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The c.5242C>A *BRCA1* missense variant induces exon skipping by increasing splicing repressors binding

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Abstract Several unclassified variants (UV) of *BRCA1* can be deleterious by affecting normal pre-mRNA splicing. Here, we investigated the consequences at the mRNA level of the frequently encountered c.5242C>A UV in *BRCA1* exon 18. We show that the c.5242C>A variant induces skipping of exon 18 in UV carriers and in vitro. This alteration predicted to disrupt the first BRCT domain of *BRCA1*. We show that two splicing repressors, hnRNP A1 and hnRNP H/F, display a significant preference toward binding with the mutated exon 18 and assemble into a protein complex. Sequence analysis of the region surrounding the c.5242C>A change reveals the presence of hnRNP A1 and hnRNP H/F binding sites, which are modified by several UVs. Mutation of these sites alters the RNA binding ability of both splicing regulators. In

conclusion, our work supports the model of the pathogenicity of the c.5242C>A *BRCA1* variant that induces exon skipping by creating a sequence with silencer properties. We propose that other UVs in exon 18 interfere with splicing complex assembly by perturbing the binding of hnRNP A1 and hnRNP H/F to their respective *cis*-elements. RNA analysis is therefore necessary for the assessment of the consequences of UVs on splicing of disease-associated genes and to enable adequate genetic counseling for breast/ovarian cancer families.

Keywords Hereditary breast cancer · *BRCA1* ·
Unclassified variant · Splicing mutation · Aberrant splicing

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Introduction

Five to ten percent of breast and ovarian cancers are hereditary mainly due to mutations in the susceptibility genes, *BRCA1* and *BRCA2*. Since the discovery of the *BRCA1* gene, more than 1,500 distinct variants have been identified by extensive mutational analysis (listed at the Breast Cancer Information Core (BIC); <http://research.nhgri.nih.gov/bic>). However, only frameshifts, splice sites, nonsense mutations and a few missense variants are accepted as disease-associated genetic alterations. In consequence, most of missense mutations cannot be readily distinguished as either disease-associated mutation or benign polymorphism, and are classified in the BIC database as variants of uncertain pathological significance or unclassified variants (UV).

It is now admitted that exonic missense or nonsense mutations can be deleterious by perturbing normal pre-mRNA splicing. The underlying mechanism of aberrant splicing is the disruption of positive *cis*-regulatory elements known as Exonic Splicing Enhancers (ESEs) and/or

the creation of negative *cis*-regulatory elements known as Exonic Splicing Silencers (ESSs). Most ESEs are known to interact with members of the serine/arginine-rich (SR) family, whereas, ESSs have often been found to interact with factors of the heterogeneous nuclear ribonucleoprotein (hnRNP) family. The two models have been explored to elucidate the mechanism by which the nonsense c.5199G>T variant induces exon skipping of *BRCA1* exon 18 in vivo [1] and in vitro [2]. This variant, which was initially predicted to disrupt a putative binding site for the SR protein, ASF/SF2 [2], has been recently shown to create a sequence silencer element that binds the splicing inhibitor, hnRNP A1/A2, and Deleted in Azoospermia-Associated Protein 1 (DAZAP1) [3, 4]. Skipping of exon 18 does not alter the reading frame, but results in removal of 26 amino acids, thus disrupting the first BRCT (for *BRCA1* C Terminus) domain of *BRCA1*, which is essential for the *BRCA1* function [1]. Several other variants have been detected in the exon 18 of *BRCA1*; one of the most frequent being the missense c.5242C>A variant, A1708E (or c.5123C>A according to the current nomenclature). Over the last several years, several works have attempted to classify the A1708E variant as a probable deleterious mutation [5, 6]. Yet, to date, no modification of exon splicing has been linked to the presence of this variant.

We investigated whether the c.5242C>A *BRCA1* variant may affect exon 18 splicing, and we have demonstrated that this variant causes inappropriate skipping of the entire constitutive exon in vivo. This abnormal exon skipping is correlated with a preferential binding of the splicing inhibitory factors, hnRNP A1 and hnRNP H/F, with the variant. This work provides additional evidence that the c.5242C>A UV should be considered as a deleterious mutation usable as a genetic test for pre-symptomatic genetic testing.

Materials and methods

Families

Breast/ovarian cancer families were ascertained at the Institute Claudius Regaud in Toulouse in the South of France. Blood samples were collected following informed consent.

Genotyping analysis

Peripheral blood samples were collected for *BRCA1* mutation screening. DNA was extracted using a EasyOne DNA blood (Qiagen) according to the manufacturer's instructions.

Total RNA was extracted from peripheral-blood leukocytes using TRIzol reagent (Invitrogen, Paisley, United Kingdom) according to the manufacturer's protocol. cDNA was synthesized using iScript cDNA Synthesis kit (Biorad). Primers (5'-GAAAGTGTGAGCAGGGAGAAG-3' for exon 16 and 5'-TACCATCCATTCCAGTTGATC-3' for exon 21) were used to amplify the *BRCA1* coding region from exon 16 to exon 21 (GenBank accession number U14680).

In silico analysis

The variant cDNA sequence was screened by ESE finder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/ese_finder.cgi?process=home) and Splicing Rainbow (<http://www.ebi.ac.uk/asd-srv/wb.cgi>) and compared with the wild-type sequence to identify any loss or gain of predicted SR or hnRNP binding sites.

BRCA1 minigenes and in vitro splicing

BRCA1 WT and c.5242C>A minigenes were constructed by splicing PCR as previously described [2]. ³²P uniformly labeled, m7G capped, T7 runoff transcripts were spliced in 25- μ l splicing reactions containing HeLa nuclear extracts (3 μ l) in buffer D [7] including ATP (1.5 mM), RNasin (40 U), DTT (5 mM) and MgCl₂ (2 mM). After incubation at 30°C for 2–4 h, the RNA was extracted and analyzed on 6% denaturing polyacrylamide gels, followed by autoradiography. For the time-course experiments, a splicing assay equivalent to three samples was assembled and three aliquots were taken after 2, 3 and 4 h incubation. We calculated exon inclusion as percentage of the total amount of spliced mRNA, that is included mRNA \times 100/(included mRNA + skipped mRNA) [8].

Templates for in vitro transcription

DNA templates for in vitro transcription were obtained by annealing of forward and reverse oligonucleotides (described in Supplementary information), followed by exo-klenow DNA polymerase reaction. Mutations within the hnRNP A1 and hnRNP H/F binding sites (Fig. 4) were the following: the GGG motif of the H/F site was changed to GCG, the TAG motif of A1(a) was changed to CAG and the TAGTTAG motif of A1(b) site was changed to CAGTCAG.

RNA- and protein-protein interactions

RNA affinity chromatography and UV cross-linking assays were performed as described in Refs. [9, 10] with minor modifications (see Supplementary information).

Results

The c.5242C>A, A1708E, variant of *BRCA1* is associated with abnormal splicing of *BRCA1* mRNA in patients and induces *BRCA1* exon 18 skipping in vitro

Full *BRCA1* and *BRCA2* sequencing were performed on the proband's germline DNA of the different families in which the single base-pair substitution c.5242C>A (A1708E) (RefSeq: NM_000059.2, GeneBank: DQ889340) has been identified and no other deleterious mutation has been detected (Table 1). About 13 relatives in family 1 and 11 in family 2 were screened for the presence of this variant. Respectively, four and two unaffected relatives in each family carried the UV c.5242C>A, while seven relatives in family 1 and two in family 2 were wild-type (WT) for this variant. All the affected members tested were shown to be carriers of the variant (UV) thus suggesting a co-segregation.

In order to evaluate the consequence of this sequence variant on *BRCA1* pre-mRNA splicing, we performed RT-PCR on freshly extracted RNA from proband's

peripheral lymphocytes of family 1 and in cancer-free control using primers located on exon 16 and 21. As shown in Fig. 1a (upper gel), while one band is detected by RT-PCR on RNA from a cancer-free patient (WT), the RT-PCR product of proband carrying the c.5242C>A UV presented an additional band. We then performed the same analysis on freshly extracted RNA from four families either carrying or not carrying the c.5242C>A UV carriers (Fig. 1a, lower gel), confirming the presence of an additional band only for the c.5242C>A UV carriers. The sequencing of the RT-PCR products revealed the presence of a dual sequence at the exon 17–18 junction for the forward sequence (and at the exon 18–19 junction for the reverse sequence), witness of an heterozygosity of exon 18 splicing for the patient carrying the UV (Fig. 1b). These results demonstrated that the presence of the c.5242C>A UV was associated with the skipping of exon 18, strongly suggesting that the presence of the substitution C to A on nucleotide 5242 might modify the splicing pattern of exon 18.

In order to confirm this hypothesis, we then constructed WT and c.5242C>A mutant *BRCA1* minigenes, which comprised exons 17–19 and shortened introns 17 and 18

Table 1 Cancer history of the families carrying the c.5242C>A *BRCA1* variant

Family number	Age at diagnostic in proband	Diagnosis of the proband	Number of relatives affected with breast cancer in family (age at diagnosis)	Number of relatives affected with ovarian cancer in family (age at diagnosis)	Number of relatives screened (number of affected relatives)	Number of unaffected relatives carrying c.5242C>A UV	Other cancers (age at diagnosis)
1	50	Ovarian	1 FDR (40) 1 SDR (44) 2 RSL (31,44)	1 FDR (56) 2 SDR (39,72)	13 (2)	4	Leukemia (62)
2	43	Ovarian	1 FDR (39) 6 RSL (49, 49, 36, 46, 39,44)	1 SDR (69) RSL (58)	11 (2)	2	Colon (33)
3	46, 57	Breast, ovarian	2 FDR (50,54)	0	1 (0)	1	Colon (53) Oesophagus (64) Head and neck (49)
4	28, 30	Bilateral breast	1 RSL (50)	0	0	0	
5	46, 47	Bilateral breast	1FDG (bilateral 46 and 62) 1SDR (35) RSL	0	0	0	Leukemia; melanoma (51)
6	52, 61	Breast, ovarian	1 SDR (58)	1 RSL (42)	1 (0)	1	Endometrial (60)
7	38	Ovarian	2 RSL (30, 39)	0	2 (1)	1	Hepatic (60)
8	49	Breast	2FDR (bilateral (37,38) and 44) 1 SDR (77) 1RSL (55)	0	1 (1)	0	Colon (62); Oesophagus (70)

FDR first degree relative, SDR second degree relative, RSL relatives of the same lineage

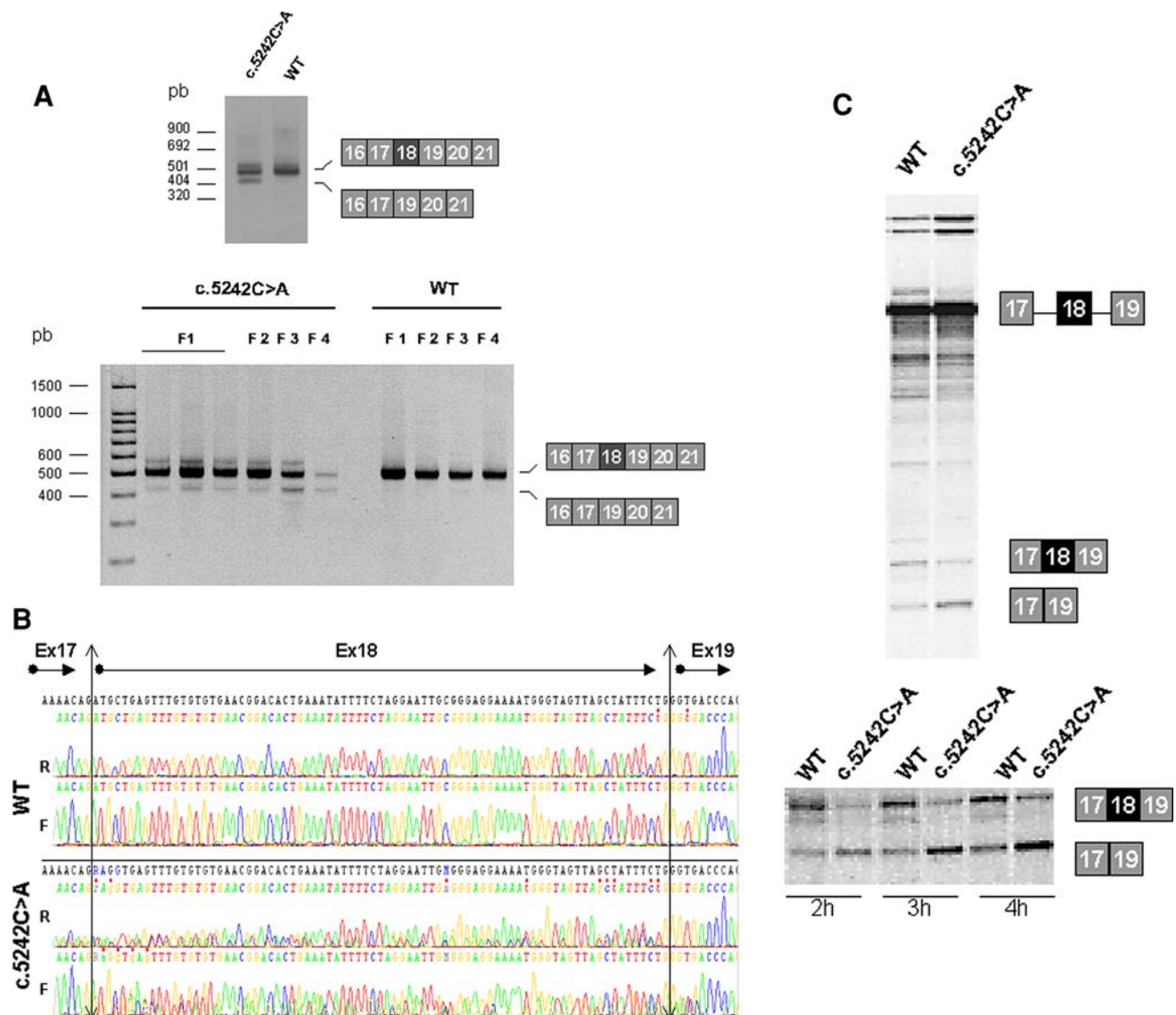


Fig. 1 The c.5242C>A variant is associated with aberrant splicing of BRCA1 exon 18. **a** RT-PCR analysis of exon 16–21 of BRCA1 on family 1 proband and cancer-free control patient RNA (*upper gel*) or on RNA extracted from blood patients samples belonging to family 1 (F1), family 2 (F2), family 3 (F3) and family 4 (F4) (*lower gel*). Two bands of 497 bp and 419 bp respectively, corresponding to the full-size mRNA (*upper band*) and the exon18 skipped mRNA (*lower band*) were observed for patients presenting the c.5242C>A variant (c.5242C>A), while only one band corresponding to the full-size mRNA was observed for wild-type (WT) BRCA1 carriers patients. The identity of each band is indicated schematically on the *right*. The results of RT-PCR analysis of three different relatives of family 1 and one of family 2, 3 or 4 carrying c.5242C>A UV BRCA1 variant and

one WT patient from family 1–4 are shown (*lower gel*) **b** Forward (F) and reverse (R) sequencing analysis of the RT-PCR products of exon 16–21 of BRCA1 for patients carrying the c.5242C>A variant and wild-type sequence (WT). The positions of exons 17, 18 and 19 are mentioned on the sequence. While the sequence of the WT patient is homozygous, the sequence of the variant becomes heterozygous at the exon junctions. **c** In vitro splicing assays of WT and c.5242C>A variant BRCA1 minigenes comprising exons 17–19 and shortened introns 17 and 18 were performed using 32 P-labeled transcripts in the presence of HeLa nuclear extracts (NE) for 4 h (*upper gel*) or for the indicated *times* (*lower gel*). The identity of each band is indicated schematically on the *right*. Exons are shown as *boxes* and shortened introns as *lines*

[2]. In vitro splicing assays using 32 P-labeled transcripts corresponding to the two minigenes were performed in presence of HeLa nuclear extracts. As shown in Fig. 1c (*upper gel*), mRNAs containing exon 18 were the predominantly spliced product (63% inclusion) with the WT substrate, whereas, exon skipping was favored with the

mutated exon 18 (30% inclusion). The differential splicing pattern of the WT and mutated exon 18 was detectable at any time of the splicing reaction (Fig. 1c, *lower gel*). Taken together, our results demonstrate that the c.5242C>A variant induces exon 18 skipping in vivo and in vitro.

The *BRCA1* c.5242C>A variant increases the RNA binding activity of hnRNP A1 and hnRNP H/F

One possible explanation of mutation-associated exon 18 skipping is that the c.5242C>A variant lies and eventually abrogates an ESE motif in the region encompassing the mutated residue. We tested this possibility by analyzing the WT and mutated exon 18 *BRCA1* sequence using the ESE finder algorithm (<http://rulai.cshl.edu/cgi-bin/tools/ESE3>), which predicts functional ESE motifs recognized by the SR proteins ASF/SF2, SC35, SRp40 and SRp55. This in silico approach revealed that the c.5242C>A might eliminate the high score of both SC35 and SRp55 motifs without affecting the scores for ASF/SF2 and SRp40 motifs (Fig. 2a). In order to further identify *trans*-acting factor(s) differentially bound to *BRCA1* exon 18 WT and c.5242C>A UV sequences, we performed RNA affinity chromatography using HeLa nuclear extracts and biotinylated transcripts corresponding to the exon 18 WT or c.5242C>A UV, each immobilized on streptavidin agarose beads. As shown in Fig. 2b, comparison of the two RNA–protein interaction profiles revealed that a protein of about 35 kDa was preferentially bound to the c.5242C>A UV RNA. We also detected another minor difference in the two protein profiles corresponding to a 50 kDa protein band which was more intense in the presence of UV RNA. Based on this result, it seemed plausible that the c.5242C>A variant does not interfere with the ability of proteins to bind an ESE but instead creates a sequence element which binds more tightly *trans*-acting factors playing an inhibitory function in splice site recognition. In order to test this hypothesis, we performed western blot analysis of the proteins bound to the WT and c.5242C>A UV RNAs using antibodies specific for SC35 and for two hnRNPs, hnRNPA1 and hnRNP H/F. These last two proteins function as splicing inhibitors and have respective molecular weight similar to the protein species whose binding to the exon 18 was increased by the c.5242C>A UV. As shown in Fig. 2c, hnRNP H/F and hnRNP A1, but not SC35 or the control proteins U2AF65 and PTB (both of which have the ability to bind exonic sequences [11–13] and might interact with the pyrimidine rich element upstream of the UV; see also Fig. 4), preferentially bound to the c.5242C>A UV RNA.

In order to fully demonstrate that the two hnRNPs are recruited directly to the RNA, we performed UV cross-linking assays using a ³²P-labeled RNA substrate corresponding to the WT or c.5242C>A UV in the presence of HeLa nuclear extracts (Fig. 2d). The protein–RNA interaction profile was similar for the two RNAs except for a protein of about 35 kDa that preferentially cross-linked to the mutated RNA. Immunoprecipitation of the UV cross-linked complexes showed that while SC35 similarly bound to the two RNAs, hnRNP H/F and hnRNP A1 displayed a

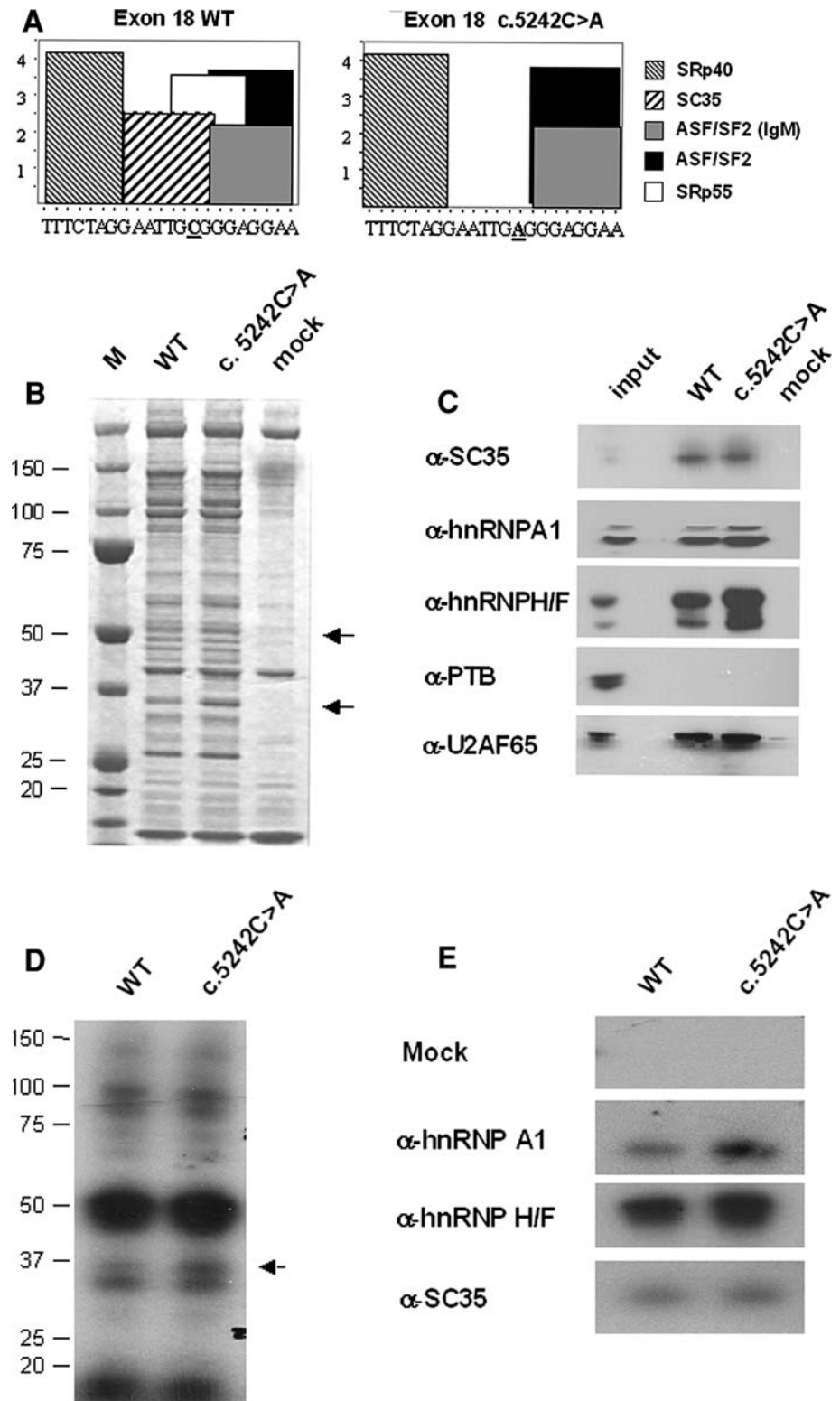
preferential binding activity to the c.5242C>A UV RNA (Fig. 2e). Taken together, our results showed that the c.5242C>A UV of *BRCA1* does not alter the binding of SR proteins but increases interaction of both hnRNP A1 and hnRNP H/F with the RNA.

In order to investigate the possibility that these two factors might form a regulatory splicing complex, we performed GST pull-down assays using recombinant hnRNP A1 in the presence of HeLa nuclear extracts. Western blot analysis revealed that recombinant hnRNP A1, but not the control protein R17, was able to bind both hnRNP H and F and, as expected, hnRNP A1 but not PTB from HeLa nuclear extract ([14]; Fig. 3a). The experiments were carried out in the presence of RNase A, suggesting that the interaction was not mediated through RNA binding. In order to understand whether hnRNP A1 could independently interact with both hnRNP H and F, and to provide evidence of a direct protein–protein interaction, we performed GST pull-down assays using purified recombinant hnRNP H and F proteins (Fig. 3b). Both recombinant hnRNP H and F similarly bound to hnRNP A1 and the R17 control protein. These results suggest that hnRNP A1 and hnRNP H/F do not interact directly and might assemble in a complex through the involvement of additional factor(s).

Other *BRCA1* exon 18 missense variants target hnRNP A1 and hnRNP H/F binding sites

Sequence alignment analysis revealed that, in addition to the c.5242C>A variant, other UVs of *BRCA1* exon 18 replaced very conserved nucleotides and co-localized with putative hnRNP A1 and hnRNP H/F binding sites (Fig. 4a). In particular, we found two motifs, TAGG (named A1a) and TAGTTA (named A1b), known to bind hnRNP A1 [15, 16] that are respectively located upstream and downstream of the c.5242C>A variant. A third motif, composed of three consecutive G (named H/F), which may serve as a hnRNP H/F binding site [17] is located just downstream of the c.5242C>A variant (Fig. 4a). In order to test whether the three sequence elements constitute binding sites for hnRNP A1 or hnRNP H/F, we performed UV cross-linking assays using exon 18 RNA substrates WT or mutated in each of the putative binding sites (named M A1(a), M A1(b) and M H/F; Fig. 4b, c). As positive controls of mutations affecting hnRNP A1 and/or hnRNP H/F binding, we included in the assay an exon 18 transcript containing either the c.5199G>T [3, 4] or the c.5242C>A (this study) variant. Immunoprecipitation of the UV cross-linked complexes showed that both the c.5199G>T and the c.5242C>A variants improved the hnRNPA1–RNA interaction, while mutations in the A1a and A1b elements abrogated the binding of hnRNP A1. This result is consistent with

Fig. 2 The c.5242C>A variant increases the RNA binding activity of hnRNP A1 and hnRNP H/F. **a** Analysis of the sequence encompassing the nucleotide at position 5242 in the WT and the c.5242C>A exon 18 *BRCA1* sequence using the ESEfinder software. The height of each *bar* indicates the score for each SR protein, the position along the *x* axis indicates its location along the exon 18, and the width of the *bar* represents the length of the motif. The C to A nucleotide change is *underlined*. **b** RNA affinity chromatography using biotinylated transcripts corresponding to the WT or UV sequence in the presence of HeLa nuclear extracts. Eluted proteins were visualized by Coomassie staining of SDS-PAGE gels. *Arrow* indicates the position of the 35 and 50 kDa proteins preferentially bound to the mutant RNA. Positions of size marker proteins (*M*) are indicated on the *left*. **c** Western blot analysis of the eluted proteins bound to the WT and UV RNAs was performed using antibodies against SC35, hnRNP A1, hnRNP H/F, PTB and U2AF65. The input accounts for the 5% of HeLa nuclear extract used in the assay. **d** UV cross-linking assays using a 32 P-labeled RNA substrate corresponding to the WT or the c.5242C>A variant were performed in the presence of HeLa nuclear extracts. *Arrow* indicates the position of the 35 kDa protein that preferentially cross-linked to the mutated RNA. **e** Immunoprecipitation of the UV cross-linked complexes was performed using antibodies directed against SC35, hnRNP H/F and hnRNP A1 for the WT and c.5242C>A mutant followed by SDS-PAGE and autoradiography



previous observations showing that the two TAG-containing elements can function as splicing silencers [4]. Similarly, a mutation in the H/F element depressed the RNA binding activity of hnRNP H/F. These results suggest that

missense UV affecting three hnRNP A1 and/or hnRNP H/F binding sites on exon 18 may affect splicing of *BRCA1* exon 18 by perturbing the binding of the two factors to their respective *cis*-elements.

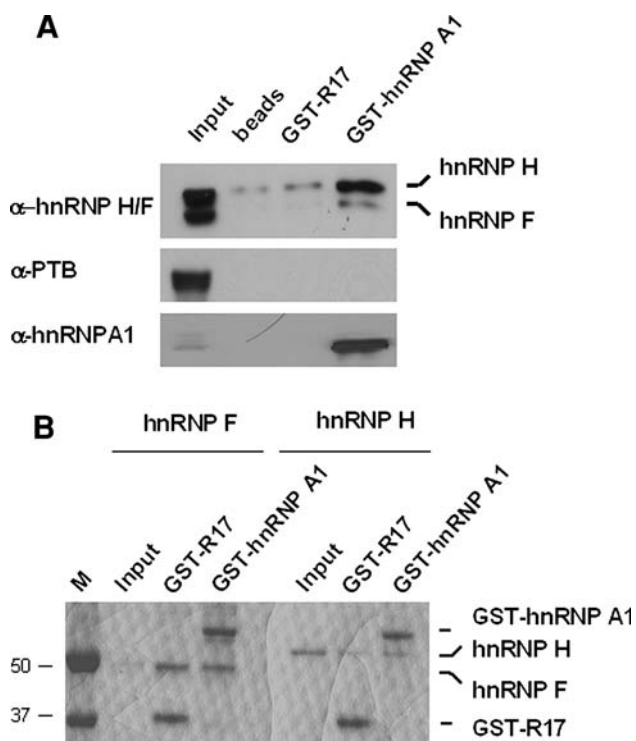


Fig. 3 Interaction between hnRNP A1 and hnRNP H/F. **a** In vitro GST pull-down assay using GST-tagged hnRNP A1 or R17 proteins and HeLa nuclear extracts, followed by western blot analysis using antibodies against hnRNP H/F, PTB and hnRNP A1. A 1/20th equivalent of the input nuclear extract is shown (*input*). Mock column for pull-down assay in the absence of a GST-tagged protein is shown. **b** Coomassie stained gel of an in vitro GST pull-down assays using GST-tagged hnRNP A1 or R17 proteins and purified recombinant His-tagged hnRNP H and F proteins. The positions of size marker proteins (*M*) are indicated on the *left*

Discussion

We showed that the c.5242C>A variant of *BRCA1* induces exon 18 skipping in vivo and in vitro, and that this is correlated with an increased binding of the splicing inhibitors hnRNP A1 and hnRNP H/F to the mutated exon 18. If translated, skipping of the in-frame exon 18 removes 26 amino acids (R1692-F1717) and changes the very conserved aromatic residue W1718 to G because of the junction of exon 17 to exon 19. This results in the disruption of the first BRCT domain. Since the BRCT region of *BRCA1* interacts with proteins involved in transcriptional control or DNA repair [18, 19], disruption of part of this region is expected to strongly affect the *BRCA1* function.

Several works have attempted to define the impact of the c.5242C>A variant on *BRCA1* expression, localization and function. Tumors from carriers of this variant display histopathological features associated with *BRCA1* tumors [6, 20]. This finding correlates well with many studies showing that *BRCA1* encoded by a cDNA containing the A1708E missense change has altered functional and protein interaction properties [5, 6, 20–22] probably due to a modification in the folding of the BRCT domain [6, 23]. Recently, Lovelock and colleagues investigated the consequence of the c.5242C>A variant on *BRCA1* mRNA expression [6]. In this study, RT-PCR analysis from RNA extracted by cycloheximide-treated EBV transformed lymphoblastoid cell lines from heterozygous carriers of the variant, revealed no evidence of aberrant splicing. In contrast, using RNA extracted from peripheral-blood

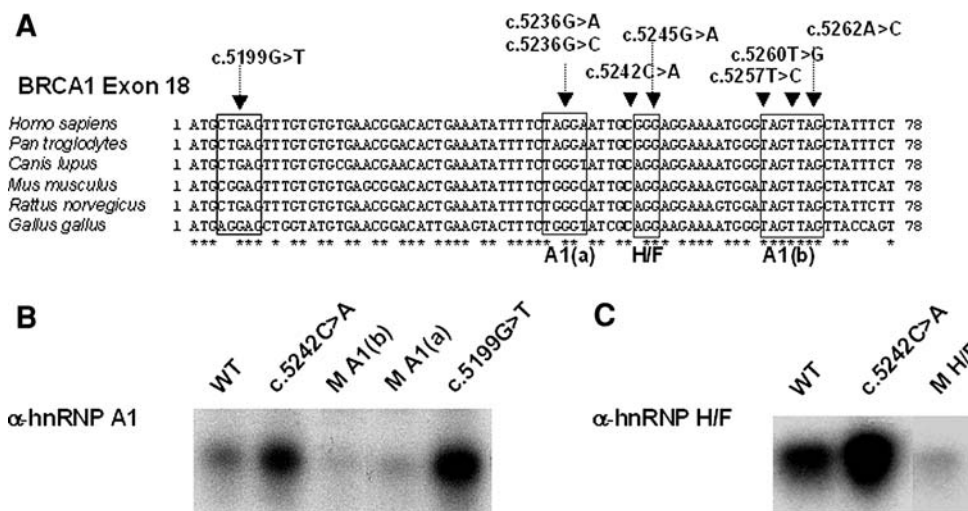


Fig. 4 Other UVs located on *BRCA1* exon 18 replace very conserved nucleotides and map to elements bound by hnRNP A1 and hnRNP H/F. **a** Sequence alignment of vertebrates *BRCA1* exon 18. The position of several UVs is indicated by an *arrow*. Analysis of the human *BRCA1* exon 18 revealed the presence of putative hnRNP A1 (named *A1a* or *A1b*) and hnRNP H/F (named *H/F*) binding sites, which might

be affected by the indicated UVs. **b** UV crosslinking of HeLa nuclear extract proteins to the uniformly ³²P-labeled transcripts corresponding to *BRCA1* exon 18 WT or containing a mutation in each of the putative binding site (called *M A1(a)*, *M A1(b)* and *M H/F*), or the c.5199G>T or c.5242C>A UVs, followed by immunoprecipitation with antibodies against hnRNP A1 or hnRNP H/F

leukocytes, we showed that the c.5242C>A variant induces skipping of the exon 18 in several variant carriers. This result was confirmed in *in vitro* splicing assays using a minigene construct comprising exons 17–19. The discrepancy between the RT-PCR analyses can be explained by the different procedures used to obtain RNA from carriers of this variant. Also, our results showing that the mutated exon 18 was predominantly but not totally skipped (Fig. 2b), do not exclude the possibility that a fraction of *BRCA1* mRNAs containing the mutated exon 18 can escape the aberrant splicing, thus encoding a misfolded protein with altered functional properties.

Several lines of evidence indicated that exonic mutations alter the expression of a disease-associated gene by inducing skipping of constitutive exons. Two models have been proposed to explain the mechanism of mutation-induced splicing defects: the disruption of an ESE and the creation of an ESS. The two models have been extensively explored in several disease-causing genes, including *BRCA1* [1, 3, 4]. In a recent report, the nonsense c.5199G>T exon 18 variant was proposed to induce exon skipping by creating a sequence with silencer properties, which bind hnRNP A1 [3, 4]. In agreement with this study, our results support a model in which the variant increases the binding of two splicing regulators, hnRNP A1 and hnRNP H/F, which may act by repressing the inclusion of the mutated exon in the *BRCA1* mRNA transcript. The increased RNA binding activity of hnRNP H/F can be explained by the fact that 5242 C to A transversion creates a AGGG motif, which is a higher affinity binding site for hnRNP H/F than the natural CGGG motif [24]. In contrast, the 5242 C to A mutation does not create a TAG motif that appears to be the hallmark of the hnRNP A1 binding site [25] but an AGGG element. Since previous studies have shown that this motif can also bind hnRNP A1 with high affinity [15, 16], we propose that the 5242 C to A variant just upstream of a GGG motif not only increases the affinity of hnRNP H/F for the RNA, but also creates an hnRNP A1 binding site. Interestingly, this novel hnRNP A1 binding motif in the mutated exon 18 is flanked by two TAG elements, which are natural hnRNP A1 binding sites (Fig. 4). In agreement with previous studies [26], the presence of consecutive hnRNP A1 binding sites might result in its propagation along the exon, thus blocking the access to the splicing machinery.

In summary, our results showing that the c.5242C>A variant of *BRCA1* exon 18 induces aberrant exon 18 skipping, provide additional evidence of its pathogenicity. The correlation between aberrant *BRCA1* exon 18 splicing and the increased interaction of the splicing regulators hnRNP A1 and hnRNP H/F to the mutated RNA strongly support the role of splicing regulation as a genetic modifier of *BRCA1*. In agreement with other studies [4, 27–29], our

report also suggests that *in silico* predictions must be used with caution when evaluating the consequence of a UV on splicing. RNA analysis is therefore necessary to the assessment of UVs as a mutation in genetic counseling.

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