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

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CHAPTER 7

Future perspectives

7

FUTURE PERSPECTIVES

According to ClinVar (as of October 2022), a clinically oriented database for genetic variants, around 75% of all germline variants in the coding sequences of *PALB2* and *CHEK2* are missense variants, whereas 17% are frameshift and 8% are nonsense. Almost all (94%) *PALB2* (n=1987) and *CHEK2* (n=1284) missense variants are classified as variants of uncertain significance (VUS), which constitute variants that cannot be used for clinical decision-making or cancer risk assessment due to insufficient available evidence that can be used for clinical interpretation. Potential biochemical and structural alterations resulting from missense VUS are often extremely challenging to predict, meaning that they cannot be used for clinical interpretation of these variants. In contrast, the concerted efforts to functionally characterize numerous *PALB2* and *CHEK2* missense VUS in distinct functional assays on a variant-by-variant basis (as reviewed in **Chapters 2** and **3**), represent milestones for clinical interpretation and clinical management of *PALB2* and *CHEK2* VUS carriers. However, some challenges still remain, such as addressing the functional effects of an overwhelming number of VUS in these genes that are yet to be functionally characterized. This also includes addressing the functional impact of variants on RNA splicing as many missense or synonymous variants that do not affect protein function in many of the cDNA-based complementation systems that have been used for functional analysis of variants, may still have a negative impact on protein function due to the potential introduction of cryptic splice sites (1). A future outlook to address these challenges, as well as the challenge of implementing functional evidence in clinical variant interpretation, is provided below.

Characterizing functional impact of genetic variants at scale

Sequencing hereditary breast cancer susceptibility genes has proven to be a powerful diagnostic tool to identify individuals at increased risk for breast cancer (2). However, each individual's genome contains millions of sites where his or her DNA differs from the reference sequence. Despite major advances in cataloging and this overwhelming number of genomic variants (e.g., in ClinVar), current clinical and functional understanding of most of the identified variants is insufficient. Even with the ongoing efforts in experimentally measuring the functional consequences of variants in *PALB2* and *CHEK2*, most missense variants in these genes are still classified as VUS (3,4). This is mainly because variant-by-variant assays (as those discussed in this thesis) are too time and resource intensive to keep up with the number of identified variants. Also, functional assays are generally performed after a variant is identified in a carrier. Results may then become available years later, when they may no longer be relevant for deciding on preventive therapeutic strategies (at least for the carrier in which the variant was identified first). Alternatively, to keep up with the large number of genetic variants

that are being detected during genetic testing, high-throughput systems can be applied to measure the functional consequences of all possible variants in disease-relevant loci, for a variety of molecular and cellular phenotypes, simultaneously. The functional data obtained by high-throughput approaches can then be presented in the form of comprehensive atlases that do not only facilitate interpretation of variants that have already been identified in carriers, but also variants that are yet to be identified during genetic testing.

For *PALB2*, we employed a cDNA-based complementation system, using a variant library for the Coiled-Coil (CC) region of *PALB2*, to assess sensitivity to PARPi treatment in a high-throughput manner (**Chapter 5**). Using this strategy, we functionally assessed 91.1% of all possible missense variants in this region. Importantly, this strategy can be extended to the WD40 domain of *PALB2*, or other regions, or even *CHEK2* (with phospho-Kap1 as a readout, **Chapter 6**), with the use of additional variant libraries. An alternative approach to our high-throughput assay may be to introduce variants endogenously. For instance, a saturation genome editing technique that relies on CRISPR/Cas9-induced DNA breaks in combination with the use of repair templates (each containing a distinct variant), was reported for *BRCA1* (5). Using a similar approach for *PALB2*, introduction of a damaging *PALB2* variant in the human haploid cell line HAP1 (6) will result in cell death since this is an essential gene (5). Thus, after introduction of a large number of variants in this cell line, cells expressing damaging *PALB2* variants should be depleted from the population and cell survival can be used as a functional readout in a high-throughput manner. In contrast, HAP1 cells expressing damaging *CHEK2* variants may gain a growth advantage, as was also evident in a recent base-editing screen in which variants were introduced endogenously by direct modification of bases with a nuclease-deficient Cas9 tethered to the cytosine deaminase APOBEC1 (7). Importantly, when variants are introduced endogenously, their effects on multiple layers of gene function (such as RNA splicing and stability, and protein stability and function), can be studied simultaneously. Another option to address functional effects of variants *en masse* is by massively parallel sequencing (VAMP-seq), which is an experimental strategy that can measure the effects of thousands of missense variants on intracellular abundance simultaneously (8). In this approach, a mixed population of cells is generated where each cell expresses one protein variant fused to a fluorescent tag. Cells can be sorted by flow cytometry based on their levels of fluorescence, which in turn corresponds to a certain degree of variant protein stability. As many variants in the WD40 domain of *PALB2*, or throughout the entire *CHEK2* sequence, have been shown to affect protein stability (9-11), such a method may enable the identification of many damaging variants. Collectively, large-scale functional data resulting from the above-mentioned approaches can eventually result in lookup tables that will aid in a more accurate ascertainment of the pathogenicity of many genetic variants.

Despite the promising utility of high-throughput assays in large scale variant interpretation, it should be noted that they can produce noisy data (12), hampering their use for clinical classification. This issue may be addressed by performing, 1) high numbers of replicate experiments, 2) cross validation with clinical data or other (high-throughput) functional studies, and 3) single variant assays for proper validation of functional effects. In addition, to further improve the clinical utility of high-throughput assays for missense variants, results can be cross-correlated with *in silico* prediction tools such as the splice predictor SpliceAI (13), which is generally valued for its accuracy and can easily provide evidence for variant interpretation at a large scale (14,15). Such a correlation could provide important insights into which missense variants affect protein function due to effects on RNA splicing or due the amino acid substitution it causes.

The possibility of examining a specific phenotype of interest at scale, such as PALB2's function in HR, in high-throughput functional assays, is a prerequisite for the ability to test large numbers of variants in a gene. However, despite the recent development of different types of high-throughput functional assays, many disease-associated genes still remain beyond reach owing to a lack of assays with a suitable read-out. Consequently, an important question is: what do we need in order to apply high-throughput functional assays to all disease-associated genes in the genome? Alternative to the aforementioned functional readouts, recent advances in microscopy-based cell sorting now allows for high-throughput examination of visual cellular phenotypes as a result of genomic variation (i.e., Visual Cell Sorting) (16). For example, this technique enables the sorting of hundreds of thousands of cells according to the nuclear localization of a fluorescently tagged protein (variant) (16). As PALB2 localizes to the nucleus in order to perform its DNA repair function, and mis-localization in the cytoplasm has been associated with impaired PALB2 protein function (11,17,18), this technique may enable the identification of damaging PALB2 variants at a large scale. This is only one example of a technique that will strongly expand the repertoire of disease-associated genes for which genetic variants can be functionally characterized. Based on this, it may be expected that high-throughput assays will soon be further adapted and optimized, allowing for an expansion of the repertoire of genes for which large-scale variant analysis is desired.

Functional analysis of splice variants

Splicing of precursor messenger RNA (pre-mRNA) is an extremely complex process, and the clinical interpretation of variants that affect splicing can take into account predictions from both computational algorithms, as well as experimental data. When these variants occur at canonical splice sites, i.e., at the 'GT' splice donor and the 'AG' splice acceptor site, they are often easy to classify. This is because predictive models, nowadays based on deep learning, perform reasonably well (13). For instance, these variants are generally considered pathogenic

when loss of function of a gene (e.g., due to expected skipping of an entire exon that may encode a region essential for protein function), is known to be causative of disease (19). However, there are many caveats that must be considered for these types of variants, as there are several scenarios in which a functional protein can be produced despite the presence of a variant in a canonical splice site. Therefore, these variants can be most problematic for clinical interpretation

Splicing assays that assess the impact of variants at the mRNA level can be highly informative and can include direct analysis of RNA, or in vitro minigene splicing assays (20). These assays have been shown to be useful for the interpretation of splice variants occurring at canonical splice sites, in coding sequences, or even in deeper intronic regions (21). However, unlike a functional readout for protein function itself, an effect on splicing (e.g., exon skipping, or intron retention) does not necessarily translate to an impact on protein function. In general, aberrant splicing can result in multiple outcomes with respect to mRNA fate and the protein-reading frame. Although it is often assumed that abnormally spliced transcripts resulting in a premature stop codon will undergo nonsense-mediated decay, this is not always the case as exemplified by normal protein levels observed for the Fanconi anemia-associated *PALB2* p.Y551X truncating variant (22). Consequently, some abnormal transcripts can lead to expression of a truncated protein with or without functional consequences (23). Thus, accurate clinical classification of variants that affect RNA splicing requires that alternative transcripts are identified, quantified, and functionally characterized.

With regards to clinical interpretation, a functional evaluation of genetic variants that includes potential effects on RNA processing, is most valuable. An advantage of the haploid HAP1 cell-based system where genome editing can be employed to introduce variants at endogenous loci (5,7), is that the effect of variants on regulatory mechanisms such as splicing can be included (24). Alternatively, complementation with a bacterial artificial chromosome (BAC) containing the complete human gene of interest (GOI), can also allow for evaluation of any type of variant. This method was, for instance, used to evaluate numerous *BRCA2* variants for their effects on splicing and their capacity to express functional BRCA2 protein isoforms after loss of the endogenous gene in mES cells. Importantly, multiple alternative transcripts encoding (partially) functional protein isoforms were identified and their altered expression attenuated the functional effects of several predicted *BRCA2* loss-of-function canonical splice variants (25). Additionally, several *BRCA2* nonsense variants in exon 12, that were initially assumed to be pathogenic, have been shown to result in enhanced expression of an alternative transcript lacking exon 12, which encodes a (partially) functional protein isoform (26). Consequently, these and other assumed loss-of-function variants in exon 12 of *BRCA2*, constitute variants for which further studies are required to estimate their associated cancer risk. These findings highlight the need of examining the effects of genetic variants on RNA

splicing and protein function. To date, however, it has been difficult to identify all the different transcripts that are expressed due to a genetic variant. Moreover, the quantification of all these distinct transcripts is extremely challenging. The advent of PacBio-based Next Generation Sequencing, however, may be able to provide a complete RNA transcript profile as it allows for the analysis of long reads up to 25kb (27). For many genes, all RNA transcripts ranging from the first to the last exon can then be captured in a quantitative manner and linked to a functional phenotype. Overall, such techniques will result in more detailed understanding of how variants can affect RNA splicing, and also a better clinical classification of these variants.

The use of functional data for clinical interpretation of variants

Most variants identified in the breast cancer susceptibility genes are exceedingly rare and it will require extremely large case-control association studies (i.e., >1 million individuals) to accurately quantify cancer risk for specific variants. Validated functional assays, are considered by the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines, as strong evidence for or against the pathogenicity of rare missense variants (28,29). Accordingly, a strong concordance was observed between two high-throughput functional studies for *BRCA1* and ClinVar classifications of pathogenicity for variants with expert panel evaluations (5,30). This supports the claim that functional characterization of genetic variants is extremely useful for clinical interpretation of variants and assessment of cancer risk. Furthermore, as shown for both *PALB2* and *CHEK2* (**Chapter 5** and **Chapter 6,** respectively), results from functional assays quantitatively correlate with the degree of breast cancer risk, as calculated based on variant frequency data from the Breast Cancer Association Consortium (BCAC) (31). That is, the degree of breast cancer risk that is associated with a certain level of PALB2 or CHK2 protein function, was established using a burden-type association analysis. In this analysis, variants are grouped based on similar impact on protein function and joint frequencies of these variants in cases and controls are used to derive odds ratios per variant group (representing a level of protein functionality). However, as especially damaging *PALB2* variants are extremely rare in occurrence, it is (for this variant group particularly) difficult to obtain high enough case-control frequencies and establish an odds ratio with a narrow confidence interval that is statistically meaningful (e.g., p-value <0.01). Thus, to make these associations more conclusive, and the burden-type association analysis more valuable, high-throughput functional assays need to be performed for (nearly) all *PALB2* and *CHEK2*, variants. Even then, it may be challenging to identify enough damaging *PALB2* missense variants to warrant an accurate association with breast cancer risk. Accordingly, there is also an urgent need for more clinical data from large case control association studies, such as that from BCAC (31), that can ultimately be combined with other large case control studies, such as for instance CARRIERS (32), in order to improve

our understanding of the quantitative relationship between PALB2 protein function and cancer risk.

Although variants in *CHEK2* occur more frequently in the general population relative to variants in *PALB2* (33), the fact that it constitutes gene that is associated with moderate risk (~2 fold increased) excludes the use of genetic approaches such as co-segregation analysis to determine the pathogenicity for variants as is done in genes with high penetrance like *BRCA1* or *BRCA2*. To date, there are no *CHEK2* missense variants that are classified as benign or pathogenic based on clinical data. As a consequence, setting a functional threshold for benignity is very complicated. In contrast, although known pathogenic missense variants cannot be used, the residual functionality seen for truncating variants can be used to calibrate a functional threshold for pathogenicity. Similar to *PALB2*, it is pivotal that more *CHEK2* missense variants are functionally characterized, with for instance, high-throughput approaches. Ultimately, it may even be possible to generate a 'continuous risk model' that allows for the calculation of a variant-specific risk. For example, based on the data presented in **Chapter 6**, we could reason that the damaging *CHEK2* missense variants, for which the functional impact is similar to that of the truncating variants, are associated with a similar breast cancer risk. Relative to that, the average decrease in protein function that we observed for intermediate missense variants is 60%, while the average decrease of the functional variants is 10%. Because we associated these variant groups with odds ratios in the burden type association analysis, we can also deduct a simplified continuous risk model from this data (Fig. 1). Assuming that the odds ratio is a function of the functional score (i.e., a decrease in CHK2 protein function inversely correlates with breast cancer risk), we can then for example calculate that *CHEK2* p.D203G, for which we could not calculate an odds ratio specifically and which showed a decrease in protein function of ~50%, associates with an odds ratio of ~1.55. This continuous risk model is currently based on only 44 out of the 388 *CHEK2* missense variants that were identified the BCAC studies (31) and that were functionally characterized as functional, intermediate or damaging in **Chapter 6**. Several relatively frequent *CHEK2* missense variants for which cancer risk estimates are already available, such as those mentioned in **Chapter 3** (Table 2), could be added as individual datapoints (i.e., not being part of the functional, intermediate or damaging variant groups) to improve the linear regression analysis. In addition, high-throughput functional analysis of *CHEK2* missense variants should allow for functional characterization of nearly all 388 *CHEK2* missense variants and should therefore results in an even more accurate estimation of the correlation between residual functionality and associated cancer risk.

With an optimistic view to the future, it is foreseeable that additional high-throughput functional and clinical studies will ultimately result in the establishment of a quantitative relationship between protein function (e.g., functional, intermediate and damaging), and the

degree of associated cancer risk. This will be a major step towards the use of functional data in personalized risk prediction and clinical decision making.

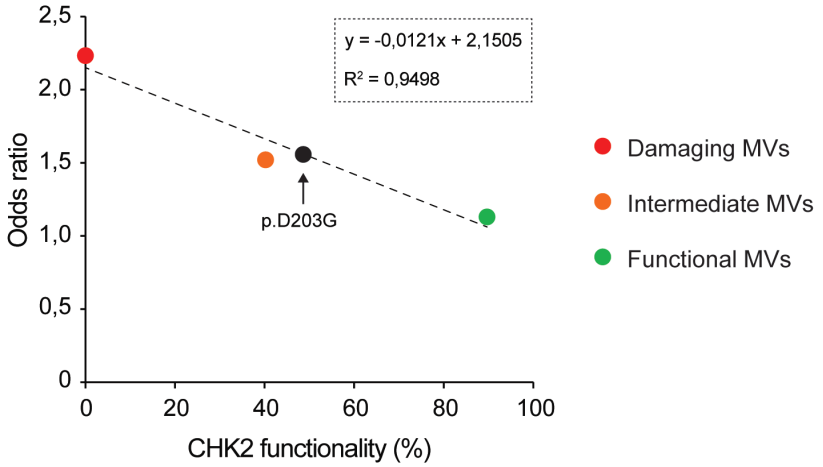

Figure 1. Quantitative relationship between CHK2 function and cancer risk. The functional defect of damaging missense variants (MVs) compares to that of truncating *CHEK2* variants and the average impact of this variant group on Kap1 S474 phosphorylation is therefore set to a 100% decrease in CHK2 protein function. The average impact of the functional and intermediate variant groups on Kap1 S474 phosphorylation are presented relative to that of the damaging *CHEK2* variant group. In **Chapter 6**, odds ratios were calculated for the three *CHEK2* variant groups (i.e., functional, intermediate and damaging). The dotted line represents the correlation between CHK2 function and associated breast cancer risk. Once the impact on CHK2 function has been established for a VUS, the OR of the variant can be estimated.

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