Two novel variants in the 3'UTR of the gene in familial breast and/or ovarian cancer

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Two novel variants in the 3'UTR of the *BRCA1* gene in familial breast and/or ovarian cancer.

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Abstract

For the majority of breast and/or ovarian cancer patients tested for *BRCA1/2* genes, mutation screening of the coding regions remains negative. MicroRNAs which negatively regulate mRNA translation by binding to 3' untranslated region (3'UTR) are implicated in cancer. Genetic changes in the 3'UTR of several genes were reported to be associated with higher susceptibility to particular tumor types. The aim of the present study was to analyze the *BRCA1* 3'UTR in patients tested negative for *BRCA1/2* deleterious mutations, in order to find variants implicated in the decrease of *BRCA1* expression through modification of miRNA binding.

Genotyping analyses were performed on genomic DNA of 70 *BRCA* negatives index cases, selected among patients with breast or ovarian cancer, less than 50 years old, with a strong family history. The co-occurrence of the identified variants with deleterious *BRCA1* mutations was then determined in a control population of 210 patients. A luciferase gene reporter assay was used to investigate the impact of the variants on the *BRCA1* gene expression.

Two novel variants, c.*750A>G and c.*1286C>A, were identified in the 3'UTR of *BRCA1* gene, in two patients. The former was found three times in the control population, whereas the latter was absent. The used functional assay did not reveal any effect on the luciferase expression.

This study reveals a weak genomic variability in the 3'UTR of the *BRCA1* gene. All together, the results led us to classify the variant c.*750A>G as probably neutral, the variant c.*1286C>A remaining unclassified.

Key words

Breast, ovarian, inherited cancer, 3'UTR, *BRCA*, miRNA

Introduction

BRCA1 and *BRCA2* genes are two inherited breast and/or ovarian cancer susceptibility genes [1;2]. A recent meta-analysis of ten studies estimated that the risk of developing breast cancer by age 70 years was 57% and 49% for *BRCA1* and *BRCA2* mutation carriers, respectively. The corresponding risks for ovarian cancer were 40% and 18% [3]. As risk-reduction strategies, including chemoprevention and prophylactic surgeries, have been demonstrated to be effective in high-risk individuals [4], genetic testing for *BRCA* gene mutations has been increasingly utilized in clinical settings. The identification of mutation in these genes is extremely beneficial for patients pursuing risk reduction strategy and more recently for their cancer treatment. In fact, a novel class of promising drug, the PARP (poly ADP-ribose polymerase) inhibitors, is particularly effective in patients with *BRCA*1/2 mutations [5]. These news drugs, like olaparib, are currently developed in this target *BRCA* positive population [6;7]. Despite our understanding of the clinical phenotypes most predictive of the presence of *BRCA1* and *BRCA2* mutations, a lot of families with both breast and/or ovarian cancers do not have identifiable mutations [8]. About 80% of the mutation screening remains negative making the identification of the risk of developing a cancer difficult for the concerned families [9] and depriving the access to clinical trials. Most of the techniques used for genetic screening of *BRCA1* and *BRCA2* genes are performed by analysing the entire-coding region, including the intron-exon junctions, while introns and proximal untranslated regions remain unexplored. However, discovery of microRNA (miRNA) has pointed out the important role of these untranslated regions in gene regulation. MicroRNAs are a class of small non-coding regulatory RNAs ranging in size from 17 to 25 nucleotides. They post-transcriptionnally repress expression of target genes by recognizing complementary sites mostly in their 3'untranslated region (3'UTR). Their expression deregulation has been implicated in the onset and progression of many diseases, including cancers [10].

In spite of the potential importance of the 3'UTR in gene regulation, the role of genomic variations in the 3'UTR of the tumor suppressor *BRCA1/2* genes remains unclear. Until now, few studies analysed the 3'UTR of *BRCA1* and most of them focused on the role of single nucleotide polymorphisms (SNP) [11-14]. From Pongsavee *et al*., one SNP c.*421T/T (rs 8176318) seems to be linked to a decrease of *BRCA1* gene expression associated with an increased risk of cancer [12]. Authors proposed that the *BRCA1* gene expression alteration was the result of the creation of a new miRNA binding site by the SNP, although the link between both events has not been clearly established. However, to our knowledge, no mutation has been already described in the 3'UTR of *BRCA1/2* genes. These observations led us to assume that unknown genomic variations in the 3'UTR of *BRCA1/2* genes could be implicated in the inherited breast or ovarian cancer through their effect on *BRCA* gene expression. In order to test this hypothesis, we analysed, in a first attempt, the *BRCA1* 3'UTR from patients tested negative for *BRCA1* and *BRCA2* mutations in highly selected families.

Material and Methods

Patient population

Genotyping analyses were performed on genomic DNA of 70 index cases selected from patients with breast or ovarian cancer diagnosed at an age of less than 50 years. These index cases had a strong history family at least on two generations: 3 breast cancers at first degree, one of which before 40 years, or 1 ovarian cancer associated

with a breast cancer before 40 years. They have been tested negative for *BRCA1* and *BRCA2* mutations. Screening for *BRCA1* and *BRCA2* mutations was performed using two techniques, depending on the exons analyzed, i.e.denaturing High Performance Liquid Chromatography (dHPLC) and High Resolution Melting (HRM), both followed by direct sequencing. The screening methods for *BRCA1* and *BRCA2* large rearrangements were performed using a combination of the multiplex ligation-dependent probe amplification (MLPA) and quantitative multiplex PCR of short fluorescent fragments (QMPSF).
All patients have been selected from a database of families undergoing genetic counseling in the Western French Network. The patient gave signed informed consent.

Control population

The control group consisted of 210 unrelated probands with a *BRCA1* deleterious mutation. This population was chosen as control population to determine the frequency of the variants found in the 3'UTR. Arguing the fact that *BRCA1* compound heterozygotes for two deleterious mutations are embryonically lethal [15], this population was used to determine the frequency of the identified variants in 210 *BRCA1* wild type alleles. Moreover, the choice for this specific control population could allow to exclude the identified variant as a deleterious mutation if found in *trans* status with an other deleterious mutation. These patients were checked in our database between 1997 and 2009 and gave signed informed consent.

Nomenclature

The DNA sequence numbering is based on the cDNA sequence for *BRCA1* (NCBI RefSeq NM_007294), following the recommendations of the Human Genome Variation Society (HGVS, translation initiation codon ATG=1).

PCR and sequencing

Patient peripheral blood samples were collected in EDTA tubes. Genomic DNA was isolated using an automated procedure (EZ1 DNA blood kit on biorobot EZ1 workstation, Qiagen, Hilden, Germany). The 3'UTR of *BRCA1* gene (1376 bp) was studied in 4 overlapping fragments about 500 nucleotides.
Each fragment was amplified by PCR (polymerase chain reaction) by a Taq Gold polymerase (Invitrogen Corporation, Madison, Carlsbad, California) with specific forward and reverse primers (50µM, Eurogentec, Liege, Belgium). These primers were tailed with M13 common sequences (50 µM, Eurogentec) (Table 1).
PCR was performed with a commercial kit (Taq Gold polymerase, Invitrogen Corporation, Madison, Carlsbad, California), from 250 ng of DNA. All amplifications were performed using a touch-down PCR program. The thermocycler parameters for PCR were 95°C for 10 minutes for the initial DNA polymerase activation, 26 cycles of 94°C for 20 seconds, 63°C for 20 seconds with a decrease of 0.5°C/cycle, and 72°C for 45 seconds, followed by 20 cycles of 94°C for 20 seconds, 50°C for 20 seconds and 72°C for 45 seconds, and a final extension at 72°C for 7 minutes. PCR products were separated by electrophoresis on a 1.5 % agarose gel containing ethidium bromide and visualized by exposure to ultraviolet light. The amplified products were purified by ExoSap kit

(USB Corp., Cleveland, Ohio, USA), according to the manufacturer's instructions. The purified templates were sequenced in both directions with the M13 forward/reverse primers. A dye terminator cycle sequencing kit (Big Dye Terminator kit v1.1; Applied Biosystems) was used and templates analysed on an ABI 3130 sequencer (Applied Biosystems, USA).

HRM

HRM has only been performed for the exploration of the fragment 3 in the control population. Twenty ng of genomic DNA was added to a reaction master mix consisting of 1×LightCycler® 480 High Resolution Melting Master (containing the proprietary ds-DNA saturating binding dye), with 2.5 mM $MgCl_2$ (Roche Diagnostics, Germany) and 0.5 µM of F3F and F3R (same PCR section). All the samples were performed under the same conditions in a 96-well plate in the LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Germany). The PCR program started with an initial denaturation step of 10 min at 95 °C, continued with 42 cycles of 15 s at 95 °C, 15 s at 65 °C with a decrease of 1°C/cycle and 25 s at 1 min followed by 10 cycles of 15 s at 95°C, 15 s at 55°C and 25 s at 72°C. To induce heteroduplexes formation, samples were heated to 95 °C for 1 min and cooled to 40 °C for 1 min. Samples were melted from 60 °C to 95 °C performing 25 acquisitions per 1 °C. HRM melting curve analysis was performed using the Light-Cycler® 480 Gene Scanning software version 1.2 (Roche Diagnostics, Germany), which allows clustering the samples into groups on the basis difference plot obtained analyzing the differences in melting curve shapes. All the samples with a late amplification, as monitored by real-time PCR or associated with fluorescence of less than the 60% of the maximum, were excluded from the analysis. According to manufacturer's recommendations, unreliable melting profiles could be generated. All samples studied were clustered at the default sensitivity setting (0.3) with a pre-melt slider settings (100% of fluorescence) between 85.9 and 86.4°C and post-melt slider settings (0% fluorescence) between 88.3 and 88.8°C. Significant differences (ie +/- 2 of relative signal differences) in fluorescence curves from the horizontal baseline were indicative of mutations. Results were confirmed by sequencing PCR products in the ABI Prism 3130 Genetic Analyser (Applied Biosystems, USA).

Determination *cis/trans* status of the doubly heterozygous c.[*750A>G (+) *1286C>A] variant

BRCA1 3'UTR was amplified using genomic DNA from the patient doubly heterozygous for the variants c.*750A>G and c.*1286C>A. Extension time and number of PCR cycles were determined in order to limit PCR-mediated recombination [16;17]. In brief, PCR mixture (50µl) contained 100ng of genomic DNA, 200µM dNTP, the forward BRF1 5'CT**ACGCGT**CCCCACAGCCACTACTGA3' and reverse BRR1 primers 5'CCC**AAGCTT**TTGCTACCAAGTTTATTTGCAG3' (300µM each) (the sequences in bold character represent the *MluI* and *HindIII* restriction sites for BRF1 and BRR1 respectively) and 2.5U of Taq PHUSION (Finnzymes). PCR program was undertaken for 1 cycle of 98°C (40s) followed by an annealing step of 58°C (30s) and an extension step of 72°C (80s). This first cycle was followed by 20 cycles of 98°C (30s), 66°C (30s), 72°C (80s) and 1 cycle of 72°C (2min30). After gel purification of PCR products, 3'-A overhangs were added to the blunt-ended 3'UTR fragment using the standard A-tailing protocol described by Promega. The 3'A-tailed 3'UTR fragment was cloned into pGEMT easy vectors (Promega). Several clones were analyzed by sequencing.

Luciferase reporter gene constructs

Contrary to what it was described above, in order to obtain the 4 possibilities of *BRCA1* 3'UTR containing the 750A or 750G and 1286C or 1286A, we performed a *BRCA1* 3'UTR amplification under experimental conditions favouring the PCR-mediated recombination. For such a purpose, we performed the PCR with a shorter extension time (50s instead of 80s) for 35 cycles instead of 20. PCR products were purified, cloned into pGEMT easy vector and sequenced. Among the sequenced clones were retrieved clones with the wild type 3' UTR *BRCA1* (*750A;*1286C) (pGEMT-BRCA1-WT), the (*750G;*1286C) (pGEMT-BRCA1-750G), the (*750A;*1286A) (pGEMT-BRCA1-1286A) and the (*750G;*1286A) (pGEMT-BRCA1-750G/1286A). The generated plasmids were digested by *MluI* and *HindIII*, releasing a *BRCA1* fragment containing the entire 3'UTR region (1376 bp). Each restricted fragment was inserted between the *MluI* and *HindIII* restriction sites of the pMIR-Report™ miRNA Expression reporter vector (Applied Biosystems). In such constructions all the *BRCA1* 3'UTR are immediately downstream of the coding sequence of firefly luciferase.

Dual luciferase assay

All transfections were performed using the JetPRIME™ transfection reagent (Poly-plus transfection). A breast cancer cell lines (MCF-7) and two ovarian cancer cell lines (SKOV3 and IGROV1) at 50-70% confluence were transiently co-transfected with each pMIR-Report-BRCA1 construction (200ng) and the control reporter plasmid pRL-TK from Promega (20ng). pRL-TK contains a cDNA encoding Renilla luciferase, the expression of which is under the control of the thymidine kinase promoter. Luciferase assays were performed after 24h using the Dual Luciferase reporter assay kit (Promega) and the luminometer Centro XS LB960 (Berthold Technologies) according to the manufacturer's instructions. Firefly luciferase activity was normalized relative to Renilla luciferase activity.
After 24h, the luciferase activity of each transfected population was compared with a wild type (*750A;*1286C) *BRCA1* 3'UTR pMIR construction (pMIR-BRCA1-WT).

Results

Study population

Three polymorphisms in the 3'UTR of *BRCA1* were identified: c.*421G>T (rs8176318) with genotype frequencies 0.53, 0.11 and 0.36 for G/G, T/T and G/T respectively, c.*1287C>T (rs12516) with genotype frequencies 0.53, 0.11 and 0.36 for C/C, T/T and C/T and c.*1332G>A (rs8176320) found heterozygous in only two patients.
The polymorphism c.*421G>T was always associated with the polymorphism c.*1287C>T in our study population.
Two novel variants c.*750 A>G and c.*1286C>A were described for the first time, confirmed on two independent blood tests. Patient A was diagnosed with bilateral breast cancer at 40 and 44 years and presented the variant c.*750A>G (Figure 1A). Patient B was diagnosed with breast cancer at 33 years and presented both

the c.*750 A>G and the c.*1286C>A variant (Figure 1B). The patients A and B were homozygous for the two polymorphisms c.*421G/G and c.*1287C/C. These results are summarized in table 2.

Control Population

The c.*750A>G variant was screened by HRM. The fragment 3 of the 3'UTR of *BRCA1* was also sequenced in the control population. Three women with three different types of deleterious mutations presented this variant in the 210 patients tested (1.4%). The c.*1286C>A variant was screened by sequencing analysis. This variant was not found in the control population.

Determination *cis/trans* status of the doubly heterozygous c.[*750A>G (+) *1286C>A] variant

The study of 19 clones from the patient B showed that the variants c.*750A>G and c.*1286C>A were localized in *cis* on the same allele (Figure 2).

Functional assay

In order to determine whether the c.*750A>G and/or the c.*1286C>A base substitutions in the *BRCA1* 3' UTR could alter gene expression, MCF-7 breast cancer cells were transfected with luciferase reporter plasmids containing either one base substitution (pMIR-BRCA1-750A, pMIR-BRCA1-1286C) or both (pMIR-BRCA1-750A;1286C). The results showed that the presence of c.*750A>G and/or c.*1286C>A does not alter the luciferase expression (Figure 3). The same results were obtained using SKOV3 and IGROV1 ovarian cancer cell lines as recipient cells (data not shown).

Bioanalysis

Analyses of miRNA binding sites on *BRCA1* 3'UTR were performed using microRNA.org (http://www.microrna.org/microrna/home.do), microinspector (http://www.imbb.forth.gr/microinspector.) and RegRNA (http://regrna.mbc.nctu.edu.tw) softwares. No human miRNA binding site has been identified in the zone of interest for the variant c.*750A>G. For the variant c.*1286C>A, the base substitution increased slightly the binding of several miRNAs. The potential miRNAs were different according to the bioinformatic softwares and the defined thresholds for the free energy change.

Discussion

This is one of the first published studies which identified variants in the 3'UTR of *BRCA1*. Two novel variants c.*750A>G and c.*1286C>A were identified in the 3'UTR of *BRCA1* gene in 2 patients from 70 index cases non *BRCA1/2* mutation carriers selected for high probability of inherited predisposition. For the doubly heterozygous patient, we showed clearly that the variants c.*750A>G and c.*1286C>T were localized in *cis* on the same allele.

The variant c.*750A>G was found three times in the control population associated to three different deleterious mutations, thereby probably localized in *trans* with each mutation. It induced no alteration of luciferase expression in the functional assay and did not create any potential miRNA binding sites. All these results suggest that this variant has probably no impact on the *BRCA1* expression.
The lack of association of the variant c.*1286C>A with another deleterious mutation was evocative of a potential deleterious character. This hypothesis was reinforced by the bioinformatics analysis which shows that it increases the binding of several miRNAs on the *BRCA1* 3'UTR. Conversely, its presence immediately downstream of the coding sequence of firefly luciferase gene, either alone or in association with the variant c.*750A>G, did not induce any effect on the expression of this reporter gene in three cell lines. This observation would suggest this variant is not able to modulate *BRCA1* expression through modified miRNA binding. However, although the functional testing was carried out on three different cancer cell lines, one cannot completely exclude that the miRNAs identified by bioanalysis could be absent from the tested cell lines thus leading to a false negative result. Such cell specific miRNA expression was already observed by Pongsavee *et al*. [12]. The authors tested the *BRCA1* gene expression alteration induced by the SNP c.*1287T/T (rs12516) on three different breast cell lines. A weak inhibitor effect was only observed in MCF-7 cells (p=0.044) [12]. Finally, one cannot also rule out the hypothesis that the absence of the variant c.*1286C>A in the control population or its presence in one patient among 70 is the result of chance. Thus, without further studies, it seems difficult to conclude on the potential biological effect of the c.*1286C>A variation.

Nevertheless, one has to notice that the sequencing of the 3'UTR of *BRCA1* from 68 over 70 high selected patients did not reveal any variant in this region. This weak genomic variability, as also observed by Puget *et al*. [14], suggests that the creation of new miRNA binding sites in this region is not a major mechanism to alter the *BRCA1* gene expression. Recently, a study revealed that a SNP, associated with an increased risk of breast cancer, could positively regulate the expression of the *BRCA1* gene through modified binding of a miRNA in the coding region of this gene [13]. Futhermore, genetic variants in miRNA genes, targeting the 3'UTR of *BRCA1*, can potentially alter the regulation of this gene although their impact on the genetic susceptibility to breast cancer remains to be determined [18-20].

It is a real challenge to identify inherited mutations in such patients with a strong family history, to propose an adaptive follow-up for them and their family. Therefore, in families with a strong history of breast cancer, other studies must be performed on the other regions of *BRCA* gene which are still unexplored, including the 3'UTR of *BRCA2*. The increased risk of hereditary breast cancer is not only the result of the alteration of the *BRCA* genes expression [21]. The search for the implication of other genes would beneficiate from high throughput methods of analysis [22].

In conclusion, the screening of the 3'UTR of *BRCA1* in 70 patients with a strong family history of breast or ovarian cancer revealed two novel variants. The variant c.*750A>G alone could be identified as neutral. The variant c.*1286C>A remains unclassified and did not allow us to bring a clear element decision-making for use in routine.

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Tables captions

Table 1: **Primers for the amplification of 4 overlapping fragments from the 3'UTR of the *BRCA1* gene**
The primers were designed in order to have specific sequences of the 3'UTR of the *BRCA1* gene (in bold) downstream to M13 sequences (in italic).

Table 2: Genomic variations in the 3'UTR of the *BRCA1* gene
The sequencing of the 3'UTR allows the identification of three polymorphisms (for comparison the frequency in the European panel is shown) and 2 novel variants.

Figures captions

Fig 1: The identification of the novel variants c.*750A>G and c.*1286C>A by sequencing analysis
(A) Forward sequencing of patient A fragment 2 containing the c.*750A>G variant; **(B)** Reverse sequencing of patient B fragment 4 containing the c.*1286C>A variant.

Fig 2: *Cis/trans* status determination of the doubly heterozygous c.[*750A>G (+) *1286C>A] variant
The 3'UTR of the *BRCA1* gene was amplified from the genomic DNA of the patient doubly heterozygous for the variants c.*750A>G and c.*1286C>A. Amplified 3'UTR was cloned and sequenced.
(V): variant; (WT): wild type

Fig. 3: Determination of the effect of c.*750A>G and/or c.*1286C>A variants on gene expression by the luciferase gene reporter assay.
Luciferase levels were measured from MCF-7 cells transfected with pMIR-BRCA1-WT, pMIR-BRCA1-750G, pMIR-BRCA1-1286A and pMIR-BRCA1-750G/1286A plasmids as described. Data are expressed as Means +/- Standard Deviation of three independent experiments.

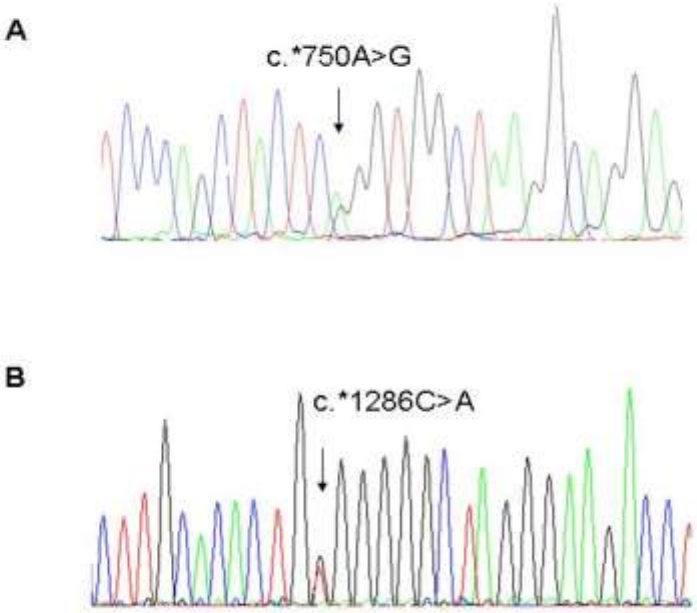

Fig 1

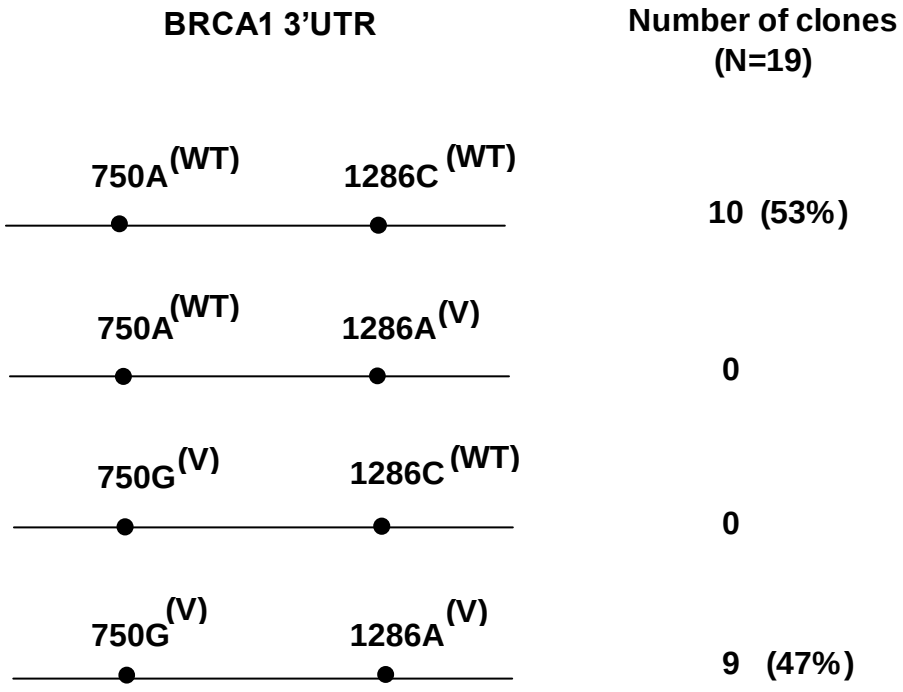
BRCA1 3'UTR
Number of clones
(N=19)
750A(WT)
1286C (WT)
10 (53%)
750A(WT)
1286A(V)
0
750G(V)
1286C (WT)
0
750G(V)
1286A(V)
9 (47%)

Fig 2

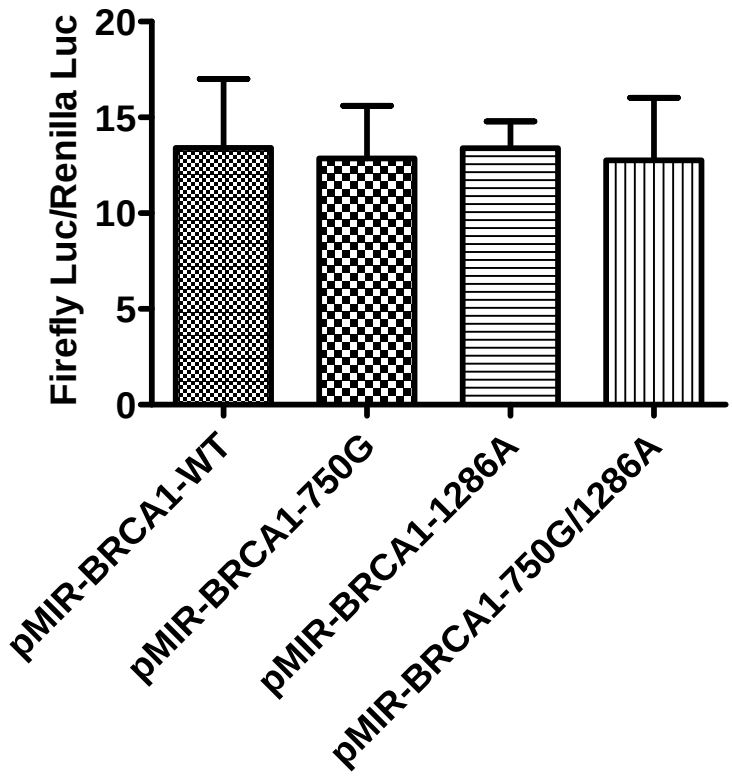
Firefly Luc/Renilla Luc
20
15
10
5
0
pMIR-BRCA1-WT
pMIR-BRCA1-750G
pMIR-BRCA1-1286A
pMIR-BRCA1-750G/1286A

Fig 3

Fragment	*M13 primer* **Specific primer**
Fragment 1 Forward	*GTAAAACGACGGCCAGT***ATCCCCCACAGCCACTACT**
Fragment 1 Reverse	*AACAGCTATGACCATG***TGAGAACTGCCCAAGGACT**
Fragment 2 Forward	*GTAAAACGACGGCCAGT***GCTGTTGTTGGCTTAGGGC**
Fragment 2 Reverse	*AACAGCTATGACCATG***CTGGAGTGCCGTGGTATGA**
Fragment 3 Forward	*GTAAAACGACGGCCAGT***TGAAACCCCATCTCTACTAA**
Fragment 3 Reverse	*AACAGCTATGACCATG***ATCACCTCAAAGAAAGCAAC**
Fragment 4 Forward	*GTAAAACGACGGCCAGT***ATCATACCACGGCACTCCAG**
Fragment 4 Reverse	*AACAGCTATGACCATG***TGTTTGCTACCAAGTTTATT**

Table 1

	Genomic variation	Frequency (70 patients)	Frequency in European panel

SNP	c.*421G>T	25 (35,7%)	45,8%
SNP	c.*421G/G	37 (52,9%)	37,5%
SNP	c.*421T/T	8 (11,4%)	16,7%
SNP	c.*1287C>T	25 (35,7%)	47,8%
SNP	c.*1287C/C	37 (52,9%)	39,1%
SNP	c.*1287T/T	8 (11,4%)	13%
SNP	c.*1332G>A	2 (2,9%)	2,4%
Variant	c.*750A>G	2/70 (2.8%)	
Variant	c.*1286C>A	1/70 (1.4%)	

Table 2