sensitivity to iPARP-1 [4, 18, 19].

CE, a technique of high efficiency, high resolution and short analysis time, is successfully used for mutation and polymorphism analysis [20, 21]. Considering the analytical (sizing resolution of ± 5% and sizing accuracy of ± 10%) and technical specifications of the microchip CE devices, we applied this powerful approach for the rapid genotyping the *BRCA1 c.4964_4982del19* variant on both germline and FFPE samples. Our laboratory frequently receives many samples belonging to women from Calabria and Sicily, where *BRCA1 c.4964_4982del19* accounts for 21.7–33.3% of all *BRCA1* PVs [13]. Nevertheless, our group has recently published as the *c.4964_4982del19* PV cannot be always detected using NGS pipelines which are not completely validated [22]. Therefore, the need of an improvement of *BRCA1/2* laboratory protocols should be take into account to overcome these issues and pitfalls. In order to better standardize our method, we also compared the performance of two micro-device platforms: the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and the Experion system (Bio-Rad Laboratories, Hercules, CA, USA).

Materials and methods

Samples and DNA extraction

The study was performed in accordance with the Declaration of Helsinki.

A total of 20 ovarian cancer patients, who were referred to department of Clinical Molecular and Personalized Diagnostics of the Hospital 'Agostino Gemelli' Foundation, were selected for the study after signing the appropriate informed consent.

All the samples selected to set-up this method were previously amplified by *BRCA* Tumor MASTR Plus (Multiplicom, Niel, Belgium), fully genotyped by NGS on the Illumina MiSeq® platform (Illumina, San Diego, CA, USA), and lastly confirmed by Sanger sequencing. Furthermore, the evaluation of *BRCA1* and *BRCA2* copy number changes was performed as previously reported [23, 24]. The samples were genotyped as follows: 10 wild-type (WT) and 10 patients carrying the *BRCA1 c.4964_4982del19* allele.

In addition, we also evaluated the performance of CE assay on 20 additional FFPE DNA from ovarian cancer patients (10 WT and 10 mutated).

Total genomic DNA was isolated by using the Magcore® Nucleic Acid Extractor (RBC Bioscience, Taiwan) following the manufacturer's protocols: MagCore® Genomic DNA Whole Blood Kit for the blood samples and MagCore® Genomic DNA FFPE One-StepKit for the FFPE samples.

The DNA concentration and purity were determinate by NanoPhotometer™, (Implen, Munchen Germany).

Primer design and PCR conditions

Primer design was performed using the freely available software Primer3 (http://primer3.ut.ee/) in order to generate an amplicon of 213 bp surrounding the *c.4964_4982del19* variant. Both primers were checked for primer–dimer interactions, both for self-dimers and cross-dimers using software DINAMelt (http://unafold.rna.albany.edu/?q=dinamelt). In addition, we used the BLAST software to verify the primers' specificity (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The resulting designed primers were the following: forward (F) 5′-GGTATAATG CAATGGAAGAA-3′ and reverse (R) 5′-CTTCCTCTA GGTTATTAATTGAC-3′ (provided by Eurofins Genomics GmbH, https://www.eurofinsgenomics.eu/).

PCR amplification was performed in 25-µL reactions and the mixture contained: 12.5 µL of MasterMix 2× [Promega, Madison, USA, (http://www.promega.com), containing buffer (pH 8.5), dNTPs (400 µM), Taq Polymerase

(50 U/μL) and Mg^{2+} (3 mM)], 3–4 μL of genomic DNA as template (about 120–170 ng), 0.5 μL of both primers (200 nM) and ultrapure H_2O until reaching the final volume. The amplification was performed using 1 cycle of 95 °C for 2 min followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and ending with 1 cycle of 72 °C 10 min.

At the end of the PCR step, 5 μL of PCR was used for electrophoresis on 4% agarose gel in order to establish the efficiency of the amplification.

Mutation detection by Agilent and Experion systems

Two automated CE platforms for the identification of the *c.4964_4982del19* variant were used: the Agilent 2100 and the Experion™ instruments, with the DNA 1000 kit (Agilent Technologies, Santa Clara, CA) and the DNA 1K Bio-Rad Laboratories, Hercules, CA, USA kits, respectively. Both kits contain: DNA ladders, microfluidics chips and the reagents required to perform capillary electrophoresis of DNA amplicons ranging from 15 to 1500 bp (Table 1).

Both systems include a microfluidic apparatus, combined with computer-controlled instruments and measurements along with software-based reporting and analysis.

The ladder and sample wells were subsequently loaded with 5 μL of the size marker mixture plus 1 μL of either the molecular size ladder or sample. After mixing by vortex, the chip was immediately insert into the instruments and processed.

The use of two DNA internal markers (lower and upper) allows the peak alignment. In addition, the presence of DNA ladders during every electrophoresis course provides an accurate sizing and quantification of each DNA sample.

The results obtained can be reported as an electropherogram or a gel-image, while the dataset can be exported in various plain text tabulated formats.

All experiments were run using 2100 Expert software version B.02.08 (Agilent Technologies, Santa Clara, CA) and Experion software (Bio-Rad Laboratories, Hercules, CA, USA) depending on platform used.

Tabular data of the samples were then imported in KaleidaGraph v4.1.3 (Synergy Software Inc, Reading, PA, USA) and plotted using *double xy* chart option.

Table 1 Analytical and physical specifications Agilent 2100 Bioanalyzer versus Experion Automated Electrophoresis systems

	Agilent DNA 1000 Kit	Experion DNA 1K Assay
Analytical specification		
Separation range (bp)	25–1000	15–1500
Physical specification		
Analysis time	35 min	40 min
Sample per chip	12	11
Gel-dye mix wells	3	4
Priming mode	Manual	Automated

Results

On overall samples, both automated CE systems were able to unambiguously distinguish the WT alleles from those carrying the *c.4964_4982del19* PV. In fact, the WT samples is characterized by only one peak (corresponding to 213 bp), while the presence of the *c.4964_4982del19* variant determines two fragments [one of 194 bp (the mutant allele) and the alternative by 213 bp (WT allele)].

Furthermore, in order to compare the performance of the two platforms, we also evaluated some parameters such as aligned migration time (s) and time corrected area The detailed results, obtained by the two platforms, are summarized in Table 2.

Moreover, we also evaluated the ratio between the peak area corrected by time of migration of wild-type and mutant

Table 2 Summary of results of Agilent 2100 Bioanalyzer versus the Experion Automated Electrophoresis systems

	Agilent		Experion	
	Wild type	Mutant[c]	Wild type	Mutant[c]
Germline sample				
Fragment size PCR (bp)[a]	214 ± 0.5	215 ± 0.5 199 ± 0.5	211 ± 0.5	215 ± 0.5 199 ± 0.5
Aligned migration time (s)	66 ± 0.1	66 ± 0.1 64 ± 0.1	61 ± 0.1	62 ± 0.1 60 ± 0.1
Time corrected area	150 ± 0.5	64 ± 0.5 61 ± 0.5	216 ± 0.5	87 ± 0.5 85 ± 0.5
Ratio time corrected area[b]		1 ± 0.1		1 ± 0.1
Somatic sample				
Fragment size PCR (bp)[a]	219 ± 0.5	220 ± 0.5 204 ± 0.5	213 ± 0.5	210 ± 0.5 195 ± 0.5
Aligned migration time (s)	67 ± 0.1	67 ± 0.1 65 ± 0.1	60 ± 0.1	60 ± 0.1 58 ± 0.1
Time corrected area	29 ± 0.5	13 ± 0.5 42 ± 0.5	30 ± 0.5	10 ± 0.5 85 ± 0.5
Ratio time corrected area[b]		0.3 ± 0.1		0.3 ± 0.1

[a]Values are expressed as means and DS

[b]Ratio were calculated using the mean value of time corrected area between allele wild-type and allele mutated. Ratio values differ between germline and somatic amplicons

[c]The mutant sample are indicated with the two mean value belonging to wild-type and mutated alleles

allele. Clearly, the peak ratio calculated on DNA extracted from blood was about 1.0, while the same ratio on DNA extracted from FFPE was indeed approximately 0.3.

The electropherograms of three selected samples are reported in Fig. 1, showing data from both Agilent and Experion systems. In detail, wild-type and heterozygous germline samples are shown in panels a and b, respectively. The somatic profile of *c.4964_4982del19* variant (panel c), clearly shows the loss of heterozygosis (LOH) of the wild-type allele compared to the germline pattern (reported in the panel b).

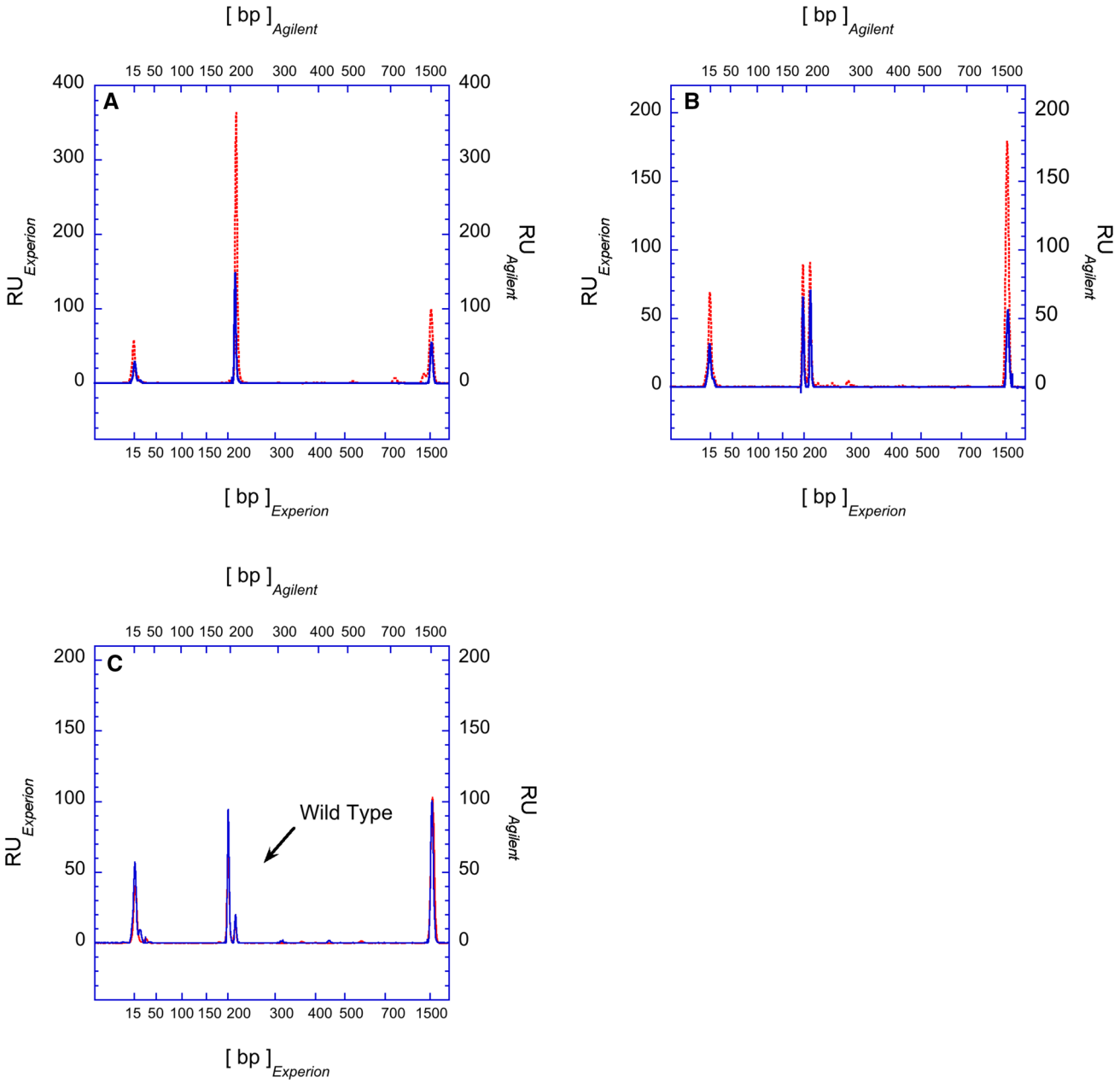

Fig. 1 Electrophoretic datasets obtained on both Agilent and Experion systems shown in plotted and overlapping modality. The CE profiles for each given sample are reported in the same panel as blue and red lines for Agilent and Experion systems, respectively. The electropherograms of wild-type is reported in **a**, while the germline and somatic *c.4964_4982del19* heterozygote variant in **b** and **c**, respectively. As indicated by the arrow within **c**, the peak height of WT allele strongly differs from the germline one, thus confirming the different peaks ratios obtained comparing germline (1.0 ± 0.1) and somatic (0.3 ± 0.1) datasets. This behavior can be dependent on possible somatic LOH of the wild-type allele, above all considering that this pattern is not present at germline level (**b**)

Evaluation of CE sensitivity

We evaluated the sensitivity of our assay by mixing the *c.4964_4982del19* heterozygote sample with a reference WT DNA at following ratio: 50, 30, 10, 5 and 2%.

A sensitivity of 100% was achieved since we were capable to detect the mutated allele even on the sample containing 2% of allele fraction (Fig. 2).

Discussion

BRCA genes can show both germline and somatic PVs. When related to HGSC, PVs show prognostic value and also predict the response to molecular targeted drugs, like iPARP-1. In this context, molecular screening of both germline and somatic *BRCA* mutations has become to be incorporated in clinical routine settings [22]. The large sizes of the *BRCA* genes, along with the large pattern of mutations reported and spanning the entire sequence, do not facilitate the molecular analysis above all when performed with linear sequencing. The proportion of women needing this testing is rapidly raising worldwide, due to the increasing number of new diagnoses of HGSOC in many countries.

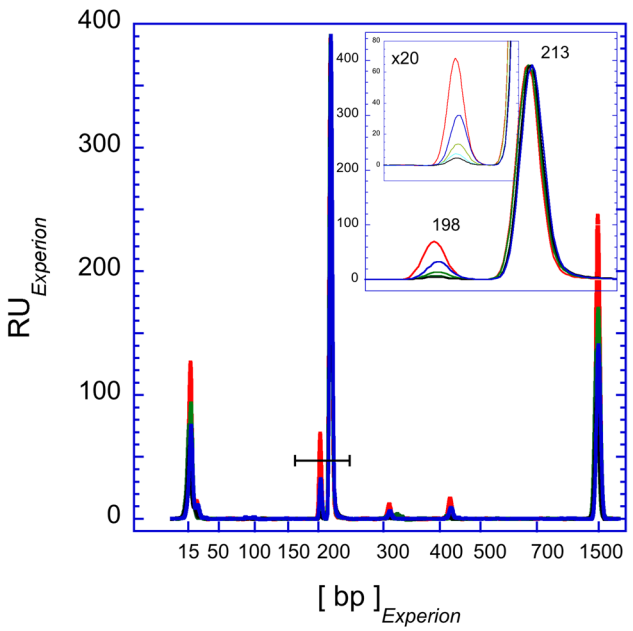

Fig. 2 CE profiles of different ratios of wild-type/*c.4964_4982del19* tumor samples. In the upper-right inset, a ×20 magnification of the main electropherogram representing the *c.4964_4982del19* allele (bp198) at different ratio: 50% (red), 30% (blue), 10% (green), 5% (light blue) and 2% (black) is reported. It shows that 2% ratio is still detectable. The mid-size inset shows the interval underlined by the solid line (ca. 190–220 bp). *Arbitrarily, only the electropherograms obtained on Experion system were reported due to the highly similarity with the Agilent BioAnalyzer system. (Color figure online)

However, the presence of founder mutations (reduced genetic variability explaining a disease) in a specific population gives a good opportunity to design low-expensive and feasible tests, improving the capability to early screen peculiar regions worldwide [25, 26]. In Italy, significant regional founder effect has been demonstrated for few PVs: among these, the *c.4964_4982del19* in exon 16 of *BRCA1* is representative of Sicily and Calabria Regions of South Italy, where it accounts for about 22–33% of all Italian *BRCA* PVs [13]. Furthermore, the *BRCA* screening of 3000 ovarian cancer women at Gemelli hospital, pointed out as about 6% of these women carried the *c.4964_4982del19*, in addition to those of Sicilian and Calabrian origins. As a consequence, due also to pitfalls found using NGS pipelines [22], we decided to set-up this rapid screening to improve the quality of our analysis particularly on Sicilian Calabrian women resulting mute at BRCA1/2 NGS analysis.

In light of this, we show how this variant can be simply identified with high sensitivity, cost-effectiveness method, above all when PCR and CE are performed on both peripheral blood and FFPE tumor samples.

In fact, we unambiguously characterized the wild-type from mutated alleles by using two distinct electrophoretic systems, with 100% concordant with DNA Sanger and NGS sequencing.

The correct identification of particular *indels* by massively parallel sequencing (MPS) could be mainly affected by library design (e.g. amplicon length), MPS platform as well as bioinformatics algorithms [27]. Similarly, the variant *c.4964_4982del19* herein discussed was missed due to a 'bioinformatics bug' while testing an Ion5S Oncomine pipeline as reported in a previous study by our group [22].

Consequently, the bioinformatics pipeline was tailored and validated in our diagnostics routine settings to correctly identify this peculiar type of mutation (*rs80359876*).

In addition, CE allowed the detection up to 2% of the mutated allele on FFPE tumor samples, showing higher sensitivity compared to Sanger sequencing (where the theoretical LOD is about 10%). Therefore, CE could be used not only as a screening test but also as a confirmation technique of *rs80359876* variant in FFPE tumor samples. Notably, FFPE mutated alleles may be present in a lower proportion of the total DNA, that could be not detected by Sanger sequencing [6, 28].

The differences found between behaviors of germline and tumor wild type and mutated *BRCA1* exon 16 alleles (1.0 vs. 0.3 ratio, respectively) could be dependent on possible LOH of wild-type allele on tumor cells [29]. In light of the above, CE could be an alternative more powerful strategy for LOH evaluation in tumor samples: its use could be extended also to other types of *BRCA1/2 indel*s in the future.

Finally, the performances of two micro-device platforms, 2100 Bioanalyzer and Experion system, resulted

as completely superimposable in terms of specificity and sensitivity, as well as for the workflow, time of analysis and costs. Consequently, we can confirm as CE represents a valid, highly sensitive and reliable mutation scanning technology also for the identification of *BRCA1 c.4964_4982del19* variant.

Given the high prevalence of this variant in Italian HBOCs Calabrian and Sicilian patients, the development of an efficient screening test represents an improvement of the *BRCA* molecular workflow. In this case, the analysis of the *c.4964_4982del19* mutation via CE-based technique allows for the rapid identification of about 30% of the overall *BRCA1* positive patients, greatly reducing costs and turnaround time of the genetic analysis. This *c.4964_4982del19* screening, offered as first level test, represents a population-focused surveillance strategy and could facilitate the rapid identification of patients eligible for targeted treatment with iPARP-1 inhibitors [30].

Concluding remarks

Identification of founders and common mutations is an extremely important step towards the improvement of genetic counseling since molecular testing can be targeted to those mutations allowing for a more rapid and less expensive test.

We developed a faster, cheaper and easier molecular test able to detect an Italian *BRCA1* founder variant. Particularly, this approach allows for the efficient identification of the most frequent PV within Calabrian and Sicilian individuals. The diffusion of such screening method can also facilitate the admission of *BRCA*-positive ovarian cancer women to iPARP-1 target therapy. Finally, the strategy reported herein could represent also a methodological model useful in the routine clinical-diagnostic workflow. We also suggest to perform it as a valid confirmatory test for the *indels* ranging between 8 and 20 bp, especially in tumor FFPE-deriving DNA, where the mutated allele may be represented in a lower proportion of the total DNA and potentially loosed by Sanger sequencing.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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