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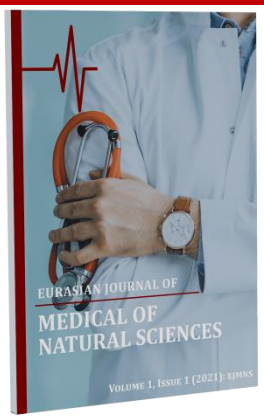

CONNECTING FUNCTIONAL CONSEQUENCES TO CANCER RISK THROUGH CHEK2 VARIANTS

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ABSTRACT

Protein-truncating variations in the breast cancer susceptibility gene CHEK2 exhibit a moderately heightened risk of breast cancer. In contrast, the associated breast cancer risk for missense variants of uncertain significance (VUS) in CHEK2 often remains ambiguous. To aid in their classification, functional assays assessing the impact of missense VUS on CHK2 protein function have been conducted. This discussion delves into these functional analyses, consistently revealing a connection between impaired protein function and an increased risk of breast cancer. Overall, these findings imply that damaging CHEK2 missense VUS are linked to a breast cancer risk similar to that of protein-truncating variants. This underscores the importance of expanding the functional characterization of CHEK2 missense VUS to gain a deeper understanding of the associated cancer risk.

INTRODUCTION

CHEK2 and Cancer Predisposition

Initially identified as the mammalian homolog of the Saccharomyces cerevisiae Rad53 and Schizosaccharomyces pombe Cds1 protein kinases, the checkpoint kinase 2 (CHK2) plays a crucial role in cell-cycle control and apoptosis triggered by exposure to DNA-damaging agents. Activation of CHK2 by the ataxia-telangiectasia mutated (ATM) kinase leads to the phosphorylation of downstream substrates like p53, cell division cycle (CDC) 25A and CDC25C, KAP1, and breast cancer type 1 susceptibility protein (BRCA1). This signaling cascade collectively serves to prevent genome instability and cancer development by prompting cells to halt proliferation and repair DNA damage or induce apoptosis in response to inefficient or improper repair (refer to Figure 1). Shortly after its discovery, frameshift variants, including the well-known c.1100del;p.T367Mfs variant, were identified in the CHEK2 gene, linking it to a cancer susceptibility disorder known as Li–Fraumeni syndrome (LFS). LFS, a rare hereditary autosomal dominant disorder, is characterized by a broad spectrum of malignancies that manifest at an unusually early age. Analogous to CHK2, the tumor-suppressor protein p53 regulates cell division following DNA damage, and inherited mutations in the corresponding

gene (tumor protein 53, TP53) are implicated in most LFS cases. The connection between CHK2 and p53 became apparent when it was demonstrated that CHK2 phosphorylates p53 at S20, leading to the dissociation of preformed p53–Mdm2 complexes and resulting in p53 stabilization. These findings suggest that CHK2 acts as a tumor-suppressor protein within the p53 signaling pathway.

In recent years, numerous studies have substantiated the tumor-suppressive role of CHK2. Truncating variants in the CHEK2 gene, such as c.1100del;p.T367Mfs, have been consistently linked to a moderate risk of breast cancer, with an increased risk ranging from two to threefold. For female carriers with heterozygous CHEK2 truncating variants, this translates to a lifetime risk of approximately 25% for developing breast cancer before reaching the age of 80. Additionally, CHEK2 has been identified as a multi-organ cancer susceptibility gene, a classification supported by various studies (summarized in [13]). Consequently, these discoveries have prompted a significant rise in genetic testing for CHEK2, leading to the identification of numerous rare missense variants whose clinical significance remains uncertain. Apart from the well-established high-risk breast cancer susceptibility genes, namely BRCA1, BRCA2, and partner and localizer of BRCA2 (PALB2), it is now clear that CHEK2, in conjunction with ATM, stands out as one of the most frequently mutated genes in the germline of individuals affected by breast cancer [6].

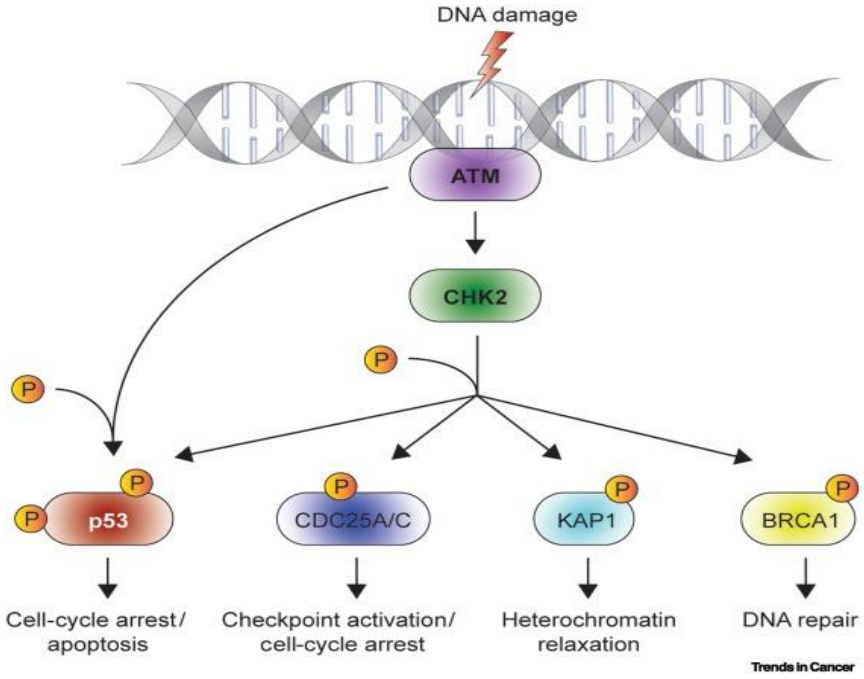

Figure 1. Schematic model showing the regulation and function of CHK2 kinase.

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 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. Abbreviations: ATM, ataxia-telangiectasia mutated; BRCA1, breast cancer type 1 susceptibility; CHK2, checkpoint kinase 2.

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As of February 2023, ClinVar reports a total of 1148 distinct missense Variants of Uncertain Significance (VUS) in CHEK2. Collectively, many of these rare missense variants, also referred to as missense VUS, exhibit an association with breast cancer (odds ratio (OR) 1.42; 95% confidence interval (CI), 1.28–1.58; P = 2.5 × 10^11) [6]. Notably, this association appears to be independent of their specific location within the gene and, consequently, their impact on any of the functional domains of CHK2. These domains include a N-terminal SQ/TQ cluster domain (SCD) (amino acids (aa) 19–69), a Forkhead-associated (FHA) domain (aa 92–205), a serine/threonine kinase domain (aa 212–501), and a nuclear localization signal (NLS; aa 515–522) (refer to Figure 2). Understanding which missense variants affect protein function and to what extent becomes pivotal in discerning variants linked to an elevated risk of breast cancer. Consequently, the results from quantitative and well-validated functional assays for CHEK2, aligning with American College of Medical Genetics and Genomics (ACMG) guidelines [15], play a crucial role in guiding the clinical classification of genetic variants in this gene, thereby enhancing the counseling of carriers. Several recent studies have undertaken the functional characterization of CHEK2 variants, offering insights into diverse approaches, outcomes, potential pitfalls of functional assays, and the correlation between functional results and breast cancer risk.

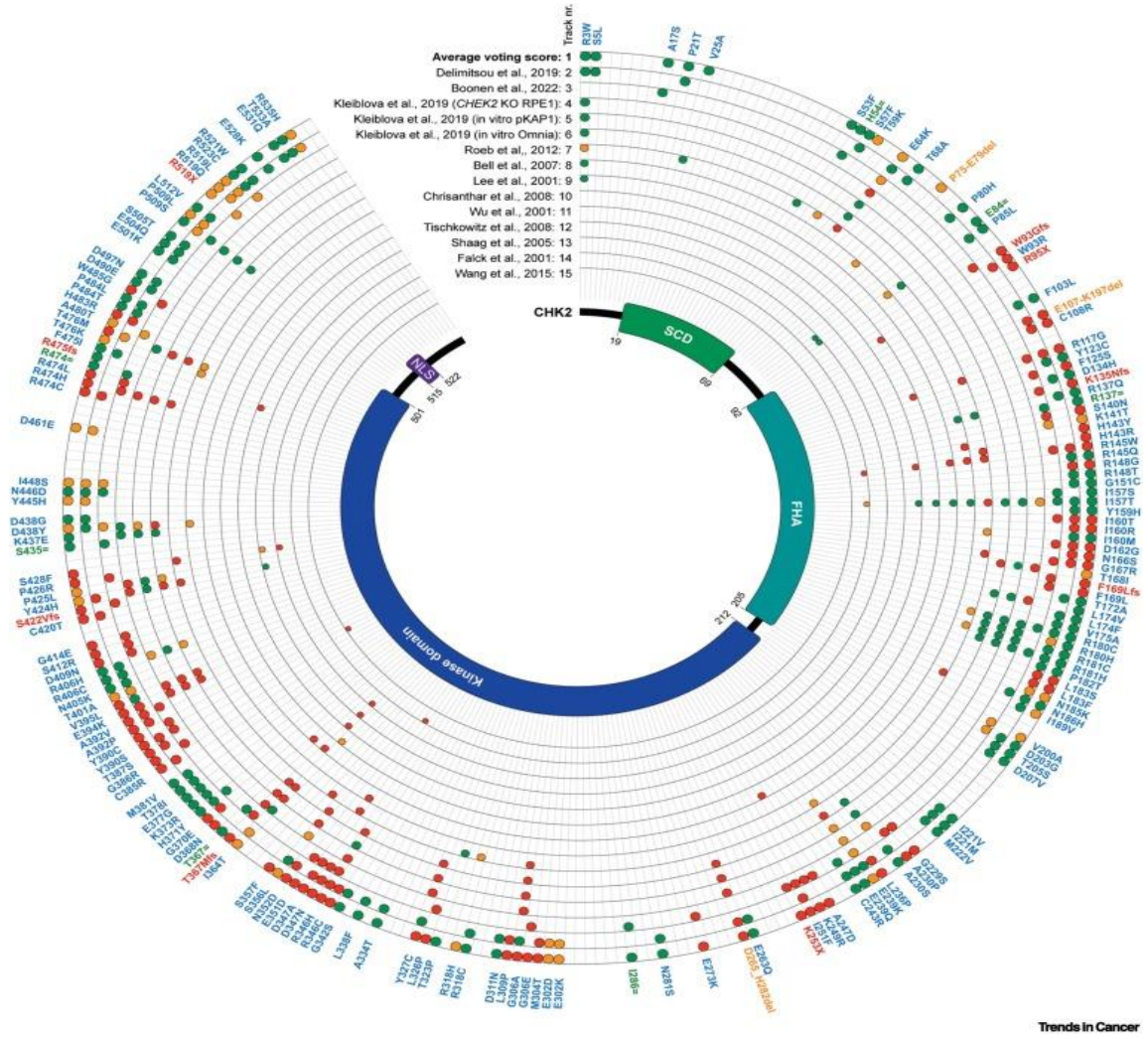

Figure 2. Circos plot of CHK2 protein displaying the functional classification of 179 variants, including truncating (nine), deletion (three), synonymous (seven), and missense variants (160).

CHK2 variants are represented in the outer ring, depicted clockwise from the N-terminus of the CHK2 protein, with the domain structure displayed in the middle. Variants are color-coded based on their type: green for synonymous variants, red for truncating variants, orange for deletion variants, and blue for missense variants. Each track, excluding track 1, illustrates the functional classification of variants from the specified study (refer to Table 1 in the main text): 'functional' (depicted as a green sphere), 'intermediate' (depicted as an orange sphere), or 'damaging' (depicted as a red sphere). Track 1 displays the average voting score, calculated based on all available functional classifications for a particular variant. In tracks 2–15, each classification was assigned the following weights: 'functional' = 100%, 'intermediate' = 50%, 'damaging' = 0%. Utilizing these weights, the average voting score was determined, resulting in a classification of 'functional' (depicted as green; 81 variants) for scores ≥66.7%, 'intermediate' (depicted as orange; 28 variants) for scores ranging from 33.4–66.6%, or 'damaging' (depicted as red; 70 variants) for scores ≤33.3%. The data presented in this figure are also accessible in Table S1. Refer to [16,17,20., 21., 22., 23., 24., 25., 26., 27., 28.] for additional details. Abbreviations: CHK2, checkpoint kinase 2; FHA, Forkhead-associated domain; NLS, nuclear localization signal; SCD, SQ/TQ cluster domain.

Functional analysis of CHEK2 VUS

Numerous studies have been undertaken to assess the functional consequences of rare variants in the CHEK2 gene, aiming to enhance clinical interpretation (refer to Table 1) [16., 17., 18., 19., 20., 21., 22., 23., 24., 25., 26., 27., 28.]. Ideally, a functional assay for a cancer predisposition gene should measure a function linked to the cancer phenotype. While it is established that CHK2 phosphorylates a broad range of substrates involved in cell-cycle regulation, DNA repair, and apoptosis [29., 30., 31., 32., 33., 34.], precisely identifying which modifications are relevant for cancer development remains largely unclear. Nevertheless, CHK2's ability to phosphorylate any of these substrates may reflect its activity towards all other substrates, providing insights into its functionality in general. In the following sections, we delve into the various functional assays and readouts employed for the classification of missense Variants of Uncertain Significance (VUS) in CHEK2 (refer to Table 1).

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

Track[b]	Study	Model system	Functional assay	Number of variants[c]
N/A	Cuella-Martin *et al.* [19]	MCF7 and MCF10A cells	Growth after DNA damage induction using cisplatin, olaparib, doxorubicin, or camptothecin	~159
2	Delimitsou *et al.* [20]	*RAD53*-null yeast strains	Growth after DNA damage induction using MMS	122
3	Boonen *et al.* [17]	*Chek2* KO mES cells	Kap1 S473 phosphorylation	63
N/A	Boonen *et al.* [17]	*Chek2* KO mES cells	Protein stability	30

N/A	Boonen *et al.* [17]	*Chek2* KO mES cells	Growth after DNA damage induction using phleomycin	8
4	Kleiblova *et al.* [22]	*CHEK2* KO RPE1 cells	KAP1 S473 phosphorylation	28
5	Kleiblova *et al.* [22]	*In vitro*	Phosphorylation of KAP1 peptide (aa 467–478)	28
6	Kleiblova *et al.* [22]	*In vitro*	Omnia kinase assay	28
7	Roeb *et al.* [24]	*RAD53*-null yeast strains	Growth after DNA damage induction using MMS	26
8	Bell *et al.* [16]	*In vitro*	Phosphorylation of BRCA1 peptide (aa 758–1064)	9
N/A	Bell *et al.* [16]	*In vitro*	Protein stability	
9	Lee *et al.* [23]	*In vitro*	Phosphorylation of CDC25C peptide (aa 200–256)	6
N/A	Lee *et al.* [23]	*In vitro*	Protein stability	
10	Chrisanthar *e t al.* [18]	*In vitro*	Phosphorylation of CDC25C peptide	4
N/A	Chrisanthar *e t al.* [18]	*In vitro*	Autophosphorylation	
11	Wu *et al.* [28]	*In vitro*	Phosphorylation of CDC25C peptide (aa 200–256)	4
N/A	Wu *et al.* [28]	*In vitro*	CHK2 T68 phosphorylation	
12	Tischkowitz *e t al.* [26]	*RAD53*-null yeast strains	Growth	4
13	Shaag *et al.* [25]	*RAD53*-null yeast strains	Growth	4
14	Falck *et al.* [21]	*In vitro*	Phosphorylation of CDC25A peptide	3
15	Wang *et al.* [27]	*Eμ–Myc p19Arf*$^{-/-}$ B cells	Growth after DNA damage induction using cisplatin, olaparib, or doxorubicin	1
N/A	Wang *et al.* [27]	*Eμ–Myc p19Arf*$^{-/-}$ B cells	p53 S20 and CDC25A phosphorylation	1
N/A	Wang *et al.* [27]	*Eμ–Myc p19Arf*$^{-/-}$ B cells	p53 protein levels	1

A Abbreviations; aa, amino acids, KO, knockout; mES cells, mouse embryonic stem cells; MMS, methyl methanesulfonate; N/A, not applicable.

B Tracks correspond to rings in the Circos plot (see Figure 2 in the main text). Track numbers only apply to a functional readout that resulted in a functional classification by the authors (i.e., functional, intermediate, and damaging).

C The number of variants indicates the number of unique variants that were assessed in a model system with a specific functional readout.

Shortly after the identification of the CHK2 protein [1], the functional impact of the initial reported missense variants found in patients was assessed through functional assays [3,21,23,28]. These investigations revealed the first damaging missense variants in CHEK2 (e.g.,

p.R145W) by demonstrating a significant effect on CHK2 protein stability and/or kinase activity. This was assessed through in vitro kinase assays utilizing CDC25A [21] or CDC25C peptides [23,28] as substrates. Subsequent studies adopted a similar approach, utilizing in vitro assays with CDC25C [18], BRCA1 [16], and KAP1 peptides [22] as substrates. These studies predominantly relied on the immunoprecipitation of activated and tagged CHK2 from cells (i.e., post DNA damage induction) [16,18,21,23,28] or the purification of recombinant CHK2 [22]. In total, these efforts led to the functional characterization of 39 distinct variants in the CHEK2 gene (Figure 2, Table 1, and see Table S1 in the supplemental information online) [16,18,21., 22., 23.,28].

Another system employed for functional analysis of CHEK2 variants utilized budding yeast S. cerevisiae strains null for RAD53 (and SML1 to rescue viability), which is the homolog of human CHEK2 [1] and the functional analog of CHEK1 [35]. The expression of human wild-type CHEK2 cDNA in RAD53-null yeast strains restored their slow growth phenotype, indicating the restoration of functions in cell-cycle checkpoints [36]. This system effectively differentiated the damaging effect of the truncating c.1100del;p.T367Mfs variant from wild-type CHEK2, as the expression of the variant resulted in reduced growth 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], inducing cell-cycle arrest due to stalled replication forks. Through this approach, two independent studies reported the functional characterization of 132 distinct CHEK2 variants (Figure 2, Table 1, and Table S1). Notably, 35 missense Variants of Uncertain Significance (VUS) identified in patients, two control deletion variants (p.E107_K197del and p.D265_H282del), and a catalytically inactive variant (p.D347A) that impairs kinase activity [20,24] were classified as damaging.

A third system for functional analysis relied on mammalian cell lines where endogenous CHK2 protein was depleted before complementation with human CHEK2 cDNA carrying specific variants [17,22,27]. Depletion of endogenous CHK2 was achieved through siRNA/short hairpin (sh)RNA-mediated silencing of CHEK2 expression (i.e., knockdown) [27] or by CRISPR/Cas9-based loss of CHEK2 expression (i.e., knockout) [17,22]. CHEK2 knockout is viable as it is a non-essential gene, promoting mammalian cell growth [17,19]. After the loss of endogenous CHK2, the functional effects of CHEK2 variants were measured using various readouts, including 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] (refer to Table 1). In total, these three studies functionally characterized 81 distinct CHEK2 variants (Figure 2, Table 1, and Table S1), identifying numerous missense variants with a damaging impact [17,22,27].

The studies resulted in the functional characterization of 179 distinct CHEK2 variants, including seven synonymous, nine truncating, three deletion and 160 missense VUS. An average voting score (Figure 2 and Table S1) revealed that 81 variants (i.e., seven synonymous variants and 74 missense VUS) were functional, 28 variants (i.e., one deletion variant, one truncating variant, and 26 missense VUS) were intermediate in function, and 70 variants (two deletion variants, eight truncating variants, and 60 missense VUS) were damaging. Mechanistic follow-up studies showed that some of the damaging CHEK2 missense variants impaired autophosphorylation and thus activation of CHK2, whereas 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, the most damaging missense variants were located in the FHA domain (aa 92–205) and the kinase domain (aa 212–501) of CHK2, which is perhaps not surprising because these together make up most of the protein (Figure 2 and Table S1). However, to gain a comprehensive view on the damaging impact of variants throughout CHK2, a more extensive functional assessment of variants located in the SQ/TQ cluster domain (SCD; aa 19–69) as well as outside functional domains is needed.

Challenges in the functional characterization of CHEK2 VUS

Each system employed for the functional analysis of genetic variants in CHEK2 comes with its own set of strengths and weaknesses, leading to potential discrepancies in outcomes and, consequently, the functional classification of CHEK2 variants. Here, we examine these strengths and weaknesses and outline some future challenges.

The initial functional analysis of CHEK2 variants primarily relied 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]. However, this approach has limitations, as upon activation by DNA damage, CHK2 variant proteins can form dimers with endogenous wild-type CHK2 protein. This interaction may impact the results of the assay, potentially obscuring the functional impact of variant CHK2 proteins. This concern may also apply to systems in which the depletion of endogenous CHK2 relied on knockdown [27] rather than knockout, as residual wild-type CHK2 protein may still be present. Conversely, the purification of recombinant CHK2 variant proteins from Escherichia coli for use in in vitro kinase assays could influence the functional impact of the variants due to the lack of post-translational modifications typically induced in response to DNA damage in human cells [22]. Moreover, in vitro assays may not capture potential defects in CHK2 protein stability or intracellular localization and often utilize artificial substrates to measure CHK2 kinase activity [16,18,22,23,28], which may differ from that on full-length substrates.

A significant number of CHEK2 variants have been characterized using a yeast-based system [20,24,26]. Despite the overall structural similarity of CHK2 in all eukaryotes, human CHK2 only shares 28% amino acid identity with the S. cerevisiae Rad53 protein [39]. Such sequence differences may affect the functional analysis of human CHEK2 variants in a yeast cell context. Additionally, yeast cells grow at 30°C instead of 37°C, potentially diminishing the impact of some variants on the thermodynamic stability of CHK2. Consequently, certain unstable CHK2 variants with intermediate functional effects in mammalian cells (e.g., p.D203G, p.E239K, and p.D438Y) [17] were classified as functional in a yeast-based system [20]. Therefore, the growth temperature of a model system is an important consideration in the functional characterization of human CHEK2 variants.

Given the potential limitations of a yeast-based system, a mammalian cell-based approach may be preferred for the functional analysis of CHEK2 variants. Two studies employed such a system based on stable and physiological CHK2 expression levels, avoiding 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. Generally, functional outcomes were consistent, with minor inconsistencies observed for only 3 (p.E64K, p.I157T, and p.D438Y) of the 10 variants studied. A potential limitation of this

approach is that some CHEK2 missense variants may disrupt CHK2 activity on one substrate but not on another. Consequently, this approach may not precisely measure the overall impact of a variant on CHK2 activity following DNA damage induction. However, correlating the results from phospho-Kap1 S473 assays with a broader functional readout (e.g., cell growth after DNA damage induction) for 8 variants indicated a strong and significant correlation [17]. While the role of CHK2 in regulating cell growth after DNA damage induction likely arises from its ability to phosphorylate multiple downstream targets, these findings suggest that phosphorylation of Kap1 S473 may serve as a suitable readout to assess the overall function of CHK2.

When using Kap1 S473 or another phospho-target of CHK2 as a functional readout, an additional complicating factor in the functional assessment of CHEK2 variants is the observed kinetic defect reported for some variants, such as p.E64K and p.R521W [17]. Examination of CHK2 kinase activity at different timepoints after ionizing radiation (IR) revealed that, unlike wild-type CHK2, these two variants are incapable of sustaining phosphorylation of Kap1 S473 over the course of the experiment (i.e., 6 h compared with 2 h after IR). This implies that the timing of CHK2 kinase activity assessment after DNA damage induction may influence functional classification, potentially contributing to reported discrepancies for p.E64K and p.R521W [17,20,22].

In contrast to cDNA-based complementation systems, variants can be introduced at endogenous loci using CRISPR-dependent technologies. For BRCA1, a CRISPR/Cas9-dependent saturation genome-editing technique was employed, enabling the functional characterization of nearly 4000 variants in the RING and BRCT domains of BRCA1 with cell survival as a functional readout [40]. Furthermore, a CRISPR-dependent cytosine base-editing screen was used for 86 DNA damage response (DDR) genes, including CHEK2. This approach allowed the interrogation of the functional effects of thousands of variants by examining cell growth after DNA damage induction [19]. A significant advantage of these approaches is their ability to assess the effects of variants within the context of the endogenous gene, thus reflecting physiological expression levels. Additionally, the effects of variants in noncoding regions, impacting mRNA splicing, can be functionally evaluated. While these technological advances are expected to play a crucial role in the future characterization of variants on a large scale, they may need optimization before being considered as a clinical diagnostics tool. For example, the base editor utilized by Cuella-Martin and colleagues has a six-nucleotide editing window and often introduces multiple variants therein [19]. This can make it challenging, if not impossible, to obtain and interpret results for individual variants. Moreover, the repertoire of variants that can be generated depends, among other factors, on protospacer adjacent motifs (PAMs) in the DNA targeted by the CRISPR system, limiting the number of variants that can be characterized. Finally, when a general readout like cell growth is examined, off-target effects of single guide (sg)RNAs may significantly impact the outcome of the functional assay. Nevertheless, these large-scale studies will undoubtedly expedite the path to clinically interpreting genetic variants in a high-throughput manner.

Clinical interpretation of *CHEK2* variants: functional assays to the rescue?

Over the past decade, there has been a rapid acceleration in genetic testing aimed at identifying individuals with an elevated risk of developing breast cancer. This now extends to moderate-risk genes such as CHEK2. However, the clinical classification of Variants of

Uncertain Significance (VUS) in CHEK2 is challenging due to their rarity and the moderate breast cancer risk linked to pathogenic CHEK2 variants. This complexity hinders the use of genetic approaches like cosegregation analysis, successfully employed for high-risk genes like BRCA1 and BRCA2 [41,42].

In improving the clinical classification of VUS in CHEK2, validated functional assays emerge as an attractive option. Before implementing these assays, it is crucial to establish the quantitative relationship between CHK2 protein functionality and cancer risk.

So far, reliable cancer risk estimates have only been established for a few relatively common CHEK2 variant alleles (Table 2) [6,17,43., 44., 45.]. Notably, risk estimates for these variants (e.g., p.E64K, p.R117G, p.I157T, p.R180C, p.H371Y, and p.T476M) exhibit an inverse correlation with their functional impact, indicating that variants with lower activity are associated with higher cancer risk (Table 2).

Contrary to these well-studied CHEK2 variants, the low prevalence of other missense variants hampers the empirical determination of their association with breast cancer risk. Assuming variants with similar impacts on CHK2 protein function share similar cancer risks, a burden-type association analysis based on reported protein functionality is justified (Table 3) [17,20,22].

This analysis brings to light several findings. Firstly, in vitro kinase assays generally exhibit poor correlation between functional effects and breast cancer risk, suggesting inadequacy in determining the functional effects of CHEK2 variants. Secondly, the yeast-based system excels in classifying damaging variants (OR ~2) but struggles to differentiate functional variants from intermediate variants (both having ORs ~1.3). Lastly, mammalian cell-based systems [17,22] demonstrate an inverse correlation between CHK2 protein function and breast cancer risk, aligning with findings for unique variant alleles (Tables 2 and 3).

While the dataset of variants with functional data is still limited, the Odds Ratios (ORs) derived from variant-specific or burden analysis underscore a subset of CHEK2 missense variants with cancer risks akin to truncating CHEK2 variants. These variants can be identified through functional analysis. Furthermore, the available data indicate that certain CHEK2 variants, not associated with clinically relevant cancer risks up to ORs of 1.3 (e.g., p.I157T and p.R180C), exhibit no discernible functional impact.

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

Nucleotide change	**Amino acid change**	**Average voting score (see Figure 2 in the main text)**	**Odds ratio[a]**	**95% CI**	***P* value**	**Refs**
c.190G>A	p.E64K	Intermediate	1.78	1.14–2.77	0.0112	[6,17]
c.349A>G	p.R117G	Damaging	2.22	1.34–3.68	0.002	[6,17]
			2.26	1.29–3.95	0.003	[44]
c.470T>C	p.I157T	Functional	1.37 (iCOGS[a] array)	1.21–1.55	<0.0001	[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	[44]

c.1100del	p.T367Mfs	Damaging	2.66	2.27–3.11	<0.0001	[6]
c.1111C>T	p.H371Y	Functional	1.01	0.64–1.59	0.9618	[6,17]
c.1427C>T	p.T476M	Damaging	1.60	1.10–2.35	0.0145	[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[a]

Study	Variant group based on function	Number of cases	Number of controls	OR	95% CI	*P* value
Boonen *et al.* [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 (excluding 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 (excluding p.R117G)	71	33	2.23	1.48–3.38	<0.0001
Delimitsou *et al.* [20]; *RAD53*-null yeast strains	Functional variants	397	304	1.36	1.17–1.58	0.0001
	Functional variants (excluding 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 (excluding 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 (excluding p.R117G)	69	36	1.99	1.33–2.98	0.0008
Kleiblova *et al.* [22]; *CHEK2* KO RPE1 cells	Functional variants	173	133	1.35	1.08–1.69	0.0092
	Functional variants (excluding 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 (excluding p.E64K)	38	23	1.72	1.02–2.88	0.0411
Kleiblova *et al.* [22]; pKap1 *in vitro*	Functional variants	153	107	1.48	1.16–1.90	0.0017
	Functional variants (excluding 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 (excluding p.T476M)	36	22	1.7	1.00–2.89	0.0501
Kleiblova *et al.* [22]; *in vitro* Omnia assay	Functional variants	131	90	1.51	1.16–1.98	0.0017
	Functional variants (excluding 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 (excluding p.T476M)	82	61	1.4	1.00–1.94	0.0487

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

Presently, there is a lack of established guidelines for reporting Variants of Uncertain Significance (VUS) in CHEK2 missense mutations, primarily due to insufficient evidence of disease association. However, recent insights (Table 3) [17,20] suggest the presence of CHEK2 missense variants linked to breast cancer risk comparable to that of CHEK2 truncating variants, including the c.1100del;p.T367Mfs variant (Table 2). Given this likelihood, it is imperative to employ functional assays to differentiate between missense variants affecting protein function, associated with an elevated breast cancer risk, and those that do not. This approach ensures a vital contribution to accurate variant classification and enhanced clinical management for carriers and their families.

In tandem with functional assays, computational tools may offer utility in the clinical interpretation of CHEK2 missense variants on a broader scale. One such tool, Helix [46,47], has demonstrated effectiveness in predicting the functionality of CHEK2 missense variants [17]. However, caution is warranted in handling these in silico predictions, as studies indicate a tendency to overestimate the number of damaging variants [38,48,49]. Consequently, computational tools can assist in interpreting missense variants with inconsistent functional outcomes across studies or those lacking functional analysis. They may also pinpoint missense variants requiring further scrutiny of their functional impact, especially when the predicted impact diverges from that measured in functional assays.

Concluding remarks and future perspectives

Given the rapidly increasing discovery of germline CHEK2 variants, there is a pressing need to identify which variants are linked to elevated cancer risk. In response, functional assays have been developed and employed to characterize a substantial array of CHEK2 missense variants, leading to the identification of rare variants with damaging effects on protein function (Figure 2). This progress has laid the groundwork for a burden-type association analysis, enabling the correlation of the functional impact of these rare CHEK2 missense variants with breast cancer risk (Table 3) [17].

Crucially, expanding the current cDNA-based methodologies to genome editing-based approaches will shed light on the impact of both coding and noncoding variants on RNA splicing and subsequent functional consequences. This extension holds the promise of refining the clinical classification of CHEK2 variants. Anticipated assays that comprehensively assess the functional effects of every conceivable nucleotide change in CHEK2, akin to those conducted for BRCA1 [50], are expected to result in publicly accessible resources showcasing quantitative functional outputs derived from validated and calibrated assays for all CHEK2 variants.

In conclusion, a ClinGen variant curation expert panel (VCEP) is poised to establish CHEK2-specific specifications for the ACMG-based clinical variant interpretation guidelines. This panel will also offer recommendations for incorporating results from functional analyses in the classification of CHEK2 missense variants. Ultimately, the incorporation of functional data from well-validated assays is anticipated to enhance the clinical interpretation of these variants, facilitating counseling for carriers and their families.

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