Missense Variants of Uncertain Significance (VUS) Altering the Phosphorylation Patterns of BRCA1 and BRCA2

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Abstract

Mutations in BRCA1 and BRCA2 are responsible for a large proportion of breast-ovarian cancer families. Protein-truncating mutations have been effectively used in the clinical management of familial breast cancer due to their deleterious impact on protein function. However, the majority of missense variants identified throughout the genes continue to pose an obstacle for predictive informative testing due to low frequency and lack of information on how they affect BRCA1/2 function. Phosphorylation of BRCA1 and BRCA2 play an important role in their function as regulators of DNA repair, transcription and cell cycle in response to DNA damage but whether missense variants of uncertain significance (VUS) are able to disrupt this important process is not known. Here we employed a novel approach using NetworKin which predicts in vivo kinase-substrate relationship, and evolutionary conservation algorithms SIFT, PolyPhen and Align-GVGD. We evaluated whether 191 BRCA1 and 43 BRCA2 VUS from the Breast Cancer Information Core (BIC) database can functionally alter the consensus phosphorylation motifs and abolish kinase recognition and binding to sites known to be phosphorylated in vivo. Our results show that 13.09% (25/191) BRCA1 and 13.95% (6/43) BRCA2 VUS altered the phosphorylation of BRCA1 and BRCA2. We highlight six BRCA1 (K309T, S632N, S1143F, Q1144H, Q1281P, S1542C) and three BRCA2 (S196I, T207A, P3292L) VUS as potentially clinically significant. These occurred rarely (n<2 in BIC), mutated evolutionary conserved residues and abolished kinase binding to motifs established in the literature involved in DNA repair, cell cycle regulation, transcription or response to DNA damage. Additionally in vivo phosphorylation sites identified via through-put methods are also affected by VUS and are attractive targets for studying their biological and functional significance. We propose that rare VUS affecting phosphorylation may be a novel and important mechanism for which BRCA1 and BRCA2 functions are disrupted in breast cancer.

Introduction

Rare germline mutations of BRCA1 and BRCA2 predispose carriers to early-onset familial breast or ovarian cancers [1–3]. These genes can account for half of breast and/or ovarian familial cancer aggregates (whereas the remaining families receive inconclusive results) and are responsible for about 5–10% of all breast cancer cases and 10–15% of ovarian cancers in the general population [4,5]. Clinically informative results from BRCA screening have been mostly derived from protein-truncating mutations presenting as indels, nonsense codons and splice variants as well as large genomic rearrangements [3,6,7]. Such mutations have very apparent impacts on the normal protein function and have been widely utilized in the clinical management of familial breast and ovarian cancers. However, further analysis of a significant number of BRCA1 and BRCA2 missense variants of uncertain significance (VUS) continue to pose an important obstacle to the clinical management of a considerable portion of familial breast cancer probands and families who carry such VUS.

Previously, the need to characterize missense variants to provide risk assessment to individuals from high-risk families led to development of several approaches in classifying VUS. These include integrating interspecies sequence variation [8–10], functional analysis to uncover the consequences of VUS on protein function [11–14], genetic assessment approaches including pedigree analysis [15], likelihood models [16], structural-based approaches to model the effect of amino acid substitution [17,18] and transcriptional activity assays [19]. These studies have provided important information into the clinical significance of BRCA mutations.

Phosphorylation is an important post-translational modification that occurs at specific serine, tyrosine and threonine residues within protein sequences [20]. The phosphorylated residue is surrounded by a kinase interaction/recognition motif that is typically comprised of 7–12 amino acids [21] and that kinase specificity is determined by the identity of these residues [22,23].
Our studies have previously suggested that missense VUS and commonly occurring single nucleotide polymorphisms (SNPs) altering phosphorylation patterns of cell cycle and DNA repair proteins may contribute to human cancer risk [24,25] and our preliminary analysis showed that many of the missense variants in BIC are found within the consensus motifs of sites known to be phosphorylated in vivo. Despite this wealth of information, the potential functional impact of these rare VUS remains uncharacterized. In the present study, our goal is evaluate the potential consequences of missense VUS on kinase recognition and phosphorylation of BRCA1 and BRCA2 proteins. Accordingly, we have utilized the web-based algorithm NetworKIN 2.0 [26] and selectively tested the missense VUS listed in the BIC database that are located within 10 amino acids around the experimentally verified and biologically characterized phosphorylation sites as well as residues identified via high-throughput methods to be phosphorylated in vivo. Here, we analyzed 191 BRCA1 and 43 BRCA2 missense VUSs, which have the potential to interfere with the phosphorylation process via abolishing or creating phosphorylation sites on BRCA1 and BRCA2.

Methods

Selection of in vivo Phosphorylation motifs for analysis

A comprehensive list of known phosphorylation sites of BRCA1 and BRCA2 was obtained from the curated databases PhosphoSitePlus [27] and Phospho. ELM [28] as of August 2012. We evaluated BRCA1 and BRCA2 missense variations’ effect in relation to 44 and 11 phosphorylation sites reported in humans, respectively (Figure 1a, b). Based on the curated databases, all sites selected were reported to be phosphorylated in vivo and reported in the literature. Kinase binding and biological significance of the phosphorylation on protein function had been demonstrated for sixteen sites in BRCA1 and six sites in BRCA2. Accordingly, these experimentally characterized sites are denoted “biologically characterized” in this manuscript. The remaining sites were previously identified as phosphorylated in vivo using high-throughput methods (e.g. Mass spectrometry) where a definitive biological significance in protein function has not yet been shown and are designated as “biologically uncharacterized” in this manuscript.

Missense VUS from the Breast Cancer Information Core Database

The National Institute of Health (NIH)’s Breast Cancer Information Core (BIC) database (http://research.nhgri.nih.gov/bic/) contains 11 types of genetic variations. These genetic variations are identified by studying the tumor DNA samples and may therefore be either inherited or somatic variations. Using the most up-to-date version of the BIC database as of August 2012, 591 BRCA1 and 883 BRCA2 missense VUSs were retrieved. Only VUS located in or within a 10 amino acids sequence upstream and downstream of a phosphorylation site were selected for analysis. A total of 191/591 BRCA1 and 43/883 BRCA2 missense variants located in or near a kinase recognition motif were included in this study.

NetworKIN analysis of VUS on BRCA1 and BRCA2 phosphorylation

BRCA1 (Genbank P38393) and BRCA2 (Genbank P51587) protein sequences were queried by the NetworKIN Beta 2.0 algorithm (http://networkkin.info/version_2_0/search.php) [26], an improved version of the NetworKIN algorithm featuring more kinases. The NetworKIN tool is designed to predict in vivo kinase-substrate relations [26]. It remains up to date with the most current human phosphoproteome information derived from Phospho.ELM and PhosphoSite databases and these sites are compared with sequence motifs predicted using the Scansite [29] and NetPhosK [30] programs to predict the kinase families that potentially bind and phosphorylate such sequences. The algorithm takes into account also the biological context of a kinase through the use of probabilistic functional associations from the STRING database [31].

The BRCA1 or BRCA2 protein sequences carrying each VUS substitution was queried by NetworKIN and the output matched to predictions made for the wild-type protein sequence. VUS which result in abolishing kinase binding at the phosphorylation motif or create a site at the altered residue are included in this report. Furthermore only the predictions for kinase-phosphorylation motif interactions with a NetworKIN score ≥5 were considered reliable (Dr. Rune Linding, personal communication). In cases where multiple kinases are predicted to bind a phosphorylation site with a NetworKIN score ≥5 we arbitrarily assumed the abolition of 80% or more of the kinase binding to be the equivalent to the complete abolition of a phosphorylation motif.

Evolutionary conservation analyses

To determine whether the missense VUSs substitute functionally critical residues we have investigated their evolutionary conservation status using: (1) Sorting Intolerant From Tolerant (SIFT; http://blocks.fhcrc.org/sift/SIFT.html). SIFT (V.2) is a multiple sequence alignment tool that was developed based on the idea that amino acids which play an important role tends to be conserved in the protein family, so changes at these sites would be deleterious to protein function [32]. SIFT analysis was performed using algorithms to find homologous sequences from database SWISS-PORT version 51.3 and TrEMBL 34.3, and selecting median conservation score sequence score 3.00. Predictions out of the accepted median sequence conservation score of 2.75–3.25 were also considered not reliable and thus were considered “not informative”–. (2) PolyPhen (Phenotypic Polymorphism); (http://genetics.bwh.harvard.edu/pph2/). PolyPhen-2 v.2.2.2(398) predicts the impact of an amino acid substitution on the structure and function of a human protein [33]. (3) Align-grantham variation grantham deviation (A-GVGD) specific weighted evolutionary conservation analysis was carried out for BRCA1 and BRCA2 (http://http://agvgd.iarc.fr/agvgd_input.php) to determine the A-GVGD class of each variants presented [10]. A-GVGD uses the biochemical characteristics of amino acids together with protein sequence alignments of multiple species to determine whether a missense mutation could be neutral or deleterious to protein function. A-GVGD was used with all default settings. Library alignments for BRCA1 and BRCA2 were selected and analysis was performed using the longest evolutionary depth (Human to Sea Urchin).

Although PolyPhen also uses other assessment criteria such as protein 3-dimensional structure, both SIFT and PolyPhen use alignment of similar proteins to determine whether an amino acid is conserved and whether its substitution by a VUS has potential functional consequences. To standardize the predictions made by these two tools, we have annotated the “affecting protein function” prediction of SIFT and both the “probably damaging” and “possibly damaging” predictions of PolyPhen as “damaging” in this report. Similarly, the “tolerated” prediction of SIFT and the “benign” prediction of PolyPhen are collectively annotated as “benign”. For any predictions that include a “damaging” and...
“benign/tolerated” output of either program, we have annotated such VUS as “likely damaging”.

Results

Study design and overall findings

Using NetworKIN Beta 2.0, we investigated the impact of 191 BRCA1 and 43 BRCA2 missense VUS found within or around 44 BRCA1 and 11 BRCA2 phosphorylation sites, respectively (Figure 1a, b, Tables S1 & S2 in File S1). Our analysis indicated that 13.09% (25/191) BRCA1 and 13.95% (6/43) BRCA2 VUSs impact an existing phosphorylation site, and/or create a new site at the altered residue (Table 1, 2). Specifically six BRCA1 and three BRCA2 VUS resulted in deleterious NetworKIN predictions at experimentally and biologically characterized phosphorylation sites while nineteen BRCA1 and three BRCA2 VUS similarly affected biologically uncharacterized phosphorylated sites. In cases where NetworKIN predictions of kinases differ from those identified experimentally, we found in most cases the prediction fell within the same family of protein kinases. The Leiden Open Variation Database (LOVD v.2.0 build 35; http://chromium.liacs.nl/LOVD2/cancer/home.php) was accessed and VUS highlighted by this study and included in previous studies are summarized in Table S3 and S4 in File S1.

VUS impacting biologically characterized phosphorylation sites

Six BRCA1 VUS (K309T, S632N, S1143F, Q1144H, Q1281P, S1542C) were predicted to affect the phosphorylation status of BRCA1 by abolishing kinase interaction at experimentally verified sites Ser308, Ser632, Ser1143, and Ser1281 (Table 1). Three of the aforementioned substitutions (S632N, S1143F, S1542C) directly altered the Serine residue of the phosphorylated sites Ser308, Ser1143, and Ser1281, resulting in the complete abolition of their respective kinase binding without creating new kinase binding. In BRCA2, S196I and P3292L VUS altered the consensus kinase motif for Ser193 and the sequence for CDK2 binding for Ser1291, respectively and T207A directly altered the phosphorylated Threonine residue and completely abolished kinase binding at Thr207 (Table 1).

VUS impacting biologically uncharacterized phosphorylation sites

A total of nineteen BRCA1 and three BRCA2 VUS were found to affect biologically uncharacterized phosphorylation sites. These sites were shown to be phosphorylated in vivo experiments; however their potential roles on protein and subsequent cellular function have not been investigated yet. Affecting BRCA1 were twelve VUS associated with the complete abolition of kinase binding motif without creating binding sites for kinases. These VUS included the S1217P, S1218C, T1550I, S1577P, and T1720A, which removed the phosphorylated residues at Ser1217, Ser1218, Thr1550, Ser1577, and Thr1720, respectively (Table 2). Additionally, seven VUS substituted the wild-type residue with Y, S or T resulting in the creation of putative kinase binding site at the altered residue. In BRCA2, three VUS, D1923A, D1923V and P3194Q, were all predicted to abolish kinase binding while none was predicted to create a new kinase binding site (Table 2).

Evolutionary conservation of VUS

SIFT and PolyPhen analyses were performed to evaluate whether the residues altered by VUS disrupting protein phosphorylation are damaging to protein function. Multiple sequence
| Protein | Mutation\(^a\) | Nucleotide Change\(^b\) | SNP Id\(^c\) | Exon | BIC Freq\(^d\) | NetworKIN Results\(^e\) | SIFT/Polyphen/A-GVGD | Biological Significance of Affected Phosphorylation Motif |
|---------|---------------|------------------|-------------|------|----------------|-----------------|------------------|------------------------------------------------------|
| BRCA1   | p.K309T       | c.926A>C         | rs80356877  | 11A  | 1              | T309 abolishes STK6 binding at S308 in FCNK5|Q2QG and creates ATM binding to T309 in FCNK5|Q2QG | Damaging (C0) | Loss of STK6 binding decreases G2 to M transition of the cell cycle in cells \((50)\) |
| BRCA1   | p.S632N       | c.1895G>A        | rs80356983  | 11B  | 1              | N632 abolishes CDK2 binding to S632 in VSRNL5|Q2QNPCTNT and creates CDK2 binding to T633 in VSRNL5|Q2QNPCTNT | Likely Damaging (C0) | S632A affects BRCA1-dependent regulation of transcription \((48)\) |
| BRCA1   | p.P633T       | c.1897C>A        | N/A         | 11B  | 1              | T633 abolishes CDK2 binding to S632 in VSRNL5|Q2QNPCTNT and creates CDK2 binding to T633 in VSRNL5|Q2QNPCTNT | Likely Damaging (C0) | S632A affects BRCA1-dependent regulation of transcription \((48)\) |
| BRCA1   | p.P633S       | c.1897C>T        | rs80356902  | 11B  | 1              | S633 abolishes CDK2 binding to S632 in VSRNL5|Q2QNPCTNT and creates CDK2 binding to T633 in VSRNL5|Q2QNPCTNT | Likely Damaging (C0) | S632A affects BRCA1-dependent regulation of transcription \((48)\) |
| BRCA1   | p.S1143F      | c.3428C>T        | rs80357434  | 11D  | 1              | F1143 abolishes ATM binding to S1143 in SSHA5|SQVCSE | Likely Damaging (C0) | S1143 inactivation reduces intracellular localization of BRCA1 into MMTS-induced loci \((46)\) |
| BRCA1   | p.Q1144H      | c.3432G>T        | rs80356922  | 11D  | 1              | H1144 abolishes ATM binding to S1143 in SSHA5|SQVCSE | Likely Damaging (C0) | S1143 inactivation reduces intracellular localization of BRCA1 into MMTS-induced loci \((46)\) |
| BRCA1   | p.Q1281P      | c.3842A>C        | rs80357483  | 11D  | 2              | F1281 abolishes ATM binding to S1280 in LAKA5|Q2QEHHL | Damaging (C0) | S1280 inactivation reduces intracellular localization of BRCA1 into MMTS-induced loci \((72)\) |
| BRCA1   | p.S1542C      | c.4625C>G        | rs41293457  | 15   | 2              | C1542 abolishes CSNK2A2, CK2A1 binding to S1542 in QLEELS|QPHDL | Likely Damaging (C0) | S1542 phosphorylated by ATM and possibly involved in response to DNA double-strand breaks produced by ionizing radiation \((49)\) |
| BRCA2   | p.S196I       | c.587G>T         | rs80358818  | 7    | 1              | I1961 abolishes TGFBR2, ACVR2B binding to DAMaging (C65) | S193 in VDPDMSAWSSS | Phosphorylation of S193 regulates BRCA2 interaction with p300/CBP-associated factor (P/CAF) \((56)\) |
| BRCA2   | p.T207A       | c.619A>G         | rs80358858  | 7    | 2              | A207 abolishes NEK2 binding to T207 in Damaging (C55) | TLS5TV1|LIR | Phosphorylation of T207 regulates BRCA2 interaction with p300/CBP-associated factor (P/CAF) \((56)\) |
| BRCA2   | p.P3292L      | c.9865C>T        | rs56121817  | 27   | 7              | P3292 abolishes CDK2, MAPK11, MAPK13, MAPK14 binding to S3291 at CTPVS|PAQQK | Damaging (C0) | S3291 phosphorylation necessary for recombinatory repair \((44,45)\) |

\(^a\)In **bold** are BRCA1 mutations that directly mutate an experimentally identified phosphorylation site. \(^b\)The position and change at the amino acids specified by the missense variant is as reported in the BIC database. \(^c\)The nucleotide change conforms to the HGVS nomenclature. \(^d\)SNP IDs correspond to the dbSNP database \((73)\) SNP identifiers. \(^e\)Frequency represents the number of times reported in the BIC database. The ten-residue long biologically uncharacterized kinase recognition motifs are shown. The biologically uncharacterized Serine (S), and threonine (T) residues shown to be phosphorylated by NetworKIN are underlined.

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| Protein | Mutation | Nucleotide Change | SNP Id | Exon | BIC | NetworKIN Results | SIFT/Polyphen/A-GVGD | Biological pathway of Phosphorylation site |
|---------|----------|-------------------|--------|------|-----|-------------------|----------------------|------------------------------------------|
| BRCA1   | p.S403F  | c.1208C>T         | rs80356934 | 11A  | 1   | F403 abolishes CK2A1 and CSNK2A1 binding to S403 in HDGESESNAK | Benign (C0) | Cell cycle regulation by protein phosphorylation by cyclin-dependent kinases (CDK) [65] |
| BRCA1   | p.N417S  | c.1250A>G         | rs80357113 | 11A  | 2   | S417 creates CK2A1, CSNK2A1 binding to S417 in VLDVLNEVDE | Benign (C0) | |
| BRCA1   | p.D420Y  | c.1258G>T         | rs80357488 | 11A  | 3   | Y420 creates IGF1R, INSR binding to Y420 in VLNVEYESG | Damaging (C15) | |
| BRCA1   | p.S454N  | c.1361G>A         | rs80357181 | 11A  | 1   | N454 abolishes CK2A1 and CSNK2A1 binding to S454 in KSVE3IEDK | Benign (C0) | DNA damage response following ionizing radiation (IR) [66] |
| BRCA1   | p.N609S  | c.1826A>G         | rs80357326 | 11A  | 1   | S609 creates PRKDC binding to S609 in APKK5ILRKR | Likely Damaging (C0) | |
| BRCA1   | p.R612G  | c.1834A>G         | rs80357245 | 11A  | 1   | G623 abolishes RP56R81 binding to S615 in LRRK3STIRI | Likely Damaging (C0) | Cell growth, proliferation via Akt-RSK-56 signaling network [42] |
| BRCA1   | p.D749Y  | c.2245G>T         | rs80357114 | 11B  | 1   | Y749 abolishes CK2A1 and CSNK2A1 binding to S753 in KDLMLSGERVL | Damaging (C0) | Phosphorylation site occupancy during Mitosis [65,67] |
| BRCA1   | p.G1201S | c.3601G>A         | rs55725337 | 11D  | 3   | S1201 creates NEK2, PRKCD, PRKC1, PRKCQ, PRKCZ, PRKCA, PRKCG binding at HLQ2YRIRGA | Benign (C0) | |
| BRCA1   | p.E1214K | c.3655G>A         | N/A     | 11D  | 9   | K1214 abolishes CK2A1 and CSNK2A1 binding to S1211* in AKKLESSEEN and S1212 in KKLESSEENL | Damaging (C0) | |
| BRCA1   | p.S1217P | c.3649T>C         | N/A     | 11D  | 1   | P1217 abolishes CK2A1 and CSNK2A1 binding to S1218 in EENL5SEDEE | Damaging (C65) | |
| BRCA1   | p.S1218C | c.3652A>T         | rs80356894 | 11D  | 2   | C1218 abolishes CSNK2A2, CK2A1 binding to S1218 in EENL5SEDEEL | Damaging (C25) | Phosphorylation site occupancy during Mitosis [65,67] |
| BRCA1   | p.T1550I | c.4649C>T         | rs80357076 | 15   | 3   | I1550 abolishes NEK2 binding to T1550 in HDLTE5YLP | Benign (C0) | Phosphorylation sites in cellular proteins sensitive to rapamycin [74] |
| BRCA1   | p.S1577P | c.4729T>C         | rs80356909 | 16   | 1   | P1577 abolishes CSNK2A2, CK2A1 binding to S1577 in SDDPESOSPE | Likely Damaging (C0) | Phosphorylation site occupancy during mitosis [67] |
| BRCA1   | p.A1584S | c.4750G>T         | rs80357070 | 16   | 1   | S1584 creates CDK2, MAPK8, MAPK10, MAPK9, MAPK14, MAPK11, MAPK13 binding at S1584 in PSEDPSPEA | Benign (C0) | |
### Table 2. Cont.

| Protein | Mutation* | Nucleotide Changeb | SNP Idc | Exon | BIC Freqd | NetworKIN Results* | SIFT/Polyphen/A-GVGD | Biological pathway of Phosphorylation site |
|---------|-----------|--------------------|--------|------|-----------|--------------------|-----------------------|------------------------------------------|
| BRCA1   | p.F1695L  | c.5085T>A          | rs80357837 | 18   | 1         | L1695 abolishes TGFBR2, ACVR2B, PRKCD, PRKC, PRKCG, PRKCA, PRKCA, PRKCG, MST2 binding at T1700 in FVCERTILKP | Likely Damaging (C0) | DNA damage response [55] |
| BRCA1   | p.R1699L  | c.5096G>T          | rs14293459 | 18   | 1         | L1699 abolishes PRKCD, PRKC, PRKCG, PRKCA, PRKCA, PRKCG, MST2 binding at T1700 in FVCERTILKP | Damaging (C65) | DNA damage response [55] |
| BRCA1   | p.R1699W  | c.5095C>T          | rs55778010 | 18   | 13        | W1699 abolishes PRKCD, PRKC, PRKCG, PRKCA, PRKCA, PRKCG, MST2 binding at T1700 in FVCERTILKP | Damaging (C65) | DNA damage response [55] |
| BRCA1   | p.T1720A  | c.5158A>G          | rs56195342 | 19   | 15        | A1720 abolishes ATM binding to T1720 in YFWVTSQIKE | Likely Damaging (C0) | DNA damage response [55] |
| BRCA2   | p.D1923A* | c.5768A>C          | rs45491005 | 11E  | 9         | A1923 abolishes CSNK2A2, CK2A1 binding to S1926 in ADIQSEILQ | Damaging (C0) | General Mass Spec screen [61] |
| BRCA2   | p.D1923V* | c.5768A>T          | rs45491005 | 11E  | 1         | V1923 abolishes CSNK2A2, CK2A1 binding to S1926 in ADIQSEILQ | Damaging (C0) | General Mass Spec screen [61] |
| BRCA2   | p.P3194Q  | c.9581C>A          | rs28897760 | 26   | 6         | Q3194 abolishes CDK2 binding and creates ATM binding to T3193 in PKWSTPTKDC | Damaging (C0) | General Mass Spec screen [61] |

In **bold** are BRCA1 mutations that fall within an experimentally identified but biologically uncharacterized phosphorylation site. *The position and change at the amino acids specified by the missense variant is as reported in the BIC database. The nucleotide change conforms to the HGVS nomenclature. SNP IDs correspond to the dbSNP database [73] SNP identifiers. Frequency represents the number of times reported in the BIC database. The ten-residue long, biologically uncharacterized kinase recognition motifs are shown. The biologically uncharacterized Serine (S) and threonine (T) residues shown to be phosphorylated by NetworKIN are underlined. * Sites that retained a score but was considered to be “abolished” due to score falling below 5 with the presence of the VUS.

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alignment retrieved from Polyphen results were also organized to visualize if the VUSs affect evolutionarily conserved residues. We also used A-GVGD to assign classes of C0 (neutral) to C65 (likely deleterious) to each variant. A-GVGD classified the 6 BRCA1 VUS affecting biologically characterized sites as C0 or neutral while 66% (2/3) BRCA2 VUS were designated a higher class (Table 1). On the other hand 26.3% (5/19) of BRCA1 affecting uncharacterized sites were classified as possibly deleterious with 73.7% (14/19) and 100% (3/3) BRCA2 variants being C0 (Table 2).

Multiple sequence alignment from Polyphen demonstrated that 6 BRCA1 VUS affecting biologically characterized sites were highly conserved (Figure 2) and the substitutions were predicted as either likely damaging or damaging to the protein function (Table 1). Of the 19 BRCA1 VUS affecting biologically characterized sites, 68.42% (13/19) were predicted to be likely damaging or damaging to protein function while 31.58% (6/19) VUS were benign (Table 2).

In this study, we applied a prediction strategy based on the NetworKIN algorithm [26] to investigate the impact of VUS on the kinase-binding ability and phosphorylation patterns of BRCA1 and BRCA2 proteins. By targeting sites phosphorylated in vivo with clearly defined biological roles, NetworKIN analysis permits inference on biological and possibly clinical significance for any VUS that abolish kinase association at that residue. This is a significant advantage over predictions based on consensus sequence motifs recognized by active sites of enzymes alone. Therefore the method provides an effective way to identify VUS altering kinase association at key residues of biologically characterized phosphorylation sites and their potential impact can be inferred via validation assays in the literature. An added advantage of our approach is that NetworKIN can shed light on potential kinases that interact with phosphorylation sites confirmed to be phosphorylated in vivo using proteomic discovery methods but for which no additional experiments have yet been done to characterize their role in BRCA function.

VUS impacting the phosphorylation of BRCA1 and BRCA2

The sixteen biologically characterized phosphorylation sites for BRCA1 (Table S1 in File S1) studied are involved in activation or deactivation of the BRCA1 protein function including its stability, protein-interactions and sub-cellular location [34–36], its regulation of DNA repair [37–40] and its transcriptional activity [41–43]. The phosphorylation pattern of BRCA2 is less well known but it is shown to be essential in the regulation of BRCA2-mediated DNA recombination repair [44,45].

In this study, we applied a prediction strategy based on the NetworKIN algorithm [26] to investigate the impact of VUS on the kinase-binding ability and phosphorylation patterns of BRCA1 and BRCA2 proteins. By targeting sites phosphorylated in vivo with clearly defined biological roles, NetworKIN analysis permits inference on biological and possibly clinical significance for any VUS that abolish kinase association at that residue. This is a significant advantage over predictions based on consensus sequence motifs recognized by active sites of enzymes alone. Therefore the method provides an effective way to identify VUS altering kinase association at key residues of biologically characterized phosphorylation sites and their potential impact can be inferred via validation assays in the literature. An added advantage of our approach is that NetworKIN can shed light on potential kinases that interact with phosphorylation sites confirmed to be phosphorylated in vivo using proteomic discovery methods but for which no additional experiments have yet been done to characterize their role in BRCA function.

**Discussion**

BRCA1 interacts with many proteins to serve its function in the cell. Protein kinases have been shown to be critical in BRCA1-phosphorylation, where they are involved in activation or deactivation of the BRCA1 protein function including its stability, protein-interactions and sub-cellular location [34–36], its regulation of DNA repair [37–40] and its transcriptional activity [41–43]. The phosphorylation pattern of BRCA2 is less well known but it is shown to be essential in the regulation of BRCA2-mediated DNA recombination repair [44,45].
191) of BRCA1 and 6.98% (3/43) of BRCA2 VUS studied represent variants of potentially high clinical significance because they occur only very rarely (n^-2 in BIC) and are predicted to disrupt in vivo phosphorylated sites whose role in regulating BRCA1/2 functions have been biologically characterized. Lastly our results also suggest that VUS impacting phosphorylated sites tend to occur at evolutionarily conserved residues. Using the SIFT, Polyphen, and A-GVGD algorithms concurrently we ensured that all true positives were captured. This is important since the VUS impact in vivo phosphorylated sites and that the vast majority of the variants identified in this study do not fall within the functional domains of BRCA1 and BRCA2 where most pathogenic mutations to date are found.

Candidate BRCA1/2 VUS for disease association studies

Six BRCA1 VUS affected phosphorylation of BRCA1 at a biologically characterized site by altering the kinase motif and thus eliminating kinase binding. In particular, three of the VUS S632N, S1143F, and S1542C directly removed the S residue and completely abolished the biologically characterized phosphorylation sites at Ser632, Ser1143, and Ser1542, respectively. Although the remaining three VUS K309T, Q1144H, and Q1281P did not directly impact the phosphorylated residue, they were predicted to alter the consensus kinase binding motif, resulting in the abolition of a phosphorylation site. For BRCA2, S196I, T207A, and P3292L affected phosphorylation of previously biologically characterized phosphorylation sites at Ser691, Thr207, and Ser3291, respectively. Given that the biological function of the affected phosphorylation sites are known, these BRCA1 and BRCA2 VUS are excellent candidates for further association studies into pathogenicity. In the following section, we discuss the potential biological consequences of these VUSs based on studies demonstrating their functions.

BRCA1-K309T promotes aberrant chromosome segregation

Aurora-A/STK6 localizes to the centrosome in the G2-M phase, and its kinase activity positively regulates the G2 to M transition of the cell cycle [50]. It physically binds to and phosphorylates BRCA1 in vivo at Ser308 and that this interaction is required for the regulation of progression from G2 to M transition. As it has been shown that centrosome maturation from late S to M phase is essential in the completion of mitosis [51] and that Aurora-A has a role in inhibiting BRCA1-mediated centrosome nucleation in the late G2-M phase [52], the K309T VUS identified in breast cancer patients is a candidate mutation that may promote aberrant chromosome segregation resulting in multi-nucleation and multi-centrosomes often associated with breast cancers [53,54].

BRCA1-S632N affects BRCA1-mediated transcription

In vivo phosphorylation of BRCA1 at Ser632 by cyclin D1/cdk4 complex has been shown by Kehn et al [48] to inhibit DNA binding activity of BRCA1 to gene promoters during G0–G1 phase of the cell cycle. Among these gene promoters are involved in tumor suppression (RBP, APEX, SST, OAS1) as well as oncogenes involved in positively aiding tumor progression (ARGH,
rather than ATM for Ser1542 this may be explained by the fact that while NetworKIN predicted CSNK2A2 and CK2A1 binding cell proliferation and recovery processes. It should be noted that growth inhibition by ionizing radiation compared to wildtype identified from the same study were significantly more sensitive to abolished ATM binding to Ser1143 and Ser1280, suggesting these are likely to contribute to the tumorigenic process by interfering with BRCA1-mediated SSB DNA repair.

BRCA1-S1143F, Q1144H and Q1281P interfere with BRCA1-mediated single strand repair
Phosphorylation of Ser1143 and Ser1280 play a role in single strand break (SSB) DNA repair following alkylating agent methyl methanethiosulfonate (MMTS) exposure by contributing to the localization of BRCA1 to nuclear foci [46]. The authors showed that site-directed mutagenesis of Ser1143 and Ser1280 reduced the targeting of BRCA1 to MMTS-induced foci. Indeed, our results showing three VUS, S1143F, Q1144H and Q1281P, completely abolished ATM binding to Ser1143 and Ser1280, suggesting these are likely to contribute to the tumorigenic process by interfering with BRCA1-mediated SSB DNA repair.

BRCA1-S1542C deregulates BRCA1-mediated double stranded break repair
ATM phosphorylates BRCA1 at Ser1542 in vivo in response to double stranded breaks (DSB) induced by γ irradiation [49,55]. While it is unknown how phosphorylation at this site contributes to BRCA1 function, Cortez et al. demonstrated that site-directed mutagenesis of two of the seven sites (Ser1423 and Ser1542) identified from the same study were significantly more sensitive to growth inhibition by ionizing radiation compared to wildtype BRCA1 owing to the altered function of BRCA1 in post-exposure cell proliferation and recovery processes. It should be noted that while NetworKIN predicted CSNK2A2 and CK2A1 binding rather than ATM for Ser1542 this may be explained by the fact that in contrast to Ser1423 and Ser1542 along with four other sites identified in the study (Ser1143, Ser1280, Ser1457, Ser1466) were phosphorylated only when kinase reaction was allowed to proceed longer with higher concentrations of adenine triphosphate and ATM [49]. Nevertheless NetworKIN found that ATM was the predicted kinase for three of the four sites (Table S1 in File S1). This suggests that ATM is the most likely kinase for Ser1542 and that double-strand break DNA repair following ionizing radiation may be compromised by this VUS.

BRCA2-S196I and T207A disrupt interaction with P/CAF
Phosphorylation of highly conserved Ser196 and/or several Ser/Thr residues between codons 203–207 by the polo-like 1 (Plk1) kinase modulates BRCA2 dissociation from the p300/CBP-associated factor (P/CAF) [56]. Interestingly, while PLK1 was not associated with the predicted kinase for these sites, S196I and T207A VUSs were phosphorylated and kinase reaction was allowed to proceed longer with higher concentrations of adenine triphosphate and ATM [49]. Nevertheless NetworKIN found that ATM was the predicted kinase for three of the four sites (Table S1 in File S1). This suggests that ATM is the most likely kinase for Ser196 and that double-strand break DNA repair following ionizing radiation may be compromised by this VUS.

BRCA2-P3292L affects interaction with RAD51
BRCA2 Ser3291, the most well characterized phosphorylation site for BRCA2 located at the carboxy-terminal region, interacts with the recombination protein RAD51 [57]. It has been shown that phosphorylation of Ser3291 by CDKs blocks interaction between BRCA2 and RAD51 serving as a molecular switch for the regulation of recombination activity [44]. P3292L occurs at a highly conserved residue and abolishes CDK2 binding to Ser3291. This strongly suggests that this VUS is of high clinical significance and impact breast cancer by negatively affecting the interaction between BRCA2 and RAD51.

Candidate VUS for BRCA1/2 functional studies
In this study we have also identified 19 BRCA1 and 3 BRCA2 VUS (Table 2) that were predicted to alter known in vitro and in vivo phosphorylated sites, however, not yet characterized for their biological role in protein function or in breast cancer development. Overall, our findings indicated casein kinase II (CK2) and ATM to be important kinases that bind to many biologically uncharacterized but phosphorylated sites that are affected by VUS as discussed below.

Casein Kinase II (CK2) is a ubiquitous protein serine/threonine kinase involved in SSB repair of chromosomal DNA [50]. It was first described to bind and phosphorylate the carboxyl region of BRCA1 (amino acids between 1345–1863) at Ser1577 [59]. In cell cycle regulation it is required in the transition from G0 to G1 and G1 to S [60]. NetworKIN prediction showed that the predicted kinase for the biologically uncharacterized sites Ser454, Ser455, Ser479, Ser1214, P1502S and Ser1577 to be CK2 and CSNK2A1. In support of the functional significance of this observation, four of the five BRCA1 VUS (S454N, S1217P, S1218C and S1577P) which directly mutated serine residues at Ser454, Ser455, Ser479, Ser1214, P1502S and Ser1577 are predicted to be phosphorylated by CK2/CSNK2A1 binding to these sites. In fact 35% (7/20) BRCA1 VUS (S403F, S454N, D749Y, E1214K, S1217P, S1218C and S1577P) are predicted to result in the abrogation of CK2A1 and CSNK2A1 interaction on these sites while N417S and P1502S created a binding site for these two kinases at Ser417 and Ser1502, respectively.

These variants likely play a role in breast cancer predisposition by deleteriously affecting BRCA1-mediated cell cycle regulation and thus warrant further investigation. Interestingly in BRCA2, the biologically uncharacterized sites Ser1923 and Thr1919 identified from a general mass spectrometry screen in prostate cancer cells [61] and non-small cell lung cancer from the CST research group [62–64] are also predicted to be phosphorylated by the CK2 kinases. Two of the three BRCA2 VUSs (D1923V and D1923A), were predicted to abolish the CK2 kinase binding at Ser1923 which is a highly evolutionarily conserved residue, also making these variants valid targets for functional analyses in breast cancer.

Several phosphorylation sites were identified via mass spectrometry to detect phosphorylation in response to DNA damage [55,65–67]. Thr1708 and Thr1720 were identified from an ATM/ATR kinase analysis and NetworKIN also predicted ATM to be the kinase for Thr1720. Thr1708 in the C-terminal BRCT domain of BRCA1 is part of a hydrogen bonding network with the DNA helicase BACH1 and DNA resectioning factor CtIP [68,69] and our results show that VUSs (F1695L, R1699L and R1699W) reduce the consensus motif of Thr1700 to abolish the majority of kinase affinity. Interestingly R1699W is a variant known to be clinically significant as it reduces peptide binding to the pSer-x-x-Phe motifs in partner proteins that regulates the response to DNA damage [12]. These results suggest that a significant change in phosphorylation pattern of Thr1700 may also contribute to their clinical significance by altering the DNA damage response of BRCA1.

T1720A was the subject of several analyses including structural [70,71], transcription [11], transactivation [71] and phospho-peptide binding assays [70] because it was the sole BRCA1 alteration in individuals considered to be at high risk for breast or ovarian cancer. These analyses suggested T1720A to be of
neutral/low clinical significance. In our study, however, Networ-KIN predicted ATM binding to this site, which was removed by T1720A, therefore warrants further attention with respect to kinase recognition and binding.

Future Studies

In silico analysis greatly enhance our ability to make predictions on genetic variations for which currently no experimental evaluation is available. BRCA1 and BRCA2 variations found to affect kinase binding to these sites will be invaluable in the prioritization for further functional characterization and/or association studies in breast cancer. A follow-up study covering more comprehensive list of VUS compiled from various databases and literature sources will be a great value for the clinical management of disease in the families carrying them.

Conclusion

The results of this study suggest for the first time that missense VUS can influence the phosphorylation patterns of BRCA1 and BRCA2. The variants identified using in silico methods here are based on in vivo phosphorylated sites and the functional evidence for the corresponding observation were also supported by the literature. Therefore the VUSs highlighted in this study are key candidate mutations that alter phosphorylated motifs to prevent kinase interactions essential for the biological functions of BRCA1 and BRCA2, and represent important candidates for further analysis into disease susceptibility. Our approach and data provide novel insights into how mutations can alter the function of BRCA1 and BRCA2 through post-translational modifications such as phosphorylation. As new phosphorylation sites are identified and their kinase specificities and biological role are elucidated, it is likely that missense variants affecting this important process will significantly contribute to the clinical management of breast cancer.

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Supporting Information

File S1 Table S1, Summary of the BRCA1 phosphorylation motifs studied. A list of all BRCA1 phosphorylation sites studied. Bolded phosphorylation site represents in vivo phosphorylated residues. *STK6 score fell below the cut-off value of 5 but since it has previously been shown experimentally (Ouchi et al., 2004) it is included. ** S405 and S1286 were excluded from the study due to wildtype predictions below the score of 5. Table S2, Summary of the BRCA2 phosphorylation motifs studied. A list of all BRCA2 phosphorylation sites studied. Bolded phosphorylation site represents in vivo phosphorylated residues. * S206, S384, Y3009 were excluded from the study due to wildtype predictions below the score of 5. Table S3, BRCA1 and BRCA2 variants identified in this study to affect biologically characterized phosphorylation sites and were also previously reported in other publications (retrieved from the Leiden Open Variation Database 2.0 (Build 35)). Table S4, BRCA1 and BRCA2 variants identified in this study to affect biologically uncharacterized phosphorylation sites and were also previously reported in other publications (retrieved from the Leiden Open Variation Database 2.0 (Build 35)). (DOCX)

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Author Contributions

Conceived and designed the experiments: ET HO. Performed the experiments: ET. Analyzed the data: ET SS. Wrote the paper: ET.
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