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Functional redundancy of exon 12 of *BRCA2* revealed by a comprehensive analysis of the c.6853A>G p.I2285V variant

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Abstract

Variants of Unknown Significance (VUS) in *BRCA1* and *BRCA2* are common, and present significant challenges for genetic counseling. We observed that *BRCA2*: c.6853A>G (p.I2285V) (Brest cancer Information Core [BIC] name: 7081A>G; <http://nhgri.nih.gov/bic/>) co-occurs in trans with the founder mutation c.5946delT (p.S1982RfsX22) (BIC name: 6174delT), supporting the published classification of p.I2285V as a neutral variant. However, we also noted that when compared with wild-type *BRCA2*, p.I2285V resulted in increased exclusion of exon 12. Functional assay using allelic complementation in *Brca2*-null mouse embryonic stem cells revealed that p.I2285V, an allele with exon 12 deleted and wild-type *BRCA2* were all phenotypically indistinguishable, as measured by sensitivity to DNA-damaging agents, effect on irradiation-induced Rad51 foci formation, homologous recombination and overall genomic integrity. An allele frequency study showed the p.I2285V variant was identified in 15/722 (2.1%) Ashkenazi Jewish cases and 10/475 (2.1%) ethnically-matched controls, odds ratio: 0.99 (95% confidence interval: 0.44–2.21), *P* = 0.97. Thus the p.I2285V variant is not associated with an increased risk for breast cancer. Taken together, our clinical and functional studies strongly suggest that exon 12 is functionally redundant and therefore missense variants in this exon are likely to be neutral. Such comprehensive functional studies will be important adjuncts to genetic studies of variants.

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Keywords

BRCA2; unclassified variants; co-occurrence; exon splicing enhancer; exon skipping; in-frame deletion; neutral variant; Embryonic Stem (ES) cells

Introduction

Variants of unknown significance (VUS), also known as unclassified variants (UV), account for a small, but nevertheless significant, proportion of all variants identified in *BRCA1* (OMIM#113705) and *BRCA2* (OMIM#600185). Interpreting such variants pose significant challenges for both clinicians and patients. The unclassified variant NM_000059.3: c.6853A>G (p.I2285V) appears to be restricted to the Ashkenazi Jewish population and has been reported 79 times in the BIC database (<http://nhgri.nih.gov/bic/>) (BIC name: 7081A>G) [Mutation Nomenclature used in this paper: nucleotide numbering reflects cDNA numbering, with +1 as the A of the ATG initiation codon in the reference sequence (BRCA2: NM_000059.3) (www.hgvs.org/mutnomen). The initiation codon is codon 1. Traditional numbering based on the BIC mutation database is also provided. It is the most frequently reported variant in exon 12, and was identified 97 times in 68,000 tests performed by Myriad Genetics Laboratories, Salt Lake City, UT. Several studies of *BRCA1/2* mutations in women with breast or ovarian cancer have identified p.I2285V in affected women and have designated p.I2285V as a VUS (Shih et al., 2000; Pal et al., 2005). We were requested to offer genetic counseling to an unaffected member of a family who carries this variant. In this context, it was reported to us (by a Canadian clinical molecular genetics laboratory) to be possibly associated with aberrant splicing of exon 12 of BRCA2, resulting in a truncated protein. If exclusion of exon 12 occurs, the result is an in-frame deletion of 32 amino acids (codon 2281–2312). This isoform is known as BRCA2ΔE12.

Splice site mutations are a common cause of exclusion of an exon and many have been identified in *BRCA1* and *BRCA2* (Frank et al., 2002). In addition, variants that do not alter the consensus splice site sequences but affect splicing have been identified (Liu et al., 2001). These mutations result in the interruption of exon splicing regulators and have been increasingly recognized as important causes of exon exclusion or intron inclusion (Cartegni et al., 2002). Since the BRCA2: p.I2285V was reported to be associated with increased exclusion of exon 12 in one family, we were interested to establish if this were the case in other individuals carrying this variant, and whether disruption of an ESE was a likely mechanism. Moreover, we wondered if exclusion of exon 12 was associated with an identifiable phenotype.

Interestingly, it had been previously reported that breast cancer tissues show increased amount of E12 skipping transcript (BRCA2ΔE12) (Bieche and Lidereau, 1999), but no specific function has been attributed to this polypeptide. Notably, several cell lines such as MDA-MB-468, MCF-7, OVCA420, DOV13 and SKOV3 (among others) have increased expression of this variant. In addition, the prostate cancer cell line DU-145 expresses BRCA2 ΔE12 at much greater levels than the full-length isoform (Rauh-Adelmann et al., 2000). Despite these findings, co-segregation and family history analyses have shown that p.I2285V is unlikely to be a highly penetrant allele (Easton et al., 2007). This view is supported by the recently published work of Tavtigian and colleagues, which predicts that BRCA2: p.I2285V is a neutral variant, because the two independent measures used (missense mutations outside of the DNA binding domain and family history likelihood ratio) place this variant in a group that has a prior probability of <0.05 (Tavtigian et al., 2008). The potential effect of a variant on splicing, however, is not measured in these analyses. Taking all the observations together, it seems possible that this variant affects splicing and thus has

a subtle effect on risk, perhaps akin to the risk associated with so-called low penetrance breast cancer genes identified recently (Pharoah et al., 2008). Here, we describe the (1) investigation of the relationship between this variant and exclusion of exon 12; (2) the functional consequences of exclusion of exon 12 of *BRCA2*; and (3) the potential effect of this variant on breast cancer risk.

Materials and Methods

BRCA2: p.I2285V pedigrees

We collected information from four families where at least one individual carried the BRCA2: p.I2285V variant. These pedigrees are shown in supplementary figures 1a–d. Lymphocyte RNA and DNA was extracted from one carrier of the variant from each family and were used in the experiments described below. All participants signed an informed consent form. The details of each family are described in the figure legend accompanying supplementary figure 1.

Testing for inclusion or exclusion of exon 12 in humans

We confirmed the co-occurrence of *c.6853A>G* (p.I2285V) (BIC name: *7081A>G*) with another deleterious BRCA2 mutation *c.5946delT* (p.S982RfsX20) (BIC name: *6174delT*), using a blood sample from a patient carrying both alleles (see supplementary figure 2 and accompanying legend). We used RNA extracted from blood samples from this proband and three others (supplementary figure 1) using PAXgene (Qiagen, Valencia, CA) kit and cDNA was synthesized using Sensiscript (Qiagen). We used the following PCR primers to semi-quantitatively compare the degree of exon 12 skipping between the cells lines the wild-type line (referred to as WLT) and the double variant line (referred to as DBV): 1) for exon 12 inclusive transcript: Forward (located in exon 12): 5′ – CCCTTATCTTAGTGGG AGAACCCTCA-3′, Reverse (located in exon 13): 5′ – AGTTGTGCGAAAGGGTACACAGGT-3′; 2) for constitutive exon 11: Forward (located in exon 11): 5′ – TTGAGGTAGGGCCACCT GCATTTA-3′, Reverse (located in exon 11): 5′ – ATCCAATGCCTCGTAACAACCTGC-3′; 3) for exon 12 skipping transcript: Forward (located at junction of exon 11 and exon 13): 5′ – ATCTTAGTGGGCACAATAAAAG-3′; Reverse (located in exon 13): 5′ – CAGAAATTCTTGA CCAGGTGCGGTA-3′. The relative intensity of the PCR products was measured by using GeneTool software (Syngene, Frederick, MD).

Testing for the functional significance of exclusion of BRCA2 exon 12 in mouse embryonic stem (ES) cells

To test the functional significance of p.I2285V and BRCA2ΔE12 variants, mutant BAC DNAs were generated in the human *BRCA2* BAC RP11-777I19 by recombineering. A mutant BAC DNA containing the p.I2285V variant was constructed by two step ‘hit and fix’ method as described previously (Yang and Sharan, 2003). The BRCA2ΔE12 variant was generated in the BAC by using *galK* selection and counter-selection method as previously described (Warming et al., 2005). The sequences of the oligonucleotides are available upon request. Mutant BAC DNAs were then introduced in PL2F7 mouse ES cells followed by deletion of the endogenous mouse *Brca2* gene by Cre-mediated recombination (Kuznetsov et al., 2008). Recombinant clones lacking endogenous *Brca2* were selected in HAT media as described previously (Kuznetsov et al., 2008). We tested the efficiency of the DNA repair function by challenging the recombinant cells with DNA-damaging agents: N-methyl-N′-nitro-N-Nitrosoguanidine (MNNG), mitomycin C (MMC), methylmethane sulfonate (MMS), cisplatin, ultra-violet light and γ -irradiation. Significance of the survival difference between mutant and wild-type cells was assessed by two-tailed t-test at drug concentrations corresponding to LD₅₀. We tested the efficiency of homologous recombination, RAD51 foci

formation and karyotype of the wild-type and the recombinant cells as described previously (Kuznetsov et al., 2008).

For the RT-PCR, we synthesized cDNA using RNA isolated from mouse ES cells expressing wild-type, I2285V and Δ exon12 mutant human *BRCA2* gene using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Region flanking exon 12 was amplified with a forward primer B2ex11FRT (5'-CCAAGTCATGCCACACATTC-3') and a reverse primer B2ex14RRT (5'-ATTCTTGACCAGGTGCGGTA-3') from exons 11 and 14, respectively, and the PCR conditions included: one cycle of 94°C for 5 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, followed by incubation at 72°C for 5 min. Amplification products were separated in a 1.6% agarose gel.

Allelic association study

As controls, the study enrolled 475 healthy women collected through the New York Cancer Project (NYCP). The NYCP is a cohort study involving consent for biospecimen collection and follow-up of 8,000 healthy volunteers in the same geographical region as the cases used in this study (Mitchell et al., 2004). For the cases, we genotyped 722 consecutive breast cancer cases unselected for family history or BRCA1/2 status seen at Memorial Sloan-Kettering Cancer Center (New York, NY) and collected for the study under IRB approved protocols. All individuals in the study were self identified as Ashkenazi Jewish. For genotyping of BRCA2: p.I2285V we used TaqMan allelic discrimination assay under standard manufacture's protocol (Applied Biosystems, Foster City, CA). The alleles were called using SDS 2.1 software (Applied Biosystems, Foster City, CA).

Results

Conservation and co-occurrence

When we examined the amino acid sequence at position p.2285 of human *BRCA2* with Align-GVGD (<http://agvgd.iarc.fr/alignments/>) (Tavtigian et al., 2006; Mathe et al., 2006), we found it to be relatively conserved across vertebrate *BRCA2* orthologs. This residue is located within a conserved motif encoded by exon 12. From humans through to pufferfish (tetraodon), this position is either Ile or Leu. Val at this position can be considered to be a relatively conservative substitution because the three amino acids are structurally similar, each with a nonpolar side chain.

We confirmed that *c.6853A>G* co-occurs with a deleterious variant by testing 85 Ashkenazi Jewish individuals from Montreal and Toronto who had previously been found to carry the *6174delT* (*c.5946delT*) variant in *BRCA2*. We identified one living person who carries both variants (supplementary figure 1). We re-contacted this person and established a lymphoblastoid cell line (LCL), hereon referred to as DBV (for double variant). Being unable to study other family members, we could not establish phase genetically. Instead, by using RNA from this cell line, we showed that exon 12 inclusion and exclusion transcripts were produced from both alleles, but the degree of exclusion was much greater with for the I2285V-associated allele than for the *c.6174delT*-associated allele. We were able to demonstrate that these variants occur in *trans* (supplementary Figure 2). This supports the previous segregation and family history data showing that p.I2285V is very unlikely to be a highly penetrant allele (as discussed above). Nevertheless, we were left with the question of the splicing out of exon 12 – is it frequently associated with BRCA2:p.I2285V, and if so, what is the consequence of this?

Effect of c.6853A>G on the BRCA2 mRNA

The clinical protein truncation test (PTT) of *BRCA2* on one individual carrying this variant indicated that exon 12 skipping occurs to a greater degree in this individual than the level in the control samples (data not shown). We searched for splicing regulatory elements located in exon 12 by using the following on-line prediction tools: ESEFinder: <http://rulai.cshl.edu/tools/ESE/>; RESCUE-ESE: <http://genes.mit.edu/burgelab/rescue-eese/ESRsearch:http://ast.bioinfo.tau.ac.il/ESR.htm> and PESX: <http://cubweb.biology.columbia.edu/pesx/>. Using RESCUE-ESE, we identified putative ESEs at the region where the *BRCA2*: c.6853A>G variant is located. Notably, the variant “G” allele disrupts three of overlapping putative ESEs (Figure 1A). By studying exon 12 retention in 4 individuals who carry the c.6853A>G variant and two controls, we were able to show quantitatively that this variant significantly increases the exclusion of exon 12 of *BRCA2* (Figure 1B). The test we used here was to see if the variant was retained in exon 12 by capillary sequencing of exon 12 inclusion transcripts, as a variant promoting exon 12 skipping would result in a diminished signal of itself in the retained exon 12. As shown in Figure 1B, among 3 heterozygotes (I2285V-a, I2285V-b and I2285V-c), the c.6853A>G variant (resulting in p.I2285V) is barely detectable in retained exon 12, indicating the “G” variant strongly promotes exon 12 skipping. This causes extremely skewed A:G allele ratio in retained exon 12, to a degree that the G allele (C as sequenced with a reverse primer) is below the detection power of capillary sequencing. The interpretation of the results is somewhat more complicated for the lymphoblastoid cell line DBV (Figure 1B, fifth sample), as this cell line is derived from individual compound heterozygote for c.6853A>G and c.6174delT (discussed above and shown in supplementary figure 2). It is possible that the ratio of A:G has been reverted by the presence of a degree of nonsense-mediated decay of the A allele intact transcript, resulting from the upstream c.6174delT mutation on this allele, which introduces a premature stop codon. Despite the fact that the upstream c.6174delT may equally causes some decay of the *BRCA2* E12 transcript produced from this allele in individual DBV (in which c.6853A>G and c.5946delT are present in *trans*), a greater degree of exon 12 skipping was observed in this cell line (as the sum of the skipping products from both alleles) than in the wild-type counterpart lymphoblastoid cell line WLT (Figure 1C), suggesting that c.6853A>G enhances exon 12 skipping. Therefore, the biologically relevant outcome of this single base substitution is likely to be the disruption of a putative ESE, resulting in increased exclusion of exon 12. The creation of a novel amino acid could also have some biological significance, but this seems less likely as the change of I to V is only just outside the tolerated range. Our next goal was to establish if this splicing out of exon 12 has any functional consequences.

Functional test of c.6853A>G variant and complete deletion of exon 12

To test the functional significance of deletion of exon 12, mouse ES cell based functional assay was used. The *BRCA2*ΔE12 variant was generated in the BAC DNA coding for human *BRCA2* and introduced into mouse ES cells containing a single functional copy of *Brca2* that can be deleted by Cre-mediated recombination. Mouse ES cells are not viable without a functional copy of *Brca2*. After Cre-mediated deletion of endogenous mouse *Brca2*, we tested the ability of human transgene to rescue the lethality of *Brca2*-null ES cells. We tested 6 independent ES cell clones expressing the *BRCA2*ΔE12 transgene and 5 clones expressing c.6853A>G variant for viability and found no difference compared to the wild-type (data not shown). RT-PCR analysis of mouse ES cells expressing *BRCA2*ΔE12 variant confirmed the deletion of 96 bp encoded by exon 12 (Figure 2, lanes 5 and 6). These cells do not express the wild-type transcript. Similarly, ES cells expressing the p.I2285V variant resulted predominantly in transcripts lacking exon 12 (Figure 2, lanes 3 and 4, lower band). However, this variant also resulted in reduced amount of transcripts that retained the exon 12 (Figure 2, lanes 3 and 4, upper band). (Note the presence of some *BRCA2*ΔE12 product, mirroring the situation *in vivo* in humans). Taken together, these results show that

the deletion of exon 12 of *BRCA2* does not affect the viability of mouse ES cells. We then tested the *Brca2*-null ES cells expressing the *BRCA2* Δ E12 variant for other known functions of *BRCA2*. In all experiments, we compared the results for the *BRCA2* Δ E12 variant with those obtained for clones that expressed p.I2285V and wild-type *BRCA2*.

Two clones were then tested extensively for sensitivity to DNA-damaging agents and we found no significant difference for either the *BRCA2* Δ E12 allele or the p.I2285V allele compared to the control cells for any of the six agents used (Figure 3, panels A–F). As *BRCA2* has a role in the recruitment of RAD51 to the DNA repair sites (Yuan et al., 1999), we tested the effect of the deletion of exon12 of *BRCA2* and of the p.I2285V allele on radiation induced RAD51 foci formation. Both the *BRCA2* Δ E12 and the p.I2285V allele-containing cells formed similar a number of foci as the cells expressing wild-type *BRCA2* (Figure 4). The known role of *BRCA2* in homologous recombination and genomic stability (Khanna and Jackson, 2001) prompted us to test these cells for their efficiency of homologous recombination and genomic instabilities. For the former, we examined the efficiency of homologous recombination using a gene-targeting vector as described previously (Kuznetsov et al., 2008). This targeting vector targets *Gt(Rosa)26Sor* (abbreviated here as *Rosa26*) locus. We observed similar targeting efficiency in the ES cells expressing *BRCA2* Δ E12, p.I2285V or wild-type *BRCA2* (Figure 5A). The karyotyping of these cells also did not reveal any significant increase of chromosomal abnormalities due to deletion of exon12 (Figure 5B and C). Based on these results we conclude that the deletion of exon12 of *BRCA2* does not significantly affect any of its known functions. We have previously demonstrated the utility of the ES cell based assay to detect subtle functional defects in variants that are predicted to be of low risk (Kuznetsov et al., 2008). However, the possibility of clinically relevant effects of this allele on breast cancer risk that were not due to the known functions of *BRCA2* that we assayed here still remained, so we probed the function of p.I2285V from a clinical perspective.

Allelic association study

The loss of exon 12 could have an effect on breast cancer risk that might be insufficiently large to be evident from pedigree analysis, but might still be clinically relevant. To address this question, we assessed the frequency of *BRCA2*: c.6853A>G in on-going series of Ashkenazi Jewish breast cancer cases and ethnically matched controls (total n = 1197). We identified 10 carriers in 475 controls, and 15 carriers in 722 cases, resulting in an odds ratio for the association between this allele and breast cancer of 0.99, 95% confidence interval 0.44–2.21, P = 0.97. When we adjusted for age, the odds ratio became 0.97 (95% CI: 0.41–2.12), P = 0.88. Setting the alpha at 5%, a study of this size has an 80% power to rule out an allele frequency of 0.053 or greater in the cases, when the allele frequency in controls is 0.021.

Discussion

Here, we describe the analysis of *BRCA2*: c.6853A>G (p.I2285V) (BIC nomenclature, 7081A>G), a previously reported VUS. We first confirmed that *BRCA2*: c.6853A>G co-occurs in *trans* with another deleterious allele in *BRCA2*. We also showed that this variant promotes exclusion of exon 12 of *BRCA2*, but is not associated with a clinically relevant increase in breast cancer risk. Importantly, we have carried out a comprehensive analysis of the functional consequences of loss of exon 12 of *BRCA2*, using a murine ES cell system, wherein functional *BRCA2* is required to rescue *Brca2*-null murine ES cells (Kuznetsov et al., 2008).

Inappropriate splicing out of functionally critical exons is associated with disease. Most of the mutations that affect splicing are those that disrupt splice sites (Hastings and Krainer,

2001). However, missense mutations that affect splicing have also been identified (Cartegni et al., 2002). Our results show that *BRCA2: c.6853A>G* is one such variant, most likely disrupting ESEs located in exon 12. It is notable that I2285, and the surrounding amino acids (KRRGEPS_I_KRNLLNEFDR) are well conserved through vertebrate evolution. Thus, the question of the significance of loss of exon 12 is an interesting one. As noted, loss of exon 12 results in a BRCA2 protein that is shorter than the wild-type form only by 32 amino acids, and this part of BRCA2 does not contain any known important functional motifs. Nevertheless, *BRCA2ΔE12* has been reported to be more commonly found in breast cancers than in normal tissues from the same individuals (Bieche and Lidereau, 1999). In this study of breast cancer and matched normal tissue from 12 women, the proportion of *BRCA2ΔE12* transcripts was higher in the tumor in four pairs, and in no pairs was the *BRCA2ΔE12* higher in the normal tissue than in the tumor tissue. Similarly, in a study of 7 ovarian cancer cell lines, 5 were found to have a two- to five-fold increase in *BRCA2ΔE12* compared with mean values found in cultures of normal human ovarian surface epithelium. The same over-expression in tumor cell lines, rather than normal epithelium, was seen in one of four breast cancer cell lines and one of four prostate cancer cell lines. In the case of DU-145, the wild-type isoform of BRCA2 was barely detectable, whereas the truncated isoform, *BRCA2ΔE12*, was strikingly over-expressed (Rauh-Adelmann et al., 2000). Despite these findings, the functional analyses presented here indicate that *BRCA2ΔE12* can assume the biological role of full-length BRCA2. Very likely, the increased level of *BRCA2ΔE12* in cancer cells is an epiphenomenon rather than a causative mechanism.

We do not think *BRCA2ΔE12* represents a clinically important splice variant of BRCA2. Nevertheless, it is important to investigate all potentially deleterious intra-exonic VUS using several approaches. For example, the *BRCA2* variant *c.8393C>G* (p.T2722R) was initially found to result in an out-of-frame fusion of exons 17 and 19 of *BRCA2* (Fackenthal et al., 2002). Later, it was found that the skipping of exon 18 is not complete (erratum, Am J Hum Genet, December 2003) and thus the significance of the original finding was questioned. Subsequently, both genetic (Easton et al., 2007) and functional (Kuznetsov et al., 2008) analyses have confirmed that this variant is deleterious.

Although the allelic association study we have carried out has not ruled out a small effect on risk, 14,199 Ashkenazi Jewish cases and an equal number of controls would be required to rule out an odds ratio of 1.25 (with the same α and $1-\beta$ as above). Therefore, it remains possible that the splicing out of exon 12 does result in some subtle perturbations in risk for breast cancer, but at the current time, we cannot detect such an effect, if it exists at all.

The Breast Cancer Information Core (BIC) (<http://research.nhgri.nih.gov/bic/>) provides easily accessible information on numerous variants, but is dependent upon timely entry of new information about all variants. Based on a new classification system for VUS that was determined by an IARC working group and recently published (Plon et al., 2008), we suggest that this variant should now be referred to a class 1 variant (probability of being pathogenic of less than 0.1%). This conclusion is amply supported by other recently-published model-based evidence (Spearman et al., 2008).

Here, we show for the first time, that loss of an exon of a tumor suppressor gene has no measurable consequences across a range of activities. The technique described here could be used to comprehensively assess the separate functionality of each exon of *BRCA2*, and the findings raise the question of a possible class of exons that are dispensable for *BRCA2* tumor suppressing functions. Indeed, earlier biochemical studies have revealed that heterologous fusion proteins consisting of BRCA2 domains interacting with Rad51 and Dss1 are capable of restoring DNA repair in *BRCA2* deficient cells. The BRCA2 domains mediating association with Rad51 and Dss1 are the BRC repeats (central) and OB

(oligosaccharide-binding, C-terminal) domains, respectively (Saeki et al., 2006). Notably, the peptide encoded by exon 12 is not involved in either of these domains. Thus the murine ES system used here could be used to investigate which functions, if any, are impaired in cells that lack various combinations of *BRCA2* exons. It is interesting to note that exons 10 and 12 of *BRCA2* do not encode functionally critical peptides and are in-frame (i.e. the reading frame will not be altered when these exons are not represented in the transcript). Therefore, missense variants located in these two exons that alter an amino acid and/or cause skipping of the entire exon are likely neutral. This statement should be tempered by the possibility that variants could exist that may have unpredictable effect on the stability or structural integrity of *BRCA2* protein. However, such variants are likely to be rare. In support of this hypothesis, on reviewing the published literature (Easton et al., 2007; Spearman et al., 2008; Gomez Garcia et al., 2009) and the BIC <http://research.nhgri.nih.gov/bic/>, we were unable to identify any known pathogenic missense variants in exons 10 or 12 of *BRCA2*. While not conclusive, this observation supports our contention that exon 12 (and possibly exon 10) is functionally dispensable. Using the murine ES cell system reported here, it will be interesting to see, if such redundancy is seen in other multi-exon tumor suppressor genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Bieche I, Lidereau R. Increased level of exon 12 alternatively spliced *BRCA2* transcripts in tumor breast tissue compared with normal tissue. *Cancer Res.* 1999; 59:2546–2550. [PubMed: 10363970]
2. Cartegni L, Chew SL, Krainer AR. Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat Rev Genet.* 2002; 3:285–298. [PubMed: 11967553]
3. Easton DF, Deffenbaugh AM, Pruss D, Frye C, Wenstrup RJ, Ien-Brady K, Tavtigian SV, Monteiro AN, Iversen ES, Couch FJ, Goldgar DE. A systematic genetic assessment of 1,433 sequence variants of unknown clinical significance in the *BRCA1* and *BRCA2* breast cancer-predisposition genes. *Am J Hum Genet.* 2007; 81:873–883. [PubMed: 17924331]
4. Fackenthal JD, Cartegni L, Krainer AR, Olopade OI. *BRCA2* T2722R is a deleterious allele that causes exon skipping. *Am J Hum Genet.* 2002; 71:625–631. [PubMed: 12145750]
5. Frank TS, Deffenbaugh AM, Reid JE, Hulick M, Ward BE, Lingenfelter B, Gumpfer KL, Scholl T, Tavtigian SV, Pruss DR, Critchfield GC. Clinical characteristics of individuals with germline mutations in *BRCA1* and *BRCA2*: analysis of 10,000 individuals. *J Clin Oncol.* 2002; 20:1480–1490. [PubMed: 11896095]
6. Gomez Garcia EB, Oosterwijk JC, Timmermans M, van Asperen CJ, Hogervorst FB, Hoogerbrugge N, Oldenburg R, Verhoef S, Dommering CJ, Ausems MG, van Os TA, van der Hout AH, Ligtenberg M, van den OA, van der Luijt RB, Wijnen JT, Gille JJ, Lindsey PJ, Devilee P, Blok MJ, Vreeswijk MP. A method to assess the clinical significance of unclassified variants in the *BRCA1* and *BRCA2* genes based on cancer family history. *Breast Cancer Res.* 2009; 11:R8. [PubMed: 19200354]
7. Hastings ML, Krainer AR. Pre-mRNA splicing in the new millennium. *Curr Opin Cell Biol.* 2001; 13:302–309. [PubMed: 11343900]

8. Khanna KK, Jackson SP. DNA double-strand breaks: signaling, repair and the cancer connection. *Nat Genet.* 2001; 27:247–254. [PubMed: 11242102]
9. Kuznetsov SG, Liu P, Sharan SK. Mouse embryonic stem cell-based functional assay to evaluate mutations in BRCA2. *Nat Med.* 2008; 14:875–881. [PubMed: 18607349]
10. Liu HX, Cartegni L, Zhang MQ, Krainer AR. A mechanism for exon skipping caused by nonsense or missense mutations in BRCA1 and other genes. *Nat Genet.* 2001; 27:55–58. [PubMed: 11137998]
11. Mathe E, Olivier M, Kato S, Ishioka C, Hainaut P, Tavtigian SV. Computational approaches for predicting the biological effect of p53 missense mutations: a comparison of three sequence analysis based methods. *Nucleic Acids Res.* 2006; 34:1317–1325. [PubMed: 16522644]
12. Mitchell MK, Gregersen PK, Johnson S, Parsons R, Vlahov D. The New York Cancer Project: rationale, organization, design, and baseline characteristics. *J Urban Health.* 2004; 81:301–310. [PubMed: 15136663]
13. Pal T, Permuth-Wey J, Betts JA, Krischer JP, Fiorica J, Arango H, LaPolla J, Hoffman M, Martino MA, Wakeley K, Wilbanks G, Nicosia S, Cantor A, Sutphen R. BRCA1 and BRCA2 mutations account for a large proportion of ovarian carcinoma cases. *Cancer.* 2005; 104:2807–2816. [PubMed: 16284991]
14. Pharoah PD, Antoniou AC, Easton DF, Ponder BA. Polygenes, risk prediction, and targeted prevention of breast cancer. *N Engl J Med.* 2008; 358:2796–2803. [PubMed: 18579814]
15. Plon SE, Eccles DM, Easton D, Foulkes WD, Genuardi M, Greenblatt MS, Hogervorst FB, Hoogerbrugge N, Spurdle AB, Tavtigian SV. Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. *Hum Mutat.* 2008; 29:1282–1291. [PubMed: 18951446]
16. Rauh-Adelmann C, Lau KM, Sabeti N, Long JP, Mok SC, Ho SM. Altered expression of BRCA1, BRCA2, and a newly identified BRCA2 exon 12 deletion variant in malignant human ovarian, prostate, and breast cancer cell lines. *Mol Carcinog.* 2000; 28:236–246. [PubMed: 10972993]
17. Saeki H, Siaud N, Christ N, Wiegant WW, van Buul PP, Han M, Zdzenicka MZ, Stark JM, Jasin M. Suppression of the DNA repair defects of BRCA2-deficient cells with heterologous protein fusions. *Proc Natl Acad Sci U S A.* 2006; 103:8768–8773. [PubMed: 16731627]
18. Shih HA, Nathanson KL, Seal S, Collins N, Stratton MR, Rebbeck TR, Weber BL. BRCA1 and BRCA2 mutations in breast cancer families with multiple primary cancers. *Clin Cancer Res.* 2000; 6:4259–4264. [PubMed: 11106241]
19. Spearman AD, Sweet K, Zhou XP, McLennan J, Couch FJ, Toland AE. Clinically Applicable Models to Characterize BRCA1 and BRCA2 Variants of Uncertain Significance. *J Clin Oncol.* 2008; 26:5393–5400. [PubMed: 18824701]
20. Tavtigian SV, Byrnes GB, Goldgar DE, Thomas A. Classification of rare missense substitutions, using risk surfaces, with genetic- and molecular-epidemiology applications. *Hum Mutat.* 2008; 29:1342–1354. [PubMed: 18951461]
21. Tavtigian SV, Deffenbaugh AM, Yin L, Judkins T, Scholl T, Samollow PB, de SD, Zharkikh A, Thomas A. Comprehensive statistical study of 452 BRCA1 missense substitutions with classification of eight recurrent substitutions as neutral. *J Med Genet.* 2006; 43:295–305. [PubMed: 16014699]
22. Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG. Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res.* 2005; 33:e36. [PubMed: 15731329]
23. Yang Y, Sharan SK. A simple two-step, ‘hit and fix’ method to generate subtle mutations in BACs using short denatured PCR fragments. *Nucleic Acids Res.* 2003; 31:e80. [PubMed: 12888532]
24. Yuan SSF, Lee SY, Chen G, Song MH, Tomlinson GE, Lee EYHP. BRCA2 is required for ionizing radiation-induced assembly of rad51 complex in vivo. *Cancer Res.* 1999; 59:3547–3551. [PubMed: 10446958]

Figure 1. Splicing at *BRCA2*: c.6853A>G in humans

A) Potential exonic splicing enhancer (ESEs) disrupted by *BRCA2*: c.6853A>G (p.I2285V) RESCUE-ESE (<http://genes.mit.edu/burgelab-rescue-ese/>) identified putative ESEs at the region where the *BRCA2*: c.6853A>G variant is located. Notably, the variant “G” allele disrupts three of overlapping putative ESEs.

B) Splicing of *BRCA2* exon 12 in humans carrying the c.6853A>G allele

The amplicon of exon 12–13 was sequenced using a reverse primer located at exon 13. The position of c.6853 in the sequencing chromatogram is indicated by a dashed box. The wild-type A allele (shown here as T on the reverse strand) is the major signal in the three c.6853A>G carriers (I2285V-a, I2285V-b and I2285V-c) and a non-carrier control (control). The cell line DBV carries double variants of c.5946delT and c.6853A>G. The result shown for DBV suggests that there is no technical allele bias in the PCR. WLT is a wild-type control line carrying neither variant.

C) Relative quantification of exon 12 skipping in WLT and DBV LCLs

Three different amplicons were produced: E12–13 with exon 12 inclusion, E12 with exon 12 skipping and E11 as a control of endogenous expression level. The primers are listed in the methods section. C: Semiquantitative comparison of exon 12 skipping in WLT and DBV: normalization was done by considering E11 expression as 1.00. The relative expression level of E12 intact vs. ΔE12 is shown as a percentage bar. Red bars: exon 12 skipping transcripts, blue bars: exon 12 intact transcripts. The error bar indicates the variance seen following four measurements.

Figure 2. RT-PCR demonstrating 96-bp deletion in p. I2285V and BRCA2ΔE12 mutants
 Ethidium Bromide stained gel showing RT-PCR results of two mouse embryonic stem cell clones generated independently for each variant. WT: wild-type, negative control: water control, FL: fragment of a full length product, ΔE12: transcript with deletion of exon 12. Numbers below the gel picture are lane numbers. Marker is 1 kb plus ladder (Invitrogen).

Figure 3. Sensitivity of ES cells expressing mutant and wild-type BRCA2 to different DNA-damaging agents

Survival of ES cells expressing p.I2285V, BRCA2 Δ E12 mutants compared to ES cells expressing wild type BRCA2 after exposure to different DNA-damaging agents: Methyl-N'-nitro-N-Nitrosoguanidine (MNNG, A), mitomycin C (MMC, B), cisplatin (C), methyl-methanesulfonate (MMS, D), ionizing radiation (IR, E) and ultraviolet light (UV, F). Drug sensitivity was expressed as a percentage of surviving cells compared to untreated cultures. Two independently generated clones for each variant were tested and their average values are shown here. Error bars indicate standard deviations. *P* values were equal 0.19693 (I2285V); 0.16019 (BRCA2 Δ E12) for 5 μM MNNG, 0.09178 (I2285V); 0.08212

(BRCA2 Δ E12) for 10 ng/ml MMC, 0.5494 (I2285V); 0.4110 (BRCA2 Δ E12) for 0.4 μ M cisplatin, 0.11846 (I2285V); 0.74637 (BRCA2 Δ E12) for 15 μ g/ml MMS, 0.1131 (I2285V); 0.14066 (BRCA2 Δ E12) for 200 Rad IR and 0.3964 (I2285V); 0.4238 (BRCA2 Δ E12) for 10 J/m² UV.

Figure 4. Radiation-induced RAD51 foci formation in mouse ES cells expressing BRCA2 transgenes

A) RAD51 foci formation before (-IR) and after (+IR) ionizing radiation. RAD51 foci are shown in green, γ -H2AX foci marking sites of DNA damage are shown in red, nuclei are stained with DAPI (blue). The right-most panel shows the merged picture. Two independent clones showed similar results and only one of them is shown in each case.

B) Quantification of RAD51 and γ -H2AX foci before (-IR) and after (+IR) ionizing radiation. 30 nuclei were counted in each case and their mean values are shown. Error bars indicate means $\pm$ s.d.

Figure 5. Effect of BRCA2 variants on homologous recombination and genomic integrity in mouse ES cells

a) Homologous recombination efficiency shown by gene targeting at *Rosa26* locus. Numbers above the bars indicate the actual number of colonies undergoing homologous recombination compared to total number of colonies tested.

b) Karyotype analysis of p.I2285V, BRCA2ΔE12 mutants compared to WT control cells. Representative metaphases of the mutants and WT control cells are shown. Ten metaphases were counted in each case and scored for different chromosomal abnormalities. **c)** The identified chromosomal aberrations are tabulated.