Functional analysis of genetic variants in PALB2 and CHEK2: linking functional impact with cancer risk

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CHAPTER 3

3

CHEK2 variants: linking functional impact to cancer risk

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ABSTRACT

Protein-truncating variants in the breast cancer susceptibility gene *CHEK2* are associated with a moderate increased risk of breast cancer. In contrast, for missense variants of uncertain significance (VUS) in *CHEK2* the associated breast cancer risk is often unclear. To facilitate their classification, functional assays that determine the impact of missense VUS on CHK2 protein function have been performed. Here we discuss these functional analyses that consistently reveal an association between impaired protein function and increased breast cancer risk. Overall, these findings suggest that damaging *CHEK2* missense VUS associate with a similar risk of breast cancer as protein-truncating variants. This indicates the urgency for expanding the functional characterization of *CHEK2* missense VUS to further understand the associated cancer risk.

KEYWORDS

Breast Cancer; *CHEK2*; Variant of Uncertain Significance (VUS); Functional Assay; Variant Classification; Cancer Risk

CHEK2 and Cancer Predisposition

The CHK2 (see Glossary) protein kinase was initially identified as the mammalian homolog of the *Saccharomyces (S.) cerevisiae* Rad53 and *Schizosaccharomyces pombe* Cds1 protein kinases (1). Its characterization revealed an important role in cell cycle control and apoptosis following exposure of cells to DNA damaging agents (1,2). This involves the phosphorylation and activation of CHK2 by ataxia-telangiectasia mutated (ATM) kinase, and the subsequent modification of downstream substrates such as p53, CDC25A, CDC25C, KAP1 and BRCA1, Collectively, this may prevent genome instability and cancer development by instructing cells to stop proliferating and repair the DNA damage, or promote apoptosis as a response to inefficient or improper repair (Fig. 1). It is perhaps not surprising that shortly after its identification, frameshift variants such as the well-known c.1100del; p.T367Mfs variant, were identified in the *CHEK2* gene and were linked to a cancer susceptibility disorder called Li-Fraumeni syndrome (LFS) (3). LFS is a rare hereditary autosomal-dominant disorder that is characterized by a wide range of malignancies that appear at an unusually early age (4). Similar to CHK2, the well-described tumor suppressor protein p53 also halts cell division in response to DNA damage and inherited mutations in the corresponding gene (TP53), account for most cases of LFS (5). Interestingly, a link between CHK2 and p53 became evident when it was shown that CHK2 phosphorylates p53 on S20, resulting in dissociation of preformed p53-Mdm2 complexes and consequently in p53 stabilization (2). These observations suggested that CHK2 is a tumor suppressor protein that acts within the p53 signaling pathway.

In recent years, several studies have confirmed CHK2's tumor suppressive function by showing that truncating variants in the *CHEK2* gene (e.g., c.1100del; p.T367Mfs) are associated with a moderate-risk for breast cancer (two- to three-fold increased risk) (6-11). For heterozygous female carriers of *CHEK2* truncating variants, this translates to a lifetime risk of ~25% to develop breast cancer before the age of 80 years (6). Furthermore, Cybulski et al. characterized *CHEK2* as a multi-organ cancer susceptibility gene (12), which was confirmed by numerous other studies (reviewed in (13)). These findings have resulted in a significant increase in genetic testing for *CHEK2,* and consequently the identification of many rare missense variants for which clinical relevance is unclear. It is now evident that besides the high-risk breast cancer susceptibility genes *BRCA1*, *BRCA2* and *PALB2*, *CHEK2*, together with *ATM*, appear to be the most commonly mutated genes in the germline of breast cancer patients (6). In fact, 1148 distinct missense VUS in *CHEK2* have currently (as of February 2022) been reported in ClinVar (14). In aggregate, many of these rare missense variants, also termed missense variants of uncertain significance (VUS), also associate with breast cancer (odds ratio [OR], 1.42; 95% CI, 1.28-1.58; p=2.5x10^{11}) (6). This association appears to be independent of their position within the gene and thus their impact on any of the functional domains of CHK2; N-terminal SQ/TQ cluster domain (residues 19-69), a fork head-associated

(FHA) domain (residues 92-205), a serine/threonine kinase domain (residues 212-501), and a nuclear localization signal (NLS) (515-522) (Fig. 2). Knowing which missense variants impact protein function, and to what extent, can help distinguish which variants associate with increased breast cancer risk. To this end, the outcomes of quantitative and well-validated functional assays for *CHEK2*, in line with ACMG guidelines (15), can help to guide clinical classification of genetic variants in this gene, thereby improving the counseling of carriers. Indeed, several recent studies described the functional characterization of *CHEK2* variants. Here we review these studies by providing an overview of the different approaches and outcomes, discussing the potential pitfalls of functional assays, and associating the functional outcomes with breast cancer risk.

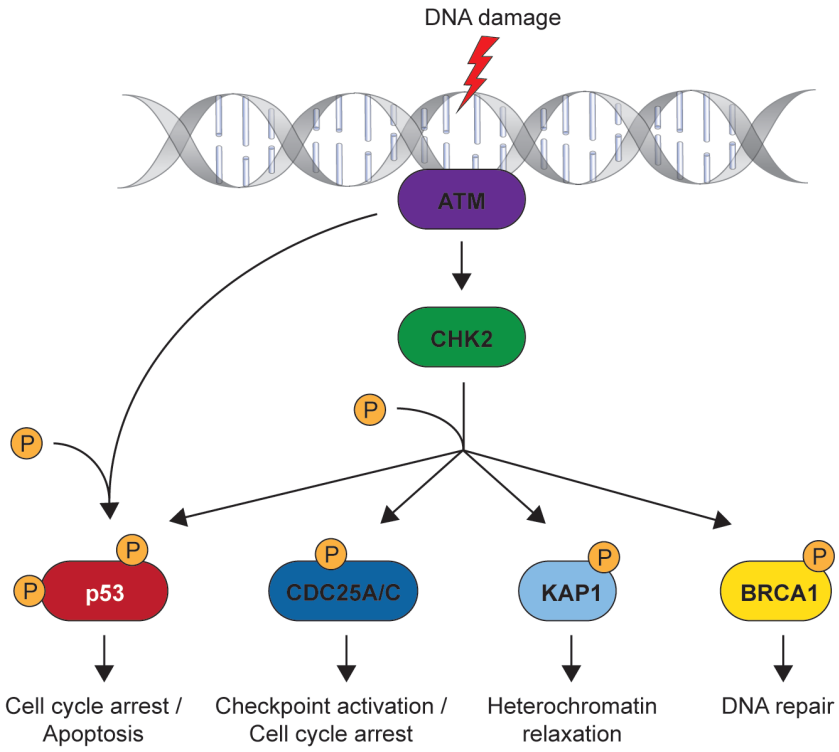

Figure 1. Schematic model displaying the regulation and function of CHK2 kinase. In response to DNA damage, ATM phosphorylates (indicated by the sphere 'P') both CHK2 and p53. ATM-dependent CHK2 phosphorylation promotes the activation of CHK2, and the subsequent CHK2-dependent phosphorylation of numerous downstream substrates such as p53, CDC25A/C, KAP1 and BRCA1. In this way, the CHK2 kinase regulates several cellular processes such as cell cycle regulation/checkpoint activation, apoptosis, heterochromatin relaxation and DNA repair.

Functional Analysis of *CHEK2* VUS

Numerous studies have set out to test the functional consequences of rare variants in the *CHEK2* gene to aid in their clinical interpretation (Table 1) (16-28). Ideally, a functional assay for a cancer predisposition gene measures a function that has been linked to the cancer phenotype. However, although it is known that CHK2 phosphorylates a wide spectrum of substrates involved in cell cycle regulation, DNA repair and apoptosis (29-34), precisely which modifications are relevant for cancer development is largely unclear. Nonetheless, CHK2's ability to phosphorylate any of these substrates may reflect its activity towards all other substrates and thus inform on its functionality in general. In the remainder of this section we discuss the different functional assays and readouts that have been used for the functional classification of missense VUS in *CHEK2* (Table 1).

Shortly after the identification of the CHK2 protein (1), the effect of the first reported missense variants that were identified in patients, were tested in functional assays (3,21,23,28). This work identified the first damaging missense variants in *CHEK2* (e.g., p.R145W), by showing a profound impact on CHK2 protein stability and/or kinase activity, as measured by *in vitro* kinase assays using CDC25A (21) or CDC25C peptides (23,28) as substrates. Three later studies similarly employed *in vitro* assays using CDC25C (18), BRCA1 (16) and KAP1 peptides (22) as substrates. These studies mostly relied on the immunoprecipitation of activated and tagged CHK2 from cells (i.e., after the induction of DNA damage) (16,18,21,23,28), or the purification of recombinant CHK2 (22). Overall, these studies resulted in the functional characterization of 39 distinct variants in the *CHEK2* gene (Fig. 2, Table 1, Supplementary table) (16,18,21-23,28).

A second system that was used for the functional analysis of *CHEK2* variants relied on the use of budding yeast *S. cerevisiae* strains that are null for *RAD53* (and *SML1* to rescue viability), which is the homolog of human *CHEK2* (1) and functional analog of *CHEK1* (35). Expressing human wild type *CHEK2* cDNA in *RAD53*-null yeast strains rescued their slow growth phenotype, likely by restoring its functions in cell cycle checkpoints (36). Accordingly, this system efficiently distinguished the damaging effect of the truncating c.1100del; p.T367Mfs variant from wild type *CHEK2*, whose expression resulted in reduced growth when compared to the wild type control (25,26). This system was later adapted by treating the cells with the DNA damaging agent Methyl methanesulfonate (MMS) (20,24), which results in cell cycle arrest due to the induction of stalled replication forks. Using this approach, two independent studies reported on the functional characterization of 132 distinct *CHEK2* variants (Fig. 2, Table 1, Supplementary table). Specifically, 35 missense VUS, which were identified in patients, two control deletion variants (p.E107_K197del and p.D265_H282del) and a catalytic-dead variant (p.D347A) that impairs kinase activity (20,24), were classified as damaging.

Figure 2. Circos plot of the CHK2 protein displaying the functional classification of 179 variants, including truncating (9), deletion (3), synonymous (7) and missense variants (160). CHK2 variants are indicated in the outer ring and are depicted clockwise, starting from the N-terminus of the CHK2 protein for which the domain structure is shown in the middle (SCD = SQ/TQ cluster domain; NLS = nuclear localization signal; FHA = forkhead-associated domain). Variants are color-coded based on type: green (synonymous variants), red (truncating variants), orange (deletion variants), and blue (missense variants). Each track, except track 1, shows the functional classification of variants from the indicated study (see also Table 1): "functional" (green sphere), "intermediate" (orange sphere), or "damaging" (red sphere). Track 1 shows the average voting score, which was calculated based on all functional classifications available for a given variant. To this end, every classification indicated in track 2-15 was given the same weight, meaning "functional" = 100%, "intermediate" = 50%, "damaging" = 0%. Using this weight, the average voting score was calculated, resulting in a classification as "functional" (green; 81 variants) ≥ 66.7%, "intermediate" (orange; 28 variants) 33.4 - 66.6%, or "damaging" (red; 70 variants) ≤ 33.3%. The data shown in this figure are also available in the Supplementary table (online manuscript only).

Table 1. List of functional studies for variants in the *CHEK2* gene.

	Study	Model system	Functional assay	Nr. of variants
n/a	Cuella-Martin et al., 2021 (19)	MCF7 and MCF10A cells	Growth after DNA damage induction using cisplatin, olaparib, doxorubicin or camptothecin	~159
2	Delimitsou et al., 2019 (20)	*RAD53*-null yeast strains	Growth after DNA damage induction using methyl methanesulfonate	122
3	Boonen et al., 2022 (17)	*Chek2* KO mES cells	Kap1 S473 phosphorylation	63
n/a	Boonen et al., 2022 (17)	*Chek2* KO mES cells	Protein stability	30
n/a	Boonen et al., 2022 (17)	*Chek2* KO mES cells	Growth after DNA damage induction using phleomycin	8
4	Kleiblova et al., 2019 (22)	*CHEK2* KO RPE1 cells	KAP1 S473 phosphorylation	28
5	Kleiblova et al., 2019 (22)	In vitro	Phosphorylation of KAP1 peptide (aa 467-478)	28
6	Kleiblova et al., 2019 (22)	In vitro	Omnia kinase assay	28
7	Roeb et al., 2012 (24)	*RAD53*-null yeast strains	Growth after DNA damage induction using methyl methanesulfonate	26
8	Bell et al., 2007 (16)	In vitro	Phosphorylation of BRCA1 peptide (aa 758-1064)	9
n/a	Bell et al., 2007 (16)	In vitro	Protein stability	
9	Lee et al., 2001 (23)	In vitro	Phosphorylation of CDC25C peptide (aa 200-256)	6
n/a	Lee et al., 2001 (23)	In vitro	Protein stability	
10	Chrisanthar et al., 2008 (18)	In vitro	Phosphorylation of CDC25C peptide	4
n/a	Chrisanthar et al., 2008 (18)	In vitro	Autophosphorylation	
11	Wu et al., 2001 (28)	In vitro	Phosphorylation of CDC25C peptide (aa 200-256)	4
n/a	Wu et al., 2001 (28)	In vitro	CHK2 T68 phosphorylation	
12	Tischkowitz et al., 2008 (26)	*RAD53*-null yeast strains	Growth	4
13	Shaag et al., 2005 (25)	*RAD53*-null yeast strains	Growth	4
14	Falck et al., 2001 (21)	In vitro	Phosphorylation of CDC25A peptide	3
15	Wang et al., 2015 (27)	Eμ-Myc p19Arf −/− B cells	Growth after DNA damage induction using cisplatin, olaparib or doxorubicin	1
n/a	Wang et al., 2015 (27)	Eμ-Myc p19Arf −/− B cells	p53 S20 and CDC25A phosphorylation	1
n/a	Wang et al., 2015 (27)	Eμ-Myc p19Arf −/− B cells	p53 protein levels	1

Tracks correspond to rings in the Circos plot (Fig. 2). Track numbers only apply to a functional readout that resulted in a functional classification by the authors (i.e., functional, intermediate and damaging). Number of variants indicates the number of unique variants that were assessed in a model system with a specific functional readout. Abbreviations: n/a, not applicable; aa, amino acid.

A third system used for functional analysis relies on the use of mammalian cell lines that were depleted of endogenous CHK2 protein, prior to complementation with human *CHEK2* cDNA carrying specific variants (17,22,27). Depletion of endogenous CHK2 was achieved through siRNA/shRNA-mediated silencing of *CHEK2* expression (i.e., knockdown) (27), or by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-based loss of *CHEK2* expression (i.e., knockout) (17,22). *CHEK2* knockout is compatible with life, since

CHEK2 is a non-essential gene, whose absence promotes mammalian cell growth (17,19). Following loss of endogenous CHK2, the functional effects of *CHEK2* variants were measured using different readouts; i.e., CHK2 kinase activity on substrates such as CDC25A (27) or KAP1 (17,22), CHK2 protein stability (17), cell growth after DNA damage induction (17,27), or p53 protein levels (27) (Table 1). Overall, these three studies functionally characterized 81 distinct *CHEK2* variants (Fig. 2, Table 1, Supplementary table), resulting in the identification of numerous missense variants with a damaging impact (17,22,27).

Overall, the aforementioned studies resulted in the functional characterization of 179 distinct *CHEK2* variants, including 7 synonymous, 9 truncating, 3 deletion and 160 missense VUS. Importantly, an average voting score (Fig. 2, Supplementary table), revealed that 81 variants (i.e., 7 synonymous variants and 74 missense VUS) were functional, 28 variants (i.e., 1 deletion variant, 1 truncating variant and 26 missense VUS) were intermediate in function, and 70 variants (2 deletion variants, 8 truncating variants and 60 missense VUS) were damaging. Mechanistic follow-up studies further showed that some of the damaging *CHEK2* missense variants impaired autophosphorylation and thus activation of CHK2, while most of the other variants impaired function by causing protein instability (17), a mechanism also reported for pathogenic variants in other genes (37,38). Generally, most damaging missense variants were located in the FHA domain (residues 92-205) and Kinase domain (residues 212-501) of CHK2, which is perhaps not surprising as they together make up most of the protein (Fig. 2, Supplementary table). However, to gain a comprehensive view on the damaging impact of variants throughout CHK2, a more extensive functional assessment of variants located in the SCD domain (residues 19-69) and outside functional domains is needed.

Challenges in the Functional Characterization of *CHEK2* VUS

The systems that have been used thus far for the functional analysis of genetic variants each have their strengths and weaknesses, which can result in discrepancies in the outcomes and consequently the functional classification of *CHEK2* variants. Here we review these differences and highlight some future challenges.

The initial functional analysis of *CHEK2* variants relied mostly on *in vitro* kinase assays involving the expression of *CHEK2* variants in cells that still express endogenous wild type *CHEK2* (16,18,21,23,28). A limitation of such an approach is that upon activation by DNA damage, CHK2 variant proteins can form dimers with endogenous wild type CHK2 protein. This may obscure assay results as the association of CHK2 variant proteins with wild type CHK2 may impact CHK2 function. This may also apply to systems in which depletion of endogenous CHK2 relied on knockdown (27) rather than knockout, since residual wild type CHK2 protein may still be present. In contrast, purification of recombinant CHK2 variant proteins from *Escherichia coli* for use in *in vitro* kinase assays, likely influences functional

impact due to lack of posttranslational modifications that are otherwise induced in response to DNA damage in human cells (22). Moreover, *in vitro* assays are unable to detect potential defects in CHK2 protein stability or intracellular localization, and often measure CHK2 kinase activity using artificial substrates (16,18,22,23,28), which may differ from that of full-length substrates.

Most *CHEK2* variants have thus far been characterized using a yeast-based system (20,24,26). Although the overall structure of the CHK2 protein is similar in all eukaryotes, human CHK2 shows only 28% amino-acid identity with the *S. cerevisiae* Rad53 protein (39). Such differences in sequence similarity may affect functional analysis of human *CHEK2* variants in a yeast-cell context. Furthermore, yeast cells grow at 30°C rather than at 37°C, which may reduce the effect of some variants on the thermodynamic stability of CHK2. Accordingly, several unstable CHK2 variants exhibiting intermediate functional effects in mammalian cells (i.e., p.D203G, p.E239K and p.D438Y) (17), were classified as functional in a yeast-based system (20). Thus, growth temperature of a model system may therefore be an important aspect to take into account with regards to the functional characterization of human *CHEK2* variants.

Given the potential limitations of a yeast-based system, a mammalian cell-based system may be favored for the functional analysis of *CHEK2* variants. Indeed, two studies employed such a system based on stable and physiological CHK2 expression levels, rather than transient overexpression of CHK2, in *CHEK2*-deficient cells (17,22). Both studies used DNA damage-induced phosphorylation of KAP1 S473 as a functional readout for CHK2 kinase activity. Functional outcomes were generally accordant and only minor inconsistencies were observed for three (i.e., p.E64K, p.I157T and p.D438Y) out of ten variants studied. A potential limitation of this approach, however, may be that some *CHEK2* missense variants disrupt CHK2 activity against one substrate but not another. Consequently, this approach may not accurately measure the overall impact of a variant on CHK2 activity following DNA damage induction. However, correlating the results from phospho-Kap1 S473 assays to a more general functional readout (i.e., cell growth after DNA damage induction) for eight variants, showed that there is a strong and significant correlation (17). Thus, although CHK2's role in regulating cell growth after DNA damage induction likely stems from its ability to phosphorylate multiple downstream targets, these data suggest that the phosphorylation of Kap1 S473 may be a suitable readout to assess the overall function of CHK2.

When using Kap1 S473, or any other phospho-target of CHK2 as a functional readout, another aspect that also complicates the functional assessment of *CHEK2* variants is the observed kinetic defect reported for some variants, e.g., p.E64K and p.R521W (17). Examination of CHK2 kinase activity at different timepoints after IR showed that, in contrast to wild type CHK2, these two variants are unable to maintain phosphorylation of Kap1 S473 over

the course of the experiment (i.e., 6 hours compared to 2 hours after IR). This suggests that the chosen timepoint at which to asses CHK2 kinase activity after DNA damage induction, may influence functional classification. Accordingly, this may have resulted in some of the reported discrepancies for p.E64K and p.R521W (17,20,22).

In contrast to cDNA-based complementation systems, variants can also be introduced at endogenous loci using CRISPR -dependent technologies, For *BRCA1*, a CRISPR/Cas9-dependent saturation genome editing technique was used that enabled the functional characterization of nearly 4000 variants in the RING and BRCT domains of BRCA1, using cell survival as a functional readout (40). Moreover, for 86 DNA damage response genes, including *CHEK2*, a CRISPR-dependent cytosine base editing screen has been used to interrogate the functional effects of thousands of variants by examining cell growth after DNA damage induction (19). This strategy has major advantages in that it assesses the effects of variants in the context of the endogenous gene and thus at physiological expression levels. Moreover, the effects of variants located in non-coding regions can also be analyzed. Thus, potential effects on mRNA splicing from variants located in both coding and non-coding regions can be functionally assessed. Although such technological advances are anticipated to become important in the future characterizion of variants at scale, they may require optimization before they can be considered a clinical diagnostics tool. For instance, the base editor employed by Cuella-Martin and colleagues has an editing window of 6 nucleotides and often results in the introduction of multiple variants therein (19). This makes it sometimes difficult, if not impossible, to obtain and interpret results for individual variants. Moreover, the repertoire of variants that can be generated is, among others, dependent on protospacer adjacent motifs (PAM) in the DNA that is targeted by the CRISPR system, thus limiting the number of variants that can be characterized. Finally, when a general readout such as cell growth is examined, off-target effects of sgRNAs may have a major impact on the outcome of the functional assay. Nonetheless, these en masse studies will undoubtedly accelerate the path to clinical interpretation of genetic variants in a high-throughput manner.

Clinical Interpretation of *CHEK2* Variants: Functional Assays to the Rescue?

Genetic testing to identify individuals at increased risk of developing breast cancer has accelerated rapidly over the past decade and now also includes moderate-risk genes such as *CHEK2*. The clinical classification of VUS in *CHEK2*, as either pathogenic or benign, is hampered by their rare nature and the moderate breast cancer risk that is associated with pathogenic *CHEK2* variants. This precludes the use of genetic approaches, such as co-segregation analysis, that have been successfully applied in the classification of VUS in high-risk genes such as *BRCA1* and *BRCA2* (41,42). The use of validated functional assays is therefore a very attractive option to consider for improving the clinical classification of VUS in

CHEK2. Before these assays can be used for variant classification, it is essential to establish the quantitative relationship between CHK2 protein functionality and cancer risk.

To date, reliable cancer risk estimates have only been established for a few *CHEK2* variant alleles which are relatively frequent in the population (Table 2) (6,17,43,44) (6,43-45). Interestingly, the risk estimates for these variants (i.e., p.E64K, p.R117G, p.I157T, p.R180C, p.H371Y, p.T476M) show an inverse correlation with their functional impact, meaning that variants exhibiting less activity associate with higher cancer risk (Table 2). In contrast to these *CHEK2* variants, the prevalence of other missense variants is too low to determine their association with breast cancer risk empirically. Assuming that variants with a similar impact on CHK2 protein function associate with the same level of cancer risk, a burden-type association analysis based on reported protein functionality is warranted (Table 3) (17,20,22). This analysis first reveals that the *in vitro* kinase assays generally show poor correlation between functional effects and breast cancer risk, suggesting they may not adequately distinguish functional effects of *CHEK2* variants. Secondly, it shows that the yeast-based system is good at classifying damaging variants (with an OR around 2), but poor at discriminating functional variants from intermediate variants (both groups with ORs around 1.3). Finally, it confirms that the outcome of mammalian cell-based systems (17,22) show an inverse correlation between CHK2 protein function and breast cancer risk as was also reported for the unique variant alleles (Table 2, Table 3). Although the number of variants for which functional data are available is still modest, both the variant specific and the burden analysis derived ORs illustrate that there is a group of *CHEK2* missense variants that associate with a similar cancer risk as has been reported for truncating *CHEK2* variants and that those can be identified by functional analysis. Moreover, the available data thus far also show that *CHEK2* variants that do not associate with clinically relevant cancer risks up to ORs of 1.3 (e.g., p.I157T and p.R180C) do not show a functional impact (see outstanding questions).

Currently, standard guidelines for reporting *CHEK2* missense VUS are lacking, mainly due to the absence of convincing evidence of disease association. However, based on recently obtained insights (Table 3) (17,20), the existence of *CHEK2* missense variants that associate with a comparable risk of breast cancer as *CHEK2* truncating variants, including the c.1100del; p.T367Mfs variant (Table 2), is highly likely. It is therefore crucial that functional assays are used to discriminate between missense variants that affect protein function and are associated with breast cancer risk from those that do not. In this way, functional analysis will provide an essential contribution to reliable variant classification and improved clinical management for carriers and their families.

In addition to functional assays, computational tools may be useful in the clinical interpretation of *CHEK2* missense variants (at scale). For instance, the *in silico* prediction tool Helix (46,47) has been shown to perform well in predicting functionality of *CHEK2* missense

variants (17). These *in silico* predictions should, however, be handled with caution as they have been shown to overestimate the number of damaging variants (38,48,49). Therefore, computational tools might specifically aid in predicting functionality of missense variants that require further analysis of their impact, either because functional outcomes were inconsistent across different studies, or because functional analysis have yet to be performed.

Table 2. Breast cancer risk associated with genetic variants in *CHEK2*.

Nucleotide change	Amino acid change	Average voting score (Fig.2)	Odds ratio	95% CI	*p*-value	Reference
c .190G>A	p.E64K	Intermediate	1,78	1,14 - 2,77	0,0112	Dorling et al., 2021 (6), Boonen et al., 2022 (17)
c.349A>G	p.R117G	Damaging	2,22	1,34 - 3,68	0,002	Dorling et al., 2021 (6), Boonen et al., 2022 (17)
			2,26	1.29 - 3.95	0,003	Southey et al., 2016 (44)
c.470T>C	p.I157T	Functional	1,37 (iCOGS)	1,21 - 1,55	<0,0001	Michailidou et al., 2017 (43)
			1.26 (OncoArray)	1,11 - 1,42	0,0002	
			0.96 (GWAS)	0.72 - 1.28	0,77	
c.538C>T	p.R180C	Functional	1,33	1,05 - 1,67	0,016	Southey et al., 2016 (44)
c.1100delC	p.T367Mfs	Damaging	2,66	2,27 - 3,11	<0,0001	Dorling et al., 2021 (6)
c.1111C>T	p.H371Y	Functional	1,01	0,64 - 1,59	0,9618	Dorling et al., 2021 (6), Boonen et al., 2022 (17)
c.1427C>T	p.T476M	Damaging	1,60	1,10 - 2,35	0,0145	Dorling et al., 2021 (6)

Abbreviations: GWAS, genome-wide association study; iCOGS, International Collaborative Oncological Gene–Environment Study.

Table 3. Burden-type cancer risk association analysis for human *CHEK2* variants.

Study	Variant group based on function	Nr. cases	Nr. controls	OR	95% CI	*p*-value
Boonen et al.,. 2021 (17); *Chek2* KO mES cells	Functional variants	117	108	1,13	0,87-1,46	0,378
	Intermediate variants	110	70	1,63	1,21-2,20	0,0014
	Intermediate variants (excl. p.E64K)	57	39	1,52	1,01-2,28	0,0448
	Damaging variants	118	55	2,23	1,62-3,07	<0,0001
	Damaging variants (excl. p.R117G)	71	33	2,23	1,48-3,38	<0,0001
Delimitsou et al., 2019 (20); *RAD53*-null yeast strains	Functional variants	397	304	1,36	1,17 - 1,58	0,0001
	Functional variants (excl. p.E64K):	344	273	1,31	1,12 - 1,53	0,0009
	Intermediate variants	138	109	1,31	1,02 - 1,69	0,0329
	Intermediate variants (excl. p.T476M)	70	65	1,12	0,80 - 1,57	0,5165
	Damaging variants	116	58	2,08	1,52 - 2,85	<0,0001
	Damaging variants (excl. p.R117G)	69	36	1,99	1,33 - 2,98	0,0008
Kleiblova et al., 2019 (22); *CHEK2* KO RPE1 cells	Functional variants	173	133	1,35	1,08 - 1,69	0,0092
	Functional variants (excl. p.T476M)	105	89	1,23	0,92 - 1,63	0,1592
	Intermediate variants	31	20	1,61	0,92 - 2,82	0,0971
	Damaging variants	91	54	1,75	1,25 - 2,45	0,0011
	Damaging variants (excl. p.E64K)	38	23	1,72	1,02 - 2,88	0,0411
Kleiblova et al., 2019 (22); pKap1 in vitro	Functional variants	153	107	1,48	1,16 - 1,90	0,0017
	Functional variants (excl. p.E64K):	100	76	1,37	1,01 - 1,84	0,0404
	Intermediate variants	38	34	1,16	0,73 - 1,84	0,5282
	Damaging variants	104	66	1,64	1,20 - 2,23	0,0018
	Damaging variants (excl. p.T476M)	36	22	1,7	1,00 - 2,89	0,0501
Kleiblova et al., 2019 (22); in vitro Omnia assay	Functional variants	131	90	1,51	1,16 - 1,98	0,0017
	Functional variants (excl. p.E64K):	78	59	1,37	0,98 - 1,93	0,0404
	Intermediate variants (only p.R406H)	14	12	1,21	0,56 - 2,62	0,6258
	Damaging variants	150	105	1,48	1,16 - 1,90	0,002
	Damaging variants (excl. p.T476M)	82	61	1,4	1,00 - 1,94	0,0487

Abbreviations: KO, knockout; mES cells, mouse embryonic stem cells.

Concluding Remarks and Future Perspectives

Due to the accelerating pace by which germline *CHEK2* variants are discovered, there is a strong need to determine which variants are associated with increased cancer risk. To this end, functional assays have been developed and used to characterize a substantial set of *CHEK2* missense variants, resulting in the identification of rare *CHEK2* variants that exhibit damaging effects on protein function (Fig. 2). These analyses have allowed for a burden-type association analysis, allowing us to correlate the level of functional impact of rare *CHEK2* missense variants to breast cancer risk (Table 3) (17). Importantly, extension of the current cDNA-based methods to genome editing-based methods will provide insight into the effect of

coding and non-coding variants on RNA splicing and downstream functional consequences, further improving the clinical classification of variants in *CHEK2*. Future assays aimed to address the functional effect of every possible nucleotide change in *CHEK2* in a high-throughput manner, such as those performed for *BRCA1* (50), should ultimately result in publicly available resources displaying the quantitative functional output from validated and calibrated functional assays for all *CHEK2* variants. Finally, a ClinGen variant curation expert panel (VCEP) will establish *CHEK2*-specific specifications of the ACMG-based clinical variant interpretation guidelines and provide recommendations for the implementation of results from functional analysis in the classification of missense variants in *CHEK2*. Ultimately, the addition of functional data from validated assays will improve their clinical interpretation and aid in the counseling of carriers and their families.

HIGHLIGHTS

Functional assays have been developed that can determine the impact of missense variants of uncertain significance (VUS) on CHK2 protein function.

Functional analyses of *CHEK2* missense VUS reveal an association between impaired protein function and increased breast cancer risk.

Damaging *CHEK2* missense VUS may associate with a similar risk of breast cancer as protein-truncating variants.

A comprehensive functional characterization of *CHEK2* missense VUS is needed to determine the associated cancer risk.

Functional analysis of missense VUS in *CHEK2* will improve the clinical management of carriers and their family members.

OUTSTANDING QUESTIONS

What is an ideal system for functional analysis of genetic variants in CHEK2? The ideal system may study the functional impact of variants in human cells and in the context of the endogenous gene. With the availability of multiplex assays and CRISPR/Cas9-mediated base-editing, the introduction of all possible variants in *CHEK2* is within reach but is certainly not a standard approach yet. Whether non-cancerous or (breast) cancer cells should be used is debatable, as differences in cell type, tissue and genetic context may affect the functional impact of *CHEK2* variants. Finally, how loss of CHK2's function relates to cancer development is presently unclear. Consequently, a functional readout that captures CHK2 defects that are causally linked to cancer remains to be established.

Can functional analysis keep up with the overwhelming number of CHEK2 variants that have been, and are being, identified by genetic tests? Using a one-by-one approach for functional analysis of *CHEK2* variants is too time-consuming to address the vast number of identified variants (1148 distinct missense VUS in *CHEK2* have currently (as of February 2022 been reported in ClinVar). High-throughput approaches, such as those performed for *BRCA1* and *PTEN* (37,50), may provide answers to this challenge. As protein instability causes most *CHEK2* missense VUS to be damaging (45), using an experimental strategy such as variant abundance by massively parallel sequencing (VAMP-seq) (37) may provide a good means to identify unstable, and thus damaging *CHEK2* missense variants en masse. Alternatively, the combining FACS-based phospho-Kap1 S473 measurements with VAMP-seq (45), may be a means to identify damaging variants that rather impact CHK2's kinase function.

What about functional analysis of CHEK2 splice variants? Generating variants at the endogenous *CHEK2* locus, high-throughput or not, may allow studying their impact on RNA splicing, CHK2 expression and CHK2 functionality. Alternatively, *Chek2*^{KO} mES cells could be complemented with a bacterial artificial chromosome (BAC) containing the human *CHEK2* gene, as has also been performed for *BRCA2* (51). In such a scenario, it is imperative that RNA analysis is performed to show that splicing of human *CHEK2* RNA in mES cells is comparable to that in human cells.

Can CHEK2 functional assays be used for breast cancer risk prediction? A major challenge is to establish the quantitative relationship between CHK2 protein functionality and breast cancer risk. Association analysis (Table 2, Table 3) (45) showed that the degree of CHK2 dysfunction correlates with increased breast cancer risk and that functional analysis can identify missense variants associated with cancer risks similar to those associated with *CHEK2* truncating

variants (OR ≥ 2). However, the exact risk calculations differ slightly per study. This may be related to the fact that these variants are rare, requiring burden-type analyses to estimate cancer risk for groups of variants. Therefore, data from larger or additional case-control association studies than those currently available (e.g., from the Breast Cancer Association Consortium (6)), as well as functional analysis of additional *CHEK2* variants will be pivotal to better understand the extent to which functional defects in CHK2 associate with cancer risk. These analyses might even enable the development of a 'continuous risk' model whereby a variant-specific risk (also with OR < 2) is calculated and can serve as a risk prediction factor on the basis of its impact on functionality.

How to establish the functional threshold for pathogenicity? Guidelines published by the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) suggest the use of 'well established' functional studies that provide strong support for or against pathogenicity of a variant (52). However, since the number of pathogenic *CHEK2* missense variants is insufficient, a threshold for pathogenicity cannot be set on basis of such variants. Under the assumption that missense variants with similar levels of functionality associate with the same level of cancer risk, a burden-type association analysis can be performed using large case-control studies (6,53). This analysis will reveal if a group of missense variants (defined by similar levels of functionality) is associated with a risk similar to that of pathogenic variants (i.e., OR>2). While this threshold may be used to identify pathogenic missense variants, its reliability has to be confirmed in the future with (missense) variants that will be classified as pathogenic independent of functional analysis.

Can functional assays guide therapy choice for patients with CHK2-deficient tumors? Currently it is unclear precisely how CHK2 loss of function leads to increased cancer risk and if this deficiency leads to a targetable vulnerability in cancer cells. Consequently, the potential of *CHEK2* functional assays in guiding therapy choice or predicting therapy response for patients with CHK2 related cancer, remains to be elucidated.

GLOSSARY

Checkpoint kinase 2 (*CHEK2*): a tumor suppressor gene that encodes the serine/threonine kinase CHK2, which is involved in DNA repair, cell cycle arrest or apoptosis.

Saccharomyces (S.) cerevisiae: A unicellular eukaryotic organism that constitutes a valuable model for fundamental research.

Rad53: a serine/threonine kinase from *S. cerevisiae* required for DNA damage and replication checkpoints, promoting cell cycle arrest and DNA repair

c.1100delC/p.T367Mfs: HGVS descriptions of a genetic variant at the nucleotide and protein level. 'c' refers to cDNA sequence, while 'p' refers to protein sequence. The numbers reflect nucleotide or codon positions of the wild-type reference sequence. 'del' Refers to deletion of the nucleotide 'C' (cytosine). 'T' Refers to the original wild type amino acid Threonine. 'M' denotes the change of a Threonine to a Methionine at amino acid position 367 in this example. 'fs' Indicates that the nucleotide change results in a frameshift in codon usage at amino acid position 367.

TP53: A tumor suppressor gene that encodes the transcription factor Tumor Protein P53 (p53), which is involved in cell cycle regulation and apoptosis.

Odds ratio (OR): A measure of association between a variable (e.g., a genetic variant) and an outcome (e.g. breast cancer). An OR indicates the odds that breast cancer will occur when carrying a specific variant, compared to the odds of breast cancer occurring in the absence of that specific variant.

Variant of Uncertain Significance (VUS): Genetic variant that cannot be used for clinical decision making or cancer risk assessment due to insufficient clinical and/or functional data needed to assess pathogenicity.

BReast CAncer 1/2 (*BRCA1/2*): The two most commonly affected high-risk breast cancer susceptibility genes, which are involved in the repair of DNA double-strand breaks and the protection of (stalled) DNA replication forks.

Partner and localizer of BRCA2 (*PALB2*): A high-risk gene breast cancer susceptibility gene, which is involved in the repair of DNA double-strand breaks by linking the actions of BRCA1 and BRCA2 therein.

Ataxia telangiectasia-mutated (*ATM*): A moderate-risk breast cancer susceptibility gene that encodes *the* serine/threonine kinase ATM, which is recruited and activated by DNA double-strand breaks to regulate cell cycle progression and DNA repair. Autosomal recessive mutations in *ATM* lead to Ataxia telangiectasia, which is a rare disorder characterized by for instance neurodegeneration, immunodeficiency, radiosensitivity and cancer.

ClinVar: A freely accessible public archive that aggregates information about genomic variation and its relationship to human health (https://www.ncbi.nlm.nih.gov/clinvar/).

ACMG guidelines: Recommendations of the American College of Medical Genetics and Genomics (ACMG) for the clinical interpretation of sequence variants.

Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9: Molecular biological tool used for genomic editing with the Cas9 nuclease.

Functional assay: Molecular and cellular experiments that can produce data describing the functional impact of a variant on a gene product.

Cell Division Cycle 25 (CDC25)A/C: Two crucial cell cycle regulators and homologs that act as a phosphatase by removing the inhibitory phosphorylation of cyclin-dependent kinases (CDKs), thereby positively regulating the activity of CDKs in promoting cell cycle progression.

Methyl-methanesulfonate (MMS): An alkylating agent that induces replication fork stalling by modifying both guanine (to 7-methylguanine) and adenine (to 3-methlyladenine) bases in the DNA.

Knockdown: Experimental condition that reduces the expression of one or more genes in a cell or organism.

Knockout: Experimental condition by which the genomic DNA of a cell or organism is perturbed to permanently prevent the expression of one or more genes in a cell or organism.

Forkhead-associated (FHA) domain: A protein modular domain that binds phospho-peptides.

Kinase domain: A structurally conserved protein domain harboring the catalytic activity of protein kinases.

SQ/TQ cluster domain (SCD): A protein domain that is defined by the presence of multiple SQ/TQ motifs within a variable stretch of amino acids. SCDs are recognized targets for kinases involved in the DDR.

Escherichia (E.) coli: A gram-negative, rod-shaped bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms. It constitutes an important species in the fields of biotechnology and microbiology, where it can serve as the host organism for work with recombinant DNA.

DNA damage response (DDR): An extensive surveillance network that maintains genome integrity and stability, and is thus critical for cellular homeostasis and disease prevention.

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