Original Articles

Accurate detection and quantification of epigenetic and genetic second hits in *BRCA1 and BRCA2*-associated hereditary breast and ovarian cancer reveals multiple co-acting second hits

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ABSTRACT

Background: This study characterizes the **second hit spectrum in *BRCA1* and *BRCA2*-associated breast and ovarian cancers** at both gene loci to investigate if second hit mechanisms are mutually exclusive or able to coincide within the same tumor.

Methods: Loss of heterozygosity, somatic point mutations and copy number alterations along with promoter methylation were studied in **56 breast and 15 ovarian** cancers from *BRCA1* and *BRCA2* germline mutation carriers. A mathematical methodology was introduced to quantify the tumor cell population carrying a second hit.

Results: **Copy neutral LOH** was the most prevalent LOH mechanism in this cohort (BC 69%, OC 67%). However, only 36% of BC and 47% of OC showed LOH in all cancerous cells. Somatic **intragenic deletions** and **methylated subclones** were also found in combination with (partial) loss of heterozygosity. Un-equivocal deleterious somatic point mutations were not identified in this cohort.

Conclusion: **Different mechanisms** inactivating the wild type allele are **present within the same tumor** sample at various extents. Results indicate that *BRCA1/2*-linked breast and ovarian cancer cells are predominantly characterized by LOH, but harbor a complex combination of second hits at various frequencies.

1. Introduction

Germline mutations in the tumor suppressor genes *BRCA1* and *BRCA2* increase the lifetime risk (until the age of 80) of developing breast cancer (BC) up to 72% (*BRCA1*) or 69% (*BRCA2*) and the risk for ovarian cancer (OC) to 44% (*BRCA1*) or 17% (*BRCA2*) [1]. These genes theoretically operate under Knudson's second hit hypothesis, meaning that both alleles need to be inactivated to render the cell vulnerable to genomic instability and irregular growth. In women with a germline mutation, the risk of breast and ovarian cancer is higher due to constitutional inactivation of one allele in all cells of

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Abbreviations

BC	breast cancer
BRCA1 and 2	breast cancer susceptibility gene 1 and 2
bVAF	variant allele frequency for a variant in the germline DNA sample derived from blood
CNA	copy number alterations
cVAF	variant allele frequency of a variant within a cancerous cell showing loss of an allele
ENIGMA	evidence-based network for the interpretation of germline mutant alleles
ER	estrogen receptor
FFPE	formalin-fixed paraffin embedded
HBOC	hereditary breast and ovarian cancer
HE	hematoxylin and eosin
HER2/Neu	human epidermal growth factor receptor 2
HGSC	high-grade serous carcinoma
IDC	invasive ductal carcinoma
ILC	invasive lobular carcinoma
Lc	percentage of tumor cells in a sample that have loss of heterozygosity
Lm	percentage of tumor cells in a sample where an allele is deleted
LOH	loss of heterozygosity
mCN	mean copy number of a gene as measured with MLPA
metR	methylation rate; percentage of tumor cells where a certain CpG is methylated
MLPA	multiplex ligation-dependent probe amplification
MNV	multiple nucleotide variation
MPS	massive parallel sequencing
MS-MLPA	methylation-specific multiplex ligation-dependent probe amplification
NACT	neoadjuvant chemotherapy
OC	ovarian cancer
PR	progesterone receptor
ROI	region of interest
SNP	single-nucleotide polymorphism
SNV	single nucleotide variation
TCGA	The Cancer Genome Atlas
TCP	tumor cell percentage
TSG	tumor suppressor gene
tVAF	variant allele frequency for a variant in the tumor DNA sample derived from formalin-fixed paraffin embedded tissue
UTR	untranslated region
VAF	variant allele frequency (describes the percentage of reads containing the non-reference base pair sequence); WT, wild type

the body. A second somatic event may occur through either of three mechanisms: 1) loss of heterozygosity (LOH), 2) the occurrence of a pathogenic point mutation arising at the functional allele and 3) epigenetic alterations, like promoter hypermethylation. The most studied somatic event leading to the inactivation of the remaining wild type (WT) allele is LOH due to large deletions, genomic rearrangements, incorrect mitosis or faulty DNA repair. Reports on LOH in hereditary breast cancer date back as early as the discovery of these two genes [2–4]. Somatic inactivating point mutations have been described in a small minority of *BRCA1* and *BRCA2*-associated breast [5] and *BRCA1*-associated ovarian cancer cases [6]. Finally, data on hypermethylation of the promoter region in germline mutated tumors are scarce, but occurrences have been reported for both genes [7,8].

Understanding the driver events for each tumor and subsequent alterations guides the application of targeted therapy and personalized medicine in cancer. Yet the number of studies investigating the combination of all 3 s hit mechanisms in *BRCA1* and *BRCA2* is limited. This study aims to investigate the prevalence of LOH, point mutations, and methylation, in a cohort of breast and ovarian tumors from patients with a germline *BRCA1* or *BRCA2* mutation. Taking tumor cell percentages into account, we developed a mathematical methodology to depict a detailed landscape of somatic events combining data generated by massive parallel sequencing (MPS), multiplex ligation-dependent probe amplification (MLPA) and methylation-specific MLPA (MS-MLPA).

2. Materials and methods

2.1. Patient cohort

Formalin-fixed paraffin embedded (FFPE) tissue of 127 primary breast tumors and fifteen primary ovarian tumors, resected from patients with a germline *BRCA1* or *BRCA2* mutation, were collected from Ghent University Hospital, AZ St.-Jan Bruges-Ostend and AZ Delta Roeselare. A DNA sample extracted from EDTA blood and

relevant clinical information was available. Fig. 1 provides a flow-chart of the patient material and methodologies applied.

2.2. Sample preparation

FFPE samples were sectioned (5 μm), fixed on 1.0 PEN slides (Carl Zeiss Microscopy, Cambridge, UK) and stained with hematoxylin and eosin (HE) according to Liu et al. [9] Three consecutive sections were used for DNA extraction after laser-guided macro-dissection on PALM Microbeam equipment (Carl Zeiss Microscopy), using QIAamp Micro DNA kits (Qiagen, Hilden, Germany) according to manufacturer's protocol. Only 88 breast samples yielded sufficient amounts (>125 ng total DNA) and quality (>1.5 absorbance 260/280 nm) of DNA as measured by fluorimetry (Qubit 1.0; Thermo Scientific Fisher, Waltham, USA) and spectrophotometry (DropSense96, Trinean, Ghent, Belgium) and were included in the study. All 15 ovarian samples were included.

2.3. Sequencing of the BRCA1 and BRCA2 coding regions

The entire coding regions of both *BRCA1* and *BRCA2* and twenty flanking nucleotides (region of interest, ROI) were amplified and sequenced in DNA extracted from 103 tumor samples and the corresponding blood samples using multiplex PCR (Supplementary Methods). We always investigated both loci, independent of the locus of the germline mutation. Thirty-two BC tumor samples yielded too low coverage for adequate analysis, probably due to the age of the FFPE material (Supplementary Fig. 1) and were excluded for further analyses. Reliable sequencing results were obtained for 56 BC and 15 OC cases.

2.4. Pathology assessment

BC subtype was determined, and tumors were graded according to the Elston-Ellis modification of the Scarff-Bloom-Richardson grading system [10]. Two pathologists, scored independently
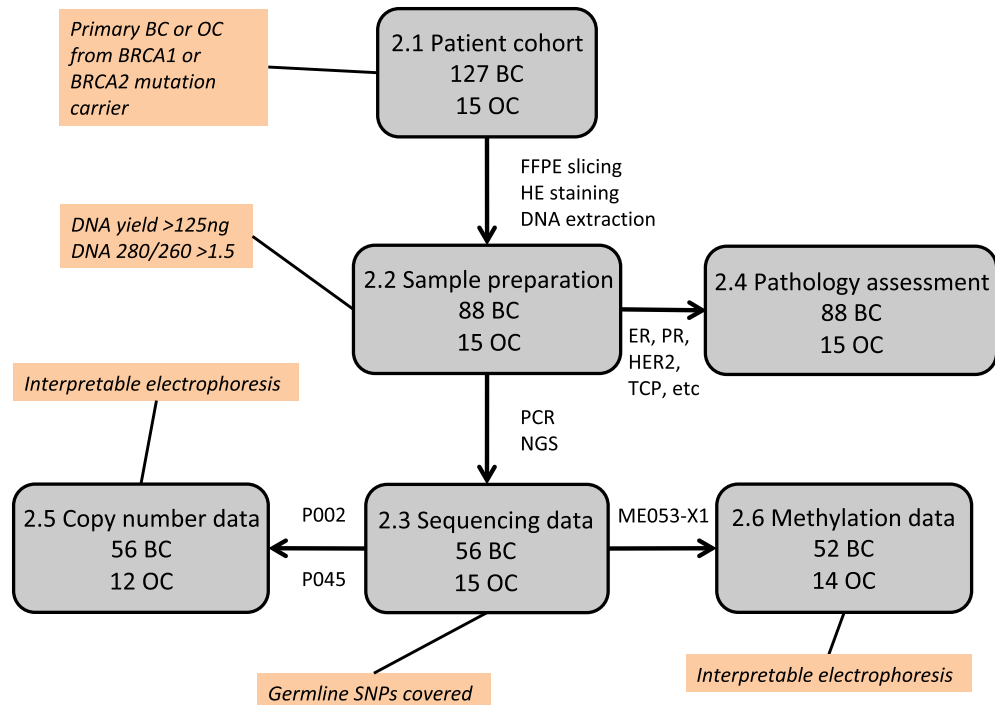
Fig. 1. Flowchart to clarify the methodology followed in the study.
The grey boxes explain each step in the workflow as described in detail in the 'Materials and methods' section. The arrows link the different steps in the procedure. Orange boxes describe the criteria to include a sample in specific steps of the workflow.

tumor cell percentages (TCP) on one HE slide per sample and the mean of both scores was taken. The mean discordance between scores was $8 \pm 2\%$. The sections scored were adjacent to the sections used for DNA extraction. Immunohistochemistry for the estrogen receptor (ER), progesterone receptor (PR) and Ki-67 and Human Epidermal Growth Factor Receptor 2 (HER2/Neu) was performed using 3.5 μm FFPE tissue sections, as previously described [11,12]. Fluorescence-in-situ hybridization was applied to determine the HER2/Neu amplification status in breast tumors with an equivocal (2+) HER2/Neu score by immunohistochemistry, according to an established protocol [11]. Supplementary Fig. 2 contains images of immunohistochemistry, while Supplementary Table 1 provides a summary of the histopathological features of all samples included in the study.

2.5. Copy number analysis

For detection of exon-spanning deletions/amplifications we used SALSA MLPA P002-D1 BRCA1 and SALSA MLPA P045-B3 BRCA2/CHEK2 probemixes (MRC-Holland, Amsterdam, the Netherlands). We always performed MLPA for both loci, independent of the locus of the germline mutation. DNA samples from individuals with normal (n = 6) or aberrant (n = 4) MLPA profiles were included in each experiment to test for inter-experimental variability. Details on data processing and normalization can be found in Supplementary Methods.

2.6. Methylation analysis

Methylation of the promoter regions of both genes was investigated with MS-MLPA (SALSA MLPA ME053-X1 BRCA1-BRCA2 probemix (MRC-Holland)) in accordance to manufacturer's recommendations and analyzed using Coffalyser.Net software (MRC-Holland). The same control samples as in 2.5 were tested. In every

experiment a no-template control, an unmethylated (Human HCT116 DKO Non-methylated DNA, Zymo Research, Irvine, USA) and methylated control (CpGenom Universal Methylated DNA, EMD Millipore, Billerica, USA) were included. Methylation rates (metR) (= percentage of tumor cells methylated at MS-MLPA probe positions) were calculated by dividing the normalized probe ratio by TCP. To evaluate the quantitative potential of the SALSA MLPA ME053-X1 BRCA1-BRCA2 probemix, dilution series were made from six artificial mixtures of the methylated and unmethylated controls, corresponding to 0, 10, 20, 50, 75 or 100% methylation.

3. Results

3.1. Somatic variants in the BRCA1 and BRCA2 coding regions detected by sequencing

In 56 BC samples 269 somatic variants were retained. Table 1 shows the distribution of these variants. The ratio in which these

Table 1
Summary of somatic variants found in breast cancer samples.

		BRCA1 n = 105 (%)[*]	BRCA2 n = 164 (%)[*]
Sequence change	C:G > T:A	91 (86.7)	131 (79.9)
	T:A > C:G	9 (8.6)	22 (13.4)
	other SNV	4 (3.8)	11 (6.7)
	MNV	1 (1.0)	0 (0)
Gene location	5′ UTR	0 (0)	3 (1.8)
	exon	101 (96.2)	160 (97.6)
	intron	4 (3.8)	0 (0)
	splice site	0 (0)	1 (0.6)

SNV = single nucleotide variation.
MNV = multiple nucleotide variation.
UTR = untranslated region.
   [*] Gene in which variants were detected.
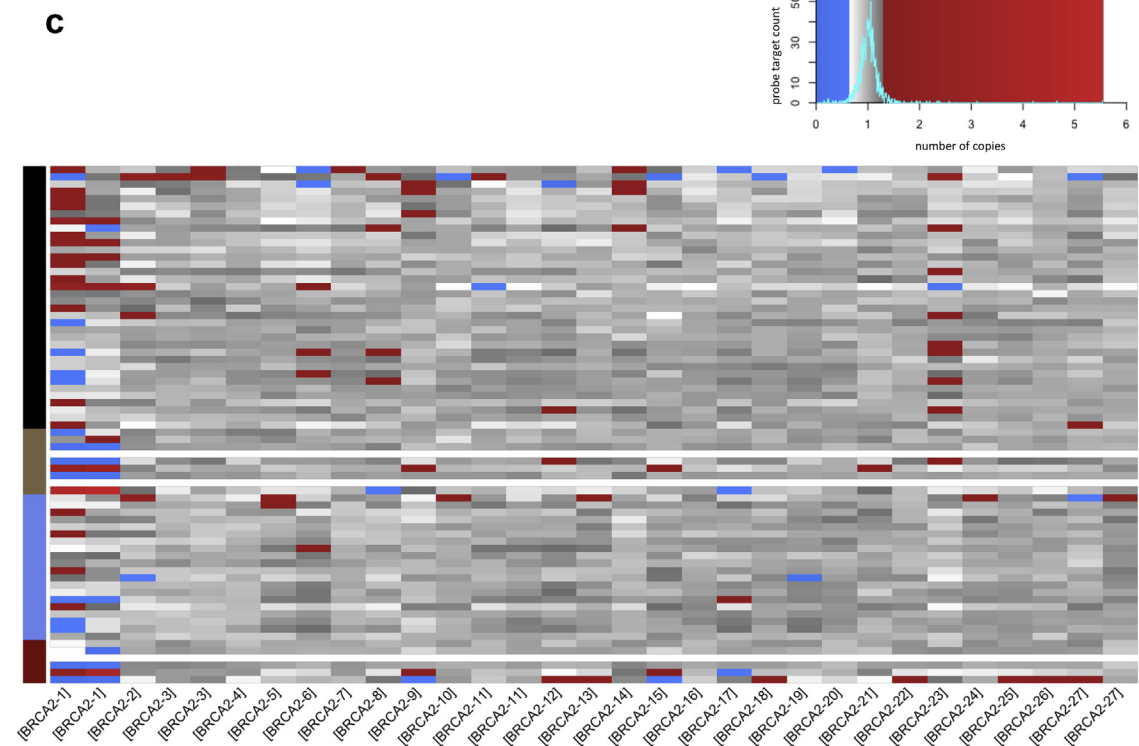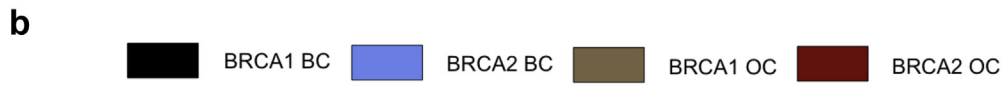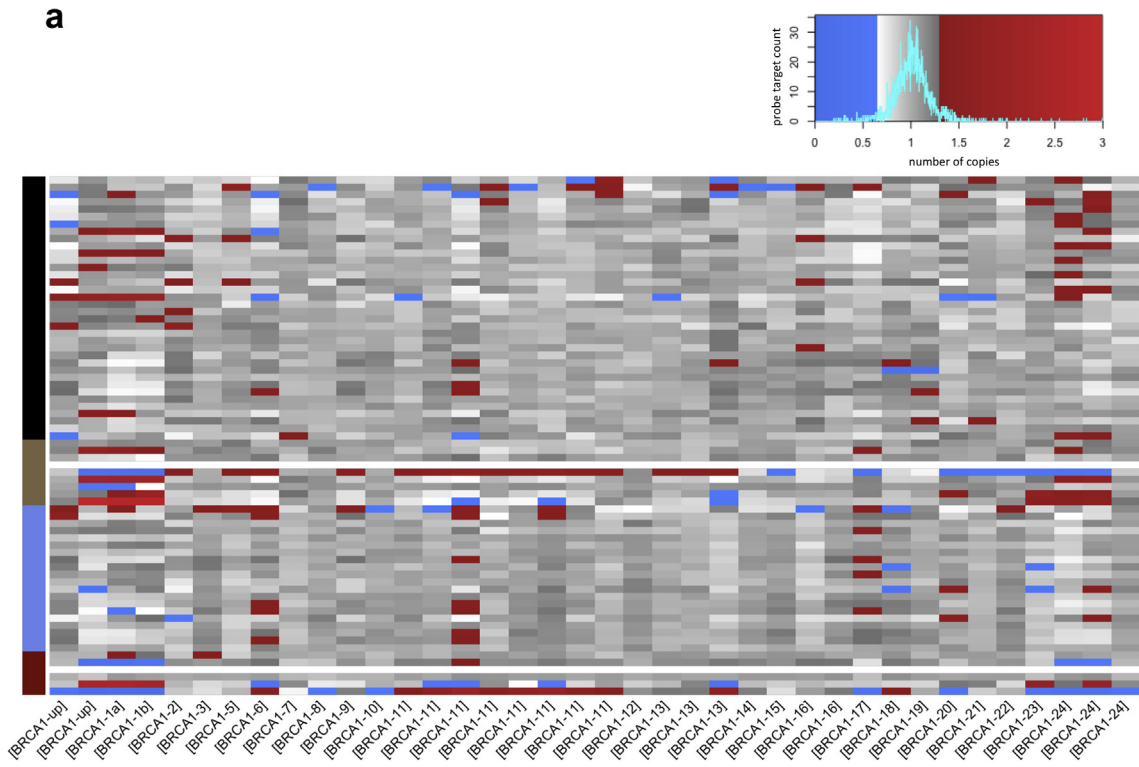
variants occurred in *BRCA1/BRCA2* was 105/164 (64%), while the ratio of number of bases in the ROI is 5759/10625 *BRCA1/BRCA2* (54%). Thirty-seven of these variants occurred in *BRCA2*-associated tumors (n = 20), while the *BRCA1*-associated cancers (n = 36) harbored 232 variants. The majority (177/269) of the variants found were missense variants and concerned C:G > T:A substitutions, indicative for fixation artefacts. In the ovarian cancers (n = 15) we found one benign somatic variant, NM_007294.3:c.4146C > T (p.Cys1382 = ; with VAF (percentage of reads containing the non-reference base pair sequence) = 23.74%).

3.2. Somatic intragenic copy number alterations in BRCA1 and BRCA2 detected by MLPA

We performed MLPA to investigate if exon-spanning deletions/amplifications in *BRCA1* or *BRCA2* occur at the somatic level. Fig. 2 shows heatmaps of the intragenic copy number data. Several samples (Table 2.1) showed signs of somatic exon-spanning deletions in *BRCA1* and/or *BRCA2* and in several OC samples there was consistent somatic loss of the first exon of *BRCA2* (independent of the locus of the germline mutation). For two BCs from patients with a deletion spanning several exons at the germline level, BC_29 (NM_000059.3:c.8332-?_8487+?del) and BC_55 (NM_007294.3:c.5075-?_5193+?del) low ratios for the germline deleted exons were suggestive for partial loss of the WT allele.

3.3. Establishment of a method to quantify the percentage of tumor cells showing loss/inactivation of the wild type allele

LOH was calculated taking into account three variables; 1) TCP, 2) the VAFs of the germline mutation and heterozygous SNPs (if present) in the blood sample (bVAF) and 3) corresponding VAFs for the same variants in the matching tumor sample (tVAF). tVAF is entirely dependent on TCP and the percentage of tumor cells exhibiting loss of the WT or mutant allele (Lc). Mathematically this is can be described as formula 1. cVAF is a theoretical value, namely the VAF of a single tumor cell that exhibits loss of one of the alleles. This cVAF can be either 0 (the allele containing the non-reference sequence is lost, meaning tVAF for the non-reference sequence reads <50%) or 1 (the WT allele is lost, tVAF non-reference sequence reads >50%). From formula 1, the Lc (percentage of tumor cells showing LOH) can be derived (formula 2). This equation is used to calculate an Lc value for each variant separately (presented as boxplots in Fig. 3). The overall Lc value for each gene was determined as the median of all Lc values (Supplementary Table 2).

Using the normalized MLPA data, the mean number of copies (mCN) was determined by dividing the value for each MLPA probe by the mean of the reference probes in the respective MLPA kits and taking the mean of the results. The percentage of tumor cells exhibiting LOH due to physical loss of one allele (Lm) was then calculated using formula 3. Both Lc and Lm represent a percentage of tumor cells with a genomic aberration. Lm/Lc gives the percentage of cells with LOH through physical loss of an allele out of all cells displaying LOH. Supplementary Fig. 3 clarifies these calculations with a hypothetical sample.

$$tVAF = (1 - TCP)bVAF + TCP \cdot ([1 - Lc] \cdot bVAF + Lc \cdot cVAF) \quad (1)$$

percentage of tumor cells that exhibit LOH = Lc

$$= \frac{tVAF - bVAF}{TCP \cdot cVAF - bVAF \cdot TCP} \quad (2)$$

percentage of tumor cells with physical allelic loss = Lm

$$= \frac{2 - 2 \cdot mCN}{TCP} \quad (3)$$

3.4. Integrated analysis of loss of heterozygosity based on sequencing and MLPA data

LOH was calculated for all samples through formula 1-3. Data for Lc and Lm calculations for both genes can be found in Fig. 3. Detailed information on every individual sample can be retrieved from Supplementary Table 2 and Table 2.2 provides a summary of these data. In our cohort copy neutral LOH turned out to be the most predominant mechanism. Remarkably, several *BRCA1*-associated BC and OC samples showed allelic loss in *BRCA2* and, similarly, several *BRCA2*-associated tumor samples displayed allelic loss in *BRCA1*. Fig. 3 shows a large number of samples with a discrepancy between the percentage of tumor cells with LOH (Lc) and the percentage of tumor cells where an allele was deleted (Lm). Possibly these samples contain more than one subclone with different copy numbers and heterozygosity.

3.5. Somatic methylation of BRCA1 and BRCA2 promoter regions detected by MS-MLPA

Using MS-MLPA the methylation status of the promoter region of *BRCA1* and *BRCA2* was examined (probe details in Supplementary Table 3). No interpretable data could be obtained for BC_01, BC_02, BC_22, BC_29 and OC_15. Supplementary Fig. 4a shows the results of the control samples. MS-MLPA is suggested to be quantitative [13]. We evaluated this by including a dilution series containing different amounts of the methylated and unmethylated controls. Linear regression showed a coefficient of determination close to 1, indicating reliable quantification for this artificial sample series (Supplementary Fig. 4b). Consequently, the probe signals detected in BC and OC samples were used to calculate the number of tumor cells that exhibit methylation at a specific CpG site (methylation rate, metR). We found a high degree of variability between probes, but for most samples metR was <20% (Fig. 4). For two probes >20% of tumor cells were methylated in a large number of BC samples (63% for BRCA1.2 and 75% for BRCA2.1). For ovarian cancers this was 14% and 21% for each probe respectively. Methylation rates for BRCA1.2 and BRCA2.1 probe targets were higher in the majority of samples compared to other CpGs. The correlation between BRCA1.2 and BRCA2.1 was better (tau = 0.65 and p-value = 3.5e-14, CpGs located on different chromosomes) than probe targets surrounding them in the promoter region of the same gene. Correlation for BRCA2.1 and BRCA2.2, separated by 215 bp, resulted in tau = 0.59 and p = 7.4e-12.

4. Discussion

The objective of this study was to paint a detailed picture of

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Fig. 2. Results of intragenic copy number analysis. a) Results obtained by the MLPA kit containing probes for *BRCA1*. b) Legend to interpret the row colors at the left of the heatmaps. Each color contains a different subset of samples. Samples ordered in the same way as in Supplementary Table 2. c) Results obtained by the MLPA kit containing probes for *BRCA2*. Data used for the heatmaps can be seen in Supplementary Table 4. Probe details can be found in the online documentation of the SALSA MLPA P002-D1 BRCA1 and SALSA MLPA P045-B3 BRCA2/CHEK2 probemixes (www.MLPA.com). Heatmaps were created using 'heatmap.2' function of the R package 'gplots'. In the top right corner of a) and c) are histograms for MLPA ratios. The light blue line shows the number of copies in function of the number of target probes.

Table 2
Overview of copy number and LOH data.

	BRCA1 carrier		BRCA2 carrier	
	BC n = 36 (%)	OC n = 9 (%)	BC n = 20 (%)	OC n = 6 (%)
2.1 Intragenic copy number alterations				
BRCA1 exon(s) loss[a]	7 (19)	5 (56)	6 (30)	3 (50)
BRCA2 exon(s) loss[a]	4 (11)	1 (11)	1 (5)	2 (33)
2.2 Gene-wide copy number alterations				
LOH in all cells	16 (44)	4 (44)	4 (20)	3 (50)
LOH in >50% of cells	29 (80)	8 (88)	8 (40)	6 (100)
no LOH in >50% of cells	7 (19)	1 (11)	4 (20)	0 (0)
copy neutral LOH[b]	28 (78)	6 (67)	3 (15)	4 (67)
LOH by deleterious event[b]	6 (17)	2 (22)	8 (40)	1 (17)

[a] Samples expected to have one or more exon-spanning deletion in this gene.
[b] Counting only samples for which interpretable data for both MLPA and sequencing were available.
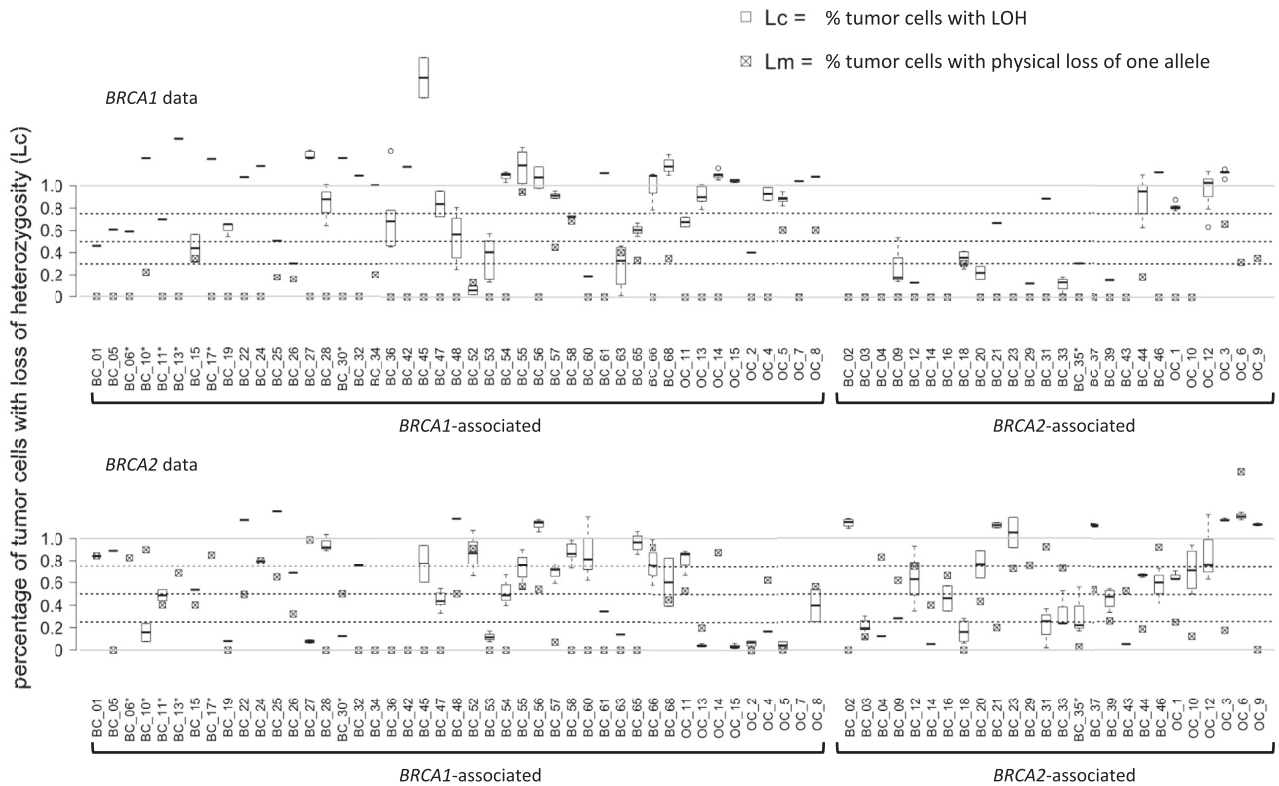

Fig. 3. Integrated LOH analysis using both sequencing and MLPA data.
Boxplots represent Lc values (percentage of tumor cells in a sample that show LOH) for all germline heterozygous markers found in each sample as determined by sequencing. No correction was done on Lc values, as it is impossible to tell which factor (tVAF, bVAF or TCP) was responsible for the over- or underestimation of Lc. BRCA1 and BRCA2 are depicted in the upper and lower plots respectively. Samples with an asterisk indicated loss of the mutant allele. Lm values (percentage of tumor cells displaying physical loss of an allele as determined by MLPA) are represented as checked square boxes.

second hits in BRCA1 and BRCA2 in breast and ovarian cancers from patients with a germline BRCA1 or BRCA2 mutation. We investigated the prevalence of LOH, copy number alterations, promoter methylation and somatic point mutations at these loci. Our study aimed at 1) uncovering whether a combination of second hits occurs in BRCA1 and BRCA2-linked HBOC and 2) the weight of each of these second hits. Quantifying these weights could prove crucial in understanding the interplay of subclones and their effects on therapy efficacy and resistance.

In our study we scored LOH as a continuous variable based on the percentage of tumor cells exhibiting LOH at a heterozygous marker. In 64% of BC and 53% of OC samples Lc was <100%, meaning that not all tumor cells displayed LOH, thought to be the predominant second hit mechanism inactivating BRCA1 and BRCA2. A recent study reported loss of the WT allele in 58/62 BC samples (30/31 97% BRCA1, 28/31 90% BRCA2) [14]. A second study observed loss of the functional allele in 29/39 BC (20/23 87% BRCA1, 9/16 56% BRCA2) and 17/21 OC cases (12/15 80% BRCA1, 5/6 83% BRCA2) [15]. However therapy may influence LOH. In BRCA1-associated OC loss of WT BRCA1 has been described to be less prevalent in tumors treated with neoadjuvant chemotherapy (NACT) [16]. Sokolenko's et al. [17] recent work on the response of BRCA1-associated ovarian tumors to NACT showed the reversion of tumors with LOH to a heterozygous state. 17/23 tumors showed LOH before NACT, but
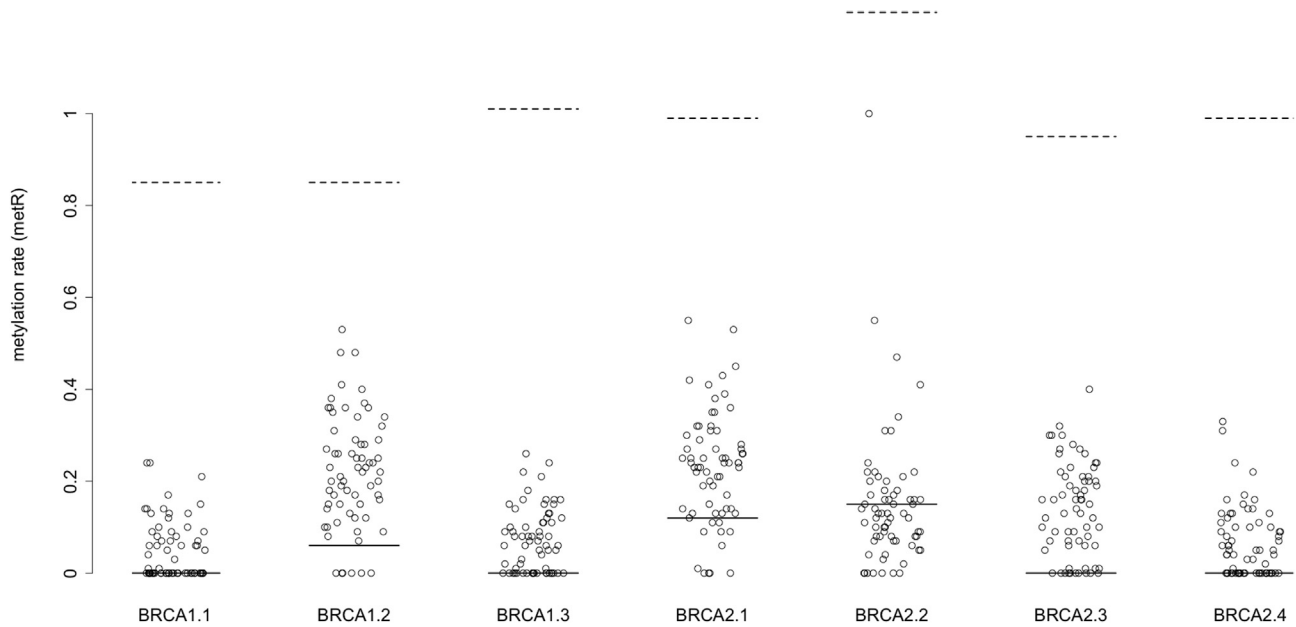
Fig. 4. Jitter plot for methylation data. The methylation rate (metR) is depicted for every sample-probe combination. Full horizontal lines represent the maximum signal detected in control samples (methylated control sample not included). The dashed horizontal lines represent the mean methylation detected in the replicates of the fully methylated control sample.

only 5/17 tumors showed LOH after NACT. For some samples evidence suggested that the reversion was due to positive selection on *BRCA1*-proficiency rather than reversion to the WT sequence. Our cohort contained three BC and seven OC cases that underwent NACT prior to resection. Two of these samples had a large LOH-free subclone (BC_12 Lc = 0.63, OC_11 Lc = 0.67). Combined, these data suggest that the subclone(s) with a heterozygous state can become the predominant subclone under NACT selection. *BRCA1* and *BRCA2* play a vital role in homologous recombination (HR) [15,17]. It could be hypothesized that the same will occur in ovarian (or breast) cancers treated with PARP inhibitors. Therefore, it appears crucial to consider at what percentage of HR-deficiency such therapies are relevant and to monitor tumor development after initiating therapy.

Remarkably, we observed a large number of samples with copy neutral LOH; 34/45 (76%) *BRCA1*-associated and 7/26 (27%) *BRCA2*-associated tumors displayed copy neutral LOH. Our data suggest that copy neutral LOH occurs more frequently in *BRCA1*-associated cancers, confirming data of Maxwell et al. In their local cohort, 34% (13/38) of *BRCA1*-associated and 23% (5/22) of *BRCA2*-associated cancers displayed copy neutral LOH. They also reported that copy neutral LOH was found in respectively 42% (23/55) and 34% (15/45) of *BRCA1*-and *BRCA2*-associated tumors from The Cancer Genome Atlas (TCGA) data.

Data for six *BRCA1*-and one *BRCA2*-associated BC suggest loss of the mutant allele (Fig. 3). King et al. were the first to describe loss of the mutant allele in *BRCA1* and *BRCA2* [18]. They concluded that LOH is a late somatic event in *BRCA1* and *BRCA2*-linked BC development, leading to heterogeneity in the LOH status within a tumor. Several samples taken from both tumor cells and normal epithelium displayed loss of the mutant allele, pointing out that loss of either allele is potentially a stochastic event [18]. Two other studies confirmed these conclusions. Clarke et al. reported loss of the WT and mutant alleles in clones taken from normal epithelial cells of *BRCA1* and *BRCA2* mutation carriers [19]. Martins et al. investigated the order of loss of BRCA1, P53 and PTEN in *BRCA1*-associated BC through immunohistochemistry [20]. Results showed that LOH at

BRCA1 was a late/absent event in the majority of tumor lineages. Tumors where LOH at the *BRCA1* locus was the earliest event, only displayed loss of the wild type allele in a portion of the tumor. These data together with ours suggest that the origin of LOH in a subclone is of a stochastic nature and the extent of LOH that is determined at time of analysis is confounded by a history of selection on each subclone in the tumor.

Consequently, inclusion of LOH-scores in BC samples in algorithms to classify germline *BRCA1* or *BRCA2* variants of unknown significance (VUS) is therefore not advised, although applied in some studies [21] Especially for the evaluation of missense variants, such assays have been suggested to be useful. For instance, Davies et al. found loss of the WT allele in 56/127 BC with a VUS in *BRCA1* or *BRCA2* [22] But using an algorithm that was designed to detect HR-deficiency, only one tumor showed a high score for HR deficiency and concurrent loss of the wild type *BRCA1* allele. It concerned a tumor with the variant NM_007294.3:c.5339 T > C (p.Leu1780Pro), a missense variant in the BRCT domain of *BRCA1* for which a deleterious effect in several functional assays has been suggested [23,24] and considered to be pathogenic based on segregation analysis by Yoon et al. [25].

Somatic exon-spanning gene deletions were found in several breast and ovarian cancer samples. These deletions did not always occur at the germline-affected locus. This again suggests that the events leading to HR-deficiency in HBOC tumors are of a stochastic nature. The occurrence of these deletions could be related to HR deficiency as it is known that HR deficient tumors display genomic instability.

Methylation was investigated using MS-MLPA, targeting three CpGs in the 5′ untranslated region (UTR) of *BRCA1* and five CpGs in the 5′ UTR of *BRCA2*. The methylation pattern across both promoters clearly varies with individual CpGs, but BRCA1.2 (at position c.-1275, relative to the *BRCA1* start codon) and BRCA2.1 (at position c.-224, relative to the *BRCA2* start codon) were more frequently methylated and both were methylated independent of the germline-mutated gene. However, methylation ratios rarely exceeded 20% for any probe, taking TCP into account. There may be

methylated subclones present in some of these cancers that contribute to the overall HR deficiency of the tumor. This is in contrast to the common perception that methylation of the *BRCA1* or *BRCA2* promoter is a rare event in *BRCA1* and *BRCA2*-associated HBOC. Our data were similar to those published by Vos et al. who used the same MS-MLPA probemix [27]. Because promoter hypermethylation, like other second hits, is hypothesized to offer therapeutic potential (e.g. PARP inhibitors) [26,27], functional studies are warranted to correlate each of these CpGs' methylation status to gene expression. In a study linking MS-MLPA data (probemix not specified) for *BRCA1* in patient-derived xenografts with BRCA1 mRNA and protein expression data, methylation ratios ranged from 60 to 100% in tumors that had little to no BRCA1 expression [28].

Somatic mutations can arise in both cancer types and could potentially inactivate the WT allele [5,14,29,30]. In the BC samples we observed a large number of somatic variants (n = 269), however, due to the quality of the FFPE samples these cannot be reliably called (Table 1: 82.5% C:G > T:A). Indeed, based on the data presented (Supplementary Fig. 1), the most important limitation of this study was the heterogeneity within the breast cancer cohort regarding the age of the FFPE samples. Age of the blocks showed to have a significant impact on the quality on the sequencing data and the number of variants detected. In the OC samples (more recent FFPE samples) one somatic variant was retained after filtering. These data lead us to believe that somatic point mutations in *BRCA1* and *BRCA2*-associated HBOC are a rare second hit mechanism. A second limitation is the manner how TCP was scored. Although it was scored by two independent pathologists and their estimations closely matched (mean discordance: 8 ± 2%), manual scoring on HE slides was shown to exhibit observer bias [31]. This may have influenced Lc and Lm calculations.

Although the correlation between each second hit and hormone receptor status, grade and other histopathological and morphological features was investigated we did not obtain statistically significant results. This supports the hypothesis that second hits are the result of random events, rather than the (in-)activation of pathways, and subsequently passed on through consecutive cell divisions.

Summarized, this study investigated all three known second hit mechanism: LOH, hypermethylation of the promoter region and somatic truncating mutations in both *BRCA1* and *BRCA2* in a cohort of 56 breast and 15 ovarian cancer samples, all from patients with a germline mutation in *BRCA1 or BRCA2*. Although most tumors display inactivation of the WT allele of the germline-mutated gene, combinations of second hits were detected within the same tumor and these second hits appear to be subjected to stochastic effects for the generation of subclones containing certain somatic alterations. The survival and relative size of the molecular subclones is then, in line with the clonal expansion cancer model, determined by selection mechanisms. The tumor composition at any given time holds key information about its origin and development, but even more crucial is the information it contains about possible prognosis and therapy effectiveness. Future cancer therapy would benefit from the initial characterization of all relevant subclones in a tumor. Subsequent molecular monitoring of these subclones during therapy will make personalized treatment tailored to each tumor's specific biology possible, increasing treatment efficacy and preventing resistance.

Acknowledgements

Conflict of interest

Lilit Atanesyan is currently employed by MRC-Holland and was involved in the development of the SALSA MLPA ME053-X1 BRCA1-BRCA2 probemix. No other authors have any conflicts of interest, financial or otherwise, to declare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.canlet.2018.03.026.

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