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Research Article

Analysis of Three Variants of *BRCA1* And *BRCA2* Genes of Unknown Clinical Significance

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Abstract

The aim of this study was to analyze and to classify three undetermined genetic variants (UVs) of *BRCA1* and *BRCA2* genes of which one was an exonic variant (c.258T>C on exon 5 of *BRCA1* gene) and two intronic variants (IVS16-3C>G on intron 16 of *BRCA1* gene and IVS14+6G>A on intron 14 of *BRCA2* gene).

We used a combination of bio-informatics and molecular biology techniques to study these variants at DNA and RNA (c.DNA) levels: *in silico* tests of splicing prediction, the simulation of the effect of the mutation on protein function (by PolyPhen), the multiple sequences alignment and *in vitro* tests: PCR, RT-PCR, PCR splicing assay, sequencing and pyrosequencing.

Our results show that:

a) The c.258T>C exonic variant on exon 5 of *BRCA1* gene is probably a pathogenic variant that affects the *BRCA1* protein function by making a substitution of a cystein by an arginine at position 47 (p.Cys47Arg or C47R). This very conserved region in several species is located at the RING Finger region which is involved in protein-protein interactions.

b) The IVS16-3C>G intronic variant on *BRCA1* gene leads to an abnormal splicing with the skipping of exon 17 which provokes a frame-shift with an early apparition of a STOP codon. This variant is classified as a splicing mutation.

c) The IVS14+6G>A intronic variant on *BRCA2* gene does not affect the splicing and is, probably, not a splicing mutation; it could be a rare polymorphism.

Keywords: Unclassified variants, *BRCA1*, *BRCA2*, c.258T>C, IVS16-3C>G, IVS14+6G>A

Introduction

Breast cancer is the most prevalent form of cancer in the industrialized countries of Western Europe, primarily affecting women. Approximately 6,000 new cases are recorded annually among women in Belgium (an incidence of 1/1000), representing about 33% of all cancers affecting women. It constitutes the second major cause of cancer-related deaths among women, following lung cancer. Its incidence in men in Belgium is lower, with about 65 cases per year (incidence: 1.3/100,000) [1-3].

The etiology of breast cancer is poorly understood Breast cancer is a multifactorial disease and likely results from an interaction between environmental and genetic factors. It is difficult to estimate the exact contribution of each risk factor. Several epidemiological and clinical studies have identified a number of factors associated with breast cancer risk. These factors include gender, age, gynecological history, geographic location, lifestyle and environment, and family history. Family history of breast cancer is the most studied risk factor and one of the most significant for developing the disease. Based on family size, structure, and age at onset, a distinction is made between "familial" and "hereditary" breast cancer, corresponding respectively to a "low to moderate" risk (less than 2 to 3 times the population risk) and a "high" risk (greater than 3 times the population risk) [4-7].

It is estimated that between 5 and 10% of breast cancers are of hereditary origin, linked to the autosomal dominant transmission of a mutated predisposition gene. A hereditary predisposition is suspected when several of the following criteria are present: at least three persons of the first or second-degree relatives on the same parental side are affected; early onset; bilaterality of breast damage; ovarian cancer; breast cancer in men [4]. These criteria are generally used in the clinical diagnosis of Hereditary Breast and/or Ovarian Cancer syndrome (HBOC). Two predisposition genes known as BRCA (BReast CAncer) have been identified: *BRCA1* (located at 17q21) and *BRCA2* (located at 13q12-13) [8-10]. Mutations in these genes account for 95% of familial cases involving breast and ovarian cancers or male breast cancer cases, 80% of familial cases with at least six cancer cases among women, and only 33% of familial cases involving four or five breast cancer cases. In addition to breast and ovarian cancer, carriers of *BRCA1* or *BRCA2* mutations may have an elevated risk of developing other gynecological or abdominal cancers such as colon, pancreatic, fallopian tube, and prostate cancers [5,11-14].

Analysis of the whole sequences of the *BRCA1* and *BRCA2* genes have led to the identification of an increasing number of nucleotide variants known as Variants of Uncertain Significance (VUSs) or unclassified ariants (UVs). The common denominator of these genetic variants is that their pathogenicity is unclear, and thus their clinical significance is uncertain. Approximately one-third of *BRCA1* genetic variants and half of those of *BRCA2* reported in the Breast Cancer Information Core (BIC) database are UVs. Several biochemical and epidemiological criteria are used to determine whether a genetic variant is pathogenic, i.e., a true mutation, or a neutral variant (a polymorphism) [1,15-21]. Biochemical criteria include alteration in protein size, expression, conformation, or properties; conservation of amino acids across species; and the function of the region affected by the genetic variation. Epidemiological criteria examine whether the variant has been previously reported in the population and its prevalence among 100 control alleles.

Loss of Heterozygosity (LOH) analyses in tumors and functional studies in cell lines can provide an appropriate test for each variant. Indeed, it has been demonstrated that loss of the wild-type allele in tumor cells is a common mechanism of tumor suppressor gene inactivation [13], providing evidence supporting the pathogenicity of the mutation. However, these tests are generally not available, and their results are often inconclusive [22]. *Radice, et al.* (2011) [20] suggest the use of integrated models in the analysis of UVs, including epidemiological and genetic data, histopathological traits, and *in vitro* and *in silico* analyses.

For more than fifteen years, the Molecular Biology Department of the Institute of Pathology and Genetics (IPG) in Gosselies has been performing a global analysis of the *BRCA1* and *BRCA2* genes in patients with breast and/or ovarian cancer. These studies are primarily conducted in affected individuals with a family history of breast and/or ovarian cancer to propose surveillance strategies aimed at preventing other cancer types in these patients, as well as offering presymptomatic diagnosis to other family members. These analyses have led to the identification of several genetic variants, some of which are of uncertain clinical significance (UVs). Given the complexity of interpreting UVs and the uncertainty regarding their clinical significance, a study was initiated to investigate some of these UV's cases identified at the IPG. The objective of this work was therefore to analyze three unclassified variants of the *BRCA1* and *BRCA2* genes identified by the Molecular Biology Department of the IPG in order to determine their pathogenicity and thereby facilitate genetic counseling for patients.

Material and Methods

The biological material used in this study primarily consists of DNA and RNA samples extracted from blood specimens collected from patients and sent to the Institute of Pathology and Genetics. Blood samples were collected in EDTA-containing tubes and delivered to IPG on the same day for nucleic acid extraction.

For the study of Loss of Heterozygosity (LOH), DNA was extracted from Formalin-Fixed, Paraffin-Embedded (FFPE) tumor tissues.

Patients selected for mutation screening and analysis should meet the following criteria:

a) Diagnosed with breast and/or ovarian cancer

b) Having a family history of breast and/or ovarian cancer

c) Carrying a genetic variant in one of the breasts/ovarian cancer susceptibility genes (*BRCA1* or *BRCA2*)

d) The variant under investigation was novel and of uncertain clinical significance based on the literature and existing databases.

The pathogenicity of UVs was investigated using bioinformatic tools and molecular biology techniques. The analysis aimed to gather supporting or opposing evidence regarding the deleterious nature of the variant. Key criteria included:

a) Predicted impact on protein structure or function (notably within critical protein domains)

b) Conservation across different species

c) Potential effect on splicing mechanisms

d) Evidence of loss of heterozygosity in tumor samples

e) Allelic expression analysis

f) Allele frequency in the general population.

Bioinformatic Analysis of UVs

The bioinformatic tools used varied according to the nature of the genetic variant—whether exonic or intronic.

Exonic Variant Analysis

All exonic variants were analyzed using the PolyPhen tool (*http://tux.embl-heidelberg.de/ramensky/*) to predict the impact of amino acid substitutions.

For exonic variants potentially affecting splicing, the ESE Finder tool (*http://www.rulai.cshl.edu/tools/ESE/*) was used to assess disruption of Exonic Splicing Enhancer (ESE) motifs [23-25].

Intronic Variant Analysis

Intronic variants were evaluated for their potential impact on splicing using several computational tools, including:

a) NNSPLICE (*http://www.fruitfly.org/seq_tools/splice.html*)

b) GeneSplicer (*http://www.tigr.org/tdb/GeneSplicer/gene_spl.html*)

c) MaxEntScan (*http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html*)

d) NetGene2 (*http://www.cbs.dtu.dk/services/NetGene2/*)

e) Splice Site Finder (*http://www.genet.sickkids.on.ca/~ali/splicesitefinder.html*)

f) Splice View (*http://l25.itba.mi.cnr.it/~webgene/wwwspliceview.html*)

Molecular Analysis of UVs

In silico predictions of splicing effects were validated *in vitro* using RT-PCR splicing assays to assess exon skipping or activation of cryptic splice sites, followed by sequencing.

Measurement of Allèles Frequency in the Population

Each variant was screened in 368 anonymized DNA samples from an unselected population using pyrosequencing technic. Allele frequency was calculated as the ratio of carriers to the total number of interpretable samples (n=368). A frequency exceeding 1% was considered indicative of a benign polymorphism [22].

Measurement of the Expression of Two Alleles

When a mutation leads to the production of a truncated protein, the RNA decay trends to degrade rapidly the mutated mRNA leaving only the normal (wild-type) mRNA.

This test is performed on cDNA to assess the expression of two alleles of one of the BRCA genes. In the case of an intronic mutation, an exonic polymorphism is first identified to serve as a 'marker', and then the expression levels of the two alleles are quantified by pyrosequencing. In the case of an exonic variant, the variant itself is used for this measurement. In the presence of a truncating pathogenic mutation, only one of the two alleles is detected (expressed at 100%), which constitutes evidence in favor of a deleterious effect of the mutation [16].

Loss of Heterozygosity (LOH) Analysis

This test is performed by pyrosequencing on DNA extracted from a tumor, to determine whether there is complete loss (deletion) of the normal (wild-type) allele in the tumor. A region of the tissue composed of 100% tumor cells is selected, and DNA is then extracted from this area. This DNA is used to assess the presence or absence of the wild-type allele. It has been demonstrated that loss of the wild-type allele in tumor cells is a common mechanism of tumor suppressor gene inactivation (*Osorio, et al.,* 2002), and this finding supports the pathogenic nature of the mutation.

Results

In this study, three BRCA genes variants were selected for in-depth analysis: one exonic variant, c.258T>C in exon 5 of *BRCA1* and two intronic variants, IVS16-3C>G in intron 16 of *BRCA1* and IVS14+6G>A in intron 14 of *BRCA2*.

The results of our search in the literature and the BIC database showed that none of these variants had been previously reported or analyzed. They are considered as UVs. The objective was then to determine their clinical significance.

Analysis of Exonic Variant c.258T>C (*BRCA1*, Exon 5)

This variant was identified in a patient designated by the pseudonym "SAV".

Bioinformatic Analysis

Prediction of the effect of the c.258T>C variant by PolyPhen

According to the prediction test performed using PolyPhen (Table 1), the amino acid substitution resulting from the c.258T>C variant is likely to be damaging (pathogenic). Specifically, this exonic variant c.258T>C, located in exon 5 of the *BRCA1* gene, leads to the substitution of a cysteine (Cys) by an arginine (Arg) at position 47 of the *BRCA1* protein (p. Cys47Arg or C47R; UniProt ID: P38398).

Prediction of Splicing Impact by ESE Finder

The potential effect of the c.258T>C variant on splicing was evaluated using the ESE Finder program (ESE = Exonic Splicing Enhancer). The results are presented in the Tables 2. They indicate that this variant causes a slight decrease in score, which could have some impact on splicing.

Table 1: Prediction of the effect of the c.258T>C variant by PolyPhen.

Query

Acc number	Position	AA_1	AA_2	Description
P38398	47	C	R	Breast cancer type 1 susceptibility protein. LENGTH: 1863 AA

Prediction

This variant is predicted to be probably damaging

Prediction	Available data	Prediction basis	Substitution effect	Prediction data
probably damaging	alignment	alignment	N/A	PSIC score difference: 3.168

Details

P38398		/bork/coot3/ramel	47

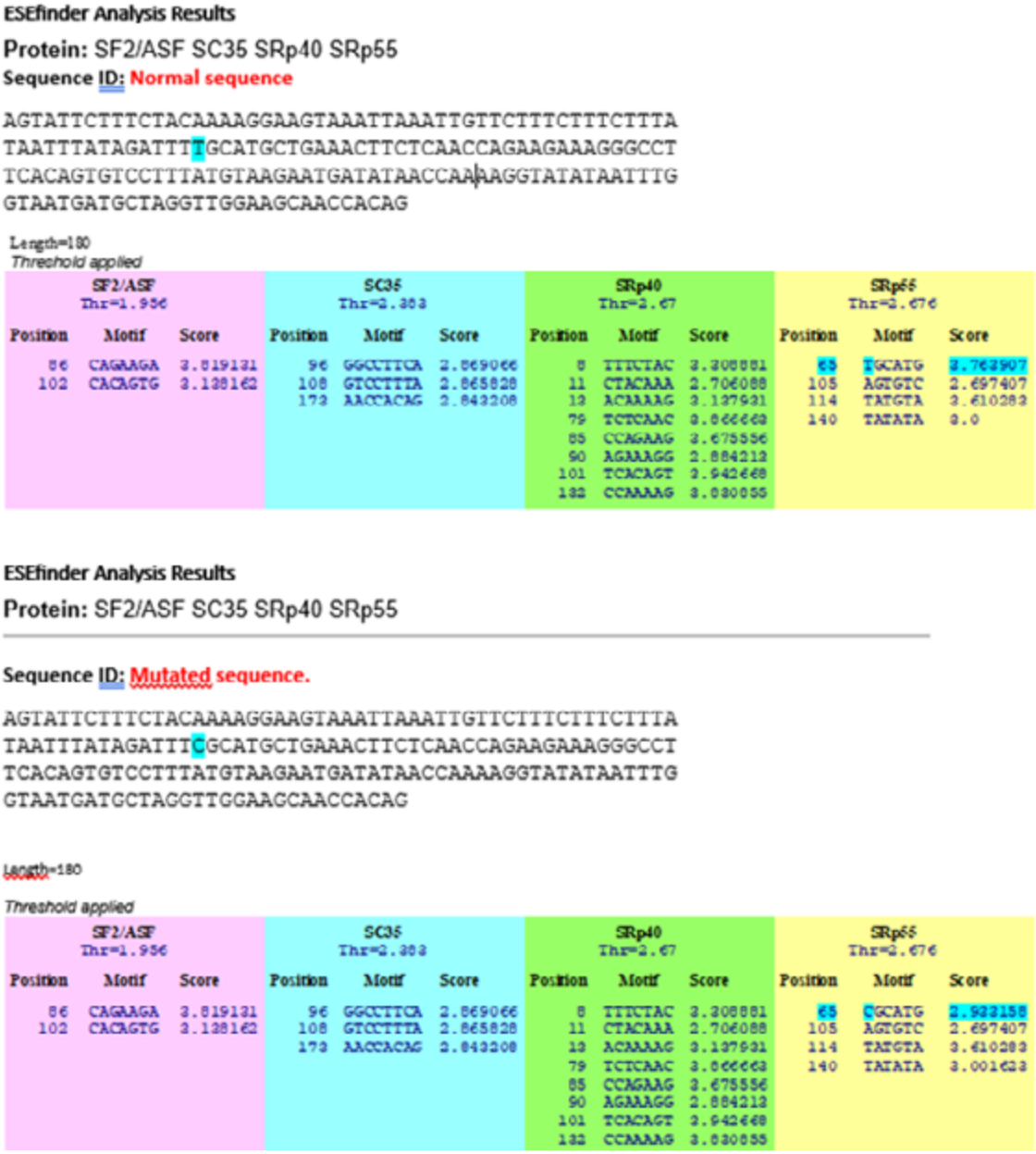
PSIC PROFILE SCORES FOR TWO AMINO ACID VARIANTS

Score 1	Score2	|Score1 Score2|	Observations	Diagnostics	Multiple alignment around substitution position
+2.415	-0.753	**3.168**	7	precomputed	Sequences: Flanks: 25 Show alignment

MAPPING OF THE SUBSTITUTION SITE TO KNOWN PROTEIN 3D STRUCTURES

Database	Initial number of structures	Number of structures
PQS	10	0

Table 2: Prediction of splicing impact of the c.258T>C variant in Exon 5 of BRCA1 gene.



ESEfinder Analysis Results

Protein: SF2/ASF SC35 SRp40 SRp55

Sequence ID: Normal sequence

AGTATTCTTTCTACAAAAGGAAGTAAATTAAATTGTTCTTTCTTTCTTTA
TAATTTATAGATTTTGCATGCTGAAACTTCTCAACCAGAAGAAAGGGCCT
TCACAGTGTCCTTTATGTAAGAATGATATAACCAAAAGGTATATAATTTG
GTAATGATGCTAGGTTGGAAGCAACCACAG

Length=180
Threshold applied

SF2/ASF Thr=1.956			SC35 Thr=2.383			SRp40 Thr=2.67			SRp55 Thr=2.676		
Position	Motif	Score	Position	Motif	Score	Position	Motif	Score	Position	Motif	Score
86	CAGAAGA	3.819131	96	GGCCTTCA	2.869066	8	TTTCTAC	3.308881	65	TGCATG	2.762907
102	CACAGTG	3.138162	108	GTCCTTTA	2.865828	11	CTACAAA	2.706088	105	AGTGTC	2.697407
			173	AACCACAG	2.843208	13	ACAAAAG	3.137931	114	TATGTA	3.610283
						79	TCTCAAC	3.666663	140	TATATA	3.0
						85	CCAGAAG	3.675556			
						90	AGAAAGG	2.884213			
						101	TCACAGT	3.942668			
						182	CCAAAAG	3.830055			

ESEfinder Analysis Results

Protein: SF2/ASF SC35 SRp40 SRp55

Sequence ID: Mutated sequence.

AGTATTCTTTCTACAAAAGGAAGTAAATTAAATTGTTCTTTCTTTCTTTA
TAATTTATAGATTTCGCATGCTGAAACTTCTCAACCAGAAGAAAGGGCCT
TCACAGTGTCCTTTATGTAAGAATGATATAACCAAAAGGTATATAATTTG
GTAATGATGCTAGGTTGGAAGCAACCACAG

Length=180
Threshold applied

SF2/ASF Thr=1.956			SC35 Thr=2.383			SRp40 Thr=2.67			SRp55 Thr=2.676		
Position	Motif	Score	Position	Motif	Score	Position	Motif	Score	Position	Motif	Score
86	CAGAAGA	3.819131	96	GGCCTTCA	2.869066	8	TTTCTAC	3.308881	65	CGCATG	2.932158
102	CACAGTG	3.138162	108	GTCCTTTA	2.865828	11	CTACAAA	2.706088	105	AGTGTC	2.697407
			173	AACCACAG	2.843208	13	ACAAAAG	3.137931	114	TATGTA	3.610283
						79	TCTCAAC	3.666663	140	TATATA	3.001623
						85	CCAGAAG	3.675556			
						90	AGAAAGG	2.884213			
						101	TCACAGT	3.942668			
						182	CCAAAAG	3.830055			

Molecular Characterization of C.258T>C Variant

This variant was analyzed at both DNA and mRNA (cDNA) levels.

Frequency of c.258T>C variant in the population

We assessed the frequency of the c.258T>C variant in DNA from 368 anonymous individuals using pyrosequencing. Of all the samples tested, 348 gave interpretable results ('passed'), while 20 were uninterpretable due to technical failure ('failed'). Among the 348 valid results, none carried this genetic variant, corresponding to a frequency of 0%.

Measurement of the Two Alleles Expression

The results of the expression of two alleles on cDNA for the c.258T>C variant are shown in Figure 1a. They indicate that both T and C alleles are equally expressed, with 47.2% for the T allele and 52.8% for the C allele in the case of the mutation Figure 1a, compared to the non-mutated control Figure 1b.

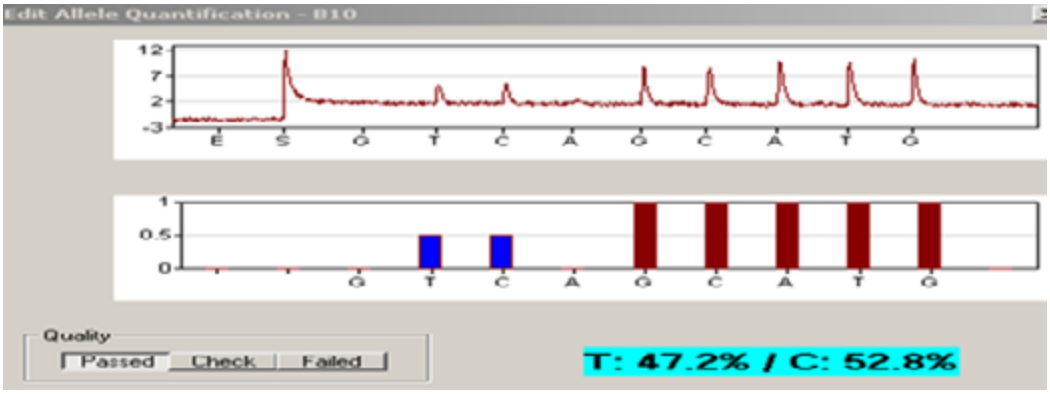


Figure 1a: Expression of two alleles in the mutation c.258T>C.

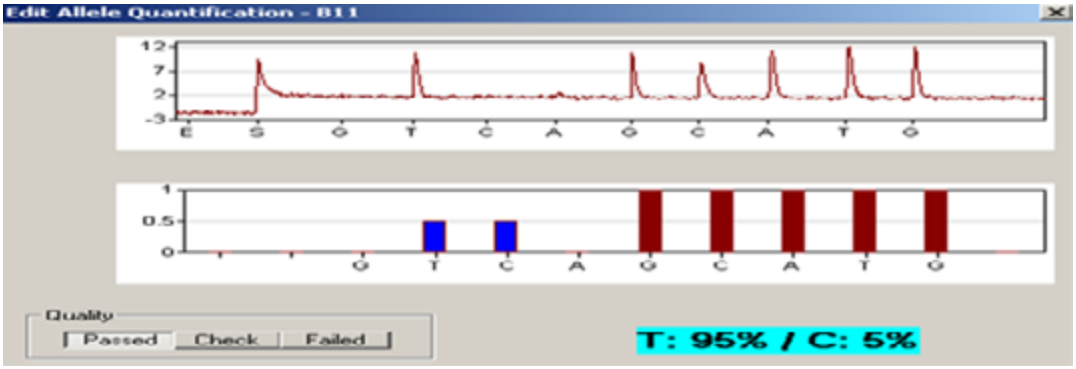


Figure 1b: Expression of two alleles in the wild type.

Loss of Heterozygosity (LOH) Analysis

The loss of heterozygosity was investigated by pyrosequencing on DNA extracted from the tumor, and compared to germline (blood) DNA. The results for the c.258T>C variant are shown in Figures 2a and 2b. These data indicate that there is no loss of heterozygosity in the tested samples.

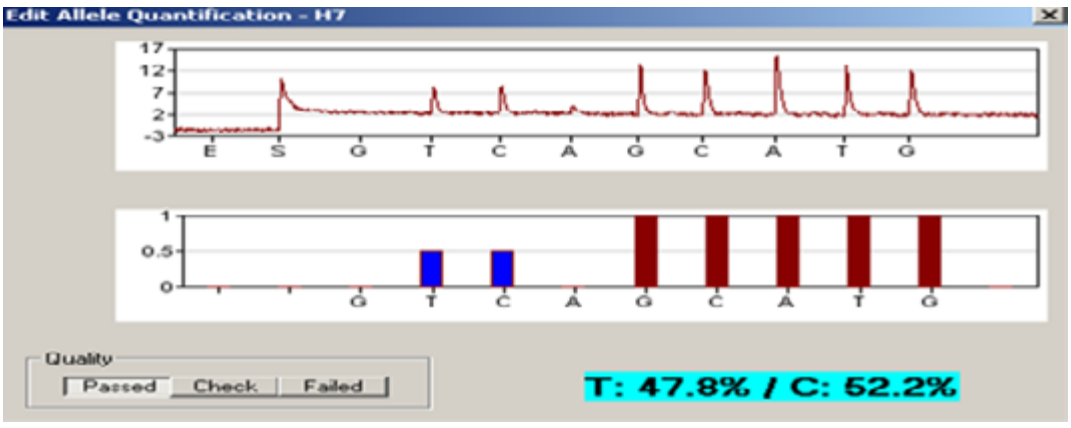


Figure 2a: Investigation of Loss of Heterozygosity (LOH) in tumor DNA.

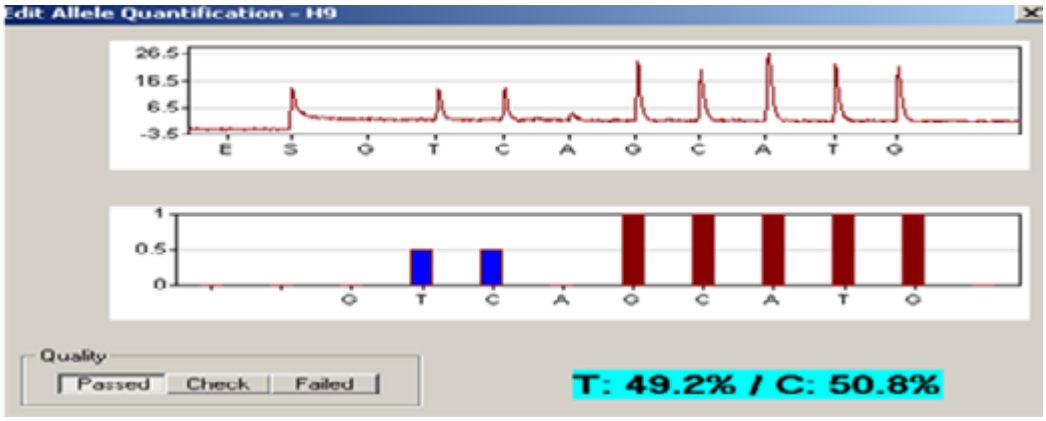


Figure 2b: Investigation of Loss of Heterozygosity in mutated blood DNA.

Analysis of Intronic Variants IVS16-3C>G and IVS14+6G>A

Two intronic variants were analyzed: IVS16-3C>G in the *BRCA1* gene and IVS14+6G>A in the *BRCA2* gene.

The IVS16-3C>G variant was identified in a female patient and the IVS14+6G>A variant, located in intron 14 of the *BRCA2* gene, was detected in a male patient diagnosed with isolated breast cancer at the age of 40. No additional cancer cases were reported in this family.

Both variants were further analyzed using bioinformatic tools and molecular biology techniques.

Bioinformatic Analysis

The potential impact of these variants on mRNA splicing was evaluated using six different splice site prediction tools. A summary of the in-silico results is presented in the Table 3.

Table 3: Evaluation of intronic variants IVS16-3C>G and IVS14+6G>A on splicing.

Programme	Variant IVS16-3C>G	Variant IVS14+6G>A
	Résultat	Résultat
NNSplice	Loss of normal site	No normal site found
Gene Splicer	Loss of normal site	No normal site found
MaxEntScan	Sensitive score fall	Very slight drop in score
NetGene2	No normal site found	No normal site found
Splice Site Finder	Very slight drop in score	Very slight drop in score
Splice View	Loss of normal site	Very slight drop in score

These simulation results suggest that the intronic variant IVS16-3C>G may affect splicing by disrupting the normal splice site, whereas the IVS14+6G>A variant is predicted to have little to no impact on normal splicing. These *in silico* predictions were confirmed *in vitro* by a splicing PCR assay.

Molecular Analysis

Splicing PCR Assay on cDNA

To confirm the computational predictions, splicing assays were performed *in vitro* using PCR on cDNA. For the IVS16-3C>G variant, the analysis focused on the potential skipping of exon 17, whereas for the IVS14+6G>A variant, exon 14 skipping was assessed.

Under normal splicing conditions, all PCR amplicons are expected to be at the same size as the wild-type (WT) transcript. Aberrant splicing typically results in two differently sized fragments due to exon skipping. Alternatively, fragments of the same size may be observed if a cryptic splice site is used.

The experimental details and expected amplicon sizes are summarized in Table 4.

Table 4: PCR Amplicon sizes for splicing assays.

Expected Size	*Variant IVS16-3C>G*		*Variant IVS14+6G>A*	
	Wild Type	Mutated	Wild Type	Mutated
	183 bp	96 bp	616 bp	190 bp

Splicing PCR Assays Results

The results of the splicing PCR assays are shown in Figures 3a and 3b. As shown in Figure 3a, samples 1, 2, and 3 exhibit abnormal splicing compared to the wild-type control. Specifically, two bands are observed: one at 96 bp, corresponding to the aberrantly spliced (mutant) transcript, and another at 183 bp, corresponding to the wild-type fragment. Figure 3b shows that all tested samples yield a single band of 616 bp, identical in size to the expected wild-type amplicon. These results suggest either normal splicing without exon 14 skipping, or the possible use of a cryptic splice site resulting in an amplicon of the same size.

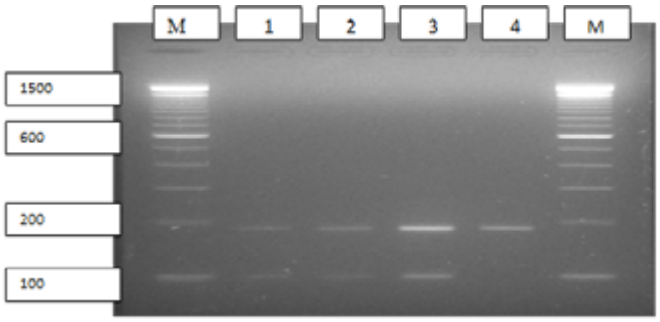


Figure 3a: PCR-splicing assay of IVS16-3C>G variant (with M = Molecular weight ladder; 1 mutated, relative of the patients; 2 = mutated, patient cDNA; 3 = mutated, a relative of the patient); 4 = Wild Type).

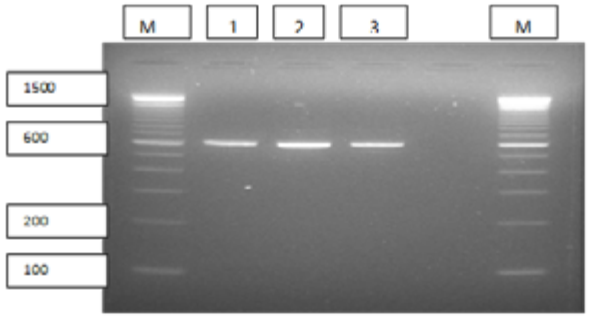


Figure 3b: PCR splicind assay of IVS14+6G>A variant (with: M = Molecular weight ladder; 1 = mutated (?) cDNA; 2 = Wild Type 1 and 3 = Wild Type 2).

Sequencing of cDNA Fragments

Following the splicing PCR assay, the resulting cDNA fragments were either directly purified or extracted from the gel and subsequently purified for sequencing. The sequencing results confirmed that the 96 bp fragments, corresponding to aberrant splicing caused by the IVS16-3C>G variant, consisted of exon 16 directly followed by exon 18, indicating skipping of exon 17. In contrast, the 183 bp fragments, consistent with normal splicing, showed the expected exon arrangement of exons 16–17–18 (Figure 4a,4b).

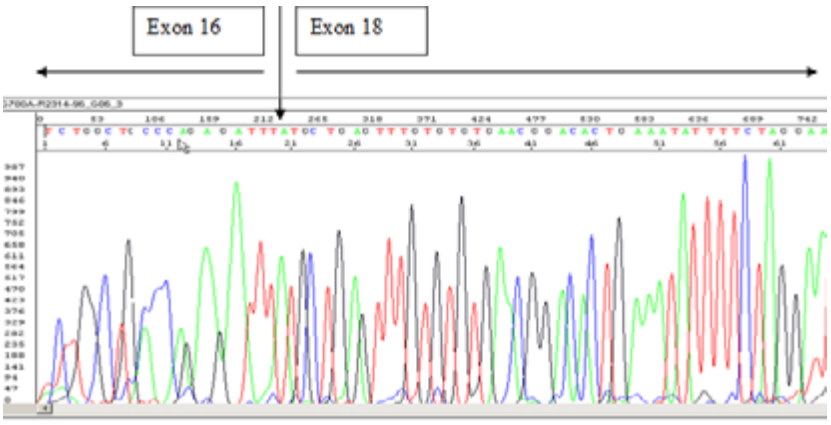


Figure 4a: Sequence of a portion of 96bp of the patient with mutation (IVS16-3C>G variant): We can see the deletion of exon 17, with the succession of exons 16-18 in BRCA1 gene.

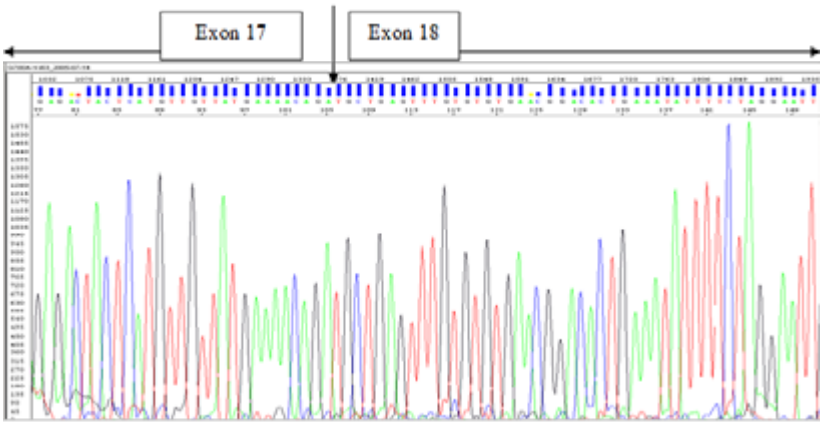


Figure 4b: A portion of the sequence of Wild-type with the succession of exons 17-18 in BRCA1 gene.

The effect of exon 17 skipping on protein translation was assessed using the translation program SHOWORF. The translation results are presented in **Figures 5a and 5b**. These results indicate that the deletion of exon 17, which comprises 88 nucleotides (a number not divisible by 3), induces a frameshift mutation, leading to an altered amino acid sequence and the premature introduction of a STOP codon.

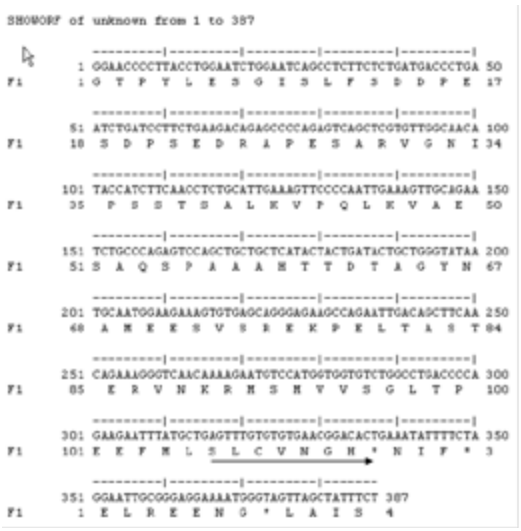


Figure 5a: Protein translation of the exons 16–18 sequence of the BRCA1 gene following an abnormal splicing due to exon 17 skipping. A change in the amino acid sequence is observed starting at the 106th amino acid (compared with Figure 5b), along with the premature introduction of a STOP codon (*) after the 112th amino acid (see arrow).

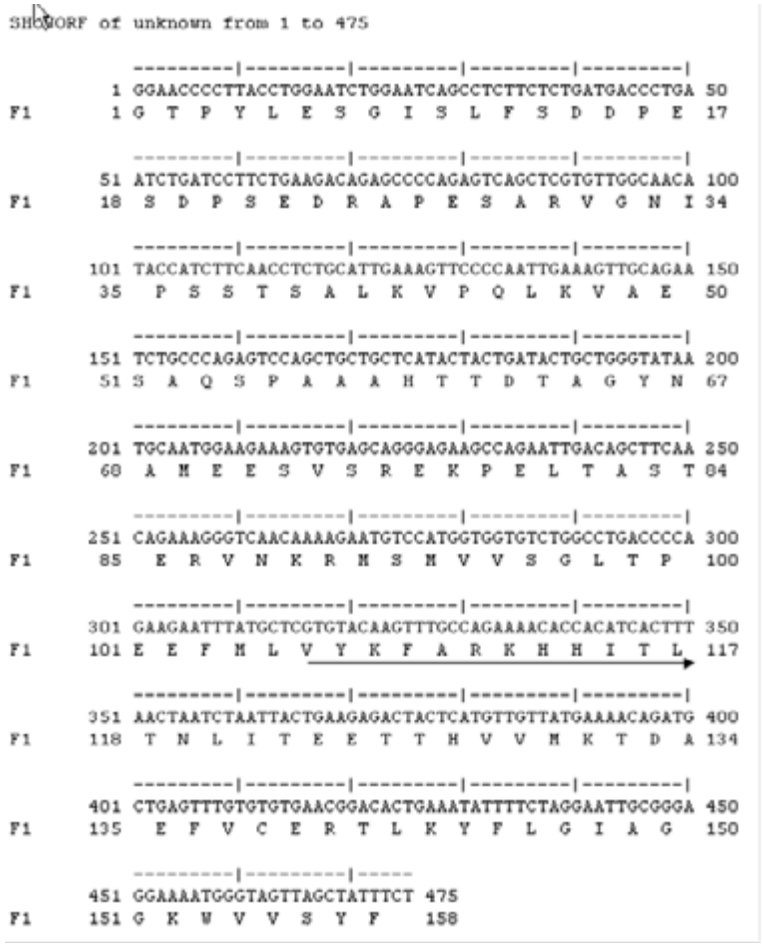


Figure 5b: Protein translation of the exons 16–17–18 sequence of the BRCA1 gene following a normal splicing.

As for the *BRCA2* IVS14+6G>A variant, it appears not to affect normal splicing. All sequences obtained displayed the normal sequential arrangement of exons 13-14-15 (Figure 6).

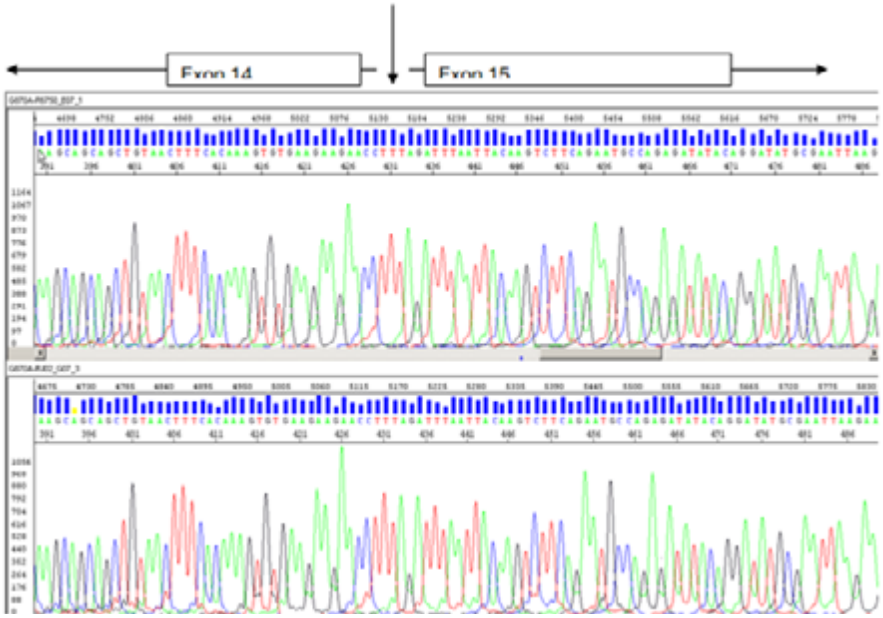


Figure 6: Sequences of exons 14 and 15 in BRCA2 gene (cDNA) of the patient with the variant IVS14+6G>A (upper part) and of the wild type (lower part) after splicing.

Frequency of the Mutations in the Population

The intronic variants IVS16-3C>G (BRCA1) and IVS14+6G>A (BRCA2) were screened by pyrosequencing in genomic DNA from a cohort of 368 anonymous individuals. For the IVS16-3C>G variant, 316 interpretable results were obtained, none of which were positive for the variant, yielding a frequency of 0%. For the IVS14+6G>A variant, 349 interpretable results were obtained, with one individual carrying the variant, resulting in a population frequency of 0.28%.

Two Alleles Expression Analysis

Allelic expression measurement was performed using cDNA samples. This required the presence of an exonic polymorphism to serve as a marker. No suitable exonic polymorphism was identified in the patient who carries the IVS14+6G>A variant in *BRCA2*, thus precluding allelic expression analysis in this case.

In contrast, for patient carrier of the IVS16-3C>G variant in *BRCA1*, two allèles expression was assessed using the exonic polymorphism c.4956A>G (S1613G) within the same gene. The cDNA samples analyzed included that of the patient as well as non-mutated control individuals heterozygous for the c.4956A>G polymorphism. The results are presented in Figures 7a and 7b.

As shown in Figures 4a and 4b, both alleles are expressed in the patient as well as in the control individuals.

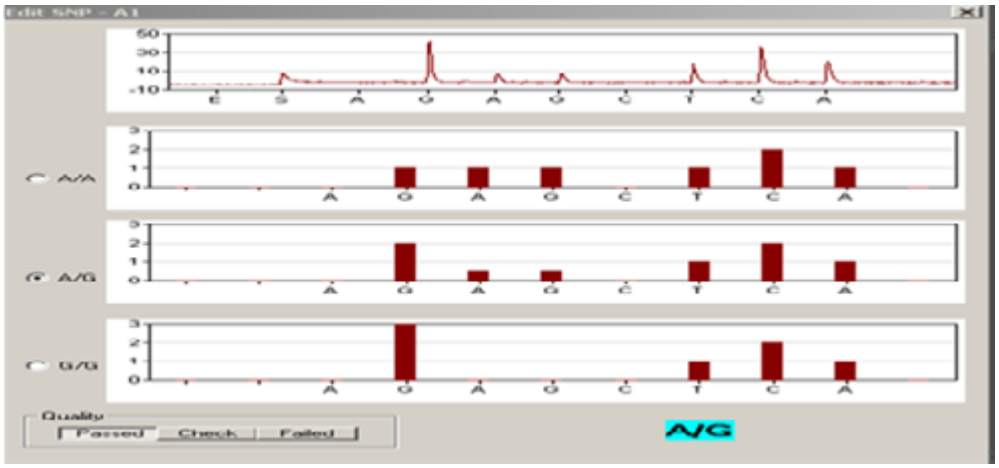


Figure 7a: Expression of the two alleles in the patient with IVS16-3C>G variant.

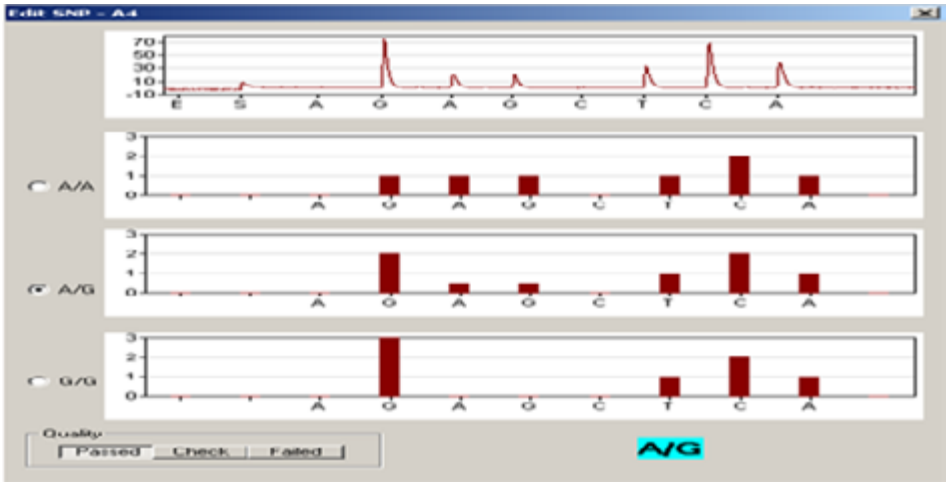


Figure 7b: Expression of the two alleles in one of the positive controls.

As shown in Figures 7a and 7b, both alleles are expressed in the patient as well as in the controls.

Loss of Heterozygosity Analysis

Due to the lack of appropriate material (DNA extracted from a tumor sample fixed in formalin and embedded in paraffin), we were not able to perform this analysis in the two patients carrying the intronic variants analyzed in this study.

Discussion

Analysis of the Exonic Variant c.258T>C

The exonic variant c.258T>C in the *BRCA1* gene was analyzed using the PolyPhen bioinformatics tool, which predicted it to

be probably damaging. This variant results in an amino acid substitution (p. Cys47Arg or C47R) located within the RING Finger motif, a highly conserved domain of the *BRCA1* protein. This domain is known to mediate protein-protein interactions. Multiple missense mutations have been identified at conserved cysteine residues within this region (e.g., C39Y, C64G, C61G) in families with HBOC syndrome, and these mutations are considered pathogenic because they disrupt the interaction of *BRCA1* with RING-finger domain-interacting proteins [17,21,26-29] reported a similar mutation affecting the same amino acid residue—C47F—which is classified as deleterious in that publication.

The potential effect of this variant on splicing was also assessed using the ESE Finder tool, which showed a slight decrease in score. However, no *in vitro* splicing experiments were performed, as the pathogenic impact on protein function was already strongly supported. It is worth noting that some exonic elements, distinct from classical splicing signals, are crucial for accurate splice site recognition. These elements can act as splicing enhancers or silencers [23,30-32].

Molecular analysis of the c.258T>C variant by pyrosequencing revealed the following:

a) This variant is extremely rare in the general population: no carriers were identified in 348 anonymous DNA samples, indicating a frequency of 0%. This supports its potential pathogenicity, as variants with a population frequency below 1% are generally not considered neutral polymorphisms (Human Genome Variation Society criteria).

b) Both the wild-type and mutant alleles were almost equally expressed: 47.2% for the wild-type (T) and 52.8% for the mutant (C) allele, which is expected since this variant does not affect splicing.

c) No Loss of Heterozygosity (LOH) was observed in the tumor DNA. This might be due to technical issues, such as contamination of tumor DNA with normal adjacent tissue. Alternatively, it could reflect a true absence of LOH. Although LOH is a common mechanism in tumorigenesis, some cancers do develop with an intact wild-type allele *Osorio, et al.,* 2002 [33]. Other mechanisms such as promoter hypermethylation or checkpoint gene inactivation may play a role instead (*Osorio, et al.,* 2002; *Venkitaraman,* 2002).

It is also important to note that the patient's family history includes breast and/or ovarian cancer (mother and sister). Unfortunately, their samples were not available for co-segregation analysis.

Taken together, all these data support the conclusion that this is a true pathogenic mutation.

Analysis of the IVS16-3C>G Variant in *BRCA1*

Most *in silico* predictions indicated that the IVS16-3C>G variant would lead to the loss of the normal acceptor splice site. This was confirmed *in vitro* by splicing assays (RT-PCR on cDNA) and sequencing, which showed *skipping of exon* 17. The impact of intronic variants on splicing is a key criterion in classifying them as pathogenic mutations rather than benign polymorphisms [1,25,34-37].

Pyrosequencing analysis of this variant showed that:

a) This variant is extremely rare or absent in the general population, with 0% frequency in 316 anonymous DNA samples. This supports its classification as a pathogenic mutation, according to Human Genome Variation Society guidelines.

b) Both mutant and wild-type alleles were expressed almost equally. However, expression levels were assessed using cDNA from total blood, which may affect RNA quality. In some research centers, allele expression is assessed using lymphoblastoid cell lines (EBV-transformed) to avoid degradation of unstable transcripts by Nonsense-Mediated Mrna Decay (NMD) [1,34]. Nevertheless, equal expression of both alleles does not exclude pathogenicity.

c) Exon 17 skipping, which removes 88 nucleotides (not divisible by 3), leads to a frameshift, resulting in altered amino acid sequence and the introduction of a premature stop codon.

d) LOH analysis could not be performed due to the lack of suitable tumor material. Only a Bouin-fixed tumor slide was available, and it was not possible to extract usable tumor DNA from it.

Family history revealed that the patient's mother and two aunts had developed breast and/or ovarian cancer at early ages. The patient herself developed breast cancer at 28 years old. Although information is limited, this supports the presence of familial co-segregation.

Based on all studied criteria, this variant is likely a true pathogenic mutation, leading to aberrant splicing and exon 17 skipping in *BRCA1. Høberg-Vetti, et al.* (2020) [37] also reports an exon skipping case.

Analysis of the IVS14+6G>A Variant in *BRCA2*

In silico prediction tools indicated that the IVS14+6G>A variant does not significantly affect normal splicing, and this was confirmed by RT-PCR and cDNA sequencing. Thus, this variant is unlikely to be a splicing mutation. It should be noted, however, that the absence of aberrant transcripts could result from mRNA decay, which removes mutant mRNA transcripts. In other research centers, cell immortalization and RNA stabilization are used to rule this out [38,39]. This study, conducted in a clinical laboratory and under time constraints, did not include such procedures. It should also be noted that position 14+6 is not a highly conserved site."

Population frequency analysis by pyrosequencing revealed a low presence of this variant—0.28% among 349 interpretable samples from anonymous individuals.

All attempts to identify an exonic polymorphism in this patient (to evaluate two allèles expression) were unsuccessful. Thus, two

alleles expression could not be assessed in this case. Furthermore, no tumor DNA was available to evaluate loss of heterozygosity.

Family history for this patient is unremarkable, with a single case of male breast cancer at age 40 and no additional cancer cases in the family.

Overall, the available data are insufficient to draw definitive conclusions about this variant. However, based on our findings, we suggest that IVS14+6G>A may be a rare polymorphism, and the cancer observed in this patient could be a sporadic event, not associated with a familial mutation.

Conclusion

As this study did not address all parameters for the three analyzed cases, we suggest that these kinds of studies be further completed, following recent guidelines and technology [20,21,27,40]. This could be achieved by performing Loss of Heterozygosity (LOH) analysis, evaluating two alleles expression after lymphoblastoid cell line culture, and conducting thorough family co-segregation studies for each patient.

Author Contributions

HP conceptualized the idea.

MBJ and BS did laboratory work.

All the authors participated in the writing and modification of this manuscript.

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Conflict Of Interest

We declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest.

Ethical Concern and Informed Consent:

All samples used in this work are from patients coming for clinical diagnosis and genetic counsel.

AI Tool Usage Declaration

We declare that no AI and associated tools were used for writing scientific content in this article.

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