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A comprehensive *BRCA1/2* NGS pipeline for an immediate Copy Number Variation (CNV) detection in breast and ovarian cancer molecular diagnosis

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ABSTRACT

Background: The aim of this study was to assess the ability of a Next Generation Sequencing (NGS)-based approach in the detection of *BRCA1/2* Large Genomic Rearrangements (LGRs).
Methods: We investigated 287 consecutive unrelated Italian women affected by breast or ovarian cancer. A NGS pipeline with a reliable Copy number Variation (CNV) prediction algorithm was applied. In addition, all samples were investigated using MAQ (Multiplex Amplicon Quantification) assay, while the MLPA (Multiplex Ligation-dependent Probe Amplification) technique was used as alternative method if necessary.
Results: Small pathogenic variants were identified in 80 samples. During NGS analysis, 11 samples were identified by Amplicon Suite Software (SmartSeq, Novara, Italy) as positive for large deletions or duplications. However, MAQ assay provided positive results in 19 patients. In particular, *BRCA2* exon 25 deletion, not revealed by NGS CNV prediction algorithm, was detected in 8 unrelated women as false positive result. Sequencing analysis showed IVS24-113T/G (c.9257-113T > G) variant in 6 of these 8 patients, while the novel IVS24-129G > A (c.9257-129G > A) variant was detected in only two samples.
Discussion: The prevalence of large rearrangements in *BRCA1/2* genes was the 12% of all disease-causing mutations detected in our patients. In particular, *BRCA1* rearrangements were the 14.5% of all *BRCA1* causing variants identified. Differently, *BRCA2* large deletions were only the 6.9% of all mutations occurring in this gene. While MAQ assay showed 2,8% of false positive results, our integrative NGS-based approach fully satisfied the sensitivity and specificity parameters required on the *BRCA1/2* LGRs detection. The workflow represents a robust and easy-to-use method for full *BRCA1/2* screening, which can be easily implemented in routine diagnostic testing.

1. Introduction

A comprehensive test for all *BRCA1/2* Large Genomic Rearrangements (LGRs) detection was introduced in 2006, about ten years after *BRCA1/2* genes identification, by main centers operating in oncologic molecular diagnostics with sequencing analysis. Over the last years, LGRs have been intensively investigated in breast and/or ovarian cancer patients from different countries. Many studies document their involvement in genetic predisposition of gynecological tumors and a significant number of different rearrangements has been reported in *BRCA1/2* genes [1–3]. Currently, if it is known that *BRCA1* LGRs may represent a significant part of all disease-causing mutations in various populations, LGRs in *BRCA2* gene are less frequently observed [3].

Multiplex ligation-dependent probe amplification (MLPA) is the most commonly used technique for the detection of large deletions/duplications in the *BRCA1/2* genes. In fact, this approach seems to be a powerful high throughput tool, although not validated for clinical diagnostics [4]. However, the accuracy of MLPA results showing single exon deletion has been widely discussed, in contrast with the results reporting multiple sequential exons deletions [5–7]. In fact, for MLPA results showing a single exon deletion, it is essential to verify the absence of variants that may affect probe hybridization. Thus, in this case the use of an alternative confirmation technique (Southern blot, Real-time quantitative PCR assay, long-range PCR) is mandatory in clinical diagnosis [5–7]. We have widely reported that Multiplex Amplicon Quantification (MAQ) technique represents a valid and reproducible

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Table 1
Detection of LGRs using NGS CNV analysis, MAQ and MLPA technique.

Patients ID	Age at diagnosis of cancer	Gene	LGR	NGS CNV analysis	MAQ	MLPA
BO35	OC 43	*BRCA 1*	Del exons 1-2	Yes	Yes	NP
BO66	BC 31 OC 42	*BRCA 1*	Del exons 1-2	Yes	Yes	NP
BO04	BC 45	*BRCA 1*	Del exons 1-2	Yes	Yes	NP
BO17	BC 37	*BRCA 1*	Del exons 1-2	Yes	Yes	NP
BO77	OC 64	*BRCA 1*	Del exon 14	Yes	Yes	NP
BO181	BC 45 OC 65	*BRCA 1*	Del exons 18-19	Yes	Yes	NP
BO61	OC 44	*BRCA 1*	Del exon 20	Yes	Yes	NP
BO106	BC51	*BRCA 1*	Dup exon 3	Yes	Yes	NP
BO165	BC42	*BRCA 1*	Del exons 4-5-6	Yes	Yes	NP
BO56	BC 36	*BRCA 2*	Del exon 3 del	Yes	Yes	NP
BO71	BR 42	*BRCA 2*	Del exon 3 del	Yes	Yes	NP
BO78	BC 46	*BRCA 2*	Del exon 25	No	Yes[a]	No
BO132	BC 42	*BRCA 2*	Del exon 25	No	Yes[a]	No
BO41	BC 37	*BRCA2*	Del exon 25	No	Yes[a]	No
BO03	BC 50	*BRCA2*	Del exon 25	No	Yes[a]	No
BO11	OC 52	*BRCA2*	Del exon 25	No	Yes[a]	No
BO220	OC47	*BRCA2*	Del exon 25	No	Yes[a]	No
BO25	BC 54	*BRCA2*	Del exon 25	No	Yes[b]	No
BO196	OC58	*BRCA2*	Del exon 25	No	Yes[b]	No

NP: Not Performed.
[a] False positive result obtained in a patient carrying the BRCA2 IVS24-113T/G variant.
[b] False positive result obtained in a patient carrying the BRCA2 IVS24-129 G/A variant.

CE-IVD tool for molecular diagnostics and it is able to provide real information [8]. In fact, the need to process a great number of samples in a short time addressed us to considered alternative methods to MLPA.

Few years ago the first NGS-based (Next Generation Sequencing) approaches were developed in order to perform comprehensive genetic testing of *BRCA1/2* genes. However, the specificity of this strategy was considered to be too low for a correct LGRs detection due to employed software, kit protocol and normalization procedures [9]. Recently, the improvements of methodologies have led many diagnostics laboratories to test NGS-based platforms as the main technology for clinical testing [10–12]. The main challenge is the development of a simple and robust pipeline, able to provide CNV (Copy Number Variation) information and sequence data using a single platform, fulfilling the requirement of quality control for diagnosis. At this purpose, here the validation of a NGS workflow involving the Illumina MiSeq platform (Illumina, San Diego, CA, USA) and the Amplicon Suite software (SmartSeq, Novara, Italy) germline CNVs in *BRCA1/2* genes calling is reported. The obtained results on 287 breast and ovarian cancer patients showed interesting data.

2. Materials and methods

2.1. Patients

In this study, 287 consecutive unrelated Italian women, affected by breast or ovarian cancer, receiving oncologic and genetic counselling between June 2017 and December 2017 are investigated. After informed consent subscription, a peripheral blood sample, collected in an EDTA tube, was received by our laboratory.

2.2. BRCA1/2 NGS analysis

Genomic DNA was extracted using an automatic station (MagCore HF16 Plus, Diatech Lab Line, Jesi, Italy); DNA concentration and quality were determined by NanoPhotometer™ (Implen, Munchen Germany) and stored at −20 °C until use. Only DNA meeting following requirements: OD260/280 ratio ≥ 1.7, concentration ≥ 15 ng/μL, no degradation signals visible on agarose gel, were processed.

Devyser BRCA NGS kit (DEVYSER, Hägersten, Sweden) was used for library preparation, according to the manufacturer's instructions. Sequencing reaction was carried out using the reagent kit V2, 500 Cycles PE, on the Illumina MiSeq System (Illumina, San Diego, CA, USA) and Amplicon Suite Software (SmartSeq, Novara, Italy) was used for NGS results interpretation. The *BRCA1* and *BRCA2* reference sequences were: NG_005905.2, NM_007294.3 and NG_012772.3, NM_000059.3.

2.3. PCR and sanger sequencing

The following primers: FW 5′-TCTGTACTCCTGTTAGCAATG-3′ and RW 5′-GACTGTCAAAATAGAAAAATACC-3′ were used in order to amplify a more wide region of *BRCA2* exon 25. PCR-amplified fragments were directly sequenced using BigDye Terminator Cycle Sequencing kit v3.1(Thermo Fisher Scientific, USA) and 3500 Genetic Analyzer (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. The results were analysed using SeqScape v3.0 software package (Thermo Fisher Scientific, USA).

2.4. LGRs detection

In order to validate the CNV prediction data provided by Amplicon Suite Software (SmartSeq, Novara, Italy), all samples were investigated using MAQ assay, as previously reported [8]. If necessary, MLPA assay was used as alternative method [8].

3. Results

On the 287 patients evaluated by Massive Parallel Sequencing (MPS), we identified deleterious small variants in 80 samples. In particular, we detected 53 small pathogenic variants in *BRCA1* and 27 in *BRCA2* gene. In addition, during NGS analysis, 11 out of 287 samples were selected by Amplicon Suite Software (SmartSeq, Novara, Italy) as positive for large deletions/duplications. On the contrary, MAQ assay provided positive results in 19 out of these 287 patients (Table 1). In particular, *BRCA2* exon 25 deletion, not detected by NGS CNV prediction algorithm, was detected in 8 unrelated women (Fig. 1a, b). This result was confirmed by repeating the assay on fresh DNA samples. Before performing a long-range PCR, we decided to test the 8 patients by MLPA technique but, since also this screening resulted mute (Fig. 1d, e), a new couple of primers was designed in order to amplify a more extensive region including exon 25 of *BRCA2* gene in our patients.