European Journal of Human Genetics (2009) 17, 1471 – 1480

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ARTICLE

Impact of *BRCA1* and *BRCA2* variants on splicing: clues from an allelic imbalance study

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Nearly one-half of *BRCA1* and *BRCA2* sequence variations are variants of uncertain significance (VUSs) and are candidates for splice alterations for example, by disrupting/creating splice sites. As out-of-frame splicing defects lead to a marked reduction of the level of the mutant mRNA cleared through nonsense-mediated mRNA decay, a cDNA-based test was developed to show the resulting allelic imbalance (AI). Fifty-four VUSs identified in 53 hereditary breast/ovarian cancer (HBOC) patients without *BRCA1/2* mutation were included in the study. Two frequent exonic single-nucleotide polymorphisms on both *BRCA1* and *BRCA2* were investigated by using a semiquantitative single-nucleotide primer extension approach and the cDNA allelic ratios obtained were corrected using genomic DNA ratios from the same sample. A total of five samples showed AI. Subsequent transcript analyses ruled out the implication of VUS on AI and identified a deletion encompassing *BRCA2* exons 12 and 13 in one sample. No sequence abnormality was found in the remaining four samples, suggesting implication of *cis*- or *trans*-acting factors in allelic expression regulation that might be disease causative in these HBOC patients. Overall, this study showed that AI screening is a simple way to detect deleterious splicing defects and that a major role for VUSs and deep intronic mutations in splicing anomalies is unlikely in *BRCA1/2* genes. Methods to analyze gene expression and identify regulatory elements in *BRCA1/2* are now needed to complement standard approaches to mutational analysis.
European Journal of Human Genetics (2009) **17,** 1471–1480; doi:10.1038/ejhg.2009.89; published online 27 May 2009

Keywords: variants of uncertain significance; *BRCA1*; *BRCA2*; allelic imbalance; mutation screening; regulatory elements

Introduction

Molecular testing is now becoming a routine part of the medical management of patients affected by genetic diseases. In some cases, interpretation of the results from mutation screening is straightforward, for example truncating mutations and large rearrangements, and leads to clinical management options. However, in other cases, a variant of uncertain significance (VUS) is found and its contribution to disease risk has yet to be defined. Classification of these VUSs is therefore a major issue in molecular diagnosis and certain guidelines have been proposed.[1] Germline mutations in *BRCA1* (MIM 113705) and *BRCA2* (MIM 600185) result in hereditary predisposition to breast and ovarian cancer (HBOC).[2] Unfortunately, one-half of the variations observed in the *BRCA1/2* genes

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Received 26 September 2008; revised 23 March 2009; accepted 15 April 2009; published online 27 May 2009

are VUSs,[3] making biological and clinical interpretation a challenging task and consequently leading to clinically and emotionally difficult situations. A striking example is that of a translationally silent nucleotide modification that would be classified as neutral despite the fact that it could be deleterious by affecting normal pre-mRNA splicing.[4] A key issue raised in molecular diagnosis in the context of VUS interpretation is therefore their putative impact on splicing.[5]

Mutant mRNAs containing premature stop codons are eliminated or destabilized by nonsense-mediated mRNA decay (NMD), leading to a state of haploinsufficiency.[6] As a result, the ratios between the expression levels from mutant alleles and the corresponding wild-type alleles are significantly decreased, resulting in allelic imbalance (AI), as shown in a number of genes, including *BRCA1* and *BRCA2*.[7–9] In an attempt to rapidly detect a deleterious impact of *BRCA1* and *BRCA2* VUSs on splicing, a SNaPshot assay has been designed to show this type of AI resulting from NMD. The SNaPshot assay is designed to interrogate single-nucleotide polymorphisms (SNPs) at known locations in a single tube. An unlabeled oligonucleotide primer is annealed to a PCR product 3′ to the SNP and extended by a single dideoxynucleotide triphosphate (ddNTP) complementary to the base of interest. The high specificity of single ddNTP incorporation makes the SNaPshot assay a powerful tool for quantitative SNP analyses. A relative quantification of allele-specific extended primers allows accurate determination of the initial ratio of the corresponding template sequences.[10–12]

Fifty-four VUSs identified in 53 HBOC patients without *BRCA1/2* mutation were screened for allelic variations. As *BRCA1/2* genes may commonly exhibit allele-specific expression differences,[13] this natural variation range was defined in a control population consisting of retinoblastoma patients. Instead of targeting each VUS individually by the SNaPshot assay, which would considerably decrease the throughput and increase the costs, AIs were screened at four frequent *BRCA1* and *BRCA2* SNPs. Following AI detection, the samples were thoroughly investigated at the cDNA level to characterize the splicing defect using a two-step strategy. Analysis initially focused on the surrounding coding regions of the VUS, as this VUS was expected to cause the out-of-frame splicing defect leading to AI. Second, when no anomaly was found, the whole coding sequence of *BRCA1* or *BRCA2* was investigated at the cDNA level to look for a deep intronic mutation that could cause the splicing defect.[14]

Patients and methods

More than 3500 consecutive patients, mostly of Caucasian descent, diagnosed with HBOC, underwent constitutional molecular analysis of the *BRCA1* and *BRCA2* genes at the Institut Curie, following appropriate genetic counseling and written informed consent.[15] In this series, 54 cases were selected on the following grounds: (i) lymphoblastoid cell line availability (ii) without *BRCA1/2* mutation (iii) bearing at least one VUS (Tables 1a and 1b) (iv) polymorphic on one SNP (at least). As Institut Curie is also a reference center for retinoblastoma (MIM180200), a panel of lymphoblastoid cell lines from retinoblastoma patients heterozygous for the SNPs of interest were used as the control group.

Routine genomic screening

Patients were screened for point mutations on *BRCA1* and *BRCA2* using denaturing high-performance liquid chromatography.[16] Large rearrangements on *BRCA1* were also investigated by using quantitative multiplex PCR of short fluorescent fragments.[17] A panel of 53 DNAs from retinoblastoma patients were screened to identify polymorphic controls for the SNPs of interest. Nucleotide position was numbered according to the HGVS guidelines on the basis of the cDNA sequences NM_007294.2 and NM_000059.3 for *BRCA1* and *BRCA2*, respectively.

RNA extraction, reverse transcription and PCR amplification (RT-PCR)

RNA extracted from lymphoblastoid cell lines was used, as validated earlier.[11,18,19] RNA was extracted from lymphoblastoid cell lines using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNAs were further processed to remove any contaminating DNA (Turbo DNA free; Ambion, Austin, TX, USA). Following quantification using a spectrophotometer (NanoDrop; Thermo Scientific, Courtaboeuf, France), 2 μg of total RNA from each sample was used for reverse transcription in a 40 μl reaction using the GeneAmp RNA PCR Core kit and according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The fragments containing the four SNPs were PCR amplified in separate vials using 100 ng of cDNA in a 10 μl final volume with standard, unlabeled primers (Table 2), and then run on a GeneAmp PCR system 9700 (Applied Biosystems). Limiting PCR conditions were used (25–28 cycles, depending on the SNPs) to remain in the exponential phase of amplification. Signal strength was therefore directly dependent on the target copy number, so that relative allelic dosage could be obtained by comparing peak heights of the two alleles. Following amplification, unincorporated primers and dNTPs were removed by ExoSAP-IT according to the manufacturer's instructions (Amersham Bioscience, Orsay, France).

Quantitative SNaPshot assay

Seven microliters of the purified RT-PCR products were used in the SNaPshot quantitative primer extension assay (Applied Biosystems) according to the manufacturer's instructions and using the primers shown in Table 2.

Table 1a Controls, patients and VUS tested on *BRCA1*

		SNP used for allelic imbalance assay and normalized ratio[a]	
Controls	*Mutations*	*c.2612C>T (exon 11) (normal range: 0.78–1.10)*	*c.4308T>C (exon 13) (normal range: 0.68–1.35)*
Control 1	c.135-2A>G/intronic retention of 58 bp	2.45	Homozygous
Control 2	c.1266T>G/p.Tyr422X	0.52	2.05
Control 3	c.1504_1508del/p.Lys503AlafsX2	0.51	2.96
Control 4	c.1961dupA/p.Tyr655ValfsX18	0.43	2.88
Control 5	c.1965C>A/p.Tyr655X	0.45	2.80
Control 6	c.2071delA/p.Arg691AspfsX10	2.11	0.53
Control 7	c.2670delG/p.Gly890GlyfsX3	2.18	0.57
Control 8	c.2776_2777insTA/p.Thr926IlefsX75	0.50	2.58
Control 9	c.2800C>T/p.Gln934X	2.44	0.47
Control 10	c.3841C>T/p.Gln1281X	0.46	2.81
Control 11	c.4088C>G/p.Ser1363X	Homozygous	2.07
Control 12	c.4484G>A/exon 14 skipping	1.65	1.92
Patient ID	*VUS*		
133A	c.1927A>G/p.Ser643Gly	0.96	1.04
3878A	c.212+23T>A	1.10	Homozygous
3885A	c.5277+48_59dup12	0.99	Homozygous
14126A	c.3608G>A/p.Arg1203Gln	0.92	Homozygous
14161A	c.1137T>G/p.Ile379Met	0.90	Homozygous
14756A	c.5194-105insG	0.92	1.10
15470A	c.5277+48_59dup12	1.70[a]	1.42[a]
16506A	c.135-49T>C	0.94	1.03
18893A	c.5074+68T>C	0.94	1.07
20958A	c.557C>A/p.Ser186Tyr	Homozygous	1.16
20958A	c.3823A>G/p.Ile1275Val	Homozygous	1.16
21665A	c.2595T>C/p.Arg866Cys	0.88	1.17
24017A	c.2315T>C/p.Val772Ala	1.05	1.53[a]
25032A	c.114G>A/p.Lys38Lys	1.10	1.23
27328A	c.1001C>T/p.Pro334Leu	1.05	1.02
27559A	c.5252G>A/p.Arg1751Gln	1.07	0.96
27559A	c.1263A>C/p.Glu421Asp	1.07	0.96
33859A	c.5277+48_59dup12	1.06	1.24

Nucleotide position was numbered on the basis of the cDNA sequence NM_007294.2 according to HGVS guidelines.
[a]See text and Table 4a for detailed description of ratio calculation.

Following purification by SAP-IT (Amersham Bioscience), separation and detection of SNaPshot products were performed on an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems). The injection cocktail, consisting of 0.5 μl of the GeneScan-120 LIZ size standard (Applied Biosystems) and 19.5 μl of formamide-EDTA, was mixed with 2 μl of SNaPshot products, heat denatured and then chilled on ice. Products were injected for 18 s at 1.2 kV, then electrophoresed for 1300 s at 15 kV using Performance Optimized Polymer 6 and a 36-cm length-to-detector uncoated capillary. Data were analyzed using the Genemapper (version 4) software program (Applied Biosystems) to collect both area and peak height for each sample.

AI measurement

The peak/area ratios were measured between the two allelic versions that is, c.2612C to c.2612T/p.Pro871Leu (rs799917) and c.4308T to c.4308C/p.Ser1436Ser (rs1060915) for *BRCA1,* and c.3807T to c.3807C/p.Val1269-Val (rs543304) and c.7242A to c.7242G/p.Ser2414Ser (rs1799955) for *BRCA2*. cDNA ratios were then normalized with respect to the values obtained on genomic DNA (cDNA ratios/gDNA ratios) to correct from putative variations in dye incorporation induced by the nucleotide sequence. As *cis*/*trans*-acting inherited variations in gene expression are known to be relatively common,[11–13] a control population was used to define the physiological expression variation at these SNPs. A panel of retinoblastoma patients heterozygous for the tested SNPs (13–20 depending on the SNPs) were screened by the SNaPshot assay and the physiological expression range was defined as the mean of the normalized ratio ±2 SD. Twenty-three samples with *BRCA1/2* truncating mutations were used as positive controls.

cDNA screening following AI detection

RNA was extracted from lymphoblastoid cell lines with and without puromycin treatment. Puromycin treatment was used to inhibit NMD[20] to allow the identification of the out-of-frame splicing defect. Following reverse transcrip-

Table 1b Controls, patients and VUS tested on *BRCA2*

		SNP used for allelic imbalance assay and normalized ratio[a]	
Controls	*Mutations*	*c.3807T>C (exon 11) (normal range: 0.92–1.61)*	*c.7242A>G (exon 14) (normal range: 0.70–1.65)*
Control 1	c.631+3A>G/exon 7 skipping	1.85	Homozygous
Control 2	c.1310_1313del4/p.Lys437IlefsX22	0.71	0.63
Control 3	c.1929delG/p.Val643ValfsX17	1.99	2.22
Control 4	c.2808_2811del/p.Gln937LysfsX23	Homozygous	0.57
Control 5	c.3455T>G/p.Leu1152X	Complete imbalance	Homozygous
Control 6	c.4285_4286insT/p.Gln1429LeufsX9	1.96	Homozygous
Control 7	c.5263G>T/p.Glu1755stop	0.42	0.60
Control 8	c.5576_5579del/p.Ile1859LysfsX3	0.67	Homozygous
Control 9	c.7322delG/p.Gly2441AlafsX28	2.16	2.80
Control 10	c.7806-7C>G/exon 18 skipping and 6 bp intronic retention	Homozygous	1.68
Control 11	c.8364G>A/p.Trp2788X	2.08	Homozygous
Patient ID	*VUS*		
3794A	c.2363G>A/p.Gly788Asp	Homozygous	1.12
3941A	c.8111C>T/p.Ser2704Phe	Homozygous	0.79
8680A	c.9116C>T/p.Pro3039Leu	1.27	Homozygous
9100A	c.7463G>A/p.Arg2488Lys	1.07	Homozygous
9408A	c.4585G>A/p.Gly1529Arg	Homozygous	1.11
10647A	c.8149G>T/p.Ala2717Ser	Homozygous	1.06
10647A	c.9226G>C/p.Gly3076Arg	Homozygous	1.06
16072A	c.1964C>G/p.Pro655Arg	Homozygous	1.10
16555A	c.9613GC>CT/p.Ala3205Leu	1.35	Homozygous
16982A	c.8632-16C>G	1.37	1.31
17073A	c.8149G>T/p.Ala2717Ser	1.37	Homozygous
19054A	c.9275A>G/p.Tyr3092Cys	1.55	Homozygous
19320A	c.3462C>T/p.Thr1154Thr	1.51	1.28
20020A	c.4187A>G/p.Gln1396Arg	1.30	1.23
20020A	c.8460A>C/p.Val2820Val	1.30	1.23
20853A	c.1792A>G/p.Thr598Ala	1.25	1.27
20909A	c.280C>T/p.Pro94Ser	Homozygous	0.94
21784A	c.8567A>C/p.Glu2856Ala	Homozygous	1.19
23324A	c.425+33A>G	1.44	Homozygous
23576A	c.3088T>G/p.Phe1030Val	1.33	1.27
23877A	c.1151C>T/p.Ser384Phe	1.68[a]	0.67[a]
23919A	c.223G>C/p.Ala75Pro	1.44	Homozygous
24311A	c.2739C>T/p.Asp913Asp	1.17	1.34
25039A	c.5312G>A/p.Gly1771Asp	1.26	1.32
25606A	c.10110G>A/p.Arg3370Arg	1.51	Homozygous
25977A	c.4570T>G/p.Phe1524Val	0.99	0.98
26355A	c.4686A>G/p.Gln1562Gln	Homozygous	0.97
26558A	c.10188T>C/p.Ser3396Ser	0.93	Homozygous
27580A	c.1514T>C/p.Ile505Thr	Homozygous	1.03
28357A	c.1786G>C/p.Asp596His	Homozygous	1.13
29050A	c.9038C>T/p.Thr3013Ile	1.40	Homozygous
29183A	c.6739A>G/p.Ser2247Gly	1.27	1.37
30709A	c.425+29T>C	1.22	1.20
30856A	c.67+57A>C	1.45	1.27
31052A	c.631+25C>T	Homozygous	1.09
31080A	c.5987C>G/p.Ala1996Gly	Homozygous	0.60[a]
31248A	c.6323G>A/p.Glu2108His	1.73[a]	Homozygous
31577A	c.4584C>T/p.Ser1528Ser	Homozygous	1.12
32536A	c.8850G>T/p.Lys2950Asn	Homozygous	1.04

Nucleotide position was numbered on the basis of the cDNA sequence NM_000059.3 according to HGVS guidelines.
[a]See text and Table 4b for detailed description of ratio calculation.

tion (see above), the surrounding coding regions of the VUS were PCR amplified (primers available on request), purified by Nucleofast 96 PCR plates (Macherey Nagel, Hoerdt, France) and sequenced on an ABI 3130 XL using the Big Dye terminator kit (Applied Biosystems) and the primers used for amplification. When no anomaly was found, the same cDNA sample was used for whole *BRCA1/2* coding sequence amplification. *BRCA1* and *BRCA2* were

Table 2 cDNA primers used for SNP amplification and SNaPshot assays

Gene	*SNP*	*Amplification forward primer (5′–3′)*	*Amplification reverse primer (5′–3′)*	*SNaPshot primer (5′–3′)*
BRCA1	c.2612C>T	CAAGGGACTAATTCATGGTTG	AACTGTCTGTACAGGCTTGAT	ACATTCAAGGTTTCAAAGCGCCAGTCATTTGCTC
	c.4308T>C	GAAACAAGCGTCTCTGAAGACT	TTCTGGATTCTGGCTTATAGGG	ACCCTTCCATCATAAGTGACTC
BRCA2	c.3807T>C	GCTGTGAAACTGTTTAGTGA	GCAAGTCCGTTTCATCTTTA	CCAATAAGTTTATCTTCAAGTAAATGTCATGATTCTGT
	c.7242A>G	TATGAACATCTGACTTTGGAA	GGCATTCTGAAGACTTGTAA	AGTCTTTGTTCCACCTTTTAAAACTAAATC

Table 3 Performance of the assay, based on serial dilutions (100:0, 80:20, 60:40, 50:50, 40:60, 20:80 and 0:100) of two DNA samples homozygous for the frequent and minor allele of two SNPs (*BRCA1*, c.2612C>T and *BRCA2*, c.3807T>C)

	Based on peak area		*Based on peak height*	
Gene, SNP	*Regression equation*	R^2	*Regression equation*	R^2
BRCA1 c.2612C>T/p.Pro871Leu	$y=1.2593x+0.0396$	0.995	$y=1.2397x+0.0662$	0.996
BRCA2 c.3807T>C/p.Val1269Val	$y=0.9986x+0.0731$	0.996	$y=1.0369x+0.0564$	0.997

Regression equations and R^2 are indicated for peak height and area. The fluorescent signal increases as a function of sample load.

studied in 7 and 10 overlapping fragments (exon 11 excluded), respectively (primer sequences available on request). Amplicons were purified and sequenced as described above.

In silico analysis

As a complementary investigation, the impact of the 56 VUSs on splicing was tested *in silico* using a set of previously evaluated web-based tools[5] that is, Splice Site Prediction by Neural Network (NNSplice available at http://www.fruitfly.org/seq_tools/splice.html),[21] Splice Site Finder (SSF no longer supported, but similar algorithms are now available on Alamut (Interactive Biosoftware, Rouen, France)), Automated Splice Site Analysis (ASSA available at https://splice.uwo.ca)[22] and MaxEntScan with the maximum entropy model (MES available at http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html).[23]

Results

Development and validation of a quantitative AI assay for *BRCA1* and *BRCA2*

Instead of targeting each VUS individually, which would be time consuming, costly and barely applicable to intronic VUSs, the presence of AI was screened at four *BRCA1/2* SNPs. These SNPS were chosen because they are relatively common (see NCBI website available at http://www.ncbi.nlm.nih.gov/SNP). RNA extracted from lymphoblastoid cell lines and a robust, widely used, primer-extension assay developed by Applied Biosystems were used.

Assay performance To determine whether our SNaPshot assay was able to quantitatively measure AI at *BRCA1* and *BRCA2*, DNAs from two individuals determined by sequence analysis to be homozygous for either *BRCA1*-c.2612C/C or *BRCA1*-c.2612T/T, and *BRCA2*-c.3807T/T or *BRCA2*-c.3807C/C, were mixed at the following ratios (100:0, 80:20, 60:40, 50:50, 40:60, 20:80 and 0:100), then SNaPshot was tested. Regression equations and R^2 were calculated for peak height and area. Measurements based on peak height were found to be more robust than measurements based on peak area, although both results were good (Table 3). Therefore, peak height was used throughout the study. Following these results, SNaPshot assaying two other SNPs on both *BRCA1* and *BRCA2* were designed for the study. However, two of them were subsequently withdrawn from further analysis (*BRCA1* SNP c.4837A>G and *BRCA2* SNP c.1114A>C) because double peaks for one allele were randomly observed in a number of samples tested (data not shown). This lack of robustness was confirmed using a serial dilution experiment (see earlier) showing $R^2=0.888$ for *BRCA1* SNP c.4837A>G and $R^2=0.786$ for *BRCA2* SNP c.1114A>C. A total of four SNPs were therefore used for this study (Tables 4a and 4b).

Detection of AI caused by NMD Before testing the putative impact of VUSs, the first step of the study consisted of checking that samples with truncating mutations inducing NMD were correctly detected with an expected AI. A total of 23 control DNAs and RNAs from HBOC patients bearing mutations leading to an

Table 4a Samples showing allelic imbalances on *BRCA1*

SNP tested and normal variation range (1): c.2612C>T – 0.78 to 1.10								
Patient and VUS	*Height peak 'C'*		*Height peak 'T'*		*Ratio 'C/T'*		*Normalized ratio cDNA/gDNA*	*Normalized ratio cDNA/gDNA following NMD inhibition (3)*
	cDNA	*gDNA*	*cDNA*	*gDNA*	*cDNA*	*gDNA*		
15470A, c.5277+48_59dup12	1522	1515	1349	2282	1.13	0.66	1.70	1.76
SNP tested and normal variation range (2): c.4308T>C – 0.68 to 1.35								
Patient and VUS	*Height peak 'T'*		*Height peak 'C'*		*Ratio 'T/C'*		*Normalized ratio cDNA/gDNA*	*Normalized ratio cDNA/gDNA following NMD inhibition (3)*
	cDNA	*gDNA*	*cDNA*	*gDNA*	*cDNA*	*gDNA*		
15470A, c.5277+48_59dup12	1224	1268	551	814	2.22	1.56	1.42	1.43
24017A, c.2315T>C/p.Val772Ala	1004	787	463	556	2.17	1.41	1.53	1.51

Normal variation range was defined as the mean of normalized ratio ± 2 SD and determined on a panel of (1) 17 and (2) 20 control samples heterozygous for the SNP of interest. Peak height is in RFU. Peak heights are not indicated for these ratios.

Table 4b Samples showing allelic imbalances on *BRCA2*

SNP tested and normal variation range (1): c.3807T>C – 0.92–1.61								
Patient and VUS	*Height peak 'T'*		*Height peak 'C'*		*Ratio 'T/C'*		*Normalized ratio cDNA/gDNA*	*Normalized ratio cDNA/gDNA following NMD inhibition (3)*
	cDNA	*gDNA*	*cDNA*	*gDNA*	*cDNA*	*gDNA*		
23877A c.1151C>T/p.Ser384Phe	2533	3762	1283	3223	1.97	1.17	1.68	1.67
31080A c.5987C>G/p.Ala1996Gly	Homozygous, not tested							
31248A c.6323G>A/p.Glu2108His	3803	2973	1926	2608	1.97	1.14	1.73	1.69
SNP tested and normal variation range (2): c.7242A>G – 0.70–1.65								
Patient and VUS	*Height peak 'T'*		*Height peak 'C'*		*Ratio 'T/C'*		*Normalized ratio cDNA/gDNA*	*Normalized ratio cDNA/gDNA following NMD inhibition (3)*
	cDNA	*gDNA*	*cDNA*	*gDNA*	*cDNA*	*gDNA*		
23877A c.1151C>T/p.Ser384Phe	4231	5858	6660	6243	0.63	0.94	0.67	0.65
31080A c.5987C>G/p.Ala1996Gly	4016	1797	7668	2077	0.52	0.86	0.60	1.12
31248A c.6323G>A/p.Glu2108His	Homozygous, not tested							

Normal variation range was defined as the mean of normalized ratio ± 2 SD and determined on a panel of (1) 12 and (2) 13 control samples heterozygous for the SNP of interest. Peak height is in RFU. (3) Peak heights are not indicated for these ratios.

out-of-frame defect and previously characterized for molecular diagnostic purposes were used (Tables 1a and 1b). Among them, the *BRCA2* splice mutation c.7806-7C>G/p.Tyr2658_Arg2659insSerPhe + p.Tyr2660PhefsX43 (6-bp intronic retention plus exon 18 skipping) was a very challenging case for detection through AI. This mutation indeed results in a mixture of in-frame and out-of-frame defects (data available on request, presented at the 2004 American Society of Human Genetics meeting http://www.ashg.org/genetics/abstracts/abs04/f537.htm). These mutated controls were heterozygous for one or both SNPs of interest and showed a strong AI, except for c.7806-7C>G, which was just above the cutoff value (see Discussion).

Sample analysis

AI measurement: BRCA1 Sixteen patients bearing 16 different VUSs were tested (Table 1a). All patients were found to be heterozygous at SNP c.2612C>T, c.4308T>C or both. AI was screened by using SNPs c.2612C>T (normal variation range: 0.78–1.10) and c.4308T>C (normal variation range: 0.68–1.35). Two samples fell outside these ranges (Table 4a). Patient 15470A (Figure 1), bearing the c.5277 + 48_59dup12 VUS, was heterozygous on both SNPs and AI was found on both SNPs, although it was much more pronounced at c.2612C>T (1.70) than at c.4308T>C (1.42). Patient 24017A, bearing the c.2315T>C/p.Val772Ala VUS, showed AI at the c.4308T>C SNP (1.53), but its normalized ratio remained in the upper normal range at the c.2612C>T SNP (1.05). All samples, apart from 15470A, showed less marked imbalance compared with the mutated controls.

AI measurement: BRCA2 Thirty-seven patients bearing 38 different VUSs were tested (Table 1b). All patients were found to be heterozygous at SNP c.3807C>T, c.7242A>G or both. AI was screened by using SNPs c.3807C>T (normal variation range: 0.92–1.61) and c.7242A>G (normal variation range: 0.70–1.65). Three samples fell outside these ranges (Table 4b). Patient 23877A, bearing the c.1151C>T/p.Ser384Phe VUS, was heterozygous on both SNPs and a slight AI was found on both SNPs (1.68 and 0.67). Patient 31080A, bearing the c.5987C>G/P.Ala1996Gly VUS, and patient 31248A, bearing the c.6323G>A/p.Glu2108His VUS, showed imbalance on one SNP (0.60 and 1.73, respectively), but this was not confirmed on the remaining SNP (homozygous state).

cDNA screening following AI detection RNA from the five patients showing AI was extracted from lymphoblastoid cell lines with and without puromycin treatment to identify an out-of-frame splicing defect. RT-PCR and subsequent sequencing of the surrounding regions of the VUSs did not show any anomaly, thus ruling out their implication in the detected imbalance. It was, therefore, postulated that AI could be due to deep intronic mutations that would generate intronic exonization and subsequent frameshifting.[14] Consequently, the whole *BRCA1* and *BRCA2* coding sequences were thoroughly investigated at the cDNA level and a *BRCA2* out-of-frame transcript

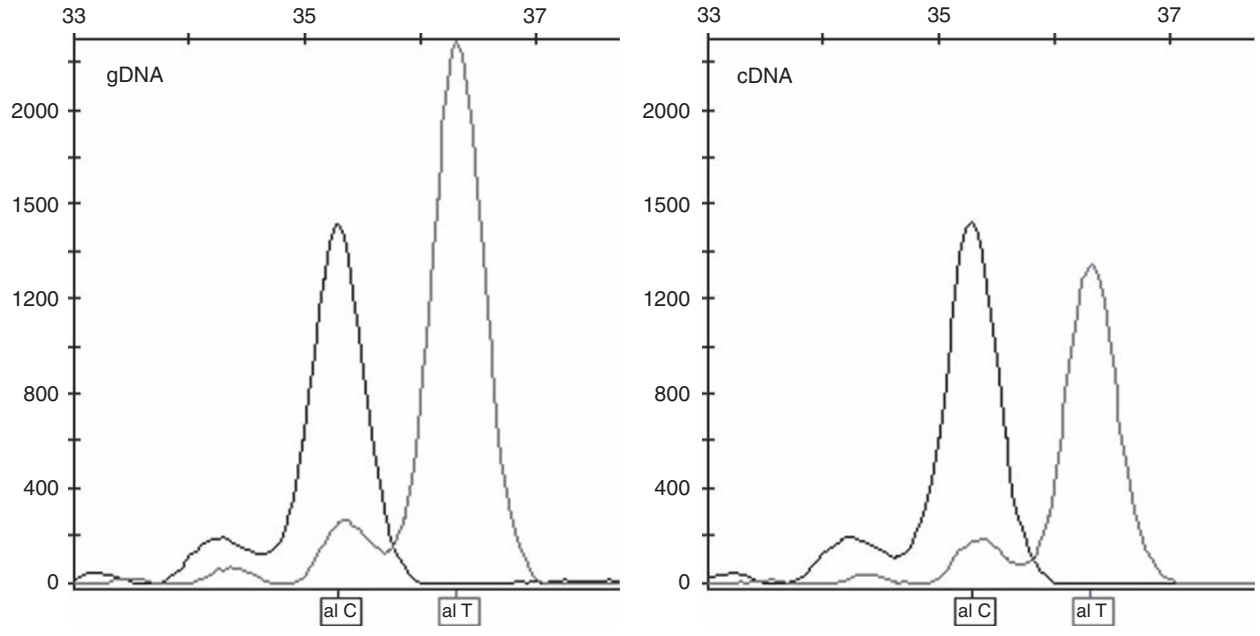

Figure 1 SNaPshot results for patient 15470A, at the c.2612C>T SNP on *BRCA1*. *x* axis: size in bp; *y* axis: fluorescence intensity (arbitrary units). Data are from cDNA (left panel) and gDNA (right panel). Allelic variants are labeled at the bottom of their corresponding peak (the rightmost peak is the 'T' peak).

lacking exons 12 and 13 was found in patient 31080A. Genomic analyses using Quantitative Multiplex PCR of Short fluorescent Fragments[24] evidenced a genomic deletion encompassing exons 12 and 13, thereby explaining our findings. No anomaly was found in the remaining four patients. To further strengthen these results, AI ratios were also measured following NMD inhibition by puromycin. Similar AI ratios were found in all but one case, patient 31080A, who recovered a corrected ratio in the normal range (1.12 for BRCA2 SNP c.7242A>G).

In silico *analyses* All VUSs were subjected to *in silico* analyses using a previously described protocol.[5] No change was observed for 5′–3′ splice sites and branchpoint consensus sequences between the wild-type and mutant sequences. More specifically, the following results were found for the imbalanced samples: VUSs p.Ser384Phe, p.Ala1996Gly, p.Glu2108his and p.Val772Ala are located in large exons of *BRCA2* and *BRCA1*, respectively, and did not lead to the creation of cryptic splice sites. Modifications of exonic splicing enhancer pattern were observed for p.val772Ala (creation of an SF2 motif scored 2.34 using ESE Finder, available at http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) and p.Ser384Phe (score increased from 2.69 to 4.04 for an SRp40 motif using ESE Finder). The intronic *BRCA1* VUS c.5277+48_59dup12 did not alter the 5′ wild-type splice site and did not create a cryptic splice site.

Discussion

In the context of molecular diagnosis, geneticists are faced everyday with a huge number of nucleotide variations of uncertain significance (VUS) in their favorite genes. One clue in VUS interpretation can be derived from current knowledge suggesting that all types of nucleotide modifications may affect normal splicing through disruption/creation of splice consensus sequences.[4,14,25,26] Unfortunately, it appears unrealistic to perform routine RNA screening targeting each VUS to detect a putative splicing anomaly, particularly for genes, such as *BRCA1* and *BRCA2,* that display a large number of VUSs. We have, therefore, developed an AI assay to rapidly detect VUSs that may have a deleterious impact on splicing by generating out-of-frame transcripts degraded by NMD. A large normal variation range was defined to avoid false positives. On the other hand, sensitivity is lowered and some imbalanced patients may have been scored normal. Fifty-four consecutively ascertained carriers of a total of 53 VUSs on *BRCA1* or *BRCA2* using four common SNPs were tested and five AIs were found. Subsequent transcript analyses ruled out the implication of these VUSs on AI and identified a deletion encompassing *BRCA2* exons 12 and 13 in patient 31080A. These findings were strengthened by AI measurements on puromycin-treated samples. The deleted patient recovered a normal ratio, but the remaining four samples displayed similar values with or without NMD inhibition (Tables 4a and 4b), further showing the absence of a hidden truncating mutation and suggesting an underlying cause outside *BRCA* genes (see below).

Overall, we believe this is a fast and easy-to-implement strategy, as the use of common SNPs allows rapid screening of a large number of patients (the same test may be used for a large number of patients). This assay should now be used on RNA extracted from fresh lymphocytes, which are the preferred source in diagnostic settings. Assaying one gene at a time prevents the burden of multiple testing and modest effects or sample size that is, low statistical power. Consequently, our mutated controls and an NMD-prone mutation were unambiguously detected. More interesting, the normalized ratio for the *BRCA2* c.7806-7C>G mutation, which produces in-frame and out-of-frame transcripts, was relatively close to the upper ratio of the normal variation range (1.68 *versus* 1.65, Table 4b), thereby illustrating the sensitivity of the assay. Unexpectedly, the same range of normal variation was not observed for the two SNPs tested on both *BRCA1* and *BRCA2*. A normal variation range of 0.32 for c.2612C>T and 0.67 for c.4308T>C was found on *BRCA1*, and this observation held true for *BRCA2* (0.69 and 0.95 for c.3807T>C and c.7242A>G, respectively). Alternative splicing may be responsible for these findings for example, the large number of *BRCA1* alternative transcripts[27] may influence expression measurement levels at a given position on the transcript. For example, discordant results were observed for patient 24017A, who showed AI on *BRCA1* for one SNP but not for the other SNP for which a normalized ratio in the upper normal range was found.

This study, designed to determine a putative impact of *BRCA1/2* VUSs on splicing, was on two aspects. First, it appears that none of the VUSs tested could be linked to a deleterious, out-of-frame defect, in contrast with earlier data.[28–32] Although very interesting, these previous reports were based on a limited number of cases and may therefore not be representative of the majority of VUSs found in *BRCA1* and *BRCA2*. This study, based on 54 VUSs, is in line with a recent report on 108 VUSs from *BRCA1* exon 11, which showed that none of the VUSs tested were found to alter splicing.[33] This is also clearly illustrated in this study for the *BRCA1* c.5277+48_59dup12 variant, which was tested in three distinct patients, but was found to be imbalanced only in one patient. Lastly, *in silico* analyses, which appear to be reliable to detect splice mutations,[5] did not show any possible effect of these VUSs on splicing. Therefore, overall data do not support a major role for *BRCA* VUS in splicing defects. Second, it also appears that deep intronic mutations are not an important part of the molecular spectrum of *BRCA* genes. To our knowledge, there is no deep intronic mutation reported to date in *BRCA1/2* and this is perfectly in line with data reported on

other genes such as *NF1*[4] and *RB1*,[34] showing that deep intronic mutations account for a mere 1% of their mutational spectrum.

The mutation detection rate for *BRCA1* and *BRCA2* among HBOC families in France was close to 14% in 2006 (http://www.e-cancer.fr/v1/fichiers/public/figures_rapport_oncogenetique_2003_2006.pdf), meaning not only that other genes are probably involved but also that a substantial number of *BRCA1/2* mutations have yet to be found outside the coding sequences and intron/exon boundaries, which are screened in routine diagnostic settings. Our study may suggest an alternative mutational mechanism because four patients without structural abnormality of *BRCA1* or *BRCA2* showed AI at transcribed polymorphisms, thereby suggesting the disruption of long-distance *cis*-regulatory elements[35,36] or mutations in the 3′-UTR that influence RNA half-life.[37] It is worth noting that these four patients have a high probability of bearing a mutation at a general autosomal dominant breast cancer susceptibility locus that is, 48–89% depending on the case, and according to the Claus model modified by Easton.[15] However, all belong to female breast cancer-only families. Although highly speculative, it might be hypothesized that disruption of regulatory elements could lead to a less severe phenotype compared with classical deleterious mutations.[38] Haplotype and co-segregation analyses need to be performed in the relevant families to confirm the transmission of an altered *cis*-regulatory element. It could also be of interest to search for *BRCA1/2* mutations in the tumor and, in the case of loss of heterozygosity, determine whether the retained allele is the one with low expression. Alternatively, it cannot be excluded that the cases identified in this study are sporadic cases with a rare combination of polymorphisms leading to decreased expression of *BRCA1/2* genes. A recent study[39] reported AI in a series of 32 HBOC patients *versus* 40 controls at SNP c.4308T>C for *BRCA1*, prompting the authors to postulate an involvement of *BRCA1* AI in HBOC. No significant difference was found in the present patient population compared with the control group at this SNP (18 and 21 cases, respectively, $P=0.59$, *t*-test). It can be concluded that AI at *BRCA1/2* genes may sometimes contribute to HBOC.

In summary, this study showed that (i) AI screening at common polymorphisms is a simple way to detect splicing defects, (ii) a major role for VUSs and deep intronic mutations in splicing anomalies is unlikely in *BRCA1/2* genes and (iii) methods for analyzing gene expression and identifying regulatory elements are now needed to complement standard approaches to mutational analysis.[40,41]

Acknowledgements

We thank Isabelle Eugène, Sandrine Miglierina, Cristelle Postel and Catherine Gilbon for their efficient assistance, the Institut Curie Breast Cancer group for referral of patients and Généthon for cell line establishment.

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