Journal of
Medical Genetics

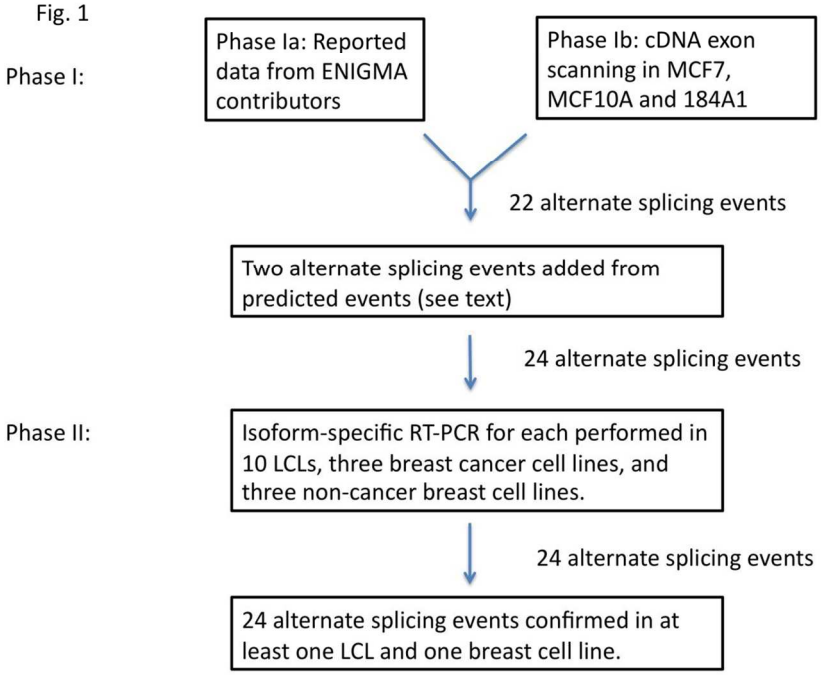


# Naturally occurring BRCA2 alternate mRNA splicing events in clinically relevant samples

| | |
|---|---|
| Journal: | *Journal of Medical Genetics* |
| Manuscript ID | jmedgenet-2015-103570.R1 |
| Article Type: | Original Article |
| Date Submitted by the Author: | 21-Jan-2016 |
| Complete List of Authors: | Fackenthal, James; University of Chicago, Medicine<br>Yoshimatsu, Toshio; University of Chicago, Department of Medicine<br>Zhang, Bifeng; University of Chicago, Medicine<br>Ruiz de Garibay, Gorka; HOSPITAL CLINICO SAN CARLOS, LABORATORIO DE ONCOLOGIA MOLECULAR<br>Colombo, Mara; Fondazione IRCCS Istituto Nazionale dei Tumori, Preventive and Predictive Medicine<br>De Vecchi, Giovanna; Fondazione IRCCS Istituto Nazionale dei Tumori, Preventive and Predictive Medicine<br>Ayoub, Samantha; University of Chicago, Medicine<br>Lal, Kumar; University of Chicago, Medicine<br>Olopade, Olufunmilayo; University of Chicago, Medicine<br>Vega, Ana; Grupo de Medicina Xenómica-USC, CIBERER, Fundación Pública Galega de Medicina Xenómica-SERGAS<br>Santamariña, Marta; University of Santiago de Compostela, CIBERER, IDIS, Grupo de Medicina Xenómica-USC<br>Blanco, Ana<br>Wappenschmidt, Barbara; University of Cologne, Gynacology and Obstetrics<br>Becker, Alexandra; University of Cologne and University Hospital Cologne, Center for Hereditary Breast and Ovarian Cancer, Center for Integrated Oncology (CIO) and Center for Molecular Medicine Cologne (CMMC<br>Houdayer, Claude; Institut Curie<br>Walker, Logan; University of Otago, Department of Pathology<br>Lopez-Perolio, Irene; Hospital Clínico San Carlos, Molecular Oncology Laboratory<br>Thomassen, Mads; Odense University Hospital, Department of Clinical Genetics<br>Parsons, Michael; Queensland Institute of Medical Research, Genetics and Population Health Division<br>Whiley, Phillip; Queensland Institute of Medical Research, Genetics and Population Health<br>Blok, Marinus; Maastricht University Medical Center, Department of Clinical Genetics<br>Brandão, Rita; Maastricht University, Maastricht Science Programme, Faculty of Humanities and Science<br>Tserpelis, Demis; Maastricht University, Department of Clinical Genetics, Maastricht University Medical Center |

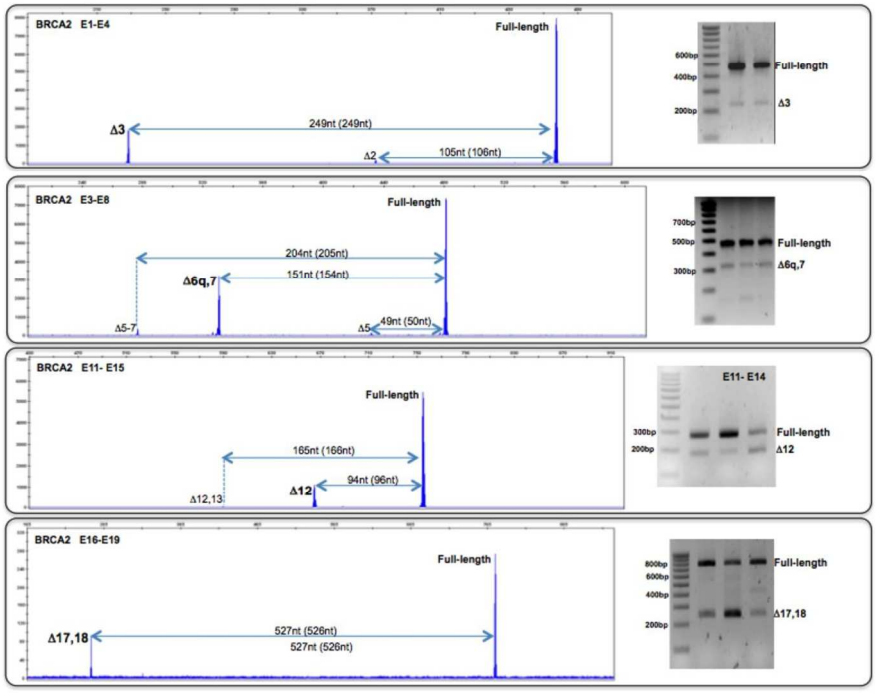
| | |
|---|---|
| | Baralle, Diana; University of Southhampton, Southhampton General Hospital, Human Development and Health Academic Unit<br>Montalban, Gemma; University Hospital Vall d'Hebron, Oncogenetics Laboratory, Vall d'Hebron Institute of Oncology (VHIO)<br>Gutiérrez-Enríquez, Sara; University Hospital Vall d'Hebron, Oncogenetics Laboratory, Vall d'Hebron Institute of Oncology (VHIO)<br>Diez, Orland; University Hospital Vall d'Hebron, Oncogenetics Laboratory, Vall d'Hebron Institute of Oncology (VHIO)<br>LAZARO, CONXI; Catalan Institute of Oncology, Hereditary Cancer Program Investigators, kConFab; Peter MacCallum Cancer Centre<br>Spurdle, Amanda; Queensland Institute of Medical Research, Genetics and Population Health Division<br>Radice, Paolo; Fondazione IRCCS Istituto Nazionale dei Tumori, Preventive and Predictive Medicine<br>de la Hoya, Miguel; Hospital Clínico San Carlos, Laboratorio OncologíaMolecular |
| Keywords: | BRCA2, breast cancer, tumor suppressor, alternate splicing |
| | |

Fig. 1



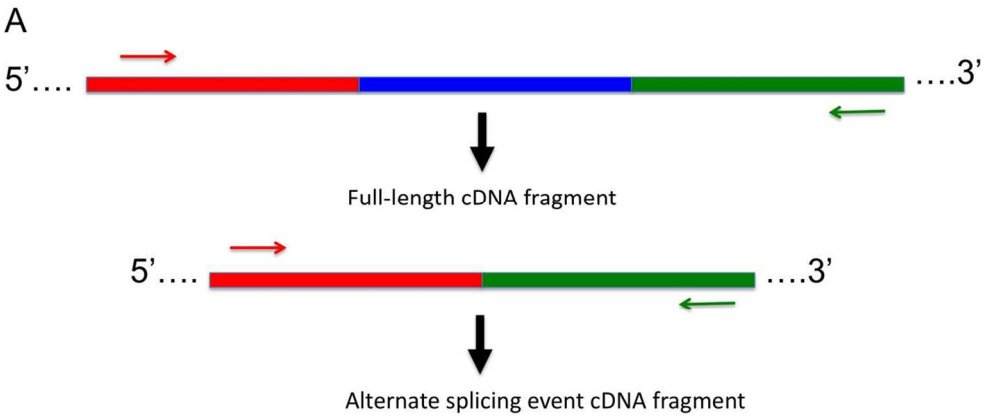

119x90mm (300 x 300 DPI)

Fig. 2



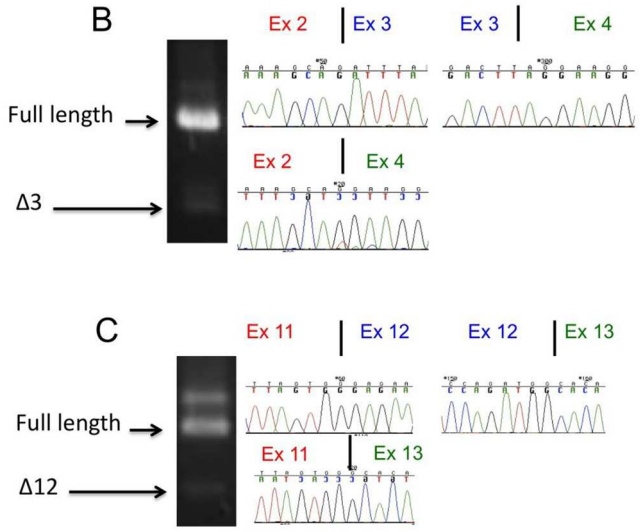

119x90mm (300 x 300 DPI)

Fig 3



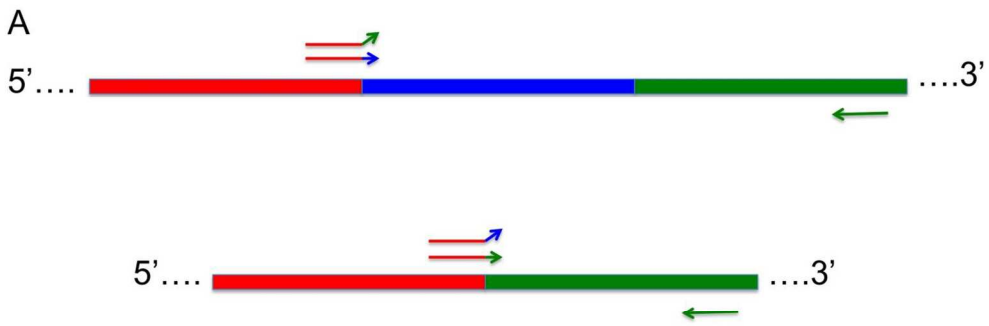

119x90mm (300 x 300 DPI)

Fig. 3



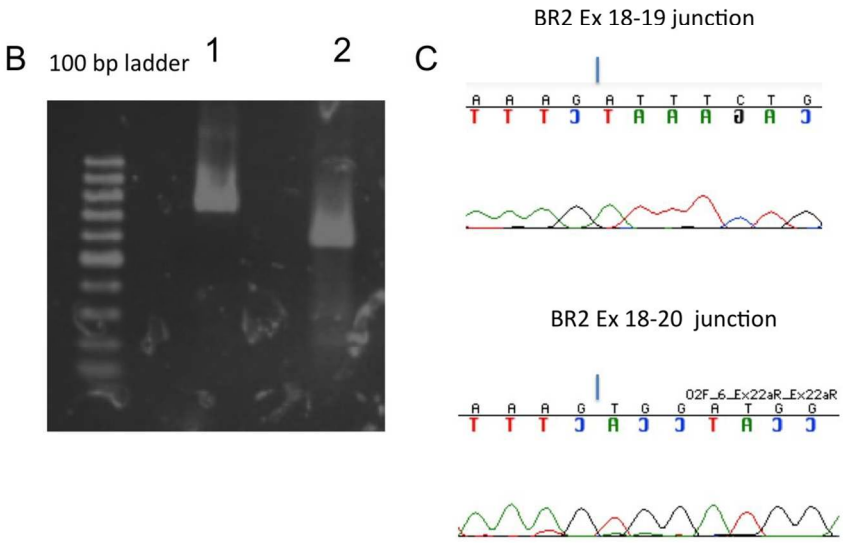

119x90mm (300 x 300 DPI)

Fig 4



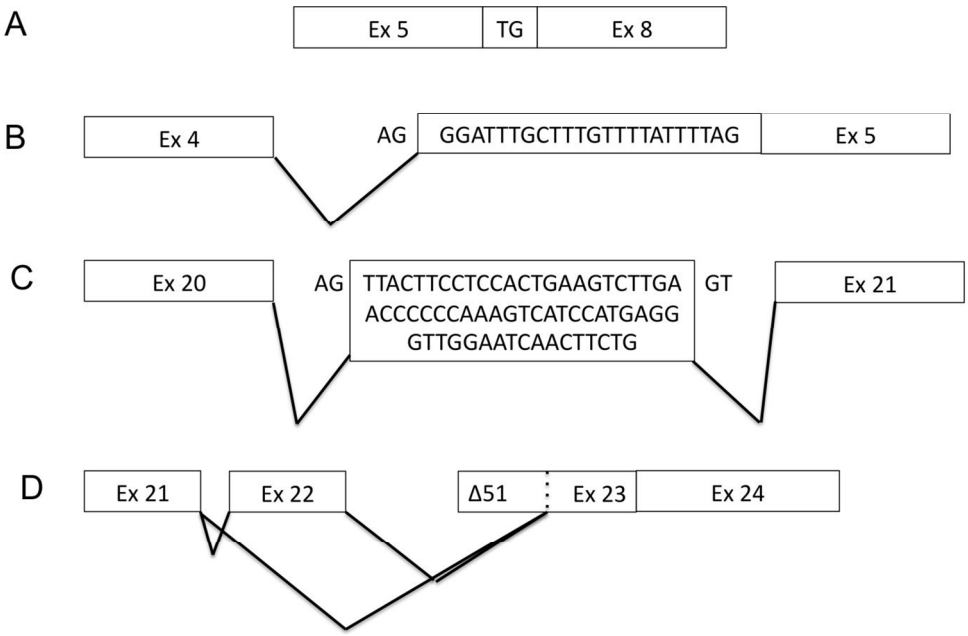

119x90mm (300 x 300 DPI)

Fig 4




119x90mm (300 x 300 DPI)

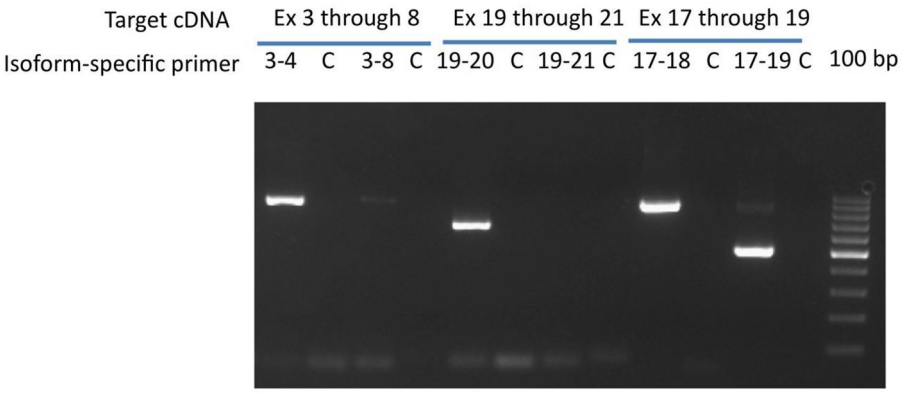
Fig 5




119x90mm (300 x 300 DPI)

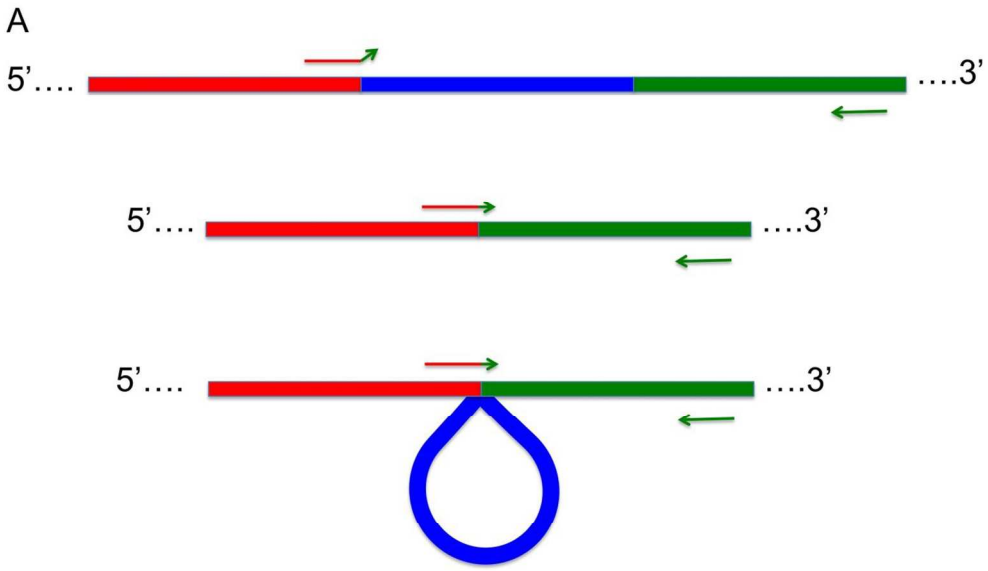
Fig S1

B




119x90mm (300 x 300 DPI)

Fig S1




119x90mm (300 x 300 DPI)

# Naturally occurring *BRCA2* alternate mRNA splicing events in clinically relevant samples.

James D. Fackenthal[1*], Toshio Yoshimatsu[1], Bifeng Zhang[1], Gorka R de Garibay[1], Mara Colombo[2], Giovanna De Vecchi[2], Samantha C. Ayoub[1], Kumar Lal[1], Olufunmilayo I Olopade[1], Ana Vega[3], Marta Santamariña[3], Ana Blanco[3], Barbara Wappenschmidt[4], Alexandra Becker[4], Claude Houdayer[5], Logan C. Walker[6], Irene López-Perolio[7], Mads Thomassen[8], Michael Parsons[9], Phillip Whiley[9], Marinus J. Blok[10], Rita D. Brandão[11], Demis Tserpelis[10], Diana Baralle[12], Gemma Montalban[13], Sara Gutiérrez-Enríquez[13], Orland Díez[13,14], Conxi Lazaro[15], kConFaB Investigators[16], Amanda B. Spurdle[9], Paolo Radice[2], Miguel de la Hoya[7]

[1]Department of Medicine, University of Chicago, Chicago, IL USA, [2]Unit of Molecular Bases of Genetic Risk and Genetic Testing, Department of Preventive and Predictive Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori (INT), Milano, ITALY, [3]Fundación Pública Galega de Medicina Xenómica-SERGAS, Grupo de Medicina Xenómica-USC, CIBERER, IDIS, Santiago de Compostela, SPAIN, [4]Center for Hereditary Breast and Ovarian Cancer, Center for Integrated Oncology (CIO) and Center for Molecular Medicine Cologne (CMMC), Medical Faculty, University of Cologne and University Hospital Cologne, Germany, [5]Service de Génétique and INSERM U830, Institut Curie and Université Paris Descartes, Sorbonne Paris Cité, Paris, FRANCE, [6]Department of Pathology, University of Otago, Christchurch, NEW

ZEALAND, [7]Laboratorio de Oncología Molecular, Instituto de Investigación Sanitaria San Carlos (IdISSC), Hospital Clínico San Carlos, Madrid, Spain, [8]Department of Clinical Genetics, Odense University Hospital, Odense C, DENMARK, [9]Genetics and Computational Biology Division, QIMR Berghofer Medical Research Institute, Brisbane, QLD, AUSTRALIA, [10]Department of Clinical Genetics, Maastricht University Medical Center, Maastricht, The Netherlands, [11]Maastricht Science Programme, Faculty of Humanities and Sciences, Maastricht University, Maastricht, The Netherlands, [12]Human Development and Health Academic Unit, Faculty of Medicine, University of Southhampton, Southhampton General Hospital, Southampton, UK, [13]Oncogenetics Group, Vall d'Hebron Institute of Oncology (VHIO) and Universitat Autonoma de Barcelona, Barcelona, Spain, [14]Clinical and Molecular Genetics Area, University Hospital Vall d'Hebron, Barcelona, Spain, [15]Molecular Diagnostic Unit, Hereditary Cancer Program, IDIBELL–Catalan Institute of Oncology, Barcelona, Spain, [16]Peter MacCallum Cancer Centre, St. Andrews Place, East Melbourne, VIC, AUSTRALIA.

*Corresponding author

Short title: *BRCA2* alternate mRNA splicing events

Key words: *BRCA2*, breast cancer, tumor suppressor, alternate splicing

## Abstract

**Background:** *BRCA1* and *BRCA2* are the two principal tumor suppressor genes associated with inherited high risk of breast and ovarian cancer. Genetic testing of *BRCA1*/2 will often reveal one or more sequence variants of uncertain clinical significance (VUS), some of which may affect normal splicing patterns and thereby disrupt gene function. mRNA analyses are therefore among the tests used to interpret the clinical significance of some genetic variants. However, these could be confounded by the appearance of naturally occurring alternative transcripts unrelated to germline sequence variation or defects in gene function. To understand which novel splicing events are associated with splicing mutations and which are part of the normal *BRCA2* splicing repertoire, a study was undertaken by members of the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) consortium to characterize the spectrum of naturally occurring *BRCA2* mRNA alternate splicing events. **Methods:** mRNA was prepared from several blood and breast tissue-derived cells and cell lines by contributing ENIGMA laboratories. cDNA representing *BRCA2* alternate splice sites was amplified and visualized using capillary or agarose gel electrophoresis, followed by sequencing. **Results:** We demonstrate the existence of 24 different *BRCA2* mRNA alternate splicing events in lymphoblastoid cell lines and both breast cancer and non-cancerous breast cell lines. **Conclusions**: These naturally occurring alternate splicing events contribute to the array of cDNA fragments that may be seen in assays

for mutation-associated splicing defects. Caution must be observed in assigning alternate splicing events to potential splicing mutations.

## Introduction

Breast cancer is among the leading causes of cancer deaths in women worldwide (http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx). While the majority of breast cancers are sporadic, some are associated with inherited "mutations" (pathogenic variants) in the *BRCA1* (MIM 113705) or *BRCA2* (MIM600185) tumor suppressor genes. Indeed, *BRCA1/2* pathogenic variants are the strongest genetic predictors of familial breast/ovarian cancer syndrome. Accordingly, individuals with strong family histories of breast and/or ovarian cancer are advised to seek genetic testing for *BRCA1*/2 sequence variants, results of which can be used to inform disease screening, disease prevention, and disease treatment options.

*BRCA1*/2 clinical testing typically involves sequence analysis of all coding exons and adjacent intronic sequences. Though there are several known pathogenic missense alterations for each gene, most pathogenic variants are "protein truncating," and include frame-shifting insertion/deletions, nonsense substitutions, and base substitutions that alter normal physiological splicing. An ongoing challenge in clinical genetic testing is characterization of DNA sequence variants of

uncertain clinical significance (VUS) [1 2]. Effective genetic counseling requires that the pathogenicity of these variants be determined [3].

To address this problem, the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) is exploring a series of approaches to characterize the potential pathogenicity of *BRCA1*/2 VUS identified during clinical screening, including those that may affect splicing [2 4 5]. These investigations can be complicated by the observation that both *BRCA1* and *BRCA2* genes are transcribed into multiple naturally occurring mRNA splice isoforms independent of any genetic variants, and that these isoforms can vary in expression level between different individuals. Thus, presumed aberrant transcript patterns seen in the presence of a VUS, in some cases, could be unrelated to the VUS and thus not necessarily pathogenic.

In order to accurately interpret clinical splicing assays, it is necessary to provide an overview of the naturally occurring alternate splicing events. ENIGMA collaborators have recently demonstrated the value of comprehensive approaches to catalogue previously unrecognized naturally occurring *BRCA1* mRNA transcripts [6]. Here, we report a complementary study conducted to provide the first systematic catalog of human alternate *BRCA2* mRNA splicing events. We have performed experiments in different blood-related RNA sources (from both healthy controls and breast cancer patients with or without known *BRCA1/2* pathogenic variants), breast cancer cell lines, and non-breast cancer cell lines to show that the

isoforms overlap in their expression profile across clinically relevant sources of mRNA.

## Materials and Methods

*Phase Ia: BRCA2 alternate splicing events reported by participating ENIGMA laboratories:*

Nine contributing ENIGMA laboratories used various cDNA amplification strategies to identify naturally occurring alternate splicing events in controls who do not have breast cancer, from whole blood leukocytes (LEUs), ficoll-isolated peripheral blood mononuclear cells (PBMCs), primary cultures of stimulated peripheral blood lymphocytes (PBLs) and lymphoblastoid cell lines (LCLs), and one healthy breast tissue (BREAST) (Fig. 1, Table 1, and Table S1). Phase Ia was a collection of independent observations by individual ENIGMA participating groups, each using samples and methodologies available to those particular laboratories prior to the coordinated effort. The contributions of each group are described in Table S1. Briefly, cDNAs were generated with *BRCA2* cDNA-specific primers or random hexamers/oligo dT, and these were amplified by PCR with *BRCA2*-specific primers designed at each contributing laboratory (all sequences are available upon request). Products were visualized on agarose gels or with capillary electrophoresis. In most cases splicing isoforms were verified by sequence analysis at the originating institution. Samples and methods have been described in detail elsewhere [6]. Details are presented in the Results section. In brief, 22 *BRCA2*

alternative splicing events were identified. Phase Ia was conducted to detect *BRCA2* splicing events, not to address quantitative aspects. Yet visual inspection of capillary electrophoresis experiments allowed us to annotate four splicing events (Δ3, Δ6q,7, Δ12, and Δ17,18) as *predominant*, a qualitative annotation indicating that they represent a non-negligible/substantial fraction of the total alternate splicing events, in particular if compared with other (*minor*) events (see representative examples in Fig.2). Predominant events are associated with much higher detection rates than minor events, regardless of the samples analyzed (Table 1).

Twenty-two candidate isoforms reported by ENIGMA collaborators were compiled and submitted to the University of Chicago where their expression in LCLs and breast normal and tumour cells was tested by isoform-specific RT-PCR and sequencing analyses as described below. Two additional potential alternate splicing events not seen in phase Ia were also tested: Δ9-11, which was reported in the ENCODE RNA-Seq dataset from the California Institute of Technology http://www.ncbi.nlm.nih.gov/geo/info/ENCODE.html, and ▼5p, which was predicted from a spliceogenic variant reported in a previous study [5].

*Phase Ib: Alternate splice product detection and sequencing:*

An exon-scanning strategy was developed at the University of Chicago to identify potential abundant *BRCA2* splicing isoforms. This strategy was designed independently of the design or results in Phase Ia. Briefly, eighteen primer pairs

were designed to amplify portions of the *BRCA2* cDNA flanking exons 2 (alone), 3 (alone), 4 through 6, 5 and 6, 5 through 7, 9 alone, 12 and 13, 13 alone, 14 and 15, 15 alone, 16 alone, 16 and 17, 18 through 20, 19 and 20, 19 through 22, 23 and 24, 23 through 25, and 23 through 26. These primers are able to generate both full-length RT-PCR products and any product resulting from intervening exon-skipping or alternate splice-site choice. RT-PCR products were run on 1% agarose gels and visualized with ethidium bromide staining and UV transillumination according to standard protocols (Fig. 3A). Gel isolated cDNA fragments were sequenced using the forward or reverse RT-PCR primer using standard protocols. The exon-scanning strategy was performed on mRNA prepared from MCF7, MCF10A, and 184A1 cell lines. This strategy covered all cDNA fragments except those containing exons 10 (1,116 bp) or 11 (4,932 bp) because their large sizes did not permit convenient analysis of full-length large-exon cDNA fragments.

*Phase II: Isoform-specific RT-PCR primer design:*

To validate potentially low-abundance *BRCA2* alternate splicing events with utmost specificity, we designed exon boundary junction-spanning isoform-specific RT-PCR primers. We used these to verify specific candidate splicing isoforms detected in phase I without competition from full-length splicing products [7-9]. These isoform-specific primers contained three to five bases of 3' sequence designed to hybridize with the 5' end of the adjacent exon in either the full length *BRCA2* mRNA or in an isoform resulting from exon skipping (Fig. 4, Table S2). As we use a one-step RT-PCR method (see below), the reverse PCR primer is also the

primer used by the reverse transcriptase to generate the single-stranded cDNA. In our experiments, when the reverse primer is the isoform-specific primer, it often amplifies both full-length and alternate splicing events, possibly because nuclease activity trims back the isoform-specific 3' end of the primer, or because of some kind of non-specific binding event. However, this occurs less frequently when the isoform-specific primer is the forward RT-PCR primer. We have therefore designed all isoform-specific RT-PCR primers as forward primers (Table S2).

*Patient samples and cell lines:*

A description of the patient samples and cell lines used in phase II is provided in the Supplementary Materials and Methods.

*RT-PCR:*

mRNA was prepared from cells using RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. In contrast to other studies of alternate splicing events, no inhibitors of nonsense-mediated mRNA decay (NMD) were used in Phase II. RT-PCR was performed using the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Life Technologies) according to the manufacturer's protocol. PCR conditions were empirically optimized for each primer pair using a standard set of mRNAs (10-50 ng per reaction) prepared from select LCLs and breast cell lines.

## Results

*BRCA2 alternate splicing isoforms reported by participating ENIGMA laboratories (Phase Ia):*

Nine groups contributed reports totaling 22 unique alternate *BRCA2* splicing events detected by RT-PCR in mRNA samples prepared from various cell types (Fig 1 and Table 1). All splicing events were supported by direct sequencing evidence, with the only exceptions of Δ5,6; Δ6; Δ12,13; and Δ23p, that were predicted from capillary EP size-calling. All events were detected by capillary EP, but only 8 events (36%) were detectable by conventional agarose gel electrophoresis/EtBR staining. We were able to detect up to 15 events (68%) in LEU samples, 17(77%) in PBMCs samples, 18 (82%) in PBL samples, and 22 (100%) in LCL samples. We tested 21 splicing variants in RNA extracted from one healthy breast tissue (BREAST), detecting 10 (48%). The low value is likely related to the fact that each alternate splicing event was tested only once due to the scarcity of biological material (see Table 1 for further details).

*Exon Scanning (Phase Ib):*

Only three isoforms (Δ3, Δ6q7, and Δ12) were detected unambiguously by exon scanning RT-PCR followed by conventional EtBr-stained gel electrophoresis (a low sensitivity detection method). The data nonetheless confirms Δ3, Δ6q7, and Δ12 as *major* alternative splicing events.

*Specificity of isoform-specific RT-PCR primers:*

To validate and survey all 22 alternate splicing events reported in phase I, an isoform-specific RT-PCR strategy was devised to amplify corresponding alternate cDNAs (see below). Specificity for individual alternate splicing event products is determined by several bases at the 3' ends of forward PCR primers that span splicing junctions being investigated. One potential source of non-specific cDNA amplification by this method is the possibility of a "loop-out" structure formation by the exon junction-spanning isoform-specific primer that could result in an exon-skipping artifact (Fig.S1). This is unlikely to be a major contributor to the detection of isoforms using isoform-specific RT-PCR used in this study because of two sets of observations. First, most alternate splicing events were not detected in every experiment, thus providing negative internal controls for most potential loop-out products. Specifically, of the 24 isoform-specific primers designed to identify exon-skipping isoforms, only one (*BRCA2* ex 2-4 F, designed to identify the *BRCA2* Δ3 isoform) amplified a product in every cell type examined. In our experiments, *BRCA2* Δ3 is easily detected with conventional flanking RT-PCR primers not subject to loop-out artifact conditions. All other isoform-specific primers designed to identify exon-skipping isoforms failed to detect the target isoform in at least one cell line, whereas all corresponding control primers (i.e., those designed amplify to full-length *BRCA2* cDNA) amplified the correct RT-PCR product in all cell lines. Thus, for these primers, starting conditions for RT-PCR were not sufficient to generate exon-skipping isoforms by looping out or other false priming events. Indeed, a loop-out structure is predicted to be energetically unfavorable given the very short exon-

spanning 3' sequence of the isoform-specific primer. Second, we tested the ability of several isoform-specific RT-PCR primers to generate forced loop-out RT-PCR products. This was done by using exon-skipping isoform-specific RT-PCR primers in PCRs with gel-isolated full-length cDNA products as templates. Under these conditions, exon-skipping cDNAs could only be generated by spanning and amplifying a loop-out intermediate. We tested twelve isoform-specific forward RT-PCR primers representing exon-skipping isoforms for their ability to generate a forced loop-out product against a full-length template (Fig. S1). Six of the twelve were unable to force the generation of exon-skipping products from the full-length template. The other six were able to generate exon-skipping products when provided with large amounts of starting template. The primer that could generate a forced loop-out amplification with the smallest amount of input template was *BRCA2* ex 11-14 F (designed to amplify the *BRCA2* Δ12,13 splicing isoform). This primer was able to amplify a *BRCA2* Δ12,13 product from 0.01 ng of full length cDNA template, but not from $5 \times 10^{-5}$ ng of cDNA, an amount over a hundred fold in excess of the corresponding full-length cDNA in the first round of an RT-PCR reaction using our conditions, assuming 100 ng RNA contains roughly $7 \times 10^4$ copies of a typical rare message [10]. We therefore conclude that exon-skipping cDNA isoforms are unlikely to be artificially generated by isoform-specific primers under our RT-PCR conditions.

*Isoform-specific RT-PCR amplification and sequence analysis (Phase II):*

The naturally occurring *BRCA2* splicing isoforms reported by contributing institutions were collected as described in Materials and Methods for Phase Ia (Table 1). These were then validated at the University of Chicago using isoform-specific RT-PCR in MCF7, HCC1937, BT20, MCF10A, 184A1, 184B5, and at least ten lymphoblastoid cell lines. Breast cell lines were included to address whether major differences in splicing events exist between clinically accessible blood-derived cells and cells derived from tissue affected by *BRCA2*-associated disease; all 24 alternate splicing events assayed were detected in the examined cell lines with variable frequencies, ranging from 29% (Δ9-11) to 100% (Δ3) positive samples (Table 2) and validated by sequence analysis. Representative cDNA fragments identified by scanning RT-PCR are shown in Fig 3B and an example of an isoform confirmed by isoform-specific RT-PCR is shown in Fig 4B. Only 7/24 (29%) of the naturally occurring splicing events (namely, *BRCA2* exon-skipping alternate splicing events Δ3; Δ5; Δ6; Δ5,6; Δ12; Δ17,18; and Δ18) were reported previously in the literature (Table 2). These were described as low-level background exon-skipping events in negative control samples used to characterize exon-skipping events associated with pathogenic sequence variants [11-17].

Of the 24 isoforms confirmed by isoform-specific RT-PCR, there are five (▼5p, Δ6q7, ▼20A, Δ22-23p, and Δ23p) that are not simple exon-skipping isoforms (Fig 5). ▼5p represents an alternate splice acceptor site and includes 23 bases of intron 4 appended to the 5' end of exon 5. Δ6q7 lacks exon 6 almost completely,

except for two nucleotides (TG), and lacks exon 7 completely. ▼20A represents a cryptic exon, in which a 64 base sequence from intron 20 is inserted between exons 20 and 21. Δ23p and Δ22,23p also represent an alternate splice site acceptor. In this case however, the acceptor lies within exon 23, truncating it by 51 bases on the 5' end.

The ▼5p finding was assayed on the presumption that it was a possible splicing event. In an unrelated study [5] an isoform containing 18 intronic nucleotides upstream exon 5 was reported in a subject carrying a c.426-12_c.426-8delGTTTT that results in the use of a cryptic acceptor site. The corresponding isoform, using the same acceptor site in a wild type allele, would be the ▼5p alternate splicing event described here. Although the ▼5p splicing event was not observed previously in RNA samples from LCLs of *BRCA1/2* pathogenic variant carriers or controls [5], or by contributing members for Phase Ia of this study, the isoform-specific RT-PCR analysis revealed this splicing event in 5/10 LCLs, BT20, MCF10A, and 184A1 (Table 2).

Another splicing event, Δ9-11, was not tested by contributing laboratories during phase I but was assayed because it appeared in the ENCODE RNASeq data. This splicing event was identified by isoform-specific RT-PCR in 2/11 LCLs as well as BT20 and 184A1.

Several *BRCA2* cDNA fragments representing alternate splicing events were detectable in most cell lines examined, though not all appeared in every cell type tested. For example, Δ3-7, Δ6q7, and ▼20A were all detectable as Phase II agarose gel bands from isoform-specific RT-PCR in all the breast cell lines tested and the majority, but not all of LCLs. In contrast, Δ9-11 was seen in only 2/6 breast cell lines and only 2/11 LCLs, while Δ20 was observed in only one breast cell line (184A1), even though it was detected in the majority of LCLs tested (Table 2). Similarly, some cell lines produced detectable levels of most *BRCA2* alternate splicing event cDNA fragments, while others did not. For example, in this survey, all alternate splicing events except Δ4 were detected in 184A1, while only 11/24 mRNA splicing isoforms were detected in 184B5, a cell line derived from the same individual as 184A1. These cell types are karyotypically and genetically similar, but not identical. Both are p16 negative, wild type for *RB1*, and originally wild type for *TP53* though some 184A1 lineages have developed deficiencies in p53 functions [18]. It is not clear whether different synthesis and degradation rates of isoforms resulting from alternate splicing events are inherent to these cell lines or are influenced by subtle differences in cell growth and RNA preparation and storage conditions. However, it is important to note that the expression of many alternate splicing events can be highly variable within and between cell types, which is an important consideration when interpreting splicing assay results in connection with potential molecular effects of a VUS. Collectively, these observations are consistent with the view that many alternate splicing isoforms may be sensitive to cell growth conditions and RNA preparation and storage conditions such that expression patterns may show

variable levels between experiments. It is also possible that some cDNAs are subject to stochastic PCR amplification, as we have demonstrated previously for *BRCA1* splicing events [6].

**Discussion:**

Clinical sequencing of *BRCA1* and *BRCA2* is recommended to anyone with a strong family or personal history of breast/ovarian cancer. To provide the most complete information for genetic counseling, it is essential to describe the likely clinical significance of VUS identified during screening. RT-PCR analysis of any variant that could affect splicing is a powerful and efficient tool for identifying high frequency exon skipping or intron retention events in mRNA that is easily prepared from peripheral blood or derivatives thereof. Cases where complete exon skipping from the mutant allele typically correspond to pathogenicity. However, it is not yet possible to know how much reduction of normal message accumulation constitutes pathogenicity, nor do we know whether any alternate splice variants might result in dominant negative effects on normal gene activity. Addressing these questions will require further research. .

The purpose of this study is to address a previously underappreciated challenge to interpreting splicing assays: the frequent occurrence of naturally occurring mRNA transcripts that can be mistaken for variant-associated splicing defects. In some cases, a potential spliceogenic variant might have modest effects on splicing and merely increase the frequency of a naturally occurring alternate mRNA

splicing event, maintaining the expression of full-length mRNA. In such cases the remaining full-length mRNA could provide some *BRCA2* function. This result is especially important as numerous *BRCA2* splicing "mutations" have been reported, supported at least in part by the appearance of "novel" RT-PCR products reported here as potential background isoforms. As shown in Table 3, 17/24 naturally occurring splicing isoforms reported in this study have been previously reported to be aberrant transcripts associated with "mutations" as determined by various assays (see references in Table 3). In addition, the analysis must capture properly the alternative splicing existing in the region of interest to avoid misinterpretations, as illustrated previously for BRCA1 alterations in exon 9 and BRCA1 splicing isoform Δ 9,10 [19]. For instance, it is not obvious whether the main outcome of impairing BRCA2 exon 17 splicing sites should be exon 17 skipping, or exon17,18 skipping (since, like BRCA1 Δ9,10, BRCA2 Δ17,18 is already a predominant event in control samples). In this regard, the effect of the BRCA2 c.7806-9T>G (IVS16-9T>G) variant on splicing has been analyzed by RT-PCR with a forward primer positioned in exons 15 and a reverse primer positioned in exon 18 [20], therefore, excluding from analysis Δ17,18 transcripts. In our opinion, an analysis with a reverse primer located in exon 19 would be more informative.

Indeed, all *BRCA2* exons except 4, 10, 11, 12, and 20 have been reported to be associated with at least one pathogenic variant near the intron/exon boundary, and designated as pathogenic due to assumed/known splicing defects by the Breast Cancer Information Core (BIC) (http://research.nhgri.nih.gov/bic). When applying

splicing assays using RT-PCR to identify aberrant splicing products associated with DNA sequence variants near intron/exon boundaries, it is important to remember that such splicing events exist as part of the natural splicing process regardless of the presence of spliceogenic variants, and that varying amounts of full-length mRNA may be produced from the same allele.

In humans, the average number of protein coding transcripts per locus is roughly four, according to the latest GENCODE release (version 24, http://www.gencodegenes.org/stats/current.html). Loci with more than 20 annotated splicing isoforms are very rare [21] Our data (up to 24 splicing events, including 4 predominant) suggests that *BRCA2* is probably in the upper limit of an average locus for the number of alternate splicing events. Yet, this is in contrast with *BRCA1*, a genomic locus similar to BRCA2 in size ( ≈81Kb vs. ≈84Kb) and number of exons (23 vs. 27), but with a notable higher level of alternative splicing (up to 63 splicing events, including 10 predominant), according to a recent study conducted by the ENIGMA consortium with a very similar methodology [6]. This difference may reflect the well establish link between protein disorder (a structural feature of BRCA1 but not BRCA2 proteins) and high levels of alternative splicing [22 23]..

In summary, we have presented an overview of 24 alternate splicing events associated with normal *BRCA2* mRNA processing. These results are of importance to the design and interpretation of mRNA splicing assays, construct-based or of

patient material, that are commonly used to assess whether VUS lead to aberrations that are phenotypically equivalent to a molecular null (ie a gene deletion). Recent studies have shown that detection of alternate splice events can be highly variable between laboratories and is quite sensitive to variations in cell types, cell growth conditions, mRNA preparation, and RT-PCR methodology [5 6 24]. Recommendations have been made to help make methodologies and reporting of alternate splicing isoforms more uniform, including inhibition of NMD, sequence confirmation, proper primer design, presentation of data from at least ten control samples of the same cell type to reveal potentially rare alternate splice isoforms, quantifying the variant allele contribution to full-length mRNA isoforms, and quantifying the level of "aberrant" transcripts relative to the full-length transcript [24]. These, however, are suggestions for achieving uniformity of alternate splicing event detection and quantification, but not directly characterizing the risk phenotype associated with a genetic variant in families. Moreover, disease-associated alternate-splicing events would likely be associated with loss of heterozygosity (LOH) in the affected tissues. Thus, examination of relative levels of "normal" and "aberrant" transcript levels in blood-derived may not reveal levels of exclusively "aberrant" transcript levels in affected tissues. Where interpretation of a splicing aberration is not straight-forward, the full phenotypic consequence of variants should preferably be assessed with additional evidence from multifactorial models that incorporate largely evidence based on the clinical characteristics of carriers of *bona fide* pathogenic variants [25 26].

Note: After the completion of this work, several contributing labs on this study reported identification of *BRCA2* cDNA fragments with combined alternate splicing events, ▼20A,Δ22, and ▼20A, Δ22,23. Others identified *BRCA2* Δ11, *BRCA2* Δ11-12, and Δ11-13 in wild type controls while investigating the splicing effects of the IVS11+1G>C spliceogenic variant (Table 2). We anticipate other alternate splicing events and combinations will be identified over time and require characterization.

**Acknowledgements:** The University of Chicago group is grateful to The Entertainment Industry Foundation, Ralph and Marion Falk Medical Research Trust, and Breast Cancer Research Foundation. Research grants and RTICC are initiatives of the Spanish Ministry of Economy and Innovation partially supported by European Regional Development FEDER Funds. We thank the following personnel of INT: Fernando Ravagnani for providing biological samples, Claudia Foglia, Donata Penso and Maria Teresa Radice for technical assistance.

We are grateful to the Spanish ISCIIII-Subdirección General de Evaluación y Fomento de la investigación (I+D+I 2008–2011) and Fondo Europeo de Desarrollo Regional-FEDER. We thank Anna Tenés for technical support and the ICO Hereditary Cancer Program team led by Dr. Gabriel Capella.

**Author Statements:**

*Funding statement:* This work was funded by the National Cancer Institute grant numbers CA-R01 89085-01A and SPORE P50 CA125183 (to OIO), US Army DOD

Grant #BC990302 (to JDF), The Italian Association for Cancer Research (AIRC) grant number 11897 (to PR), the NHMRC Senior Research Fellowship number ID1061779 (to ABS), The Cancer Council Queensland grant number ID1086286 (to MP), the Spanish Instituto de Salud Carlos III research grant PI12/00539 (to MH), Red Temática de Investigación Cooperativa en Cáncer-RTICC research grants RD12/0036/006 and RD12/0036/008 (to MH an GRG), Miguel Servet contract numbers CP10/00617 (to SGE) and PI12/02585 (to OD), and the Asociación Española Contra el Cáncer, Spanish Health Research Fund, Carlos III Health Institute, Catalan Health Institute and Autonomous Government of Catalonia, Contract grant numbers: RD12/0036/008, PI10/01422, PI10/00748, PI13/00285, PIE13/00022, 2009SGR290 and 2009SGR283.

*Competing interests statement:* the authors have no competing interests.

*Contributorship statement:* All participants in this study are university-associated members of the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) consortium, and all have provided research samples, wet lab results, literature searches, data compilation and analysis, and critical manuscript reading.

James D. Fackenthal designed the experiments executed by the team at the University of Chicago (funded by Olufunmilayo I. Olopade), performed some of the experiments, all of the analysis, and wrote the manuscript. Other experiments and critical manuscript reading were provided by the other members of the Chicago

team: Toshio Yoshimatsu, Bifeng Zhang, Gorka R de Garibay (then visiting Chicago), Samantha C. Ayoub, and Kumar Lal.

The members of Fondazione IRCCS Istituto Nazionale dei Tumori (the Milan team), headed by Paolo Radice, all participated in generating data and providing critical commentary, as did the members of the Fundación Pública Galega de Medicina Xenómica-SERGAS (Santiago de Compostela) team, Ana Vega, Marta Santamariña, and Ana Blanco.

Alexandra Becker, working under the supervision of Barbara Wappenschmidt (University of Cologne and University Hospital Cologne) provided additional data. Both provided critical commentary. Claude Houdayer (Institut Curie and Université Paris Descartes) provided leadership and critical manuscript reading, as did Logan C. Walker (University of Otago) and Irene López-Perolio (Instituto de Investigación Sanitaria San Carlos (IdISSC)).

Mads Thomassen (Odense University Hospital) provided special computational predictions as well as critical manuscript reading.

The team from the QIMR Berghofer Medical Research Institute (the Brisbane team), directed by Amanda Spurdle (chair of the Splicing Working Group of ENIGMA), included Michael Parsons, who performed extensive literature searches, and Phillip Whiley. All provided valuable data, critical manuscript reading, and insight into the development of the project.

Marinus J. Blok directed the efforts of the team from Maastricht University Medical Center (the Maastricht team) and was one of the intellectual founders of the project. The team included Demis Tserpelis and Rita D. Brandão who, along with

Prof. Blok, provided raw data, data analysis and critical reading of this manuscript. Similar work was provided by Diana Baralle (University of Southhampton).

The Vall d'Hebron Institute of Oncology (VHIO) and Universitat Autonoma de Barcelona group (the Barcelona group) was directed by Orland Díez, and included Gemma Montalban and Sara Gutiérrez-Enríquez. All members provided samples, raw data, and critical assessment of the manuscript. Likewise, Conxi Lazaro (IDIBELL–Catalan Institute of Oncology) provided data and critical reading.

Samples used in this study included those provided by kConFaB Investigators who are hereby acknowledged.

The first phase of this project was directed by Miguel de la Hoya (Instituto de Investigación Sanitaria San Carlos (IdISSC)) who, along with Gorka R de Garibay, performed much of the data generation and all of the Phase I data compilation. Both provided intellectual leadership, raw data, and critical reading of the manuscript.

*Data Sharing:* All raw data will be made available to any interested investigator. There are no unpublished data.

**Figure and Table Legends**

**Fig. 1.** Schematic diagram showing the process of identification and validation of alternate splicing events of *BRCA2* mRNA. Alternate splicing events submitted by contributing ENIGMA members were identified in healthy controls from whole

blood leukocytes (LEUs), peripheral blood mononuclear cells (PBMCs), primary cultures of stimulated blood lymphocytes (PBLs), and lymphoblastoid cell lines (LCLs) (Phase Ia). cDNA exon scanning was performed using mRNA prepared from MCF7, MCF10A, and 184A1 (Phase Ib). The two alternate splicing events added between Phases I and II are predicted events ▼5p (from a previous study) and Δ9-11, reported in ENCODE (see text).

**Fig.2.** Representative examples ofagarose and capillary electrophoresis analyses performed in Phase Ia. *BRCA2* alternative spliced events were detected by RT-PCR with forward and reverse primers located in the indicated exons. Size differences between full-length and alternative splicing transcripts are indicated in nucleotides (nt). We indicate both the difference as determined by capillary electrophoresis analyses (size-calling performed with GeneScan software), and (in brackets) the expected difference based on *BRCA2* exons size. Note the qualitative differences (in terms of relative signal compared with the full-length) between *predominant* (Δ3, Δ6q,7, Δ12, Δ17,18) an *minor* (Δ2 Δ5, Δ5_7, and Δ12,13) events .Predominant events were consistently detected in EtBr-stained agarose gels. Note that *BRCA2* Δ12 is analyzed with different RT-PCR reverse primers in capillary (left) and agarose (right) electrophoresis.

**Fig 3.** *BRCA2* alternate splice products detected by scanning RT-PCR (Phase Ib). **A**. Primers flanking one or more exons were designed to detect alternate splice products. The colored horizontal bars represent any region of a *BRCA2* mRNA

molecule with any three exons (arbitrarily colored red, blue, and green). Colored arrows represent RT-PCR and cDNA PCR primers. The top figure represents a full length mRNA molecule according to the reference sequence (NCBI Accession Number NM_000059), and the bottom figure represents a fragment of a corresponding *BRCA2* mRNA that has undergone an alternate splicing event (in this case, exon skipping). Examples of alternate splicing event detection are shown for Δ3 (**B**) and Δ12 (**C**). Full-length RT-PCR products (upper bands) and exon-deleted isoforms (lower bands) are shown next to electropherograms demonstrating the corresponding exon-exon junctions. The highest molecular weight band in B and C likely represents a heteroduplex containing some strands of Δ3 and Δ12 and full-length RT-PCR products, respectively.

**Fig 4.** *BRCA2* alternate splice products are amplified by isoform-specific RT-PCR. **A.** Illustration of isoform-specific RT-PCR primer design, with an upstream primer specific for a portion of a full length cDNA including red, blue, and green exons (top) or specific for an exon-skipping cDNA that lacks the blue exon (bottom). Note the forward primer with the green sequence 3' end is unable to amplify the full-length cDNA fragment and the forward primer with the blue 3' sequence end is unable to amplify the exon-skipping product. An example of isoform-specific RT-PCR is shown. **B.** An agarose gel shows RT-PCR products generated using primers for the exon 18-19 junction (lane 1) or the exon 18-20 junction unique to the Δ19 isoform (lane 2). **C.** Corresponding electropherograms confirming the identities of the RT-PCR products.

**Fig 5**. Structures of five *BRCA2* alternate splice products including cryptic splice donor/acceptor sites. **A**. Δ6q7, which contains an exon 6-derived TG dinucleotide between flanking exons 5 and 8. **B.** ▼5p, which results from a cryptic intronic splice acceptor site 23 bases from the 5' end of exon 5. **C.** ▼20A results from a cryptic exon within IVS 20. **D.** Δ23p and Δ22,23p both use a cryptic splice acceptor site within exon 23.

**Fig. S1**. Control for hypothetical "loop-out" artifact. **A.** Model of exon-skipping isoform-specific RT-PCR showing non-amplification (note the green sequence 3' end) (top), isoform-specific RT-PCR (middle), and a hypothetical "loop-out" product amplification (bottom). **B.** Isoform specific PCR primers in amplification reactions with gel purified full-length cDNA fragments. (Left) a *BRCA2* cDNA fragment containing exons 3 through 8 amplified with primers specific for the exon 3-4 junction or the exon 3-8 junction formed in the Δ4-7 mRNA isoform. Note the small amount of full-length cDNA amplified by the primer specific for the exon-skipping isoform. This may be due to nuclease activity removing the 3' terminal isoform-specific bases from the amplification primer or possibly non-specific priming. (Middle) a cDNA fragment containing exons 19 through 21 amplified with primers specific for the exon 19-20 junction or the 19-21 junction formed in the Δ20 mRNA isoform. (Right) a cDNA fragment containing exons 17 through 19 amplified with primers specific for the exon 17-18 junction or the 17-19 junction formed in the Δ18 isoform. Note some amplification of both full-length and Δ18 cDNA, possibly from a

loop-out artifact that might occur when PCR conditions include very large amounts of template. **C.** water control.

**Table 1.** *BRCA2* alternate mRNA splicing events identified in Phase Ia. mRNA positions (HGVS nomenclature), coverage (the number of times an alternate splicing event was assessed, including multiple assessments of the same sample), detection rates (number of positives divided by the coverage), and methods are indicated. seq, direct sequencing. QA, qualitative abundance. LEU, whole blood leukocytes. PBMC, peripheral blood mononuclear cells. . PBL, peripheral blood lymphocytes. LCL, lymphoblastoid cell lines. BREAST, breast tissue. Predominant alternate splicing events are indicated in bold. Gray boxes indicate a positive detection for alternate splicing event.

**Table 2.** *BRCA2* alternate mRNA splicing events detected during Phase II. Alternate splicing events, also indicated by their mRNA position, were detected in a subset of lymphoblastoid cell lines (LCLs) or breast cell lines as indicated. FS, frameshift; PTC-NMD, premature termination codon followed by a predicted nonsense-mediated mRNA decay. CDS, coding sequence (predicted amino acids of the portion of the protein removed by the deletion). The references for those previously reported in the literature are Bieche *et al.* (1999) [11], Claes *et al.* (2003) [12], Farrugia *et al.* (2008) [13], Hansen *et al.* (2010) [14], Santarosa et al. (1999) [15], Walker *et al.* (2010) [16], and Houdayer *et al.* (2012) [17].

**Table 3.** *BRCA2* alternate mRNA splicing events have been detected in association with mutations that affect normal splicing patterns as indicated [4 12-17 20 27-48].

Table S1. Nine different groups tested samples from eight different laboratories for the indicated alternate splicing events. For each group, the number of alternate splicing events detected/the number of cell lines tested is indicated. LEU, whole blood leukocytes. PBMC, peripheral blood mononuclear cells. PBL, peripheral blood lymphocytes. LCL, lymphoblastoid cell lines. BREAST, breast tissue. Participating groups: INT, Milano (Istituto Nazionale dei Tumori (INT), Milano); HVH, Barcelone (University Hospital Vall d'Hebron, Barcelona); HCSC, Madrid (Hospital Clínico San Carlos, Madrid); MU, Maastrich (Maastricht University, Maastricht); QIMR, Brisbane (QIMR Berghofer Medical Research Institute, Brisbane); Cologne University; Southampton General Hospital; USC, Santiago de Compostela (Grupo de Medicina Xenómica-USC, CIBERER, IDIS, Santiago de Compostela). In addition to these seven, two other groups reported sequence identification of some alternate splicing events without reporting frequencies. These were the IDIBELL–Catalan Institute of Oncology, Barcelona, and a group of ten French labs led by Claude Houdayer (Institut Curie and Université Paris Descartes).

**Table S2.** RT-PCR primers used to detect full-length *BRCA2* mRNA fragments or fragments resulting from alternate splicing events. Forward primers specific for

full-length fragments have blue sequence at their 3' ends, and corresponding forward primers specific for alternate splicing events terminate with green sequence. Both full length and alternate splicing event RT-PCRs use the same reverse primer.

## References


1. Domchek S, Weber BL. Genetic variants of uncertain significance: flies in the ointment. J Clin Oncol 2008;**26**(1):16-7 doi: 10.1200/JCO.2007.14.4154[published Online First: Epub Date]|.
2. Spurdle AB, Healey S, Devereau A, Hogervorst FB, Monteiro AN, Nathanson KL, Radice P, Stoppa-Lyonnet D, Tavtigian S, Wappenschmidt B, Couch FJ, Goldgar DE. ENIGMA--evidence-based network for the interpretation of germline mutant alleles: an international initiative to evaluate risk and clinical significance associated with sequence variation in BRCA1 and BRCA2 genes. Hum Mutat 2012;**33**(1):2-7 doi: 10.1002/humu.21628[published Online First: Epub Date]|.
3. Lindor NM, Goldgar DE, Tavtigian SV, Plon SE, Couch FJ. BRCA1/2 sequence variants of uncertain significance: a primer for providers to assist in discussions and in medical management. Oncologist 2013;**18**(5):518-24 doi: 10.1634/theoncologist.2012-0452[published Online First: Epub Date]|.
4. Thomassen M, Blanco A, Montagna M, Hansen TV, Pedersen IS, Gutierrez-Enriquez S, Menendez M, Fachal L, Santamarina M, Steffensen AY, Jonson L, Agata S, Whiley P, Tognazzo S, Tornero E, Jensen UB, Balmana J, Kruse TA, Goldgar DE, Lazaro C, Diez O, Spurdle AB, Vega A. Characterization of BRCA1 and BRCA2 splicing variants: a collaborative report by ENIGMA consortium members. Breast Cancer Res Treat 2012;**132**(3):1009-23 doi: 10.1007/s10549-011-1674-0[published Online First: Epub Date]|.
5. Whiley PJ, de la Hoya M, Thomassen M, Becker A, Brandao R, Pedersen IS, Montagna M, Menendez M, Quiles F, Gutierrez-Enriquez S, De Leeneer K, Tenes A, Montalban G, Tserpelis D, Yoshimatsu T, Tirapo C, Raponi M, Caldes T, Blanco A, Santamarina M, Guidugli L, de Garibay GR, Wong M, Tancredi M, Fachal L, Ding YC, Kruse T, Lattimore V, Kwong A, Chan TL, Colombo M, De Vecchi G, Caligo M, Baralle D, Lazaro C, Couch F, Radice P, Southey MC, Neuhausen S, Houdayer C, Fackenthal J, Hansen TV, Vega A, Diez O, Blok R, Claes K, Wappenschmidt B, Walker L, Spurdle AB, Brown MA. Comparison of mRNA Splicing Assay Protocols across Multiple Laboratories: Recommendations for Best Practice in Standardized Clinical Testing. Clin

Chem 2014;**60**(2):341-52 doi: 10.1373/clinchem.2013.210658[published Online First: Epub Date]|.
6. Colombo M, Blok MJ, Whiley P, Santamarina M, Gutierrez-Enriquez S, Romero A, Garre P, Becker A, Smith LD, De Vecchi G, Brandao RD, Tserpelis D, Brown M, Blanco A, Bonache S, Menendez M, Houdayer C, Foglia C, Fackenthal JD, Baralle D, Wappenschmidt B, Diaz-Rubio E, Caldes T, Walker L, Diez O, Vega A, Spurdle AB, Radice P, De La Hoya M. Comprehensive annotation of splice junctions supports pervasive alternative splicing at the BRCA1 locus: a report from the ENIGMA consortium. Hum Mol Genet 2014 doi: 10.1093/hmg/ddu075[published Online First: Epub Date]|.
7. Brosseau JP, Lucier JF, Lapointe E, Durand M, Gendron D, Gervais-Bird J, Tremblay K, Perreault JP, Elela SA. High-throughput quantification of splicing isoforms. RNA 2010;**16**(2):442-9 doi: 10.1261/rna.1877010[published Online First: Epub Date]|.
8. Vandenbroucke, II, Vandesompele J, Paepe AD, Messiaen L. Quantification of splice variants using real-time PCR. Nucleic Acids Res 2001;**29**(13):E68-8
9. Walton HS, Gebhardt FM, Innes DJ, Dodd PR. Analysis of multiple exon-skipping mRNA splice variants using SYBR Green real-time RT-PCR. J Neurosci Methods 2007;**160**(2):294-301 doi: 10.1016/j.jneumeth.2006.09.022[published Online First: Epub Date]|.
10. Qiagen. Qiagen OneStep RT-PCR Kit Handbook, 2010.
11. Bieche I, Lidereau R. Increased level of exon 12 alternatively spliced BRCA2 transcripts in tumor breast tissue compared with normal tissue. Cancer Res 1999;**59**(11):2546-50
12. Claes K, Poppe B, Machackova E, Coene I, Foretova L, De Paepe A, Messiaen L. Differentiating pathogenic mutations from polymorphic alterations in the splice sites of BRCA1 and BRCA2. Genes Chromosomes Cancer 2003;**37**(3):314-20 doi: 10.1002/gcc.10221[published Online First: Epub Date]|.
13. Farrugia DJ, Agarwal MK, Pankratz VS, Deffenbaugh AM, Pruss D, Frye C, Wadum L, Johnson K, Mentlick J, Tavtigian SV, Goldgar DE, Couch FJ. Functional assays for classification of BRCA2 variants of uncertain significance. Cancer Res 2008;**68**(9):3523-31 doi: 10.1158/0008-5472.CAN-07-1587[published Online First: Epub Date]|.
14. Hansen TV, Steffensen AY, Jonson L, Andersen MK, Ejlertsen B, Nielsen FC. The silent mutation nucleotide 744 G --> A, Lys172Lys, in exon 6 of BRCA2 results in exon skipping. Breast Cancer Res Treat 2010;**119**(3):547-50 doi: 10.1007/s10549-009-0359-4[published Online First: Epub Date]|.
15. Santarosa M, Viel A, Boiocchi M. Splice variant lacking the transactivation domain of the BRCA2 gene and mutations in the splice acceptor site of intron 2. Genes Chromosomes Cancer 1999;**26**(4):381-2
16. Walker LC, Whiley PJ, Couch FJ, Farrugia DJ, Healey S, Eccles DM, Lin F, Butler SA, Goff SA, Thompson BA, Lakhani SR, Da Silva LM, Tavtigian SV, Goldgar DE, Brown MA, Spurdle AB. Detection of splicing aberrations caused by BRCA1 and BRCA2 sequence variants encoding missense substitutions: implications

for prediction of pathogenicity. Hum Mutat 2010;**31**(6):E1484-505 doi: 10.1002/humu.21267[published Online First: Epub Date]|.
17. Houdayer C, Caux-Moncoutier V, Krieger S, Barrois M, Bonnet F, Bourdon V, Bronner M, Buisson M, Coulet F, Gaildrat P, Lefol C, Leone M, Mazoyer S, Muller D, Remenieras A, Revillion F, Rouleau E, Sokolowska J, Vert JP, Lidereau R, Soubrier F, Sobol H, Sevenet N, Bressac-de Paillerets B, Hardouin A, Tosi M, Sinilnikova OM, Stoppa-Lyonnet D. Guidelines for splicing analysis in molecular diagnosis derived from a set of 327 combined in silico/in vitro studies on BRCA1 and BRCA2 variants. Hum Mutat 2012;**33**(8):1228-38 doi: 10.1002/humu.22101[published Online First: Epub Date]|.
18. Stampher M. Human Mammary Epithelial Cell (HMEL) Bank Web Site. Secondary Human Mammary Epithelial Cell (HMEL) Bank Web Site 2003. hmec.lbl.gov.
19. Dosil V, Tosar A, Canadas C, Perez-Segura P, Diaz-Rubio E, Caldes T, de la Hoya M. Alternative splicing and molecular characterization of splice site variants: BRCA1 c.591C>T as a case study. Clin Chem 2010;**56**(1):53-61 doi: 10.1373/clinchem.2009.132274[published Online First: Epub Date]|.
20. Kwong A, Wong LP, Chan KY, Ma ES, Khoo US, Ford JM. Characterization of the pathogenic mechanism of a novel BRCA2 variant in a Chinese family. Fam Cancer 2008;**7**(2):125-33 doi: 10.1007/s10689-007-9155-7[published Online First: Epub Date]|.
21. Djebali S, Davis CA, Merkel A, Dobin A, Lassmann T, Mortazavi A, Tanzer A, Lagarde J, Lin W, Schlesinger F, Xue C, Marinov GK, Khatun J, Williams BA, Zaleski C, Rozowsky J, Roder M, Kokocinski F, Abdelhamid RF, Alioto T, Antoshechkin I, Baer MT, Bar NS, Batut P, Bell K, Bell I, Chakrabortty S, Chen X, Chrast J, Curado J, Derrien T, Drenkow J, Dumais E, Dumais J, Duttagupta R, Falconnet E, Fastuca M, Fejes-Toth K, Ferreira P, Foissac S, Fullwood MJ, Gao H, Gonzalez D, Gordon A, Gunawardena H, Howald C, Jha S, Johnson R, Kapranov P, King B, Kingswood C, Luo OJ, Park E, Persaud K, Preall JB, Ribeca P, Risk B, Robyr D, Sammeth M, Schaffer L, See LH, Shahab A, Skancke J, Suzuki AM, Takahashi H, Tilgner H, Trout D, Walters N, Wang H, Wrobel J, Yu Y, Ruan X, Hayashizaki Y, Harrow J, Gerstein M, Hubbard T, Reymond A, Antonarakis SE, Hannon G, Giddings MC, Ruan Y, Wold B, Carninci P, Guigo R, Gingeras TR. Landscape of transcription in human cells. Nature 2012;**489**(7414):101-8 doi: 10.1038/nature11233[published Online First: Epub Date]|.
22. Buljan M, Chalancon G, Dunker AK, Bateman A, Balaji S, Fuxreiter M, Babu MM. Alternative splicing of intrinsically disordered regions and rewiring of protein interactions. Curr Opin Struct Biol 2013;**23**(3):443-50 doi: 10.1016/j.sbi.2013.03.006[published Online First: Epub Date]|.
23. Venkitaraman AR. Cancer suppression by the chromosome custodians, BRCA1 and BRCA2. Science 2014;**343**(6178):1470-5 doi: 10.1126/science.1252230[published Online First: Epub Date]|.
24. Walker LC, Whiley PJ, Houdayer C, Hansen TV, Vega A, Santamarina M, Blanco A, Fachal L, Southey MC, Lafferty A, Colombo M, De Vecchi G, Radice P, Spurdle AB. Evaluation of a 5-tier scheme proposed for classification of sequence

variants using bioinformatic and splicing assay data: inter-reviewer variability and promotion of minimum reporting guidelines. Hum Mutat 2013;**34**(10):1424-31 doi: 10.1002/humu.22388[published Online First: Epub Date]|.
25. Lindor NM, Guidugli L, Wang X, Vallee MP, Monteiro AN, Tavtigian S, Goldgar DE, Couch FJ. A review of a multifactorial probability-based model for classification of BRCA1 and BRCA2 variants of uncertain significance (VUS). Hum Mutat 2012;**33**(1):8-21 doi: 10.1002/humu.21627[published Online First: Epub Date]|.
26. Spurdle AB, Couch FJ, Hogervorst FB, Radice P, Sinilnikova OM. Prediction and assessment of splicing alterations: implications for clinical testing. Hum Mutat 2008;**29**(11):1304-13 doi: 10.1002/humu.20901[published Online First: Epub Date]|.
27. Martinez-Ferrandis JI, Vega A, Chirivella I, Marin-Garcia P, Insa A, Lluch A, Carracedo A, Chaves FJ, Garcia-Conde J, Cervantes A, Armengod ME. Mutational analysis of BRCA1 and BRCA2 in Mediterranean Spanish women with early-onset breast cancer: identification of three novel pathogenic mutations. Hum Mutat 2003;**22**(5):417-8
28. Bonatti F, Pepe C, Tancredi M, Lombardi G, Aretini P, Sensi E, Falaschi E, Cipollini G, Bevilacqua G, Caligo MA. RNA-based analysis of BRCA1 and BRCA2 gene alterations. Cancer Genet Cytogenet 2006;**170**(2):93-101 doi: S0165-4608(06)00354-2 [pii]
10.1016/j.cancergencyto.2006.05.005[published Online First: Epub Date]|.
29. Parsons MT, Whiley PJ, Beesley J, Drost M, de Wind N, Thompson BA, Marquart L, Hopper JL, Jenkins MA, Brown MA, Tucker K, Warwick L, Buchanan DD, Spurdle AB. Consequences of germline variation disrupting the constitutional translational initiation codon start sites of MLH1 and BRCA2: Use of potential alternative start sites and implications for predicting variant pathogenicity. Mol Carcinog 2013 doi: 10.1002/mc.22116[published Online First: Epub Date]|.
30. Bonnet C, Krieger S, Vezain M, Rousselin A, Tournier I, Martins A, Berthet P, Chevrier A, Dugast C, Layet V, Rossi A, Lidereau R, Frebourg T, Hardouin A, Tosi M. Screening BRCA1 and BRCA2 unclassified variants for splicing mutations using reverse transcription-PCR on patient RNA and an ex vivo assay based on a splicing reporter minigene. J Med Genet 2008 doi: jmg.2007.056895 [pii]
10.1136/jmg.2007.056895[published Online First: Epub Date]|.
31. Sanz DJ, Acedo A, Infante M, Duran M, Perez-Cabornero L, Esteban-Cardenosa E, Lastra E, Pagani F, Miner C, Velasco EA. A high proportion of DNA variants of BRCA1 and BRCA2 is associated with aberrant splicing in breast/ovarian cancer patients. Clin Cancer Res 2010;**16**(6):1957-67 doi: 10.1158/1078-0432.CCR-09-2564[published Online First: Epub Date]|.
32. Machado PM, Brandao RD, Cavaco BM, Eugenio J, Bento S, Nave M, Rodrigues P, Fernandes A, Vaz F. Screening for a BRCA2 rearrangement in high-risk breast/ovarian cancer families: evidence for a founder effect and analysis of

the associated phenotypes. J Clin Oncol 2007;**25**(15):2027-34 doi: 10.1200/JCO.2006.06.9443[published Online First: Epub Date]|.
33. Santos C, Peixoto A, Rocha P, Pinto P, Bizarro S, Pinheiro M, Pinto C, Henrique R, Teixeira MR. Pathogenicity evaluation of BRCA1 and BRCA2 unclassified variants identified in Portuguese breast/ovarian cancer families. J Mol Diagn 2014;**16**(3):324-34 doi: 10.1016/j.jmoldx.2014.01.005[published Online First: Epub Date]|.
34. Brandao RD, van Roozendaal K, Tserpelis D, Gomez Garcia E, Blok MJ. Characterisation of unclassified variants in the BRCA1/2 genes with a putative effect on splicing. Breast Cancer Res Treat 2011;**129**(3):971-82 doi: 10.1007/s10549-011-1599-7[published Online First: Epub Date]|.
35. Zhang L, Bacares R, Boyar S, Hudis C, Nafa K, Offit K. cDNA analysis demonstrates that the BRCA2 intronic variant IVS4-12del5 is a deleterious mutation. Mutat Res 2009;**663**(1-2):84-9 doi: 10.1016/j.mrfmmm.2008.11.010[published Online First: Epub Date]|.
36. Whiley PJ, Guidugli L, Walker LC, Healey S, Thompson BA, Lakhani SR, Da Silva LM, Tavtigian SV, Goldgar DE, Brown MA, Couch FJ, Spurdle AB. Splicing and multifactorial analysis of intronic BRCA1 and BRCA2 sequence variants identifies clinically significant splicing aberrations up to 12 nucleotides from the intron/exon boundary. Hum Mutat 2011;**32**(6):678-87 doi: 10.1002/humu.21495[published Online First: Epub Date]|.
37. Colombo M, De Vecchi G, Caleca L, Foglia C, Ripamonti CB, Ficarazzi F, Barile M, Varesco L, Peissel B, Manoukian S, Radice P. Comparative in vitro and in silico analyses of variants in splicing regions of BRCA1 and BRCA2 genes and characterization of novel pathogenic mutations. PLoS One 2013;**8**(2):e57173 doi: 10.1371/journal.pone.0057173[published Online First: Epub Date]|.
38. Machackova E, Foretova L, Lukesova M, Vasickova P, Navratilova M, Coene I, Pavlu H, Kosinova V, Kuklova J, Claes K. Spectrum and characterisation of BRCA1 and BRCA2 deleterious mutations in high-risk Czech patients with breast and/or ovarian cancer. BMC Cancer 2008;**8**:140 doi: 10.1186/1471-2407-8-140[published Online First: Epub Date]|.
39. Caux-Moncoutier V, Pages-Berhouet S, Michaux D, Asselain B, Castera L, De Pauw A, Buecher B, Gauthier-Villars M, Stoppa-Lyonnet D, Houdayer C. Impact of BRCA1 and BRCA2 variants on splicing: clues from an allelic imbalance study. Eur J Hum Genet 2009;**17**(11):1471-80 doi: 10.1038/ejhg.2009.89[published Online First: Epub Date]|.
40. Li L, Biswas K, Habib LA, Kuznetsov SG, Hamel N, Kirchhoff T, Wong N, Armel S, Chong G, Narod SA, Claes K, Offit K, Robson ME, Stauffer S, Sharan SK, Foulkes WD. Functional redundancy of exon 12 of BRCA2 revealed by a comprehensive analysis of the c.6853A>G (p.I2285V) variant. Hum Mutat 2009;**30**(11):1543-50 doi: 10.1002/humu.21101[published Online First: Epub Date]|.
41. Tesoriero AA, Wong EM, Jenkins MA, Hopper JL, Brown MA, Chenevix-Trench G, Spurdle AB, Southey MC. Molecular characterization and cancer risk associated with BRCA1 and BRCA2 splice site variants identified in multiple-case breast cancer families. Hum Mutat 2005;**26**(5):495

42. Hofmann W, Horn D, Huttner C, Classen E, Scherneck S. The BRCA2 variant 8204G>A is a splicing mutation and results in an in frame deletion of the gene. J Med Genet 2003;**40**(3):e23
43. Goina E, Skoko N, Pagani F. Binding of DAZAP1 and hnRNPA1/A2 to an exonic splicing silencer in a natural BRCA1 exon 18 mutant. Mol Cell Biol 2008;**28**(11):3850-60 doi: 10.1128/MCB.02253-07[published Online First: Epub Date]|.
44. Fackenthal JD, Cartegni L, Krainer AR, Olopade OI. BRCA2 T2722R Is a Deleterious Allele That Causes Exon Skipping. Am J Hum Genet 2002;**71**(3):625-31.
45. Chen X, Truong TT, Weaver J, Bove BA, Cattie K, Armstrong BA, Daly MB, Godwin AK. Intronic alterations in BRCA1 and BRCA2: effect on mRNA splicing fidelity and expression. Hum Mutat 2006;**27**(5):427-35
46. Agata S, De Nicolo A, Chieco-Bianchi L, D'Andrea E, Menin C, Montagna M. The BRCA2 sequence variant IVS19+1G-->A leads to an aberrant transcript lacking exon 19. Cancer Genet Cytogenet 2003;**141**(2):175-6
47. Acedo A, Sanz DJ, Duran M, Infante M, Perez-Cabornero L, Miner C, Velasco EA. Comprehensive splicing functional analysis of DNA variants of the BRCA2 gene by hybrid minigenes. Breast Cancer Res 2012;**14**(3):R87 doi: 10.1186/bcr3202[published Online First: Epub Date]|.
48. Chenevix-Trench G, Healey S, Lakhani S, Waring P, Cummings M, Brinkworth R, Deffenbaugh AM, Burbidge LA, Pruss D, Judkins T, Scholl T, Bekessy A, Marsh A, Lovelock P, Wong M, Tesoriero A, Renard H, Southey M, Hopper JL, Yannoukakos K, Brown M, Easton D, Tavtigian SV, Goldgar D, Spurdle AB. Genetic and histopathologic evaluation of BRCA1 and BRCA2 DNA sequence variants of unknown clinical significance. Cancer Res 2006;**66**(4):2019-27
49. Guidugli L, Carreira A, Caputo SM, Ehlen A, Galli A, Monteiro AN, Neuhausen SL, Hansen TV, Couch FJ, Vreeswijk MP. Functional assays for analysis of variants of uncertain significance in BRCA2. Hum Mutat 2014;**35**(2):151-64 doi: 10.1002/humu.22478[published Online First: Epub Date]|.
50. Stampfer MR, Bartley JC. Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[a]pyrene. Proc Natl Acad Sci U S A 1985;**82**(8):2394-8

Table 1

| Alternate BRCA2 splice event | mRNA position | Phase Ia coverage | detection rate | Evidence | QA | LEUs | PBMCs | PBLs | LCLs | BREAST |
|---|---|---|---|---|---|---|---|---|---|---|
| Δ2 | r.38_67del106 | 61 | 28% | seq | Minor | 0/13 | 0/8 | 11/19 | 6/20 | 0/1 |
| **Δ3** | **r.68_316del249** | **102** | **97%** | seq | **Predominant** | **34/34** | **16/16** | **17/19** | **31/32** | **1/1** |
| Δ3,4 | r.68_425del359 | 85 | 51% | seq | Minor | 13/31 | 8/16 | 7/17 | 15/20 | 0/1 |
| Δ3–7 | r.68_631del564 | 30 | 70% | seq | Minor | not tested | 5/8 | 2/7 | 12/15 | not tested |
| Δ4 | r.317_425del109 | 91 | 16% | seq | Minor | 0/31 | 3/16 | 2/17 | 10/26 | 0/1 |
| Δ4–7 | r.317_631del315 | 52 | 63% | seq | Minor | 5/20 | 7/8 | 6/7 | 15/16 | 0/1 |
| Δ5 | r.426_475del50 | 89 | 24% | seq | Minor | 2/39 | 4/16 | 4/7 | 10/26 | 1/1 |
| Δ5,6 | r.426_516del91 | 97 | 3% | inferred | Minor | 2/32 | 0/16 | 0/7 | 1/41 | 0/1 |
| Δ5–7 | r.426_631del206 | 104 | 31% | seq | Minor | 3/39 | 8/16 | 5/7 | 15/41 | 1/1 |
| Δ6 | r.476_516del41 | 104 | 17% | inferred | Minor | 17/39 | 0/16 | 0/7 | 0/41 | 1/1 |
| **Δ6q,7** | **r.478_631del154** | **108** | **100%** | **seq** | **Predominant** | **39/39** | **16/16** | **11/11** | **41/41** | **1/1** |
| **Δ12** | **r.6842_6937del96** | **36** | **83%** | **seq** | **Predominant** | **8/13** | **5/8** | **10/10** | **6/6** | **1/1** |
| Δ12,13 | r.6842_7007del166 | 33 | 3% | inferred | Minor | 1/13 | 0/8 | 0/7 | 0/4 | 0/1 |
| Δ17 | r.7806_7976del171 | 95 | 8% | seq | Minor | 034 | 0/16 | 0/24 | 7/20 | 1/1 |
| **Δ17,18** | **r.7806_8331del526** | **125** | **100%** | **seq** | **Predominant** | **44/44** | **16/16** | **26/26** | **38/38** | **1/1** |
| Δ18 | r.7977_8331del355 | 124 | 99% | seq | Minor | 43/43 | 16/16 | 25/26 | 38/38 | 1/1 |
| Δ19 | r.8332_8487del156 | 101 | 34% | seq | Minor | 1/34 | 5/16 | 9/22 | 18/29 | not tested |
| Δ20 | r.8488_8632del145 | 79 | 20% | seq | Minor | 2/19 | 2/16 | 3/20 | 8/23 | 1/1 |
| ▼20A | r.8632-r.8633ins8633-1327_8633-1264 | 83 | 47% | seq | Minor | 4/21 | 7/16 | 3/20 | 25/25 | 0/1 |
| Δ22 | r.8755_8953del199 | 92 | 70% | seq | Minor | 28/33 | 16/16 | 11/20 | 9/22 | 0/1 |
| Δ22,23p | r.8755_9004del250 | 86 | 63% | seq | Minor | 27/33 | 15/16 | 3/18 | 9/19 | not tested |
| Δ23p | r.8954_9004del51 | 87 | 14% | inferred | Minor | 0/36 | 5/16 | 5/14 | 7/21 | not tested |

Table 2

| BRCA2 splice variant | HGVS nomenclature | biotype | fuctional annotation | CDS | Functional domains/biological activities predicted to be targeted ** | LCL detection | MCF7 | HCC 1937 | BT 20 | MCF 10A | 184 A1 | 184 B5 | % positive cell lines in phase II | Previously reported in literature *** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Δ2 | r.-38_67del106 | cassette | non coding | | | 9/11 | + | + | + | + | + | – | 82% | no |
| Δ3 | r.68_316del249 | cassette | no FS | p.Asp23_Leu105del | PALB2 binding. EMSY binding. Transcriptional Activation | 10/10 | + | + | + | + | + | + | 100% | yes |
| Δ3,4 | r.68_c.425del359 | multi-cassette | PTC-NMD | | | 11/11 | – | + | + | – | + | – | 82% | no |
| Δ3–7 | r.68_631del564 | multi-cassette | no FS | p.Asp23_Ile210del | PALB2 binding. EMSY binding. Transcriptional Activation | 9/11 | + | + | + | + | + | + | 88% | no |
| Δ4 | r.317_425del109 | cassette | PTC-NMD | | | 8/11 | – | + | + | – | – | – | 59% | no |
| Δ4–7 | r.317_631del315 | multi-cassette | no FS | p.Gly106_Ile210del | CEP55-TSG111 binding CEP55-Alix binding | 11/11 | + | + | + | – | + | – | 88% | no |
| Δ5 | r.426_475del50 | cassette | PTC-NMD | | | 11/11 | + | + | + | – | + | + | 94% | yes |
| Δ5,6 | r.426_516del91 | cassette | PTC-NMD | | | 7/10 | – | + | + | – | + | – | 62% | yes |
| Δ5–7 | r.426_631del206 | multi-cassette | PTC-NMD | | | 10/11 | – | + | + | + | + | + | 88% | no |
| Δ6 | r.476_516del41 | cassette | PTC-NMD | | | 4/10 | + | + | + | – | + | – | 50% | yes |
| ▼5p† | r.426-23_426-1ins 23 | cassette | PTC-NMD | | | 5/10 | – | – | + | + | + | – | 50% | no |
| Δ6q,7 | r.478-631del154 | splice donor shift+cassette | PTC-NMD | | | 7/10 | + | + | + | + | + | + | 81% | no |
| Δ9–11†† | r.682_6841del6160 | multi-cassette | PTC-NMD | | | 2/11 | – | – | + | – | + | – | 29% | no |
| Δ12*§ | r.6842_6937del96 | cassette | no FS | p.Glu2282_Gly2313 del | None**** | 6/12 | + | + | + | + | + | + | 67% | yes |
| Δ12,13 | r.6842_7007del166 | multi-cassette | PTC-NMD | | | 6/11 | + | + | + | – | + | + | 65% | no |
| Δ17§ | r.7806_7976del171 | cassette | no FS | p.Ala2603_Arg2659 del | DNA binding. DSS1 binding | 9/10 | – | – | + | – | + | – | 69% | no |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Δ17,18 | r.7806_8331del526 | multi-cassette | PTC-NMD | | | 9/10 | + | + | + | + | + | – | 88% | yes |
| Δ18 | r.7977_8331del355 | cassette | PTC-NMD | | | 9/11 | + | + | + | + | + | – | 82% | yes |
| Δ19 | r.8332_8487del156 | cassette | PTC-NMD | | | 9/10 | + | + | + | + | + | + | 94% | no |
| Δ20 | r.8488_8632del145 | cassette | PTC-NMD | | | 8/11 | – | – | – | – | + | – | 53% | no |
| ▼20A §§ | r.8633-1327_8633-1264ins64 | cassette | PTC-NMD | | | 7/11 | + | + | + | + | + | + | 76% | no |
| Δ22 | r.8755_8953del199 | cassette | PTC-NMD | | | 10/11 | – | + | – | – | + | – | 70% | no |
| Δ22,23p | r.8755_9004del250 | cassette+splice acceptor shift | PTC-NMD | | | 4/10 | – | + | + | – | + | + | 50% | no |
| Δ23p | r.8954_9004del51 | splice acceptor shift | PTC-NMD | | | 3/10 | + | + | + | + | + | + | 56% | no |
| *▼20A,Δ22* | *r.8633-1327_8633-1264ins64+8755_8953del199* | *multi-cassette* | *PTC-NMD* | | | | | | | | | | | |
| *▼20A,Δ22,23* | *r.8633-1327_8633-1264ins64+8755_9117del363* | *multi-cassette* | *PTC-NMD* | | | | | | | | | | | |
| *Δ11* | *r.1910_6841del4932* | *cassette* | *no FS* | *p.Leu638_Val2280del* | *Rad51 binding. DNA recombination.* | | | | | | | | | |
| *Δ11,12* | *r.1910_6937del5028* | *multi-cassette* | *no FS* | *p.Leu638_Gly2313del* | *Rad51 binding. DNA recombination.* | | | | | | | | | |
| *Δ11–13* | *r.1910_7008del5099* | *multi-cassette* | *PTC-NMD* | | | | | | | | | | | |

* An additional LCL was tested for the Δ12 alternate splicing event.
** Predictions have been made based on the following BRCA2 protein domain definitions[49]: binding to PALB2 and EMSY (residues 10-40), transcriptional activity (residues 24-105), binding to CEP55-TSG111 and CEP55-Alix (residues 200-600), Rad 51 binding (residues 638-2280), DNA and DSS1 binding domain (residues 2459-3190).
*** References are listed in the table legend.

**** Exon 12 has been reported to be functionally redundant [40]
† Predicted from a previous mutation analysis (see text)
†† Identified in ENCODE
§ Genescan prediction/H-Inv 7.0 GenePrediction
§§ Ensenble transcript ENST00000528762
Alternate splice variants in italics were identified after this work was completed and were not part of this study (see text).

Table 3

| BRCA2 alternate splicing events reported to be asociated with a sequence variant | Sequence variant | Reference |
|---|---|---|
| Δ2 | c.67+2T>C | Martinez-Ferrandis et al. 2003 Hum Mutat 22:417 |
| Δ2 | c.67+1G>A | Bonatti et al. 2006 Cancer Genet Cytogenet 170:93 |
| Δ2 | c.67+3A>G | Parsons et al. 2013 Mol Carcinog (Epub) doi: 10.1002/mc.22116. |
| Δ3 | c.316+5G>A | Thomassen et al. 2012 Br Ca Res Trt 132:1009 |
| Δ3 | c.316+5G>C | Bonnet et al. 2008 J Med Genet 45:438 |
| Δ3 | c.68-7T>A | Sanz et al. 2010 Clin Cancer Res 16:1957 |
| Δ3 | c.145G>T | Sanz et al. 2010 Clin Cancer Res 16:1957 |
| Δ3 | c.93G>T | Sanz et al. 2010 Clin Cancer Res 16:1957 |
| Δ3 | c.156_157insAlu | Machado et al. 2007 J Clin Oncol 25:2027 |
| Δ3 | c.231T>G | Thery et al. 2011 Eur J Hum Genet 19:1052 |
| Δ3 | c.68–7delT | Santarosa et al. 1999 Genes Chromosomes Cancer 26:381 |
| Δ3,4 | c.68-7T>A | Santos et al. 2014 J Mol Diag 16:324 |
| Δ3–7 | none reported | Not applicable |
| Δ4 | c.425G>T | Brandão et al. 2011 Br Ca Res Treat 129:971 |
| Δ4–7 | none reported | Not applicable |
| Δ5 | c.426-12_426-8del | Zhang et al. 2009 Mut Res 663:84; Whiley et al. 2011 Hum Mutat 32:678 |
| Δ5 | c.439C>T | Sanz et al. 2010 Cancer Res 16:1957 |
| Δ5 | c.475+1G>A | Colombo et al. 2013 PLoS ONE 8(2): e57173 |
| Δ5 | c.516+1G>A | Claes et al. 2003 Genes C'somes Can 37:314 |
| Δ5 | c.455C>A | Sanz et al. 2010 Clin Cancer Res 16:1957 |
| Δ5 | c.470_474del | Sanz et al. 2010 Clin Cancer Res 16:1957 |
| Δ5 | c.473C>T | Sanz et al. 2010 Clin Cancer Res 16:1957 |

| | | |
|---|---|---|
| Δ5,6 | c.476-2A>G | Colombo et al. 2013 PLoS ONE 8(2): e57173 |
| Δ5,6 | c.516G>A | Hansen et al. 2010 Br Ca Res Treat 119:547 |
| Δ5,6 | c.516+1G>A | Claes et al. 2003 Genes C'somes Can 37:314 |
| Δ5,6 | c.516+1G>T | Whiley et al. 2011 Hum Mutat 32:678 |
| Δ5–7 | none reported | Not applicable |
| ▼5p | none reported | Not applicable |
| Δ6 | c.516G>A | Hansen et al. 2010 Br Ca Res Treat 119:547 |
| Δ6 | c.516+1G>A | Claes et al. 2003 Genes C'somes Can 37:314 |
| Δ6 | c.516+1G>T | Whiley et al. 2011 Hum Mutat 32:678 |
| Δ6 | c.476-2A>G | Machackova et al. 2008, BMC Cancer 8:140; Colombo et al. 2013 PLoS ONE 8(2): e57173 |
| Δ6q,7 | none reported | Not applicable |
| Δ9–11 | none reported | Not applicable |
| Δ12 | c.6853A>G | Li et al. 2009 Hum Mutat 30:1543 |
| Δ12 | c.7007G>A | Machackova et al. 2008 BMC Cancer 8:140 |
| Δ12,13 | c.7007G>A | Machackova et al. 2008 BMC Cancer 8:140; Houdayer et al. 2012 Hum Mutat 33:1228 |
| Δ12,13 | c.7007G>C | Houdayer et al. 2012 Hum Mutat 33:1228 |
| Δ12,13 | c.5987C>G | Caux-Moncoutier et al. 2009 Eur J Hum Genet 17:1471 |
| Δ17 | c.7976G>C | Farrugia et al. 2008 Cancer Res 68:3523 |
| Δ17 | c.7976+3_7974+4del | Brandão et al. 2011 Br Ca Res Treat 129:971 |
| Δ17 | c.7976G>A | Farrugia et al. 2008 Cancer Res 68:3523; Hofmann et al. 2003 J Med Genet 40:e23 |
| Δ17 | c.7975A>G | Houdayer et al. 2012 Hum Mutat 33:1228 |
| Δ17 | c.7806-9T>G | Kwong et al. 2008 Fam Cancer 7:125 |
| Δ17 | c.7806-2A>G | Santarosa et al. 1999 Int J Cancer 83:5 |
| Δ17,18 | c.7977-1G>C | Tesoriero et al. 2005 Hum Mut #850 Online |
| Δ17,18 | c.7988A>T | Walker et al. 2010 Hum Mut 6:E1484 |
| Δ17,18 | c.8257_8259del | Bonnet et al. 2008 J Med Genet 45:438 |

| | | |
|---|---|---|
| Δ18 | c.7977-1G>C | Tesoriero et al. 2005 Hum Mut #850 Online |
| Δ18 | c.7988A>T | Farrugia et al. Cancer Res 2008 68:3523; Walker et al. 2010 Hum Mut 6:E1484 |
| Δ18 | c.8257_8259del | Bonnet et al. 2008 J Med Genet 45:438 |
| Δ18 | c.8331+6G>T | Goina et al. 2008 Mol Cell Biol 28:3850 |
| Δ18 | c.8165C>G | Fackenthal et al. 2002 Am J Hum Genet 71:625 |
| Δ18 | c.8331G>A | Sanz et al. 2010 Clin Cancer Res 16:1957 |
| Δ18 | c.7977-7C>G | Houdayer et al. 2012 Hum Mutat 33:1228 |
| Δ19 | c.8487+1G>A | Chen et al. 2006 Hum Mut 27:427; Agata et al. 2003 Can Genet Cytogenet 141:175; Acedo et al et al. 2012 Br Can Res Treat 14:R87 |
| Δ19 | c.8378G>A | Acedo et al et al. 2012 Br Can Res Treat 14:R87 |
| Δ19 | c.8486A>T | Acedo et al et al. 2012 Br Can Res Treat 14:R87 |
| Δ19 | c.8487G>A | Acedo et al et al. 2012 Br Can Res Treat 14:R87 |
| Δ19 | c.8487+3A>G | Acedo et al et al. 2012 Br Can Res Treat 14:R87 |
| Δ19 | c.8486A>G | Houdayer et al. 2012 Hum Mutat 33:1228; Chenevix-Trench et al. 2006 Cancer Res 66:2019 |
| Δ19 | c.8487G>C | Houdayer et al. 2012 Hum Mutat 33:1228 |
| Δ20 | c.8488-2A>G | Acedo et al et al. 2012 Br Can Res Treat 14:R87 |
| Δ20 | c.8488-1G>A | Acedo et al et al. 2012 Br Can Res Treat 14:R87 |
| Δ20 | c.8632+1G>A | Tesoriero et al. 2005 Hum Mut #850 Online |
| ▼20A | none reported | Not applicable |
| Δ22 | c.8755-1G>A | Machackova et al. 2008 BMC Cancer 8:140; Colombo et al. 2013 PLoS ONE 8(2): e57173 |
| Δ22,23p | c.8755-1G>A | Colombo et al. 2013 PLoS ONE 8(2): e57173 |
| Δ23p | c.8954-1_8955delinsAA | Acedo et al et al. 2012 Br Can Res Treat 14:R87; Colombo et al. 2013 PLoS ONE 8(2): e57173 |
| Δ23p | c.8969G>A | Acedo et al et al. 2012 Br Can Res Treat 14:R87 |
| Δ23p | c.9006A>T | Acedo et al et al. 2012 Br Can Res Treat 14:R87 |
| Δ23p | c.9117G>A | Acedo et al et al. 2012 Br Can Res Treat 14:R87 |

Table S1a

| Experiments performed by: | | INT, Milano | INT, Milano | HVH, Barcelone | HVH, Barcelone | HVH, Barcelone | HCSC, Madrid |
|---|---|---|---|---|---|---|---|
| Samples provided by: | | INT, Milano | INT, Milano and QIMR, Brisbane | HVH, Barcelone | QIMR, Brisbane | HVH, Barcelone | HCSC, Madrid |
| Samples (detection method): | | PBLs (capillary EP) | LCLs (capillary EP) | LEUs (agarose) | LCLs (agarose) | PBMCs (agarose) | LEUs (capillary EP) |
| Splicing event | mRNA position | | | | | | |
| Δ2 | r.38_67del106 | 0/1 | 0/6 | not tested | not tested | not tested | 0/13 |
| Δ3 | r.68_316del249 | 1/1 | 6/6 | **18/18** | **10/10** | **8/8** | 13/13 |
| Δ3,4 | r.68_c.425del359 | 0/1 | 5/6 | **0/18** | **0/10** | **0/8** | 13/13 |
| Δ3–7 | r.68_631del564 | 0/1 | 5/6 | not tested | not tested | not tested | not tested |
| Δ4 | r.317_425del109 | 0/1 | 0/6 | **0/18** | **0/10** | **0/8** | 0/13 |
| Δ4–7 | r.317_631del315 | 0/1 | 6/6 | not tested | not tested | not tested | 5/20 |
| Δ5 | r.426_475del50 | 0/1 | 0/6 | **0/19** | **0/10** | **0/8** | 2/20 |
| Δ5,6 | r.426_516del91 | 0/1 | 1/6 | **0/19** | **0/10** | **0/8** | 2/13 |
| Δ5–7 | r.426_631del206 | 0/1 | 5/6 | **0/19** | **0/10** | **0/8** | 3/20 |
| Δ6 | r.476_516del41 | 0/1 | 0/6 | **0/19** | **0/10** | **0/8** | 17/20 |
| Δ6q,7 | r.478-631del154 | 1/1 | 6/6 | **19/19** | **10/10** | **8/8** | 20/20 |
| Δ12 | r.6842_6937del96 | 1/1 | 4/4 | not tested | not tested | not tested | 5/8 |
| Δ12,13 | r.6842_7007del166 | 0/1 | 0/4 | not tested | not tested | not tested | 1/13 |
| Δ17 | r.7806_7976del171 | not tested | not tested | **0/21** | **0/10** | **0/8** | 0/13 |
| Δ17,18 | r.7806_8331del526 | 2/2 | 3/3 | **21/21** | **10/10** | **8/8** | 23/23 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Δ18 | r.7977_8331del355 | 2/2 | 3/3 | **21/21** | **10/10** | **8/8** | 19/28 |
| Δ19 | r.8332_8487del156 | not tested | 9/9 | **0/21** | **0/10** | **0/8** | 1/13 |
| Δ20 | r.8488_8632del145 | 1/2 | 1/3 | **0/16** | **0/10** | **0/8** | 2/3 |
| ▼20A | c.8633-1327_c.8633-1264ins64 | 2/2 | 3/3 | **0/16** | **10/10** | **0/8** | 4/5 |
| Δ22 | c.8755_8953del199 | 0/2 | 0/3 | **23/23** | **0/10** | **8/8** | 5/10 |
| Δ22,23p | c.8755_9004del250 | not tested | not tested | **23/23** | **0/10** | **8/8** | 4/10 |
| Δ23p | c.8954_9004del51 | 0/2 | 0/3 | **0/23** | **0/10** | **0/8** | 0/13 |

Table S1b

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| HCSC, Madrid | HCSC, Madrid | HCSC, Madrid | HCSC, Madrid | HCSC, Madrid | MU, Maastrich | QIMR, Brisbane | Cologne University | Southampton General Hospital |
| HVH, Barcelone | USC, Santiago de Compostela | MU, Maastrich | QIMR, Brisbane | HCSC, Madrid | MU, Maastrich | QIMR, Brisbane | Cologne University | Southampton General Hospital |
| **PBMCs (capillary EP)** | **PBLs (capillary EP)** | **PBLs (capillary EP)** | **LCLs (capillary EP)** | **One healthy breast tissue (capillary EP)** | **PBLs (agarose)** | **LCLs (agarose)** | **LCLs (agarose)** | **LEU detected/tested (agarose)** |
| | | | | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 0/8 | 9/16 | not tested | 6/10 | 0/1 | 2/2 | 0/4 | not tested | not tested |
| 8/8 | 14/16 | not tested | 10/10 | 1/1 | 2/2 | 3/4 | 2/2 | 3/3 |
| 8/8 | 7/16 | not tested | **10/10** | 0/1 | not tested | 0/4 | not tested | not tested |
| 5/8 | 2/6 | not tested | 7/9 | not tested | not tested | not tested | not tested | not tested |
| 3/8 | 2/16 | not tested | 10/10 | 0/1 | not tested | 0/4 | not tested | not tested |
| 7/8 | 6/6 | not tested | 9/10 | 0/1 | not tested | not tested | not tested | not tested |
| 4/8 | 4/6 | not tested | 10/10 | 1/1 | not tested | not tested | not tested | not tested |
| 0/8 | 0/6 | not tested | 0/10 | 0/1 | not tested | 0/15 | not tested | not tested |
| 8/8 | 5/6 | not tested | 10/10 | 1/1 | not tested | 0/15 | not tested | not tested |
| 0/8 | 0/6 | not tested | 0/10 | 1/1 | not tested | 0/15 | not tested | not tested |
| 8/8 | 6/6 | not tested | **10/10** | 1/1 | 4/4 | 15/15 | not tested | not tested |
| 5/8 | 6/6 | not tested | not tested | 1/1 | 3/3 | not tested | 2/2 | 3/3 |
| 0/8 | 0/6 | not tested | not tested | 0/1 | not tested | not tested | not tested | not tested |
| 0/8 | 0/12 | 0/12 | 7/10 | 1/1 | not tested | not tested | not tested | not tested |
| 8/8 | **12/12** | **12/12** | **10/10** | 1/1 | not tested | 15/15 | not tested | not tested |
| 8/8 | 12/12 | **11/12** | **10/10** | 1/1 | not tested | 15/15 | not tested | 3/3 |
| 5/8 | 3/10 | 6/12 | 9/10 | not tested | not tested | not tested | not tested | not tested |
| 2/8 | 2/6 | 0/12 | 7/10 | 1/1 | not tested | not tested | not tested | not tested |
| 7/8 | 5/6 | 3/12 | 10/10 | 0/1 | 1/1 | not tested | 2/2 | not tested |
| 8/8 | 3/6 | 8/12 | **9/9** | 0/1 | not tested | not tested | not tested | not tested |
| 7/8 | 1/6 | 2/12 | 9/9 | not tested | not tested | not tested | not tested | not tested |
| 5/8 | 5/6 | 0/6 | 7/8 | not tested | not tested | not tested | not tested | not tested |

Table S2

| Alternate splicing event to be detected | Forward or reverse | Primer name and sequence |
|---|---|---|
| Δ2 | F (full length) | BR2ex1-2F: AGAACTGCACCTCTGGAGCGGACTTATTT |
| | F (alternate) | BR2ex1-3F: AGAACTGCACCTCTGGAGCGGATTTAGGA |
| | R | BR2CA2 Ex 7R: CTAAAGAACTTGACCAAGAC |
| Δ3 | F (full length) | BRCA2 EX2-3F(a): ACACGCTGCAACAAAGCAGATT |
| | F (alternate) | BRCA2 EX2-4F: ACACGCTGCAACAAAGCAGGAA |
| | R | BRCA2 Ex 7R: CTAAAGAACTTGACCAAGAC |
| Δ3,4 | F (full length) | BRCA2ex2-3F(b): TTTTAAGACACGCTGCAACAAAGCAGATTTA |
| | F (alternate) | BRCA2ex2-5F: TTTTAAGACACGCTGCAACAAAGCAGTCCTG |
| | R | BRCA2 Ex8-7R: CTTCATTTCTGACTATGAGC |
| Δ3-7 | F (full length) | BR2ex2-3F(c): AAGACACGCTGCAACAAAGCAGATT |
| | F (alternate) | BR2ex2-8F: AAGACACGCTGCAACAAAGCAGTCA |
| | R | BRCA2 Ex 10R: GATCAGTATCATTTGGTTCC |
| Δ4 | F (full length) | BRCA2ex3-4F(a): ATAAATTCAAATTAGACTTAGGAAG |
| | F (alternate) | BRCA2ex3-5F: ATAAATTCAAATTAGACTTAGTCCT |
| | R | BRCA2 Ex8-7R: CTTCATTTCTGACTATGAGC |
| Δ4-7 | F (full length) | BR2ex3-4F(b): GATAAATTCAAATTAGACTTAGGAAGG |

| | | |
|---|---|---|
| | F (alternate) | BR2ex3-8F: GATAAATTCAAATTAGACTTAGTCAGA |
| | R | BRCA2 Ex 10R: GATCAGTATCATTTGGTTCC |
| Δ5 | F (full length) | BRCA2 Ex4-5F(a): CTAAATTCTTGTCTTAGTGAAAGTCCTG |
| | F (alternate) | BRCA2 Ex4-6F: CTAAATTCTTGTCTTAGTGAAAGTGGTA |
| | R | BRCA2 Ex8-7R: CTTCATTTCTGACTATGAGC |
| Δ5,6 | F (full length) | BR2ex4-5F(b): CCACTTCTAAATTCTTGTCTTAGTGAAAGTCCT |
| | F (alternate) | BR2ex4-7F: CCACTTCTAAATTCTTGTCTTAGTGAAAGGGTC |
| | R | BRCA2 Ex 10R: GATCAGTATCATTTGGTTCC |
| Δ5-7 | F (full length) | BRCA2ex4-5F(c): AAATTCTTGTCTTAGTGAAAGTCCTGT |
| | F (alternate) | BRCA2ex4-8F: AAATTCTTGTCTTAGTGAAAGTCAGAA |
| | R | BRCA2 Ex 10R: GATCAGTATCATTTGGTTCC |
| ▼5p | F (full length) | BR2ex4-5F(b): CCACTTCTAAATTCTTGTCTTAGTGAAAGTCCT |
| | F (alternate) | BR2ex5+23F: CTAAATTCTTGTCTTAGTGAAAGGGA |
| | R | BRCA2 Ex8-7R: CTTCATTTCTGACTATGAGC |
| Δ6 | F (full length) | BRCA2ex5-6F(a): CACCACAAAGAGATAAGTCAGTGGTA |
| | F (alternate) | BRCA2ex5-7F: CACCACAAAGAGATAAGTCAGGGTCG |
| | R | BRCA2 Ex10R: GATCAGTATCATTTGGTTCC |
| Δ6q7 | F (full length) | BRCA2 EX 5-6F(b): ACAAAGAGATAAGTCAGTGGTAT |
| | F (alternate) | BRCA2 EX 5-8+2: ACAAAGAGATAAGTCAGTGTCAG |
| | R | BRCA2 Ex10R: GATCAGTATCATTTGGTTCC |
| Δ9-11 | F (full length) | BR2ex8-9F: GTATTTCCTCATGATACTACTGCTAATG* |

| | | |
|---|---|---|
| | F (alternate) | BR2ex8-12F: GTATTTCCTCATGATACTACTGCTGAGA |
| | R | BRCA2 Ex 14R: TGCTAAATTGCTTGAAGATT |
| Δ12 | F (full length) | BR2EX11-12F(a): AGCCCCTTATCTTAGTGGGAGA |
| | F (alternate) | BR2EX11-13F: AGCCCCTTATCTTAGTGGGCAC |
| | R | BRCA2 Ex 14R: TGCTAAATTGCTTGAAGATT |
| Δ12,13 | F (full length) | BR2ex11-12F(b): AAGAAGAGGAGAGCCCCTTATCTTAGTGGGAG |
| | F (alternate) | BR2ex11-14F: AAGAAGAGGAGAGCCCCTTATCTTAGTGGCAC |
| | R | BRCA2 Ex14R: TGCTAAATTGCTTGAAGATT |
| Δ17 | F (full length) | BR2ex 16-17F(a): GGAAAAGAAGAATTTTATAGGGC |
| | F (alternate) | BR2ex 16-18F: GGAAAAGAAGAATTTTATAGATA |
| | R | BRCA2 Ex18R: TTTCAGATATATTTGCGCTC |
| Δ17-18 | F (full length) | BR2ex16-17F(b): GGAAAGGCTGGAAAAGAAGAATTTTATAGGGC |
| | F (alternate) | BR2ex16-19F: GGAAAGGCTGGAAAAGAAGAATTTTATAGATT |
| | R | BRCA2ex22R: AACATTTGCCTGTGATTATT |
| Δ18 | F (full length) | BR2ex17-18F: GCTTCTTCAACTAAAATACAGATATGA |
| | F (alternate) | BR2ex17-19F: GCTTCTTCAACTAAAATACAGATTTCT |
| | R | BRCA2 Ex23R: TGTATGTTAGCTCTTTCAGA |
| Δ 19 | F (full length) | BR2ex 18-19F: GAATCTCTTATGTTAAAGATT |
| | F (alternate) | BR2ex 18-20F: GAATCTCTTATGTTAAAGTGG |
| | R | BRCA2 Ex23R: TGTATGTTAGCTCTTTCAGA |
| Δ20 | F (full length) | BR2ex19-20F: ATTATTCAAAGAGCATACCCTATACAGTGG |

| | | |
|---|---|---|
| | F (alternate) | BR2ex19-21F: ATTATTCAAAGAGCATACCCTATACAGAAA |
| | R | BRCA2 Ex23R:TGTATGTTAGCTCTTTCAGA |
| ▼20A | F (full length) | BRCA2ex20-21F: GGAATTTGAAGAACATGAAGAAAAC |
| | F (alternate) | BRCA2ex20-i20F: GGAATTTGAAGAACATGAAGTTACT |
| | R | BRCA2 EX22R: AACATTTGCCTGTGATTATT |
| Δ22 | F (full length) | BRCA2ex21-22F(a): CAGACCCAGCTTACCTTGAGGGT |
| | F (alternate) | BRCA2ex21-23F: CAGACCCAGCTTACCTTGAGTTATA |
| | R | BRCA2 Ex23R: TGTATGTTAGCTCTTTCAGA |
| Δ22,23p | F (full length) | BR2ex21-22(b): GACCCAGCTTACCTTGAGGGT |
| | F (alternate) | BR2ex21-delex23p: GACCCAGCTTACCTTGAGAAG |
| | R | BRCA2 Ex23R: TGTATGTTAGCTCTTTCAGA |
| Δ23p | F (full length) | BR2ex 22-23F: GCTATTCAAAAAAAGAAAAAGATTCAGTTA |
| | F (alternate) | BR2ex22-delex23pF: GCTATTCAAAAAAAGAAAAAGATTCAGAAG |
| | R | BRCA2 Ex23R: TGTATGTTAGCTCTTTCAGA |

*Note: this may not always be a practical positive control as the predicted RT-PCR product is 6.5 kb.

Supplementary Materials and Methods

*BRCA2 alternate splicing event nomenclature:*

The naming of *BRCA2* alternate splicing events in this study follows the convention used by Colombo *et al.* [6]. Briefly, the exon number preceded by the letter delta (Δ) indicate alternate splicing events resulting from single exon skipping. Commas or dashes indicate events resulting in skipping of two or more contiguous exons, respectively. Events involving a shifted splice donor (distal) or splice acceptor (proximal) are indicated with p or q, respectively. The alternate splice acceptor within intron 4 that adds 23 bases to the 5' end of exon 5 is indicated by the event designation ▼5p. Exonization of a region of intron 20 is indicated by the designation ▼20A. Corresponding HGVS nomenclature for the alternate splicing events is according to the Human Genome Variation Society nomenclature guidelines (http://www.hgvs.org/rechtml).

*Identification of alternate BRCA2 splicing events in the literature:*

We define naturally occurring alternative splicing events as those resulting from alternate splice site choices during regular mRNA processing and are not caused by RNA sequence variants.

To determine which alternate *BRCA2* mRNA splicing events have been described previously, a PubMed search was performed (http://www.ncbi.nlm.nih.gov/pubmed) using search terms *BRCA2*, alternate

splicing, and mRNA. For the purpose of this study, the results were reviewed to determine whether the cDNA fragment representing the alternate splicing event had been sequenced and whether the event occurred independently of a variation in the gene sequence.

*Patient samples and cell lines:*

In phase II, lymphoblastoid cell lines were taken from archival stocks of samples from eleven breast cancer patients who were consented under a protocol approved by the University of Chicago Institutional Review Board. All patients were referred for *BRCA1/BRCA2* genetic testing by the University of Chicago Cancer Risk Clinic for reasons of personal or family history of cancer. Nine subjects were diagnosed with breast cancer, one with fallopian tube cancer, and one with no cancer diagnosis. Of the eleven subjects used for all alternate splicing events, four had no detected gene sequence variants, one had a pathogenic variant in *BRCA1*, and one had a pathogenic variant in *PALB2*. The remaining five carried *BRCA2* truncating variants: three were in exon 11 at positions not predicted to affect exon 11 splicing (c.5864C>A (p.Ser1955*), c.2808_2811del , c.5350_5351del;), one was in exon 4 (c.391delT), and another in exon 18 (c.8297delC). We note that exon skipping events involving exon 4, either alone or in combination with other exons, were detected in the majority of cell lines tested, not just the cell line with the exon 4 pathogenic variant. Likewise, the exon skipping events involving exon 18 were seen in the majority of cell lines tested. We therefore suggest that these mutations did not affect the identification of naturally occurring exon-skipping events

described below. Breast cancer cell lines were MCF7 (from an ER+ invasive ductal carcinoma with a luminal epithelial phenotype), HCC1937 (from a ductal carcinoma carrying a homozygous pathogenic variant in *BRCA1*), and BT20 (from a triple-negative invasive ductal carcinoma). None of these cell lines has any known mutation in *BRCA2*. Non-cancer breast cell lines were MCF10A (derived from mammary epithelial cells associated with fibrocystic disease), 184A1 (from chemically transformed breast epithelial cells), and 184B5 (a different isolate from the procedure that generated 184A1) [50]. Breast cell lines were obtained from the American Tissue Type Collection (ATTC). Cell lines were grown with conventional media and conditions. Multiple LCLs and breast cell lines (see above) were used to test the detectability of alternate splicing events identified in phases Ia and Ib. No attempt was made to provide definitive characterization of any single cell type with respect to specific alternate splicing events in Phase II. Thus, in most cases, each cell line was tested once per alternate splicing event (Table 2).