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PRECLINICAL STUDY

Assessment of the functional impact of germline *BRCA1/2* variants located in non-coding regions in families with breast and/or ovarian cancer predisposition

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Abstract

Purpose The molecular mechanism of breast and/or ovarian cancer susceptibility remains unclear in the majority of patients. While germline mutations in the regulatory non-coding regions of *BRCA1* and *BRCA2* genes have been described, screening has generally been limited to coding regions. The aim of this study was to evaluate the contribution of *BRCA1/2* non-coding variants.

Methods Four *BRCA1/2* non-coding regions were screened using high-resolution melting analysis/Sanger sequencing or next-generation sequencing on DNA extracted from index cases with breast and ovarian cancer predisposition (3926 for *BRCA1* and 3910 for *BRCA2*). The impact of a set of variants on *BRCA1/2* gene regulation was evaluated by site-directed mutagenesis, transfection, followed by Luciferase gene reporter assay.

Results We identified a total of 117 variants and tested twelve *BRCA1* and 8 *BRCA2* variants mapping to promoter and intronic regions. We highlighted two neighboring *BRCA1* promoter variants (c.-130del; c.-125C > T) and one *BRCA2* promoter variants (c.-296C > T) inhibiting significantly the promoter activity. In the functional assays, a regulating region within the intron 12 was found with the same enhancing impact as within the intron 2. Furthermore, the variants c.81-3980A > G and c.4186-2022C > T suppress the positive effect of the introns 2 and 12, respectively, on the *BRCA1* promoter activity. We also found some variants inducing the promoter activities.

Conclusion In this study, we highlighted some variants among many, modulating negatively the promoter activity of *BRCA1* or *2* and thus having a potential impact on the risk of developing cancer. This selection makes it possible to conduct future validation studies on a limited number of variants.

Keywords BRCA1/2 non-coding variants · Hereditary breast and/or ovarian cancer (HBOC) · BRCA1/2 transcription regulation · Breast and/or ovarian cancer risk

Introduction

At least 10% of the 14 million breast cancer diagnoses made worldwide each year are associated with hereditary predisposition. Breast cancer susceptibility gene 1 (*BRCA1*) and breast cancer susceptibility gene 2 (*BRCA2*) are the two most penetrant genes implicated in hereditary breast and/or ovarian cancer (HBOC) [1, 2]. However, a causal mutation useful for genetic counseling is identified in less than 15% of tested families and, in most cases, little is known about the underlying molecular mechanisms of cancer susceptibility. It would be particularly useful to identify inherited mutations in patients with a family history of cancers to allow

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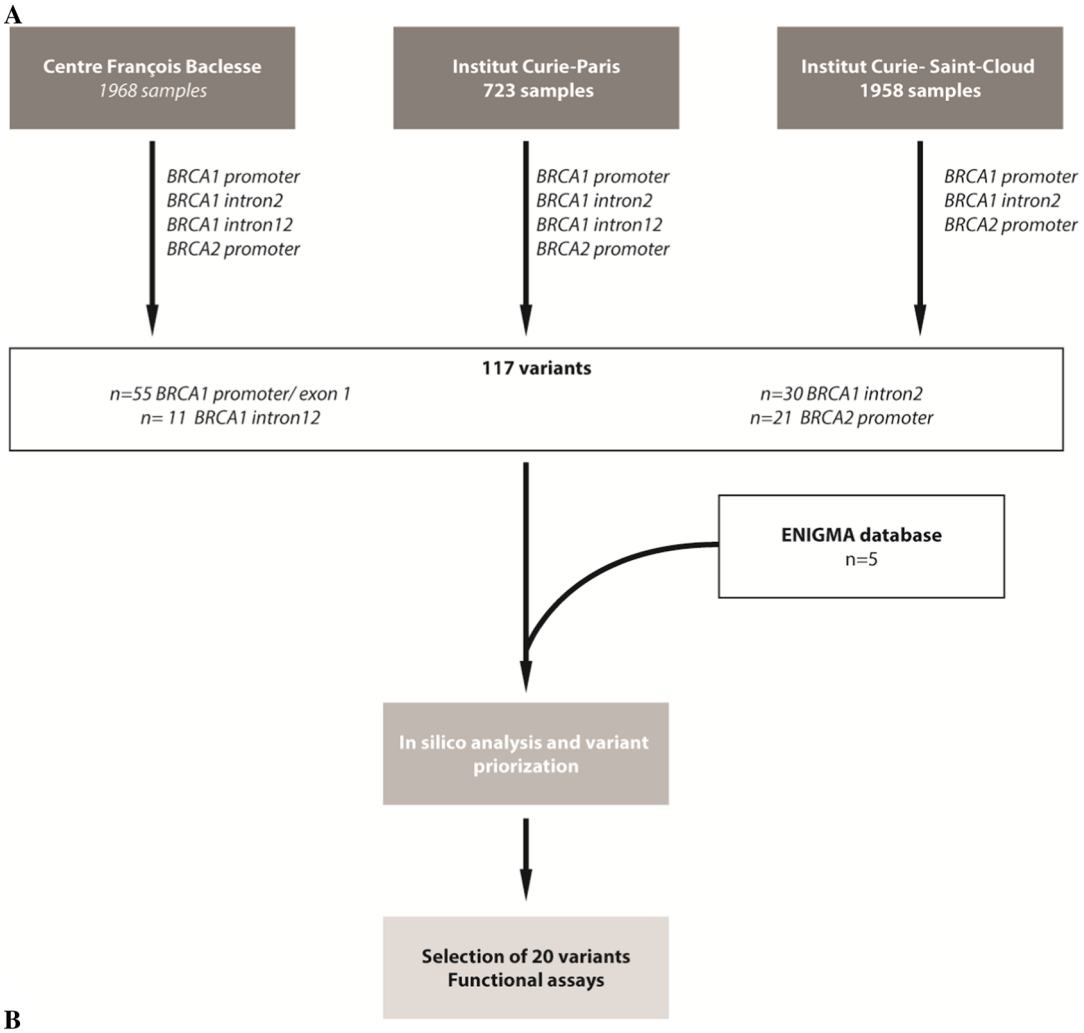
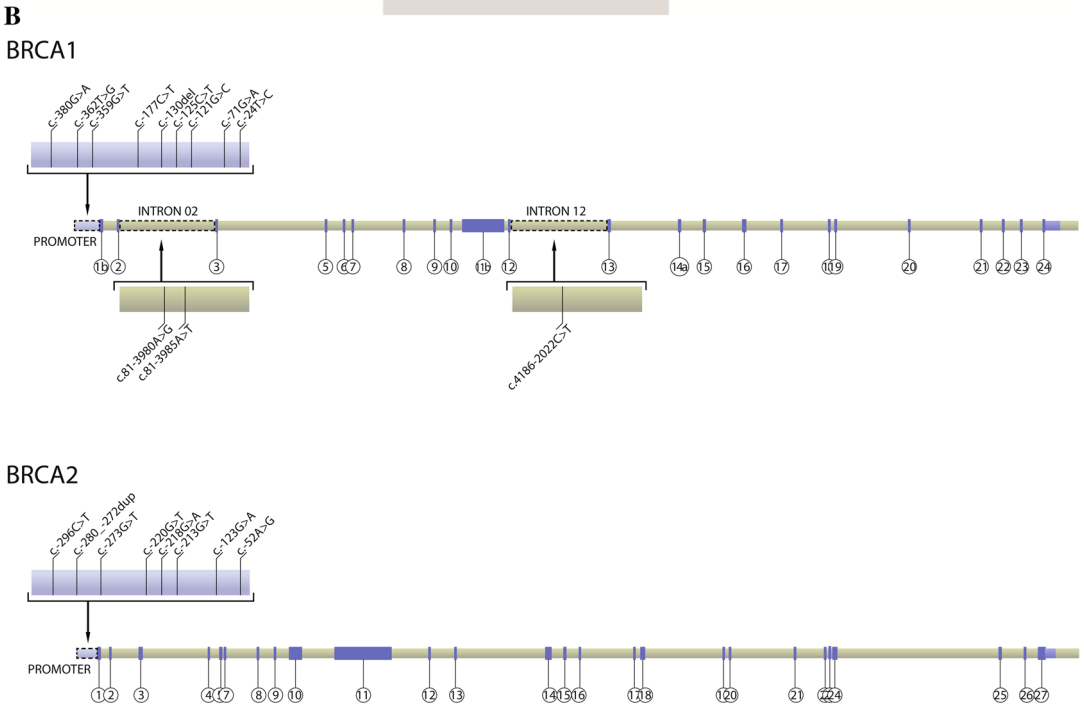

A
Centre François Baclesse
1968 samples
Institut Curie-Paris
723 samples
Institut Curie- Saint-Cloud
1958 samples
BRCA1 promoter
BRCA1 intron2
BRCA1 intron12
BRCA2 promoter
BRCA1 promoter
BRCA1 intron2
BRCA1 intron12
BRCA2 promoter
BRCA1 promoter
BRCA1 intron2
BRCA2 promoter
117 variants
n=55 BRCA1 promoter/ exon 1
n= 11 BRCA1 intron12
n=30 BRCA1 intron2
n=21 BRCA2 promoter
ENIGMA database
n=5
In silico analysis and variant priorization
Selection of 20 variants
Functional assays


B
BRCA1
c.-380G>A
c.-362T>G
c.-359G>T
c.-177C>T
c.-130delT
c.-122C>T
c.-121G>C
c.-71G>A
c.-24T>C
INTRON 02
INTRON 12
PROMOTER
c.81-3980A>G
c.81-3985A>T
c.4186-2022C>T
BRCA2
c.-296C>T
c.-280_-272dup
c.-273G>T
c.-220G>T
c.-218G>A
c.-213G>T
c.-123G>A
c.-52A>G
PROMOTER

◂**Fig. 1** **a** Work flow diagram describing the screening strategy and variant prioritization. **b** Location of the non-coding regions studied and the respective variants of each region selected for functional analysis

implementation of risk reduction strategies for these patients and their families. New technologies have been proposed to study a panel of genes known or suspected to be involved in breast and/or ovarian cancer predisposition. Other HBOC predisposition genes have also been explored but could represent less than 5% of all causative mutations [3]. *BRCA1/2* coding variants remain the major contributors to HBOC risk and the hypothesis that the remaining predisposition is also related to these genes remains plausible and could be explained by the presence of variants in non-coding regions for which the functional impact is currently unknown.

Progressing sequencing technologies and the development of bioinformatics tools now allow more informed exploration of transcriptional regulation [4, 5]. Germline mutations in the regulatory regions of the genome may represent an important tumorigenic mechanism and the impact of some non-coding regions on transcription regulation of the *BRCA1/2* genes has already been reported. Large genomic deletions involving the *BRCA1* and *BRCA2* promoters increase the risk of cancer [6–8]. Wardrop et al. described two non-coding sequences in intron 2 located 2.5 kb downstream to the *BRCA1* promoter with differential transcriptional regulatory activity [9]. Germline variants in the *BRCA1* and *BRCA2* 5′ and 3′UTRs, resulting in reduced translation efficiency, have also been described [10–15]. Moreover, several examples of variations in the non-coding sequences of other genes have also been correlated with cancer risk. Recently, two different recurrent mutations in the promoter of the telomerase reverse transcriptase (TERT) gene generating telomerase overexpression have been demonstrated to be associated with an increased risk of melanoma [16].

A reasonable mechanism to explain the impact of alterations of non-coding sequences on cancer risk is that the nucleotide change can create or disrupt a binding motif for a given transcription factor, and consequently alter the protein expression in all tissues expressing this factor. However, there is currently a lack of information about the function and polymorphisms of non-coding sequences and genetic screening of *BRCA1/2* genes is generally limited to coding regions and intron–exon junctions. The role of variants in non-coding regions with no splicing effect has not been thoroughly investigated and even less is known about their contribution to transcriptional regulation. Assessment of their impact on cancer predisposition is often more complex. The present study is a first approach to provide data to allow estimations of the impact of these variants on breast and/or ovarian cancer risk.

The primary objective of this study was to assess the significance and contribution of non-coding variants on *BRCA1/2* promoter activity and on breast and/or ovarian cancer risk.

Materials and methods

DNA samples, probands, and cohorts

In order to identify novel germline mutations that could explain hereditary predisposition, patients from three different HBOC cohorts, with eligibility criteria for familial genetic testing according to the French consensus statement and negative for *BRCA1/2* causal mutation, were enrolled [17–19]. A total of 1968 patients were tested at Centre François Baclesse, Caen, 1958 patients were tested at Institut Curie, Saint-Cloud, and 723 patients were tested at Institut Curie, Paris (Fig. 1, Table 1A). The characteristics of each cohort have been previously described [3, 20–22]. The frequency of the variants identified was also evaluated in a control cohort composed of Institut Curie patients with a cancer predisposition other than breast or ovarian cancer. The analysis was done anonymously and the frequency of the variant was only reported to compare with the cases.

DNA was extracted from lymphoblastoid cell lines and 4 *BRCA1/2* non-coding regions were screened by HRM or NGS: *BRCA1* promoter, *BRCA1* intron 2, *BRCA1* intron 12, and *BRCA2* promoter (Fig. 1, Table 1B).

In addition to the variants identified by this screening, we also selected new variants from the ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) database [23], in the context of a collaborative study.

Screening of *BRCA1/2* non-coding regions: high-resolution melting analysis and next-generation sequencing

The four regions explored had been previously defined as being regions most likely to be functional and presenting a higher probability of containing disease-associated variants. This analysis comprised bioinformatics, experimental and population-based approaches to identify and validate key non-coding regions in *BRCA1* and *BRCA2* [9, 24, 25]. For example, the regions explored in introns 2 and 12 are highly conserved among mammalian species and contain many potential binding sites for known transcription factors [9].

For HRM screening, PCR reactions were performed in duplicate in a final volume of 15 µl containing 2 ng of DNA, 0.6 µM of each primer (forward or reverse), 1 × LightCycler 480 HRM Master mix (Roche), and LightCycler® 480 Resolight Dye or LCGeen® Plus melting dye for *BRCA1* and

Table 1 Determination of variants in *BRCA1/2* promoters and *BRCA1* introns 2 and 12

A: Cohorts of this study			
Cohorts	Status of BRCA1/2	Technique	Samples or patients
Centre François Baclesse	Negative for causal mutation	NGS	*1968*
Institut Curie—Paris	Negative for causal mutation	HRM or NGS	723
Institut Curie—Saint-Cloud	Negative for causal mutation	HRM	*1958*
B: Number of variants found in each cohort for the target areas of *BRCA 1/2* genes			
Gene	Region	Cohorts (n)	Variants
BRCA1	Promoter/exon 1	3926	55
BRCA1	Intron 2	3624	30
BRCA1	Intron 12	2973	11
BRCA2	Promoter/exon 1	3910	21
Total			117

BRCA2 screening, respectively [26]. Each assay included DNA with known *BRCA1/2* mutation corresponding to the primer set as positive control. The PCR program is available on demand. The non-coding *BRCA1/2* DNA sequences evaluated and the primers selected for this purpose are specified in Supplementary Table 1.

NGS screening was performed with a dedicated panel for cancer predisposition with Illumina sequencers [3, 21, 22]. All known genetic variants detected were confirmed by sequencing PCR products (Sanger sequencing method).

In silico analysis and variant prioritization

For variant prioritization, we first applied a population frequency filter to exclude variants with an allele frequency > 1%. The minor allelic frequency (MAF) was estimated from the Ensembl project or Exome Aggregation Consortium [27, 28]. Information analysis was then performed to identify potentially pathogenic variants. This approach evaluates the effects of the variant on binding sites and whether the variant involves the creation, strengthening, weakening, or abolition of a binding site [5].

All variants were scanned with Shannon Human Splicing Mutation Pipeline, a genome-scale analysis program that predicts the effects of variants on mRNA splicing [29]. Variants were selected according to the following criteria: weakened natural site ≥ 1.0 bits or strengthened cryptic site equal to or greater than the nearest natural site of the same phase. We also analyzed the effects of variants in the 5′UTR region on TF binding using the models previously described by Mucaki et al. [5].

Finally, for functional assays, we prioritized variants located in domains most likely to be functional based on bioinformatics analysis, and for which testing tools were available.

Luciferase reporter gene constructions

Luciferase reporter plasmids containing sequences from the *BRCA1* promoter and *BRCA1* intron 2 have been described previously [9, 25]. For the *BRCA2* luciferase reporter plasmid, a 750 bp region containing the BRCA2 promoter was cloned into the pGL3-Basic vector [9, 25]. In these plasmids, promoter sequences were inserted upstream to the coding sequence of firefly luciferase in the *Xho*I site. The intronic sequences were inserted immediately downstream to the luciferase gene in the *Bam*H1 site (Fig. 2). A new construct was made in order to clone a region of *BRCA1* intron 12 downstream to the luciferase gene, using the Gibson Assembly Method [30]. Variants were introduced into the plasmids by directed mutagenesis. *BRCA1*: c.-287C > T and c.-326_324del variants were used as positive controls. As the *BRCA2* promoter has been less studied, it was not possible to model a positive control for it, and thus the wild-type promoter was used as a reference. The *BRCA2*: c.-52A > G polymorphism was used as negative control. All constructs were verified by DNA sequencing.

Cell culture, transfection, and dual-luciferase reporter assay

The triple-negative breast cancer MDA-MB-231 cell line and the estrogen receptor-positive MCF-7 breast cancer cell line were obtained from American type culture collection (ATCC). MDA-MB-231 was used in every experiment. We confirmed some of the significant results in the MCF-7 breast cancer cell line. All cells were tested regularly for

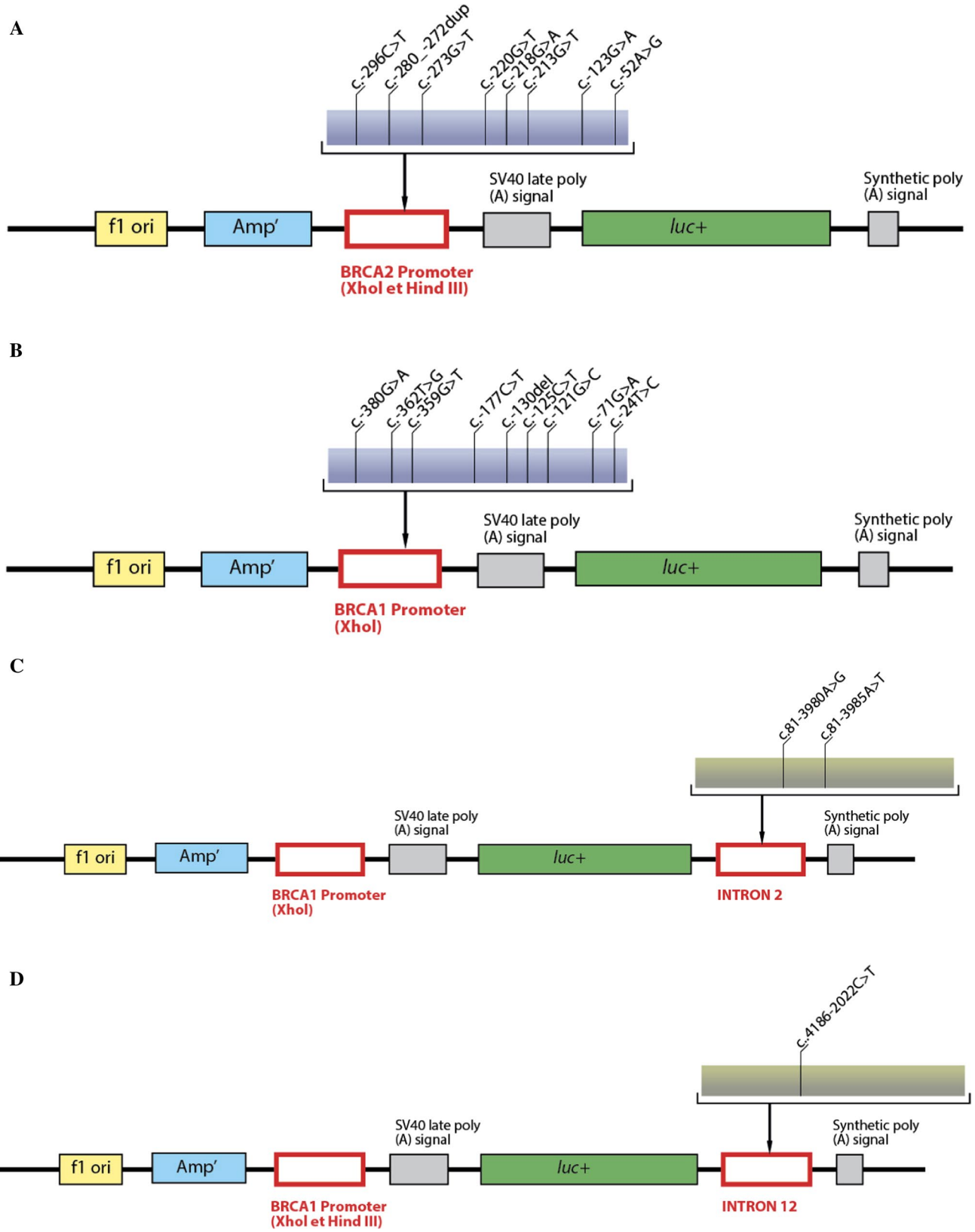


Fig. 2 Representation of the plasmids used in this study. **a** *BRCA2* promoter. **b** *BRCA1* promoter. **c** *BRCA1* promoter and BRCA1 intron 2. **d** *BRCA1* promoter and *BRCA1* intron 12

mycoplasma contamination using plasma Test (invivoGen) and authenticated using the GenePrint 10 system Kit (Promega). MCF-7 and MDA-MB-231 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and antibiotics (37 °C, 5% CO_2). To perform transient transfection, cells were seeded in 24-well plates and were subsequently transfected at 80% confluence using X-treme (QIAGEN) reagent according to the manufacturer's instructions. After 36 h, Firefly and Renilla activities were measured using the dual-luciferase kit (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity and expressed as mean ± S.D. of triplicates from a representative experiment.

All statistical calculations were performed using PASW Statistics (version 18.0; SPSS Inc., Chicago, IL). Comparisons were performed using a two-sided unpaired Student t test. *p* values less than 0.05 were considered to be statistically significant.

Clinico-pathological features of variant carriers

When a significant reduction of promoter activity was observed, more evidence for variant classification was sought. Further analysis of the patient's pedigree, allelic imbalance in RNA transcription, and tumor sample features, including Loss of Heterozygosity (LOH) and methylation, were determined, when material was available. LOH analysis was performed by Sanger sequencing or pyrosequencing. The *BRCA1* promoter methylation status was also assessed for variants with functional impact and when the material was available by pyrosequencing assay [31].

Results

Identification of new variants in *BRCA1/2* non-coding regions

The aim of this study was to identify novel germline mutations located in the non-coding regions of *BRCA1* and *BRCA2* genes that could explain hereditary predisposition for breast cancer. To do this, 4 *BRCA1/2* regions of the DNA of patients from 3 different HBOC cohorts were screened: *BRCA1* promoter, *BRCA1* intron 2, *BRCA1* intron 12, and *BRCA2* promoter (Table 1B). This approach allowed the identification of 117 variants in *BRCA1/2* non-coding regions (Fig. 1, Tables 1A, Supplementary Tables 2 and 3).

Five of these 117 variants were identified in more than 4 families: c.81-3625del, c.-20 + 11C > T, c.4186-2050A > G and c.-86C > T in *BRCA1* gene, and c.-175C > T in *BRCA2* gene. Two of them were found exclusively in our cohorts with HBOC predisposition: c.81-3625del and c.-20 + 11C > T in *BRCA1* gene. The remaining three variants were also identified in the control population.

In silico analyses

In silico analysis of these 117 variants identified 3 *BRCA1* variants with a potential impact on splicing: c.-73C > G, c.-86C > T, and c.-19-130insA; 3 *BRCA1* variants with a potential impact on UTR binding site alteration: c.-73C > G, c.-79G > T, and c.-121G > C; and twelve *BRCA1* variants with a potential impact on the TFB site: c.81-3459C > T, c.81-3510C > T, c.-19-479G > T, c.-20 + 131delGGCGTA, c.-20 + 131A > T, c.-20 + 125A > C, c.-177C > T, c.-130del, c.-125C > T, c.-20 + 486insG, c.-19-123insAT, and c.-20 + 11C > T. The impact of the variants on RNA secondary structure was also analyzed and one *BRCA1* variant, c.-130del, displayed a predicted impact on mRNA conformation (Fig. 3).

Moreover, two variants in intron 2 of *BRCA1* could have an impact on the creation of cryptic exons: c.81-4118G > A and c.81-3519G > T. Validation of these cryptic exons would require the development of a dedicated RT-PCR on mRNA. No suspected mRNA splicing effect was detected in silico for these variants.

Six *BRCA2* variants were identified with different potential impacts: c.-112G > A (UTR binding site and splicing factor binding site), c.-123G > A (splicing factor binding site), c.-171G > C (mRNA structure), c.-178insCTGCTG CGCCT (TFB site), c.-213G > T (UTR binding site), c.-296C > T (TFB site). The c.-171G > C variant also displayed a predicted impact on mRNA structure.

Based on these analyses and taking into account the available tools, twenty variants were selected for functional assays [32]. Nine of these 20 variants were located in the *BRCA1* promoter region, two variants were located in *BRCA1* intron 2, one variant was located in *BRCA1* intron 12, and eight variants were located in the *BRCA2* promoter region (Table 2).

Impact of variants on *BRCA2* promoter activity

Among the 8 *BRCA2* variants tested, only c.-296C > T induced a significant reduction (28%) of reporter gene expression, indicating that this variant inhibits the *BRCA2* promoter activity (Fig. 4). Moreover, analysis of the tumor sample harboring this variant identified LOH of the wild-type allele, and the patient's pedigree revealed that one of her 2 sisters had also a diagnosis of breast cancer at the age of 44 years (Table 3, F1), further supporting the potential pathogenic impact of this variant (Fig. 1 supplementary data).

Two variants showed an increase of promoter activity: the eventual role of this positive effect on cancer remains to be

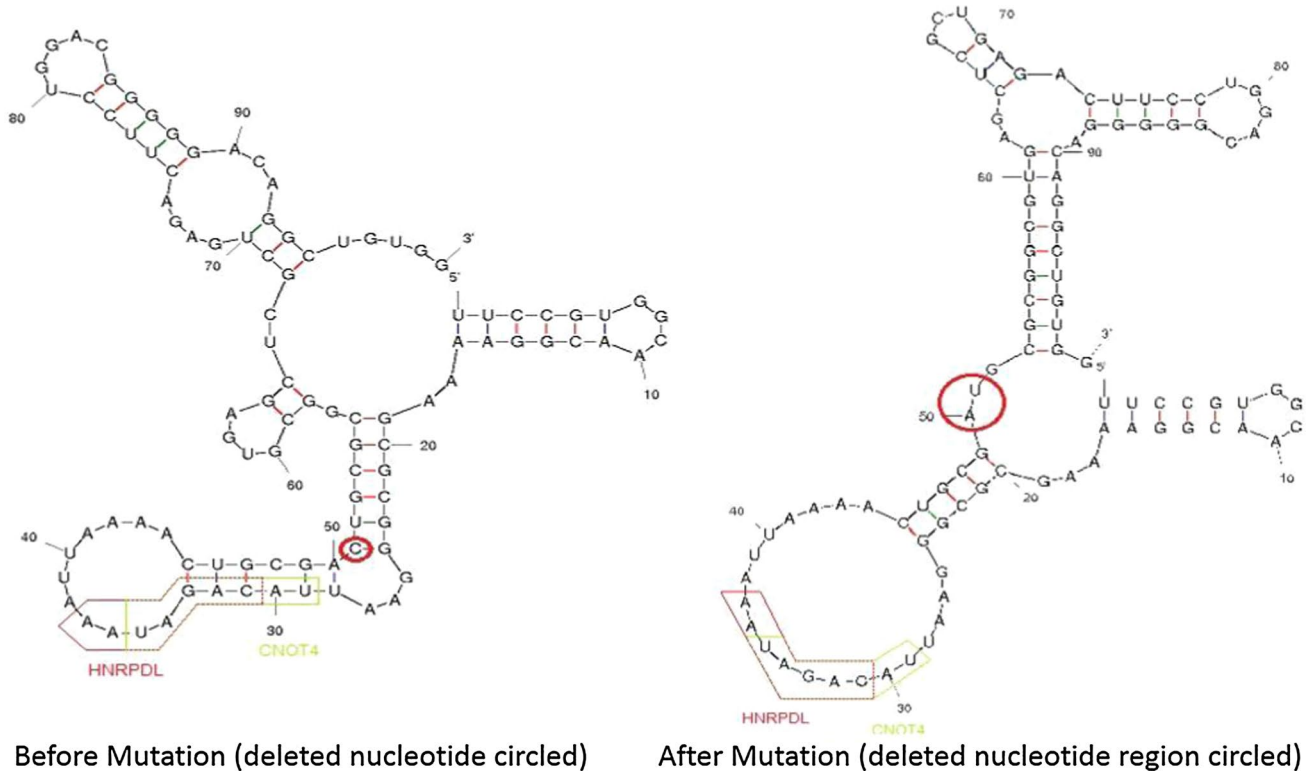


Fig. 3 *BRCA1* variant: c.-130del—structure with mFOLD is significantly changed due to loss of C-G bond

defined. The other variants demonstrated similar levels of activity to that of the wild-type sequence strongly suggesting that these variants are neutral (Fig. 4).

Impact of variants on *BRCA1* promoter activity

The *BRCA1* variants analysis revealed two neighboring variants: c.-125C > T and c.-130del, inducing a strong reduction of promoter activity (60% reduction for c.-130del $p = 0.0002$, and 56% reduction for c.-125C > T $p = 0.0025$) (Fig. 5 and Table 2B). To confirm these results, we repeated the experiment in another breast cancer cell line, MCF-7. We validated our first results (70% reduction for c.-130del, $p = 0.003$, and 30% reduction for c.-125C > T, $p = 0.003$) (Table 2B). One family was available for the *BRCA1* c.-130del with many prostate cancers (Table 3, F2). As for the *BRCA2* promoter, we also found 2 variants increasing weakly the *BRCA1* promoter activity: c.-362T > G; c.-121 G > C (Fig. 5 and Table 2A). The remaining variants were associated with similar reporter gene activity to that of the wild-type sequence (Fig. 5).

We also studied the impact of *BRCA1* intronic variants on *BRCA1* promoter activity: two detected in intron 2 (c.81-3985A > T and c.81-3980A > G) and one detected in intron 12(c.4186-2022C > T). First of all, we confirmed that the presence of a part of intron 2 and also a part of intron 12 increased the activity of the *BRCA1* promoter, 1.48- and 1.72-fold, respectively, confirming that these two introns possess important regulatory sequences (Fig. 6a). The intron 2 effect was already described contrary to the intron 12 [9]. The intronic variant c.81-3985A > T is located in a repressor region previously described in intron 2 [9]. However, we did not detect any influence of this variant on the positive effect of the intron 2 on the *BRCA1* promoter activity. Most importantly, we found that in the presence of the two intronic variants (c.81-3980A > G and c.4186-2022C > T), the introns 2 and 12 had no longer an impact over *BRCA1* promoter activity (Fig. 6b and Table 2B).

We did not detect any *BRCA1* promoter methylation for any functionally active variants.

Discussion

Results statement

Optimal management of hereditary breast and/or ovarian cancer families requires accurate identification of individuals at genuinely high risk. Although it is important to identify new breast and ovarian cancer susceptibility genes, non-coding regions are currently not investigated, with the exception of those intronic variants with an

Table 2 (A) Summary of the 20 variants tested. (B) The effect of the variants tested on luciferase activity

Gene	Variant	Localization	Record	Databases	dbSNP	1000Genomes MAF	ExAC	Conservation*	Putative TF binding site
BRCA1	c.-24T > C	Promoter	1	BIC/ClinVar	–	–	–	− 0,52	–
BRCA1	c.-71G > A	Promoter	1	No	–	–	–	0,93	–
BRCA1	c.-121G > C	Promoter	1	No		–	ALL:C = 0.0019%-	− 1,01	–
BRCA1	c.-125C > T	Promoter	1	No	rs148196794	< 0.01/4ou ALL: T = 0,1%	–	2,14	E2F1
BRCA1	c.-130del	Promoter	1	No	–	–	–	0,37	E2F1, HSF1, TEAD4
BRCA1	c.-177C > T	Promoter	1	No	–	–	–	0,85	CEBPB
BRCA1	c.-359G > T	Promoter	1	No	–	–	–	− 1,17	–
BRCA1	c.-362T > G	Promoter	1	No	–	–	–	1,25	–
BRCA1	c.-380G > A	Promoter	1	No	–	–	–	− 0,28	–
BRCA1	c.81-3985A > T	Intron 2	2	ClinVar	rs543267121	–	–	1,25	–
BRCA1	c.81-3980A > G	Intron 2	1	No	–	–	–	0,21	–
BRCA1	c.4186-2022C > T	Intron 12	1	No	–	–	–	0,85	–
BRCA2	c.-52A > G**	Promoter	1	UMD/LOVD	rs206118	ALL :G = 15%	–	− 0,12	–
BRCA2	c.-123G > A	Promoter	1	No	–	–	–	− 2,14	–
BRCA2	c.-213G > T	Promoter	1	No	rs546292946	–	–	− 0,04	–
BRCA2	c.-218G > A	Promoter	1	No	–	–	–	0,12	–
BRCA2	c.-220G > T	Promoter	1	No	–	–	–	2,38	–
BRCA2	c.-273G > T	Promoter	1	No	–	–	–	0,21	–
BRCA2	c.-280_272dup	Promoter	1	No	–	–	–	2,47	PAX5
BRCA2	c.-296C > T	Promoter	1	No	rs563971900	ALL :$T = 0.04\%$	–	− 0,28	PAX5

Gene	Variant	Localization	Effect on promoter activity MCF-7	Effect on promoter activity MDA-MB231	BRCA1 promoter methylation
BRCA1	c.-24T > C	Promoter	Not tested	NS	–
BRCA1	c.-71G > A	Promoter	Not tested	NS	–
BRCA1	c.-121G > C	Promoter	NS	↗ 1.25x ($p = 0.009$)	No
BRCA1	c.-125C > T	Promoter	↘ 0.7x ($p = 0.003$)	↘ 0.44x ($p < 0.0025$)	NA
BRCA1	c.-130del	Promoter	↘ 0.27x($p = 0.003$)	↘ 0.4x ($p = 0.0002$)↘	No
BRCA1	c.-177C > T	Promoter	Not tested	NS	–
BRCA1	c.-359G > T	Promoter	Not tested	NS	–
BRCA1	c.-362T > G	Promoter	Not tested	↗ 1.74x ($p = 0.0037$)	NA
BRCA1	c.-380G > A	Promoter	Not tested	NS	–
BRCA1	c.81-3985A > T	Intron 2	↗ 1.93x($p < 0.05$)	Not tested	No
BRCA1	c.81-3980A > G	Intron 2	Not tested	NS	–
BRCA1	c.4186-2022C > T	Intron 12	Not tested	NS	–
BRCA2	c.-52A > G	Promoter	Not tested	NS	–
BRCA2	c.-123G > A	Promoter	Not tested	↗ 1.83x ($p < 0.05$)	–

Table 2 (continued)

Gene	Variant	Localization	Effect on promoter activity MCF-7	Effect on promoter activity MDA-MB231	BRCA1 promoter methylation
BRCA2	c.-213G > T	Promoter	Not tested	NS	–
BRCA2	c.-218G > A	Promoter	Not tested	NS	–
BRCA2	c.-220G > T	Promoter	Not tested	NS	–
BRCA2	c.-273G > T	Promoter	Not tested	NS	–
BRCA2	c.-280_272dup	Promoter	Not tested	↗ 1.76x ($p = 0.00084$)	–
BRCA2	c.-296C > T	Promoter	Not tested	↘ 0.72x ($p = 0.0035$)	

NA material not available; *NS* not significant; *p* value was calculated using a two-sided unpaired Student *t* test. *p* values less than 0.05 were considered to be statistically significant

*The phyloP program was used to determinate the conservation score of the variants (http://compgen.cshl.edu/phast/)

**c.-52A > G is a polymorphism used as negative control in all *BRCA2* runs

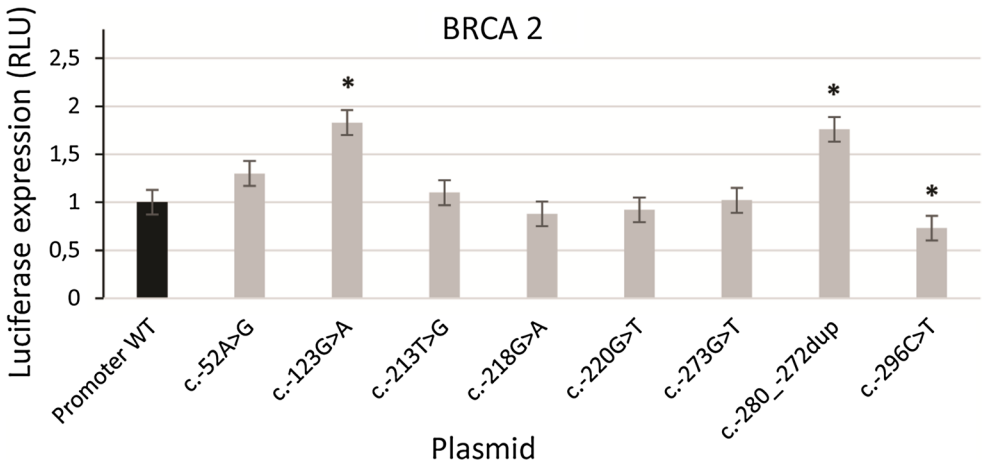


Fig. 4 Impact of different variants on *BRCA2* promoter activity. MDA-MB-231 breast cell line was transfected with the expression vector pRL-TK Renilla in combination with the luciferase reporter plasmids containing the *BRCA2* promoter wild type (Promoter WT) or possessing a variant as indicated. Twenty-four hours later, cell extracts were prepared and luciferase activities quantified

impact on RNA splicing [33, 34]. In the present study, we chose to explore these non-coding regions and carry out functional assays for these variants. Screening of the HBOC population comprising 3926 patients screened for *BRCA1* and 3010 patients screened for *BRCA2* non-coding regions revealed 117 variants (0.5 to 1.4% of the screened population).

We have validated an experimental protocol for the initial functional classification of 20 of these variants that demonstrated 10 non-coding variants with a functional impact on *BRCA1/2* promoter activity. Among these 10 variants, two decreased *BRCA1* promoter activity: c.-130del and c.-125C > T; one decreased *BRCA2* promoter activity: c.-296C > T; and two (c.81-3980A > G and c.4186-2022C > T) suppressed the positive effect of the introns 2 and 12 over the *BRCA1* promoter activity.

Limitations of functional assays for non-coding variants

Fluctuations of the basal reporter activity were observed for both the *BRCA1* and *BRCA2* promoters, which could be explained by poorly controlled parameters of the biological system as well as technical limitations, for example, the quality and conformation of transfected DNA. An internal positive control was always used to ensure correct interpretation of functional results. It is noteworthy that only minor differences were observed for PGL3 basic or Renilla luciferase activity, which confirm transfection efficiency, and that the wild-type promoter was always present to ensure correct interpretation of functional results. Moreover, the results for the potential suppressor variants, *BRCA1* c.-125C > T; *BRCA1* c.-130del; *BRCA2*

Table 3 Summary of clinical and pathological data for the non-coding variants

Gene	Variation (c.)	Localization	Index case data	Co-occurrence of variants in BRCA2 or BRCA1 or in other genes + large rearrangements	Family data	Co-segregation data
BRCA2	c.-296C > T	Promoter	C1: woman with breast cancer (diagnosed at 60)	No	F1: sister with breast cancer (diagnosed at 44)	No information
BRCA1	c.-130del	Promoter	C2: man with prostate cancer (diagnosed at 60)	No	F2: 5 Brothers with prostate cancer (diagnosed at 72, 70, 60, 65, and 64). Maternal grand-mother with breast cancer (diagnosed at 45)	No information
BRCA1	c.81-3985A > T	Intron 2	C3: woman with breast cancer (diagnosed at 48)	No	F3: mother with breast cancer (diagnosed at 60). Brother with prostate cancer (diagnosed at 50)	No information
BRCA1	c.81-3985A > T	Intron 2	C4: woman with breast cancer (diagnosed at 48)	No	F4: mother with breast cancer (diagnosed at 54). Maternal aunt with breast cancer (diagnosed at 45). Maternal cousin with breast cancer (diagnosed at 45). Paternal aunt with ovary cancer (diagnosed at 69). Paternal cousin with breast cancer (diagnosed at 51)	No information
BRCA1	c.81-3985A > T	Intron 2	C5: woman with breast cancer (diagnosed at 40)	No	F5: mother with breast cancer (diagnosed at 41). Maternal cousin with breast cancer (diagnosed at 70). Maternal grand-father with pancreatic cancer (diagnosed at 70)	No information

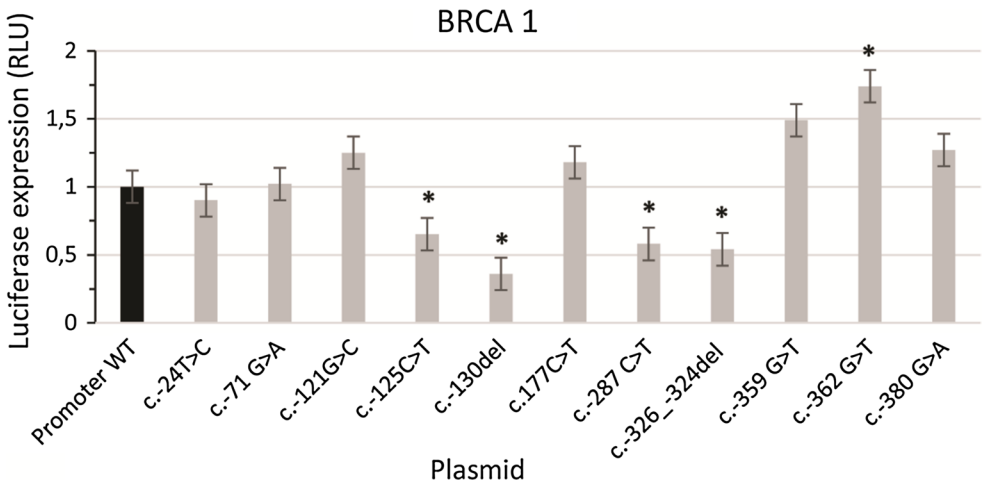


Fig. 5 Impact of different variants on *BRCA1* promoter activity. MDA-MB-231 breast cell line was transfected with the expression vector pRL-TK Renilla in combination with the luciferase reporter plasmids containing the *BRCA1* promoter wild type (Promoter WT) or possessing a variant as indicated. Twenty-four hours later, cell extracts were prepared and luciferase activities quantified. The c.-287C > T and c.-326_324del variants are artificial constructions on CAAT box and on the RIBS element, respectively, used as positive controls

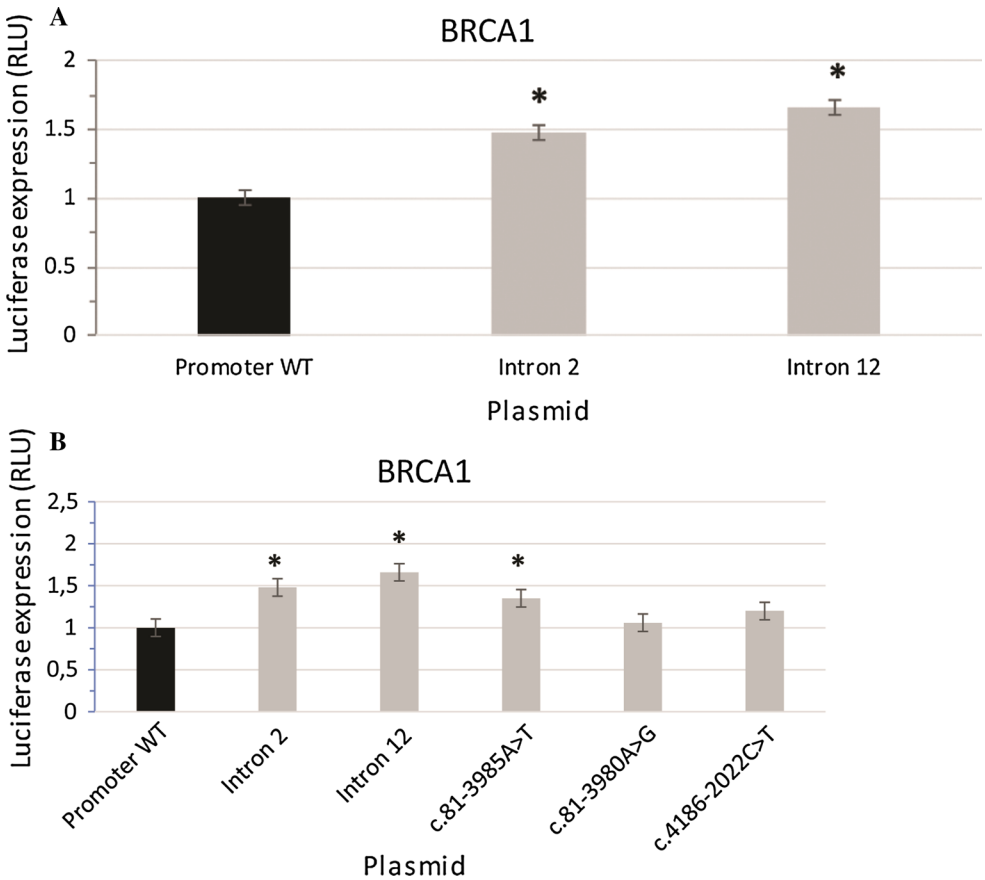


Fig. 6 **a** Impact of different intronic variants on *BRCA1* promoter activity. MDA-MB-231 breast cell line was transfected with the expression vector pRL-TK Renilla in combination with the luciferase reporter plasmids containing the *BRCA1* promoter wild type without (Promoter WT) or with the intron 2 or 12 wild type (**a**) or possessing a variant (**b**) as indicated. Twenty-four hours later, cell extracts were prepared and luciferase activities quantified

c.-296C > T, were always consistent under the various experimental conditions.

Sensitive region in promoter of *BRCA1*

We identified a sensitive region in the *BRCA1* promoter with 3 functionally active variants: c.-125C > T; c.-130del;

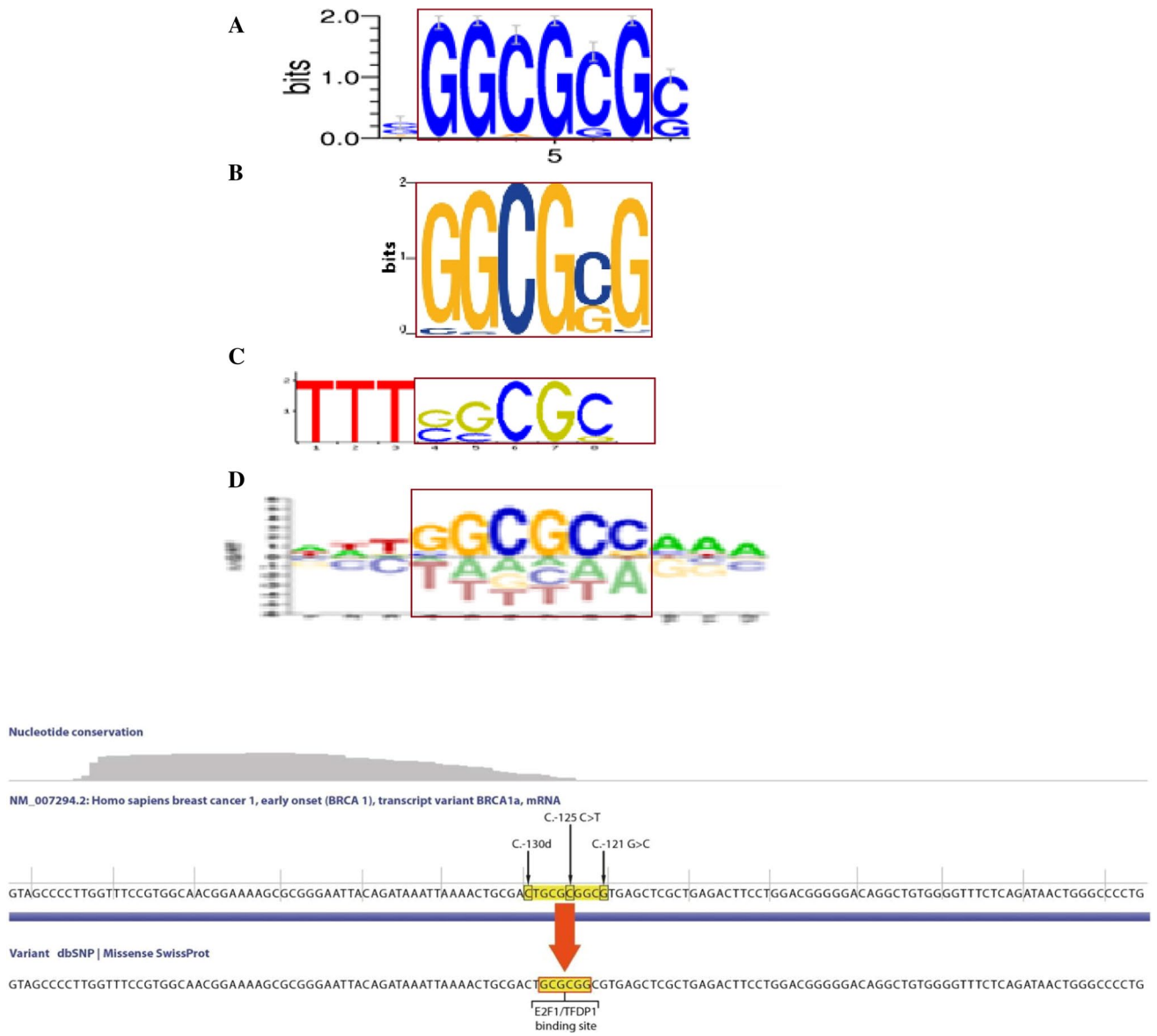


Fig. 7 Identification of a new potential E2F1 binding site in *BRCA1* promoter. **a** Information Models built from publically available ChIP-Seq data (HeLa-S3). **b** Models from SwissRegulon (Fig. 5a)

c.-121G > C, including 2 with a marked repressor impact on promoter activity (Fig. 7). Analysis of the DNA sequence region containing the neighboring *BRCA1* c.-125C > T and *BRCA1* c.-130del promoter variants, using the Swiss Regulon TF database (http://swissregulon.unibas.ch/), revealed that both variants are located in a putative E2F1 transcription factor binding site (TFBS)(Fig. 7). These two variants may thus impact the ability of E2F1 to induce BRCA1 transcription. An E2F1 information model generated using ChIP-Seq data from HeLa-S3 lysates revealed a fairly weak 3.6 bit E2F1 site on the negative strand (Fig. 7a) [35]. When the binding site was analyzed from the negative strand (the orientation of BRCA1 transcription), both mutations were predicted to decrease the strength of the predicted E2F1 site. Variant c.-125C > T was predicted to be a weak variant mainly due to the presence of a 'T' in its sequence when a C or G was expected (TGCGCG; arrow indicates the position of T relative to our model; Fig. 7a). Our analysis also revealed that the c.-130del variant is located in a putative HSF1 and TEAD4 TFBSs. Other transcription factors identified in future studies could therefore increase our understanding of the biological implications of these variants in TFBSs.

Our in silico analysis revealed that the *BRCA1*: c.-130del variant also has a potential impact on the RNA 2D structure. The RNA conformation of the first exon of the *BRCA1* gene

has been described and could have an impact on transcription, as the alternative exon 1b transcript of the *BRCA1* gene has a conformation that could reduce translation of mRNA [36]. This impact cannot be detected with the luciferase assay.

Analysis of the pedigree of the c.-130del index case, looking for more evidence for classification of variants, revealed numerous cases of prostate cancer, usually associated with alterations of the *BRCA2* gene. Patients carrying a *BRCA1* mutation usually present little or no increased cancer risk, but a more aggressive form of prostate cancer [37]. Unfortunately, sequencing of this patient's tumor sample did not reveal any additional useful for classification: neither LOH of the wild-type allele nor promoter methylation was detected. However, recent studies have demonstrated the effect of BRCA1-haploinsufficiency in various cells and tissues, which may explain how mutation in a single *BRCA1* allele conferred increased cancer risk in this patient [38].

BRCA2 promoter

For the first time, a variant of the *BRCA2* promoter has been shown to have a functional impact on transcription (c.-296C > T). This variant is also located close to a region rich in transcription factor binding sites. Analysis of the tumor sample from a carrier of this variant revealed somatic loss of the wild-type *BRCA2* allele, suggesting that loss of heterozygosity may play a role in the tumorigenesis. The other two *BRCA2* variants (c.-280_-272dup and c.-123G > A) showed an enhancer activity, the consequence of which is unknown.

Putative changes in TFBS related to the presence of the variants

The two *BRCA2* variants with a significant impact on transcription (c.-296C > T and *BRCA2*: c.-280_-272dup) were correlated with the TFBS predictions based on the variant prioritization method (Table 2A). These variants alter the binding strength of two PAX5 binding sites. ChIP-Seq experiments have shown that PAX5 binds to the *BRCA2* promoter region. Furthermore, although the *PAX5* gene has not been shown in the literature to have a direct effect on BRCA expression, it has been shown to be hypermethylated in triple-negative breast cancer [39]. Loss of a PAX5 binding site may therefore induce a similar effect to that of an overall reduction of PAX5 gene expression.

TFBS analysis showed weakening of PAX5 binding site from 12.7 to 8.5 bits in the presence of the c.-296C > T variant. Similarly, the promoter activity assay showed an increase in *BRCA2* promoter activity in the presence of the *BRCA2*: c.-280_-272dup event. TFBS analysis predicted that this duplication would create a 5.6 bit PAX5 binding site, which correlates with the reported increase in promoter activity.

Introns 2 and 12 *BRCA1*

Wardrop et al. have described the presence of regulatory regions in the intron 2 sequence of *BRCA1* gene [9]. Although these regions are situated several kb downstream to the promoter region, they regulate *BRCA1* expression at the transcriptional level, most likely via gene looping [25]. We investigated introns 2 and intron 12. Intron 12 locus has been selected for being rich on the transcription factor binding sites and interspecies conservation.

Even if the variant c.81-3985A > T was found in three families (Table 3) suspected for cancer predisposition, we did not detect any influence of this variant on the positive effect of the intron 2 over the *BRCA1* promoter activity. This result strongly suggest that the c.81-3985A > T variant do not inhibit the activity of the *BRCA1* promoter and therefore would have no effect on the breast cancer development. Furthermore, analysis of RNA from the patient's lymphoblastoid cell line showed no allelic imbalance, which support our conclusion that the c.81-3985A > T variant may have no causal impact on cancer (data not shown).

In the other hand, we found that the two intronic variants c.81-3980A > G and c.4186-2022C > T displayed wild-type devoid of intron 2 or 12, respectively. These two variants may inhibit *BRCA1* promoter activity by suppressing the positive effect of the intron 2/12 on the *BRCA1* promoter activity thereby stimulating cancer development. In this study, the regulating impact of intron 12 has been confirmed in vitro and this work highlights the importance of screening this region. Some variants were identified and a variant c.4186-2022C > T has been able to revert the enhancing impact of the intron 12 locus. Unfortunately, there was no material available to work on these variants.

Epigenetics

It is difficult to draw any solid conclusions from these results that could be used for genetic counseling of carriers of variants in *BRCA1/2* non-coding regions. Constitutional epimutation of the promoter has been described for the *MLH1* gene with a cis-acting variant, and a relationship between promoter activity and level of methylation has been established [40–42]. All of these cases presented somatic mosaicism between tissues and family members. No epimutations have been reported in the *BRCA2* gene. However, the promoter of *BRCA1* gene can also be methylated and constitutional epimutations have been reported [43]. No methylation of the promoter was identified on the c.-130del variant.

Conclusion

This study put in evidence the presence of rare variants in the non-coding regions of the *BRCA1* and *BRCA2* genes, and 5 of them induced a significant reduction of transcriptional levels. Our data raise the question whether the presence of these variants in regulatory regions may have an impact on the risk of developing cancer. To be more conclusive, it would be helpful to obtain more information about the frequency of these alterations. The model including the functional assay here described can be a useful tool to highlight the variants requiring further investigation including epimutation or co-segregation analysis, in order to ultimately establish a potential association with cancer risk.

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