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

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CHAPTER 1

General Introduction

1

GENERAL INTRODUCTION

Breast cancer etiology

Breast cancer affected roughly two million women globally in 2019, resulting in an estimated death toll of 689.000 and is thereby the most common cause of cancer-related death in women (1). Although breast cancer can occur in men as well, 98.7 percent out of all breast cancer cases in 2019 were observed in female. Furthermore, it is estimated to affect roughly one in 20 women globally, and as many as one in eight women in high-income countries by the age of 85. This makes it by far the leading cancer-related cause of disease burden in women (2).

The development of breast cancer likely involves a combination of risk factors, making it an extremely heterogeneous disorder biologically. In addition to female gender and advancing age, other breast cancer risk factors include early menarche, late menopause, and first birth at 30 years of age or later (3). Most breast cancers occur in the absence of any family history of this type of cancer, meaning the underlying cause may be a combination of demographic, behavioral and environmental factors. During life, these factors may cause to somatic gene alterations that mostly occur by chance. If so, it is not possible for a person to pass on these genomic alterations to their offspring. In contrast, inherited breast cancer can occur as a result of genetic alteration in the germline. These variants can be inherited from parent to offspring and give rise to familial predisposition (3). Many of these genetic variants occur in tumor suppressor genes, such as the well-known DNA repair genes *BRCA1*, *BRCA2*, and *PALB2* or in genome caretaker genes such as *TP53*, *ATM* and *CHEK2* (4). Notably, these genes are all involved in maintaining genomic stability by acting in the DNA damage response (DDR).

DNA damage response and cancer

The inability to respond properly to DNA damage can result in a high frequency of unwanted somatic gene alterations (i.e., genomic instability), which in turn can promote the development of cancer (5). Proper regulation of the DDR is therefore crucial for cellular homeostasis and indispensable for maintaining genomic stability (6-8). During the DDR, cells can activate cell cycle checkpoints that in turn can result in cell cycle arrest, repression or activation of transcription, DNA repair, or even programmed cell death. Depending on the type and extent of DNA damage, the site of the lesion, and stage of the cell cycle, a choice is made between several DNA repair pathways to repair the DNA damage. These pathways include nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), single-strand break repair (SSBR), canonical non-homologous enjoining (cNHEJ), alternative non-homologous enjoining (aNHEJ), single-strand annealing (SSA) and homologous recombination (HR) (8,9). Some of these pathways are more mutagenic than others, i.e., they

have a higher probability of resulting in permanent DNA changes, and careful regulation of these pathways is therefore crucial for genomic stability.

Deregulation in the repair of DNA damage can be caused by DNA variants in genes or by aberrant activities of key proteins involved in the DDR (10). Failure to faithfully repair damaged DNA can result in a high mutational frequency within the genome of a cellular lineage (11,12). In hereditary breast cancers, it is established that pathogenic germline variants in DNA damage repair genes such as the high-risk breast cancer susceptibility genes *BRCA1*, *BRCA2* and *PALB2* (odds of developing breast cancer >5), or moderate-risk genes *ATM* and *CHEK2* (odds of developing breast cancer between 2-5), lead to a significant increase in the risk for developing breast cancer (4,13) (Fig. 1). As such, it is important that these genes are sequenced in individuals that may be at risk for developing breast cancer, so that pathogenic variants in these genes can be identified early.

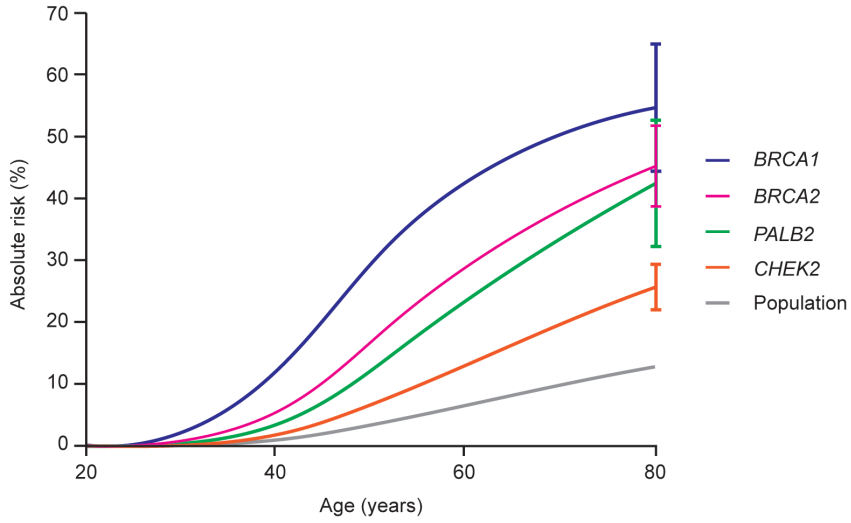

Figure 1. Estimated absolute risk of breast cancer associated with truncating variants in *BRCA1*, *BRCA2*, *PALB2* and *CHEK2* (4). The absolute risk of breast cancer is shown up to 80 years of age. The baseline estimated risk is shown in grey based on population incidences in the UK in 2016 (65). The error bars indicate 95% confidence intervals.

Homologous recombination

The three high-risk breast cancer susceptibility genes (i.e., *BRCA1*, *BRCA2* and *PALB2*) are crucial for DNA double-strand break repair by HR (Fig. 2). During HR, BRCA1 counteracts the accumulation of 53BP1, which otherwise interacts with the chromatin adjacent to the broken DNA ends to promote NHEJ (14,15). This BRCA1 activity permits DNA end-resection at the break-sites by exonucleases such as MRE11 to yield 3'-single-stranded (ss) DNA overhangs that are required for HR-mediated double-strand break repair (16). The 3'-ssDNA overhangs then become coated by replication protein A (RPA) (17), promoting the sequential accumulation of BRCA1, PALB2 and BRCA2. PALB2 is crucial in this event as it mediates the formation of the PALB2-BRCA1/2-RAD51 complex and together with BRCA2 facilitates the replacement of RPA with the RAD51 recombinase (18). The RAD51-ssDNA nucleoprotein filaments then promote the homology search using the sister chromatid and the ensuing strand exchange. As this repair pathway requires a non-damaged sister chromatid to act as a template for repair, it is mostly active during S- and G2-phase of the cell cycle and drives error-free repair of DNA double-strand breaks. As a consequence, HR is imperative for maintaining genomic stability, highlighting the importance of *BRCA1*, *BRCA2* and *PALB2* as tumor suppressor genes.

Cell cycle regulation

The moderate-risk genes *ATM* and *CHEK2* are also involved in the DNA damage response. Although their functions are linked to those of *BRCA1*, *BRCA2* and *PALB2*, they regulate the DNA damage response differently. In contrast to acting as key players in HR, their functions have predominantly been associated with the *TP53* signaling pathway. The *TP53* gene (expressing p53) represents another important tumor suppressor gene. Although somatically acquired pathogenic variants in *TP53* can be found in substantial proportions of nearly all types of cancer, germline pathogenic variants in the *TP53* gene are rare and they are associated with a significant risk for developing breast cancer (4). This is not surprising as impaired p53-mediated signaling can have a major impact on the DDR. For instance, impaired p53-mediated signaling can result in abnormal expression of numerous p53 target genes, several of which are involved in the regulation of cell cycle arrest, a process that provides cells time to repair the damaged DNA (19,20). Furthermore, defects in p53-mediated signaling may impair apoptosis. As a consequence, cells may no longer be restrained from proliferating in the presence of unrepaired DNA damage (21). It is therefore crucial for cells to have the activity of p53 carefully regulated.

Both ATM and CHK2 are important for p53-dependent signaling, as they are involved in the activation of p53 during the DDR. In fact, ATM is considered a key DNA damage signaling component in mammalian cells as it encodes a kinase that acts early in response to

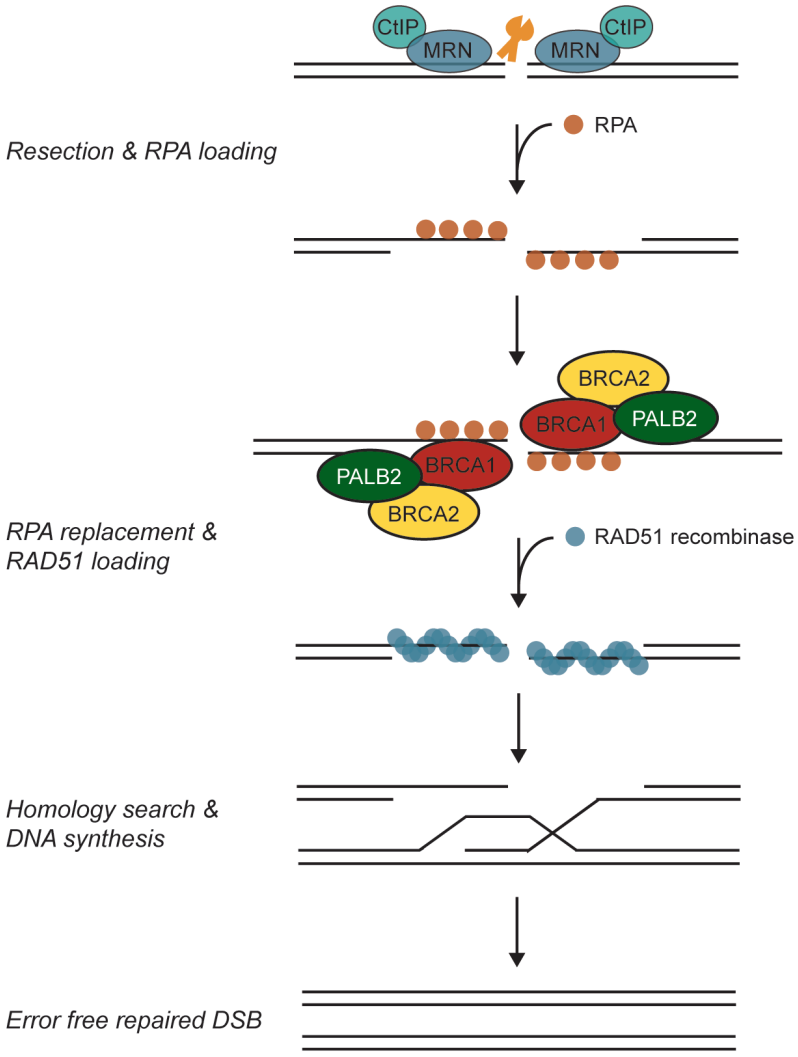

Figure 2. Schematic representation of HR-mediated repair of a DNA double-strand break. Initially, the broken DNA ends become resected by exonucleases such as MRE11 (part of the MRN complex) to yield 3'-ssDNA overhangs that are coated by RPA. Sequential recruitment of BRCA1, followed by PALB2-BRCA2 is crucial for the subsequent replacement of RPA with the RAD51 recombinase. The RAD51 nucleoprotein filaments will promote the homology search using the non-damaged sister chromatid as a template, eventually ensuing in error-free repair of the double-strand break.

DNA damage. One of the best-established downstream targets of ATM is the CHK2 kinase. CHK2, encoded by the *CHEK2* gene, functions to reduce cyclin-dependent kinase (CDK) activity by various mechanisms, including the phosphorylation and subsequent stabilization of p53. This results in an arrest in cell-cycle progression due to activation of G1-S, intra-S and possibly G2-M cell-cycle checkpoints, thereby providing time for DNA repair before cells start DNA replication and/or mitosis. These findings suggest that CHK2 and p53 act in a common pathway. Importantly, and in line with CHK2's critical role in the DDR, pathogenic variants in the *CHEK2* gene, such as the truncating c.1100delC variant, have been found to associate with a moderate risk for breast cancer (4). Consequently, it is imperative that the pathogenic potential of other type of genetic variants in *CHEK2* are well understood.

Genetic variants and clinical management

Identifying individuals who are strongly predisposed to breast cancer due to an inherited variant in a breast cancer susceptibility gene has tremendous clinical value. Such individuals may benefit from cancer prevention strategies or early detection. Several clinical features may indicate whether an individual may be at risk for breast cancer due to the presence of a genetic variant in the germline. This includes a clear positive family history (i.e., multiple (early onset) breast cancer cases), bilateral disease and distinct types of cancer (e.g., combination breast and ovarian cancer). For such cases, genetic tests can be performed that are commonly aimed at detecting variants in the *BRCA1*, *BRCA2*, *PALB2* and *CHEK2* tumor suppressor genes. Such testing can reveal the presence of different types of DNA-variants in these genes, including nonsense, frameshift, splice, missense or synonymous variants. Protein-truncating variants (PTVs, i.e., nonsense or frameshift), or variants that affect splicing, are often classified as (likely) pathogenic variants (22). These types of genetic variants are typically known to associate with high risk for breast cancer as they are expected to impair protein function. In conjunction with loss of the wildtype allele (i.e., loss of heterozygosity; LOH), which is very often seen in tumors, this means that tumor cells can no longer express a functional protein at all.

For carriers of (likely) pathogenic variants, specific recommendations for clinical management have been specified (23). For instance, measures can be taken to increase the frequency of screening for breast cancer and to consider procedures such as bilateral risk-reducing mastectomy. However, although bilateral mastectomy reduces cancer risk by at least 90% in carriers of pathogenic variants in high-risk genes such as *BRCA1* or *BRCA2* (24,25), such risk-reducing surgery is not recommended for women at moderate risk of breast cancer (e.g., due to pathogenic variants in *CHEK2*). Instead, in such carriers, annual mammography is offered on the basis that biennial screening is clinically effective in reducing advanced breast cancers and breast cancer mortality in the general population (26). Alternatively, for women

already diagnosed with breast cancer, identifying a pathogenic germline variant in *BRCA1*, *BRCA2* or *PALB2* may affect treatment options, such as surgical decisions to reduce the risk of recurrence, or the use of poly (ADP-ribose) polymerase inhibitor (PARPi) therapies. The latter option has been shown to be effective in a subset of HR-deficient tumors (27), as especially HR-deficient cells are sensitive to the inhibition of PARP (Fig. 3). It may also stimulate the testing of unaffected family members that are potentially at a similar increased risk for developing breast cancer. Taken together, it is important that carriers of (likely) pathogenic variants in the aforementioned breast cancer susceptibility genes are identified.

In contrast to PTVs or many splice variants that are clearly pathogenic, the clinical and functional impact of missense variants is often unclear. These variants are referred to as variants of uncertain significance (VUS) and for such genetic variants it is uncertain whether or not they increase the risk for developing breast cancer. This is due to the rarity of many of these missense VUS, which limits the evidence available to determine if a variant is pathogenic or benign. Accordingly, VUS cannot guide clinical decision making, complicating post-test patient counselling and clinical management (28,29). Until recently, assessment of pathogenicity of VUS relied mostly on co-segregation of the variant with cancer in families and the family history of cancer. Co-segregation is analyzed by statistical means, which usually requires multiple families to reach sufficient significance. However, the majority of VUS in breast cancer susceptibility genes occur so rarely in the general population, that they result in too few families in which the same variant can be found segregating. Hence the co-segregation as well as the associated cancer risks are difficult to assess at statistically significant levels. Furthermore, pathogenic variants in genes such as *CHEK2* are associated with a moderate risk of breast cancer. Moderate-risk alleles often cause cancer in combination with other genetic variants (such as polygenic risk alleles) and demographic, behavioral and environmental risk factors. Therefore, they can remain non-penetrant in many individuals. Accordingly, the effect of a pathogenic variant in *CHEK2* on cancer risk will often not give rise to the same sequalae seen for pathogenic variants in a high-risk gene such as *BRCA1*, *BRCA2*, and *PALB2*.

Fully realizing the clinical potential of genetic tests requires an accurate assessment of pathogenicity, even for rare genetic missense variants. To this end, additional methods for interpreting rare VUS, in both moderate- and high-risk genes, are of great value for clinical management of carriers. Knowing which VUS are damaging, or not, will help clinicians understand the test results (i.e., estimating whether a variant is pathogenic, or not) and can help to decide on the right clinical management. One powerful approach to improve the clinical classification of VUS is by using data from functional testing. Such functional evidence describes the molecular consequence of a variant on protein function and can consist of the results of either molecular or cellular experiments in vitro. When clinical data is scarce,

functional data has considerable potential to aid in variant classification, particularly VUS re-classification (30). The 2015 American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines for clinical sequence variant interpretation state that the results of a well-established functional assay can qualify as evidence to be used for clinical classification of variants (31). Aspects of the functional assay, such as calibration (by including clinically proven benign and pathogenic variants), or reproducibility of the results and the ability of the assay to reflect the tumor suppressive function of the protein, can all weigh in on the predictive power of the assay (32-34).

For *BRCA1*, *BRCA2*, and *PALB2*, assays using HR and/or resistance to DNA damaging agents have emerged as the standard for the functional characterisation of VUS in these genes (35-52). In contrast, in an effort to interpret various VUS in the moderate-risk gene *CHEK2*, several studies assessed their functional consequences, either by determining the effect on kinase activity or on cell growth (53-64). Collectively, these studies show the power of these assays in functionally characterizing many VUS, efforts that are expected to have a major impact on clinical variant interpretation.

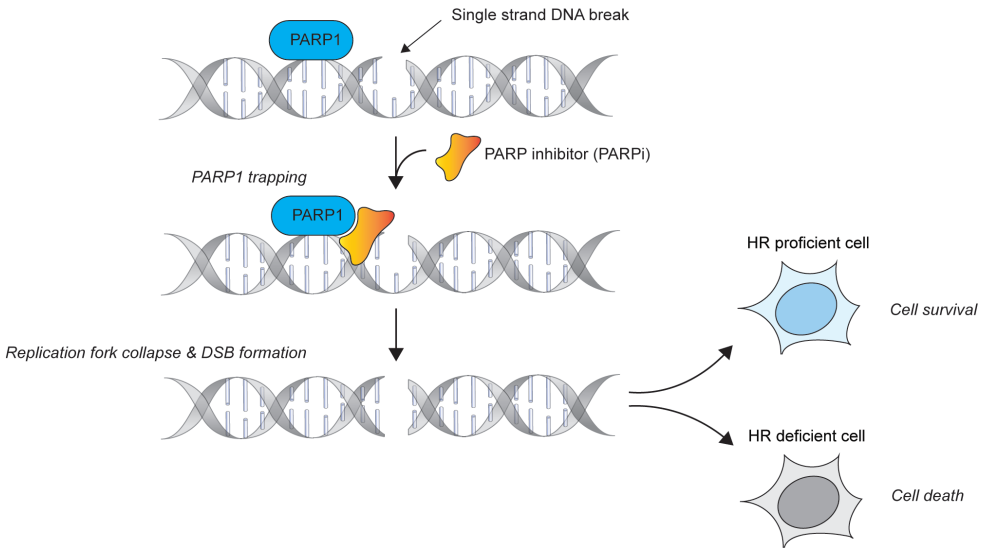

Figure 3. Mechanism of action of PARP inhibitors (PARPi). Upon the formation of single-strand breaks in the DNA, PARP1 becomes recruited and activated, resulting in the repair of these type of DNA lesions. Treatment with PARPi will result in trapping of PARP1 at the DNA lesion. This is thought to block repair of the DNA lesion and result in replication fork collapse during DNA replication, eventually ensuing in the formation of DNA double-strand breaks. This will result in lethality only in HR-deficient cells, which are unable to repair these breaks in an error free manner.

AIM AND OUTLINE OF THIS THESIS

In this thesis, I focus on the functional characterization of genetic variants in the high-risk breast cancer susceptibility gene *PALB2* and the moderate-risk gene *CHEK2.* The aim is to generate functional data for improved clinical interpretation of such variants. Quantitative assessment of the functional consequences of VUS in either gene can identify functionally damaging variants that associate with increased breast cancer risk, thereby aiding in the clinical management of patients and carriers.

In **Chapter 2** I first provide an overview of the functional analysis that have been performed by us and other research labs for variants in the *PALB2* gene. I then provide a similar overview for the *CHEK2* gene, for which different functional analysis have been used to functionally characterize numerous *CHEK2* genetic variants (**Chapter 3**). In **Chapter 4**, I present my results on the functional analysis of *PALB2* variants. Following careful validation of our newly developed functional assay, I could show that several missense VUS located in the Coiled-Coil (CC) and WD40 domains of PALB2, can result in major effects on protein function. I then present additional findings on the functional analysis of *PALB2* variants, showing that I could adapt our functional assay to allow for a high-throughput analysis of nearly all possible missense variants in the CC domain of PALB2 (**Chapter 5**). In **Chapter 6**, I discuss results on the functional analysis of *CHEK2* variants. Using a newly developed assay, I show that the degree of functional impact of variants in *CHEK2* correlates with breast cancer risk. In **Chapter 7**, I conclude my thesis with future perspectives on how the functional assays presented in this thesis can be further optimized to meet the clinical demand for functional data. Finally, I also discuss what would be required for these assays to be further implemented during clinical variant interpretation and risk assessment.

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