A Multifaceted Approach to Elucidating the Role of *BRCA1*- and *BRCA2*-Related Genes in Hereditary Breast Cancer

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One would think that after writing over 130 pages to complete this thesis, the labour involved in completing the acknowledgement section should peril in comparison. However, as I sit back and remember the countless efforts offered by so many, I find that my job here will be a great deal more difficult than I had anticipated.

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Dedicated to my family

For constantly asking, occasionally listening, rarely understanding

yet always portraying their interest in my research

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Novak DJ, Sabbaghian N, Maillet P, Chappuis PO, Foulkes WD, Tischkowitz M.

Experimental design was conceived by DJN, MT and WDF. RAP80 genotyping and analysis was conducted by DJN under the supervision of MT. Abraxas genotyping was conducted by NS under the supervision of MT. Analysis of Abraxas was conducted by DJN and NS. RAP80 M353T segregation analysis was conducted by DJN under the supervision of MT. Patient sample recruitment was conducted by MT, PM and POC. DJN drafted the manuscript, which was revised by MT.

Chapter Three: ***Identification of a novel CHEK2 variant and assessment of its contribution to the risk of breast cancer in French Canadian women.***

Novak DJ, Chen LQ, Ghadirian P, Hamel N, Zhang P, Rossiny V, Cardinal G, Robidoux A, Tonin PN, Rousseau F, Narod SN, Foulkes WD.

Experimental design was conceived by DJN, LQC, NH and WDF. Data acquisition was conducted by DJN under the supervision of WDF. Initial technical optimizations were conducted by VR and NH. Sample recruitment and implementation was carried out in collaboration with PG, PT and AR. Neonatal genotyping was performed by GC and FR. Additional French Canadian R406H genotyping was carried out by SAN and PZ. DJN drafted the manuscript, which was revised by WDF.

Chapter Four: ***The Characterization of Genomic Instability in Lymphoblastoid Cell Lines Derived from Heterozygous PALB2 Mutation Carriers.***

Novak DJ, Amrein L, Reddy J, Wark L, Aloyz R, Mai S, Tischkowitz M

Experimental design was conceived by DJN, MT, SM and RA. Patient cell lines were acquired by MT. Telomere Q-FISH was conducted by DJN under the supervision of MT and SM. Centromere FISH and cytotoxicity assays was conducted by DJN under the supervision MT. LA is responsible for teaching DJN the cytotoxicity assay and assisting in the trouble shooting of all work related to cell culture and use. Spectral karyotyping was conducted by LW and JR under the supervision of SM. DJN drafted the manuscript which was revised by MT.

Abstract

5-10% of hereditary breast cancer cases are caused by germline mutations in well-defined, dominantly acting susceptibility genes such as *BRCA1* and *BRCA2*. However, more than 50% of the genetic predisposition to hereditary breast cancer remains unexplained. In the following thesis, we present a multifaceted approach aimed at further elucidating hereditary breast cancer associated with *BRCA1* and *BRCA2* interacting genes; specifically, by analyzing the potential contribution from of two previously unscreened *BRCA1*-associating genes, *RAP80* and *Abraxas*, assessing the presence and risk associated with *CHEK2* susceptibility alleles in the previously uninvestigated French Canadian population and by investigating molecular and cellular mechanisms underlying the increased risk associated with *PALB2* susceptibility alleles.

A combination of genotyping 96 *BRCA1/2* negative, high risk breast cancer patients and segregation analysis was utilized in the determination of whether or not *RAP80* and *Abraxas* are breast cancer susceptibility genes. The contribution of *CHEK2* associated breast cancer amongst the French Canadian population was determined through the genotyping 25 *BRCA1/2* negative, high risk breast cancer and a cohort of 25 controls. Finally, the biological significance of four *PALB2* susceptibility alleles was investigated through the use of the cellular cytotoxicity assay WST-1, telomere specific Q-FISH, centromere specific FISH and spectral karyotyping.

The results presented herein suggest that both *RAP80* and *Abraxas* are not high to moderately penetrant breast cancer susceptibility genes. Further, our results suggest that alleles other than the *CHEK2* 1100delC are unlikely to significantly contribute to the hereditary breast cancer risk in the French Canadian population. Lastly, the results

obtained throughout our analysis of *PALB2* heterozygous cell lines may be suggestive of a possible chromosomal instability phenotype predisposing carriers to additional tumourgenic mechanisms.

Résumé

5-10% des cas de cancer héréditaire du sein sont causés par des mutations germinales dans des gènes des susceptibilité bien caractérisés et à l'effet dominant tel les gènes *BRCA1* et *BRCA2*. Cependant, plus de 50% de la prédisposition génétique au cancer du sein héréditaire demeure inexpliquée. Dans cette thèse, nous présentons une approche à trois volets ayant pour but d'étudier les cas de cancer héréditaire du sein associés avec des gènes interagissant avec *BRCA1* et *BRCA2*. D'abord, nous analysons la contribution potentielle de deux gènes peu caractérisés qui sont partenaires de *BRCA1* : *RAP80* et *Abraxas*. Nous étudions ensuite le risque associé avec la présence d'allèles nouveaux ou connus du gène *CHEK2* jamais encore caractérisés dans la population canadienne française. Enfin, nous examinons les mécanismes cellulaires et moléculaires responsables de l'augmentation du risque de cancer du sein conférée par des allèles à risque du gène *PALB2*.

Nous avons utilisé une combinaison de génotypage chez 96 patients souffrant du cancer du sein mais étant non porteurs de mutations dans *BRCA1/2* et d'analyse de ségrégation des mutations et des phénotypes dans leurs familles afin de déterminer si RAP80 et Abraxas sont ou non des gènes de prédisposition au cancer héréditaire du sein. La contribution au risque de cancer du sein du gène *CHEK2* fût déterminée grâce au génotypage de 25 cas à haut risque, non porteurs de mutations chez *BRCA1/2*, et de 25 contrôles sans cancer. Finalement, nous avons étudié 4 allèles nonsense du gène *PALB2* à l'aide du test de toxicité cellulaire WST-1 ainsi qu'en utilisant l'analyse Q-FISH spécifique aux télomères, l'analyse FISH spécifique aux centromères et finalement par caryotype spectral (SKY).

Les résultats présentés dans cet ouvrage suggèrent que *RAP80* et *Abraxas* ne sont pas des gènes de susceptibilité au cancer du sein à pénétrance moyenne ou élevée. De plus, il est peu probable que des allèles du gène *CHEK2* autres que l'allèle connu 1100delC contribuent de façon significative au risque de cancer du sein héréditaire dans la population canadienne française. Par contre, les résultats de notre analyse du gène *PALB2* dans les lignées cellulaires hétérozygotes pour un allèle nonsense suggèrent la possibilité que la présence de ces allèles crée de l'instabilité chromosomique chez les porteurs de mutations qui puissent prédisposer à la progression tumorale.

CHAPTER ONE: HEREDITARY BREAST CANCER

Molecular Overview of BRCA1/2 and Associated Proteins in Hereditary Breast Cancer

Despite the vast advancements in diagnostic techniques and treatments over the last decade, breast cancer is still one of the leading causes of cancer related deaths in women today. For example, world wide it was estimated there were 1,301,867 new occurrences of female breast cancer and 464, 854 breast cancer related deaths in 2007 (American Cancer Society, Global Cancer Facts & Figures, 2007). To date, approximately 5-10% of all breast cancer cases are caused by germline mutations in well-defined, dominantly acting breast cancer susceptibility genes, the majority of which can be accounted for by the major breast cancer susceptibility genes *BRCA1* and *BRCA2* [1].

However, additional susceptibility genes have been identified to confer an increased breast cancer risk, all of which can generally be classified according to the level of risk they confer: genes which confer a high risk for developing breast cancer which includes *BRCA1/2*, *TP53*, *PTEN*, *STK11* and *CDH1* and those which confer a low to moderate risk of breast cancer, including *ATM*, *CHEK2*, *BRIP1* and *PALB2* [2]. Interestingly, the majority of these genes are intimately linked by their function in DNA repair, cell cycle regulation and interaction with one or both of the *BRCA* genes.

BRCA1, originally identified by linkage analysis in 23 early-onset breast cancer families [3], is a 24 exon gene spanning a 100kb region localized on 17q21, and encodes

for a 1863-amino acid protein. *BRCA1* is classified as a tumor suppressor gene which plays a critical role in the repair of DNA damage, cell cycle regulation, chromatin remodelling, transcriptional regulation and protein ubiquitylation [4]. The BRCA1 protein is characterized by a ring finger domain, nuclear localization signals, a DNA-binding domain, SQ cluster domains and a BRCA1-carboxyl-terminal (BRCT) domain [5].

It has been shown that BRCA1 is activated in the response to DNA damage, by checkpoint kinases such as ATM, ATR and CHEK2 [6,7]. Upon activation, BRCA1, can form four distinct and mutually exclusive complexes via the BRCA1-BRCT domain with either CtIP, BACH1/BRIP1, Abraxas or PALB2. Upon formation of the BRCA1-CtIP complex, BRCA1 can bind to TP53, the MRE11-RAD50-NBS1 (MRN) complex and RAD51, resulting in the initiation of homologous recombination or non-homologous end-joining. The formation of the BRCA1-BRIP1 complex is important for normal double strand break repair function of BRCA1 [8], in addition to DNA damage-induced checkpoint control during the transition from G2 to M phase of the cell cycle [9]. The third BRCA1 complex, recently identified by the discovery of the novel BRCA1 interacting protein Abraxas, is required for the loading of BRCA1 to DNA damage foci, DNA damage repair and G2 to M checkpoint control, which is mediated through the association of RAP80 with Abraxas [10,11], the two coding genes of which are the primary focus of Chapter Two. Interestingly, these three complexes seem to be inter-related as RAP80 may also be involved in the recruiting the BRCA1-CtIP complex to DNA damage foci [11], and the BRCA1-CtIP complex may interact with the MRN complex to facilitate in HR-mediated double strand break repair [12]. The fourth and

final BRCA1-BRCT complex identified to date appears to functionally link BRCA1 with the other major breast cancer susceptibility protein, BRCA2. Recent evidence has shown that PALB2 directly binds with a COOH-terminal fragment of BRCA1 which is required for the recruitment of PALB2 to sites of DNA damage where PALB2 then recruits BRCA2, physically linking the two proteins [13].

Originally cloned in 1995 [14], *BRCA2* is a 27 exon gene, spanning a 70Kb region located on 13q12 which encodes a 3418 amino acid protein. In comparison to *BRCA1*, the functions of *BRCA2* appear to be more limited to DNA repair, specifically HR repair of DSB's, cytokinesis and meiosis [15]. The inability to date to purify the full length BRCA2 protein has significantly hindered the characterisation of specific domains in the protein. However, crystallographic studies have revealed that BRCA2 possesses both a DNA-binding domain and BRC repeats [16,17]. Many of the molecular intricacies regarding the function of BRCA2 have been illuminated by its association with three major proteins: RAD51, DSS1 and PALB2. For example, BRCA2, through its direct interaction with RAD51 via the BRCA2 BRCC repeats, is thought to bind DNA and deliver RAD51 as well as the recombination protein DMC1, to sites of DNA breaks, facilitating in the timely regulation of HR in meiosis and the repair of DSBs [18]. The ability of BRCA2 to recruit RAD51 to DNA damage foci is thought to be guided by DSS1 which binds to the BRCA2 single stranded-DNA binding domain [16]. Finally, PALB2, likely through an interaction with the BRCA2 N-terminal region, is required for the localization and stable interaction of BRCA2 with nuclear structures, protecting it from the effects of proteasome-mediated degredation [19]. In addition to these three proteins, BRCA2 is also thought to interact with TP53 [20], BRCCIP, BRAF35, EMSY,

CDKs amongst others (discussed briefly in [21]), although these interactions are currently less detailed.

Taken together, pathogenic mutations of *BRCA1* or *BRCA2* confer a 5-8 fold increased risk of breast cancer development, resulting in 50-80% risk of breast cancer development by the age of 70 [4].

DNA Repair Mechanism Disorders

As *BRCA1* and *BRCA2* are critically involved in DNA repair, candidate gene approaches to identify further breast cancer susceptibility genes have been largely successful by concentrating on genes involved in DNA repair, many of which have been associated with DNA repair disorders and breast cancer associated predisposition syndromes and examples include *PTEN* and Cowden Syndrome (MIM no. 158350), *STK11* and Peutz-Jeghers Syndrome (MIM no. 175200), and *TP53* and Li-Fraumeni syndrome (MIM no. 151623). However, perhaps the most striking evidence supporting a link between DNA repair and breast cancer susceptibility arises from the fact that homozygous mutations in *BRCA2* are known to be responsible for a subgroup of Fanconi anemia (MIM no 227650), FAND1 [22]. A brief overview of Li Fraumeni syndrome and Fanconi anemia are of specific interest.

Li-Fraumeni Syndrome

Li Fraumeni syndrome (LFS) is a rare autosomal dominant cancer syndrome which predisposes affected individuals to bone and soft tissue carcinoma, breast cancer, adrenocortical carcinomas, leukemia and brain tumors [23]. Historically, the selection

criteria for LFS were as follows: a proband with a sarcoma aged under 45 years, in addition to a first or second-degree relative in the same lineage with any cancer under the age of 45 or a sarcoma at any age [24]. Additionally, Li-Fraumeni-like syndrome (LFL) criteria have been established as: a proband with any childhood tumor or sarcoma, brain tumor or adrenocortical tumor diagnosed under 45 years of age and a first or second-degree relative in the same lineage with a typical LFS tumor at any age, as well as a first or second-degree relative in the same lineage younger than 60 with any cancer [25].

The first genetic link between DNA repair, cell cycle regulation and LFS was established in 1990 by Malkin et al. [26] who were able to demonstrate that the majority of families with classical LFS have mutations in the tumor suppressor, *TP53* gene. This landmark discovery has since been confirmed by the identification of 419 TP53-positive families (IARC mutation database, R13, November 2008 [27]). However, identified *TP53* mutations only accounts for approximately 75% of LFS families and 40% of LFL families [28], thus suggesting the existence of additional LFS susceptibility genes, likely related in function to *TP53*.

Genes involved in genomic control and cell cycles regulation such as *PTEN*, *CDKN2*, *BCL10*, *TP63* and *BAX* have all been investigated as candidates but appear to have no causal role in the syndrome (discussed in [29]). However, in 1999 Bell et al. [30] identified the first germline mutation in the cell cycle checkpoint kinase *CHEK2* (CHEK2.1100delC), in a LFS family negative for *TP53* mutations, and two families with LFL. The fact that CHEK2 is involved in DNA repair, apoptosis and cell cycle control through the stabilization of TP53 [31], made this an interesting candidate for further investigation.

Historically, the association between LFS and *CHEK2* was once largely debated focusing mainly on the CHEK.1100delC and CHEK2.I157T alleles and their contribution to LFS [32,33]. However, the present general consensus within the community is that *CHEK2* is not a LFS susceptibility gene [34,35]. As a result of this original hypothesized link, however, it is now widely accepted that *CHEK2* is indeed a low to moderately penetrant, possibly multiorgan, cancer susceptibility gene. *CHEK2* presumably contributes to familial prostate, colon, ovarian and colorectal cancer, in addition to hereditary breast cancer, which will be the focus of discussion in Chapter Three.

Fanconi Anemia

Fanconi anemia (FA), is a predominately autosomal recessive disorder [36], with one rare X-linked subtype [37]. FA is a highly heterogeneous condition characterized by a variety of abnormalities such as a propensity to develop bone marrow failure an increased incidence of early onset cancer, skeletal abnormalities and hyperpigmentation [38-40]. Additionally, incidences of aplastic anemia, myelodysplastic syndrome, acute myeloid leukemia and solid tumors are increased in FA homozygotes [41]. To date thirteen FA complementation groups, and the genes defective in these, have been identified (*FANCA, B, C, D1, D2, E, F, G, J, I, L, M* and *N*) (for review see [42]).

The wide range of clinical phenotypes presented by FA patients can be explained by the fact that FA, amongst other examples such as ataxia telangiectasia and Bloom syndrome, is a chromosomal instability disorder, predisposing biallelic mutation carriers to spontaneous chromosomal breakages, abnormal chromosome structures and an accumulation of DNA damage [43,44]. As a result, an evident cellular hallmark of FA is

hypersensitivity to DNA damaging agents that create DNA interdtrand crosslinks, such as mitomycin C (MMC) and diepoxybutane (DEB) [45,46], a phenotype common amongst *BRCA1* and *BRCA2* negative cells [47,48].

Many of the FA proteins directly interact with BRCA1 and BRCA2/FAND1 (for review see [49,50]) and can generally be classified into three distinct groups: Group 1, the upstream/core complex, Group 2, the ID complex and Group 3, the downstream or separate complex. The upstream core complex consists of FANCA, B, C, E, F, G, L and M. The primary function of this complex is thought to lay in the monoubiquitination of the ID complex, FANCD2 and FANCI, in response to DNA lesions during replication in an ATR-activated manner, as cells that are deficient in any one of the proteins in this group fail to monoubiquitylate FANCD2 and FANCI [51-53]. The two proteins making up the ID complex are interdependent with respect to their monoubiquitylation, as FANCD2-deficient cells fail to monoubiquitylate FANCI, as is the case for FANCD2 in FANCI-deficient cells [53,54]. Monoubiquitylated FANCI and FANCD2 then localize to DNA repair foci together with the final complex of FA proteins, consisting of FANCD1/BRCA2, FANCJ/BRIP1 and FANCN/PALB2 (**Figure 1.1**).

An important feature that distinguishes the third complex of FA proteins from the first two is that whereas homozygous mutations in these genes predispose carriers to FA, heterozygous mutations, predispose females to an elevated risk for breast cancer. This third complex is independent of FANCD2-I ubiquitination, thus suggesting that they function either downstream or parallel to the ID complex [55,56]. For example, FAND1/BRCA2, is a recombination mediator that facilitates the formation of Rad51 nucleofilaments [57]. Furthermore, BRCA2 is known to co-localize with FANCD2, a

protein required for BRCA2 DNA damage foci formation, linking the FA pathway to HR mediated repair [58]. FANCJ, also known as BRIP1 (or BRCA1-associated C-terminal helicase, BACH1), interacts with the C-terminal domain of BRCA1 resulting in the localization to DNA repair structures containing other proteins such as BRCA2, mediating DNA cross link repair and cell cycle progression [59,60]. Lastly, FANCN/PALB2, the center of our investigation in Chapter Four, is required for the localization and stability of BRCA2 to chromatin structures and thus, its function in HR mediated repair [19].

Rationale

To date more than 50% of the genetic predisposition to hereditary breast cancer remains unexplained [61]. It has been 14 years since the discovery of *BRCA2*, during which subsequent studies have attempted, and failed to detect a third major breast cancer susceptibility gene, "*BRCA3"* (reviewed in [4]). Furthermore, a distinct phenotype suggesting at a third class of inherited breast cancer has not emerged, an important attribute in the identification of both *BRCA1* and *BRCA2*. Rather, the remaining unresolved breast cancer risk is incrementally being associated with genes that confer low or moderate increased risk, many of those which have been discussed here, are involved in the maintenance of genome integrity, interact with either BRCA1 or BRCA2 or are involved in multiple cancer syndromes.

The loss of DNA repair is a crucial step in the formation of tumor cells, as this enables cells to progressively develop genomic instability. This instability can potentially result in the inactivation of tumor suppressor genes, upregulate cellular proliferation , a loss of contact inhibition amongst other deficiencies in the regulation of normal cellular function. Two recently identified genes, *Abraxas* and *RAP80*, appear to be involved in the BRCA1 DNA damage response, similar to the breast cancer susceptibility gene *BRIP1*: therefore implicating these genes as interesting susceptibility candidates.

In addition to identifying novel breast cancer susceptibility genes, further characterization of those already established will likely prove beneficial in the re-evaluating the full contribution of each gene and assessing the benefit of genetic testing within specific populations. For example, *CHEK2*, originally referred to as a low-

penetrance gene is now more properly associated with conferring a moderate risk (two to three fold higher) to breast cancer. Furthermore, *CHEK2* alleles seem to be more relevant in selected populations such as the Dutch, Finnish and Ashkenazi Jewish populations. Prior to our analysis, the clinical significance of *CHEK2* alleles in the French Canadian population had yet been investigated.

Identifying breast cancer susceptibility genes and characterizing the contribution of their alleles is a critical step in determining the clinical significance of genetic screening, in addition to providing carrier individuals with an informed basis on which to make decisions regarding pre-emptive preventative measures. However, determining how these susceptibility alleles abrogate normal protein function and uncovering the molecular and cellular tumorgenic mechanisms is critical in the identification of effective therapeutic targets. Such a mechanism has yet been identified to explain the risk associated with heterozygous *PALB2* mutations.

Objective

Taken as a whole, the goal of this thesis was to further our understanding of Breast Cancer arising from deficiencies in genes involved in DNA repair. In order to accomplish this, our specific aims were threefold:

I. To further elucidate the contribution to Breast Cancer as a result of a known breast cancer susceptibility gene, *CHEK2*, in addition to probing for the presence of previously unidentified founder alleles in the French Canadian population.

II. To determine whether two genes recently associated with the BRCA1-mediated DNA response pathway, *RAP80* and *Abraxas*, confer breast cancer susceptibility.

III. To explore the link between known *FANCN/PALB2* breast cancer susceptibility alleles and the potential mechanism underlying *PALB2* cancer pathogenesis.

Figures

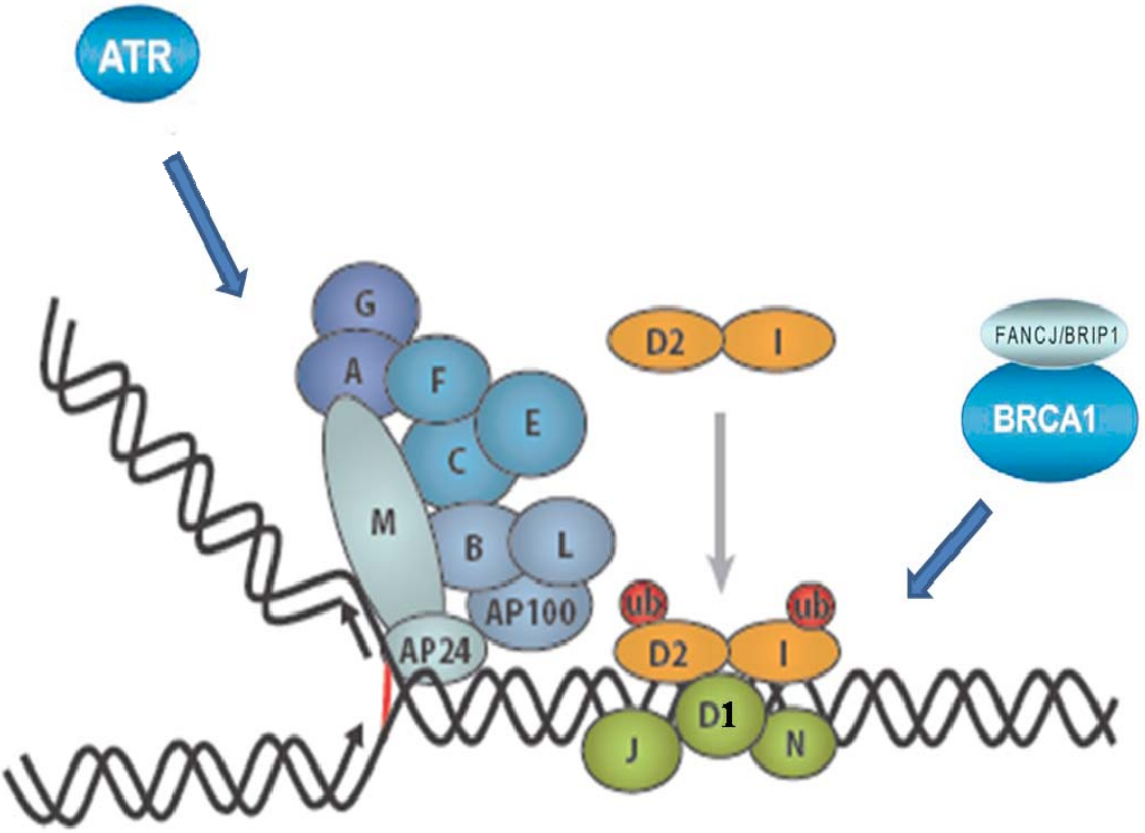

Figure 1.1: Simplified Schematic Representation of the FA/BRCA Network. This figure adapted from [42] and [15] illustrates the function of the three Fanconi anemia complexes, and their requirement for the protection against genomic instability. Upon activation by ATR, in the presence of DNA damage, the core complex, composed of FANCA, B, C, E, F, G, L and M is recruited to DNA lesions. The core complex then monoubiquitylates the ID complex, FANCD2 and FANCI. Upon monoubiquitylation, the ID complex localizes to DNA repair foci, together with FANCD1/BRCA2, FANCJ/BRIP1 and FANCN/PALB2. Additionally, BRCA1 is localized to DNA repair foci, through the direct interaction with PALB2.

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CHAPTER TWO

Analysis of the BRCA1-interacting proteins Receptor-Associated Protein (*RAP80*) / Ubiquitin Interaction Motif-Containing Protein 1 (*UIMC1*) and *Abraxas* (*CCDC98*) as Potential Breast Cancer Susceptibility Genes

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Analysis of the genes coding for the BRCA1-interacting protein, RAP80 and Abraxas (CCDC98), in high-risk, non-BRCA1/2, multiethnic breast cancer cases

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Preface

Originally identified by investigating the differerential display of RNA expressed in normal human epidermal keratinocytes, Yan et al. [1] cloned the novel 15 exon gene coding for a 719 amino acid protein with a mass of 79.6kD located on chromosome 5q35. Further analysis of this protein identified two nuclear localization signals in addition to two ubiquitin interaction motifs (UIMs) at its NH2 terminus [2]. Interestingly, UIMs are typically found in proteins with with roles in DNA repair, in addition to endocytosis, (de)ubiquitination, replication and transcription [3].

Through yeast 2-hybrid analysis, the primary function of *RAP80* was originally thought to reside in its interaction with retinoid-related testis-associated receptor (RTR) until years later when Sobhian et al. [4] demonstrated that RAP80 functionally interacts in complex with the tumor suppressor BRCA1; an interaction required for the loading of BRCA1 onto DNA damage foci [5].

The exact mechanisms resulting in the association between RAP80 and BRCA1 began to be revealed when Wang et al. [6] identified, using phosphopeptide analysis, a novel 409-amino acid protein, Abraxas, with a mass of 46.6kD located on 4q21. *Abraxas* spans 9 exons coding for a protein which contains an ABR domain and a coiled-coil domain followed by a phosphor-ser-X-X-phe motif at the C-terminal end of the protein. In this investigation, it was shown that Abraxas binds to BRCA1 to the mutual exclusion of BACH1 and CTIP, forming a third type of BRCA1 complex; a complex also containing RAP80.

The emerging picture was now becoming one of Abraxas directly binding to the BRCA1-BRCT domain linking BRCA1 with RAP80 through a direct interaction of RAP80 with Abraxas [7,8]. The association of RAP80 allows the targeting of the complex containing the BRCA1-BARD1 E3 ligase and the deubiquitinating enzyme BRCC36 to MDC1-gamma-H2AX-dependant lys6 and lys3-linked ubiquitin polymers at double strand breaks [4]. Thus, both Abraxas and RAP80 are implicated in a ubiquitin-dependant signalling pathway involved in the BRCA1-mediated repair of double strand breaks and cell cycle checkpoint regulation.

In the following study, the full coding regions of both *RAP80* and *Abraxas* were fully sequenced for variations in a cohort of high-risk breast cancer cases of varying ethnicity. The underlying assumption being that a coding variation that may alter the function of either protein, disrupt binding of Abraxas to BRCA1 or of RAP80 and Abraxas colocalization would in turn result in a disruption of the BRCA1 DNA repair response allowing a progression of genomic instability with pathogenic consequences.

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Abstract

Background

Around half of familial breast cancer cases are caused by germ-line mutations in genes which are critically involved in the maintenance of genome stability. Mutations in related genes functioning in DNA repair may account for currently unattributed cases. Two such genes, RAP80 and Abraxas, have recently been identified to be in a complex with BRCA1, and are required for the localization of BRCA1 to DNA damage foci.

Methods

RAP80 and Abraxas variants were screened for in a cohort of 95 high risk, non-BRCA1/2 breast cancer cases of varying ethnicity: those of Ashkenazi Jewish (n=35), mixed Canadian (n=34) and Swiss descent (n=26).

Results

We have identified four missense variants, four silent SNPs, three SNPs in the UTRs and seven intronic variants in RAP80. Two of the previously reported RAP80 variants were further investigated. In Abraxas, we have identified two missense, nine intronic and two variants in the 3' UTR.

Conclusions

Overall, it seems unlikely that moderate to highly penetrant alleles of either RAP80 or Abraxas, confer a significantly high relative risk of breast cancer.

Introduction

Approximately 5-10% of all breast cancers can be attributed to genetic variations of dominantly inherited alleles, with the two major breast cancer susceptibility genes, *BRCA1* and *BRCA2* accounting for the largest proportion. To a lesser extent, germ-line mutations in ten additional genes; *P53*, *PTEN*, *CDH1*, *STK11*, *CHEK2*, *ATM*, *NBS1*, *RAD50*, *BRIP1* and *PALB2* have been linked to inherited breast cancer, predisposing carriers with various increases in relative risk (for review see [1]). Interestingly, the preservation of genomic integrity is dependant to an extent, on each of these ten genes. However, to date, an estimated 50% of familial breast cancer cases remains to be elucidated. In this respect, other genes involved in the DNA repair pathway, functioning in close relationship with *BRCA1* or *BRCA2*, present as attractive candidates in the search for additional susceptibility alleles. Two such genes, *RAP80* and *Abraxas*, have recently been identified [2-4].

The receptor-associated protein 80 (RAP80), also known as ubiquitin-interacting motif containing 1 (UIMC1), is a nuclear protein with two ubiquitin-interacting motifs (UIM) at its NH2 terminus [5]. UIM's are typically found in proteins with roles in endocytosis, (de)ubiquitination, replication, transcription and DNA repair [6]. Recent evidence has shown that double strand DNA breaks induces the relocation of RAP80 to DNA damage foci [3,7,8]. More specifically, Sobhian, et al. [3] demonstrated that RAP80 is associated with a protein complex containing the tumor suppressor, BRCA1, and that this interaction is dependent on the BRCA1 COOH-terminal (BRCT) repeats.

Additionally, this interaction is necessary for the loading of BRCA1 on to DNA damage foci [7].

Further analysis of the BRCA1-RAP80 complex revealed a new protein, Abraxas or CCDC98, which has proved to be the critical mediator that links the BRCA1-BRCT domain with RAP80 [9]. Moreover, Liu, et al. [4] demonstrated the lack of BRCA1 foci formation in the absence of Abraxas, further illustrating the importance of the BRCA1-Abraxas-RAP80 complex in DNA damage localization. Together, RAP80 and Abraxas both participate in the DNA damage response and both are crucial for loading BRCA1 on to DNA damage sites. Therefore, mutations in either of these genes that result in altered protein function or expression may have an impact on genome integrity and cancer development.

In the current study, we fully sequenced a cohort of ninety-five non-*BRCA1/2*, high-risk breast cancer patients for variant alleles of either *RAP80* or *Abraxas*, to assess a potential association between such variants with a moderate to high relative breast cancer risk.

Methods

Study Population

Ninety-five women with breast cancer, all of whom are members of large, multiple case breast cancer families, have provided written informed consent to participate in this study. All patients were previously screened and found negative for the

major *BRCA1* and *BRCA2* mutations in addition to the *CHEK2* 1100delC variant. Specifically, for *BRCA1* and *BRCA2* mutations, Swiss patients were screened by sequencing of all abnormal DHPLC screening profiles, those of Mixed Canadian ethnicity were screened by sequencing and those of Ashkenazi Jewish descent were screened for the three founder *BRCA1* and *BRCA2* mutations, 187delAG, 5385insC and 6174delT, which account for 95% of all *BRCA1*/*BRCA2* mutations in this group. As stated, all patients were selected for a strong family history of breast cancer, with the group having a mean BRCAPRO score [CancerGene 4.3.1, University of Texas Southwestern Medical Center, Dallas, TX] of greater than 0.5 (range 0.11-0.99). Patient data was analyzed both as an entire set of n = 95 as well as arranged in three distinct cohorts: those of Ashkenazi Jewish descent (n = 35), mixed Canadian (n = 36) and Swiss descent (n = 26).

Molecular Methods

Genotyping: Mutation screening was performed simultaneously on all ninety-five samples by direct PCR and sequencing (sequencing was conducted by the *McGill University and Genome Quebec Innovation Center*). We searched for variants throughout the 14 coding exons of *RAP80*, the 5' and 3' UTR's as well as each intron/exon boundary, respectively. Primers used for PCR were designed using the online Primer3 program (Primer3). All primers used, annealing temperature and amplicon size are summarized in **Table 2.1**. Similarly, for *Abraxas*, all 9 coding exons as well as the intron/exon boundaries and 3' UTR, were sequenced. Primer information for *Abraxas* is

summarized in **Table 2.2**. Any variants identified were confirmed by resequencing in both the forward and reverse directions.

102A>G Restriction Assay: One identified SNP, *RAP80* 102A>G was further investigated in an extended cohort of French Canadian (cases n = 117, controls n = 82) and Ashkenazi Jewish (cases n = 67, controls n = 289) samples which were amplified using primers specified in **Table 2.1**. PCR Products were incubated overnight at 37°C with XBAI (1U/sample, New England BioLabs, USA), which cleaves a recognition site disrupted by the 102A>G variant. Digested products were visualized by gel electrophoresis. The presence of 102A>G was confirmed by direct sequencing.

Single Stranded Conformational Polymorphism (SSCP): The frequency of the M353T missense variant was further investigated in an extended cohort (n = 177) of Swiss controls, previously screened and found negative for BRCA1/2 mutations, using SSCP. P32 labeled PCR products were generated (primers listed in table 1) which were then subjected to 15min denaturation at 95°C. Samples were immediately placed on ice following denaturation and loaded into a 0.7X MDE gel. Electrophoresis was conducted at 4°C, initially at 80W for 2 minutes followed by 5 hours and 45 minutes at 25W. The SSCP gels were then dried for 45 minutes, covered with a Kodak Biomax filmsheet and tranfered to an autoradiography cassette for 24-48 hours prior to development.

Statistical Analysis

Genotype frequency is presented as a proportion of the entire sample set and Fisher's exact test was used to test for significance. In the circumstance where a sample would not amplify, it was excluded from all calculations. Two-tailed p-values are presented.

Results

RAP80

In total, we identified seven intronic variants, three single nucleotide polymorphisms (SNPs) in the untranslated regions, four silent SNPs and four missense variants (**Table 2.3**). Two variants, identified in this study and previously reported to confer a possible moderate risk to breast cancer by Akbari et al. [10] were investigated further.

102A>G (observed in 1/35, 2.9% of Ashkenazi Jewish cases), was screened for in a larger subset of cases and controls due to its close proximity to the spliceosome acceptor site (-1bp), at the 5' end of exon 2, the first coding exon of *RAP80*. No significant differences were observed between Ashkenazi Jewish (1/67, 1.5% vs 10/269, 3.7%; P = 0.7) or French Canadian (3/114, 2.6% vs 4/82, 4.9%; P = 0.46) cases and controls, respectively. Overall, the frequency of 102A>G (**Table 2.4**) was not statistically significant between cases (4/181, 2.2%) and controls (14/351, 3.99%) [P = 0.32]. Finally, cDNA, obtained from a 102A>G carrier, was analyzed for the 3' SNP, 2296A>C, which

was determined to be in complete Linkage Disequilibrium (LD=1) with 102A>G. The presence of the 2296A>C transcript was observed.

Despite the calculated mean chemical difference (Grantham Variation = 81) suggesting the neutrality of a Methionine to Threonine substitution, we genotyped for the presence and segregation of the M353T (1067 T>C) allele in the family of the identified carrier (**Figure 2.1**). DNA from eight members had been previously screened for major *BRCA1/2* mutations and were available for testing, five of which have no clinical history of cancer, two of which have been previously diagnosed with cancer of the breast and one of which had been previously diagnosed with ovarian cancer. The M353T allele was not seen in two family members diagnosed with breast cancer under 50 years suggesting that M353T is not a high penetrance allele. Additionally, the M353T allele was identified in 3/177 healthy Swiss controls. Overall the frequency of the M353T allele was not significantly elevated in breast cancer cases (1/28, 3.57%) versus controls (1/177, 0.6%) [P = 0.24].

Abraxas

We identified nine intronic, two missense and 2 variants in the 3' UTR (**Table 2.5**) within the Abraxas region. Both of the missense variants, 1042A>G (A348T) and 1117A>G (D373N), have previously been reported in the SNPdb (NCBI), and are predicted to be tolerated (Grantham scores of 71 and 23, respectively). As such, neither of these variants was subject to further investigation.

Discussion

RAP80 and Abraxas, have recently been observed to directly interact with BRCA1. These interactions are necessary for the recruitment of BRCA1 to DNA damage sites, and thus are required for DNA damage resistance [11]. In short, Abraxas, directly binds to the BRCA1-BRCT domain via its pSXXF motif in a phosphorylation dependant manner, to the mutual exclusion of BACH1 and CtIP [4,12]. The direct interaction between BRCA1 and Abraxas is the critical linker between BRCA1 and RAP80, without which, RAP80 would be unable to recruit BRCA1 to sites of DNA damage. Importantly, this interaction has been shown to be dependent on numerous regions of both RAP80 and Abraxas, which are highly sensitive to variation [4,13]. Thus, mutations which interfere with Abraxas' ability to bind to BRCA1, RAP80 and Abraxas colocalization, or the ability of RAP80 to localize to DNA damage foci could potentially hinder genomic stability and confer highly pathogenic consequences related to genomic instability, such as breast cancer.

In the current study, 95 breast cancer cases, selected for a strong family history, were fully sequenced for highly penetrant variants, in both *RAP80* and *Abraxas.* This approach provides an 80% power to detect an allele with a frequency of approximately 1% or greater, conferring a multiplicative relative risk of greater than 2.0 [14]. We have identified nine intronic, two untranslated, and two previously identified missense variants in *Abraxas*. In *RAP80*, we have identified seven intronic, four silent, three known and one novel missense variants , in addition to three variants in the UTRs.

The data presented here is in keeping with that of Osorio et al. [15], and supports their conclusion that *RAP80* and *Abraxas variants* do not make a significant contribution to hereditary breast cancer. Specifically, through extended genotyping, transcriptional and segregation analysis, we have shown that RAP80 102A>G (c.-8A>G) and M353T, two of the four variants included to statistically derive an OR=2.4 in the analysis of Akbari et al. [10], are unlikely to be candidate breast cancer susceptibility alleles.

Future investigations using a larger, less "selected" study cohort would be necessary to determine the potential combinatorial effect of low penetrant alleles. Furthermore, the functional significance of intronic variants, largely remains unknown. Thus, the *Abraxas* intronic insertions and deletions presented in this study could have an unknown impact on gene expression via disruption of intronic splicing regulatory elements (for review see [16]).

Despite these negative findings, genes implicated in DNA repair seem to be the main contributors to high penetrance breast cancer susceptibility. Thus, similar approaches as employed in the current study, directed at genes such as *BRCC36*, an additional component of the BRCA1-Abraxas-RAP80 complex, may prove successful at attributing a portion of the currently unresolved risk.

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Tables

Table 2.1 – *RAP80* Primers and Details

Exon	Size (bp)	Primers (5' – 3')	Annealing Temp (°C)
1	297	Forward: atgccggggtgtgtctct Reverse: aggtgcaaagcccaacct	61
2	342	Forward: gcaacaaagcgagaccatct Reverse: caaactcccatgggtaaag	60
3	248	Forward: gccataggggagggtagaaga Reverse: tgcaacataatcaactagtccaaa	58_5
4	272	Forward: ttctcttttctgcctccatga Reverse: tgcaactacaacaccacagaag	59
5	246	Forward: tgtccttcctgtgaccctlc Reverse: cagcatgagatcccigtgg	60
6a	500	Forward: gataacttaccaagccctcttaat Reverse: GGCAGAGAATGACCTTGGTA*	57
6b	488	Forward: CTGGGGGCACTGTGAACTAT* Reverse: atcaaggaggggaagaccac	60
7	236	Forward: tgttgagggagaacattgaaca Reverse: ctcactcatgtggttcatctcc	60
8	238	Forward: tgactcaccattgctttigg Reverse: ggtttgaagagtcagaggaaaa	58_5
9	286	Forward: ttgccaccacagtattgtcata Reverse: tgcatcagagagaggactgc	59
10	297	Forward: gcagttcgttgtagggaatga Reverse: tcccigcattgtaatgtataggat	59
11	250	Forward: gcttcttcctgtgcatttttg Reverse: gggtaagataggaattggtctttit	59
12	400	Forward: tgagtgaatctgcattggttg Reverse: gagggaaaagccagaacaga	59_5
13	248	Forward: atctgggctgtggaagttgt Reverse: ggatcacgtgtgtacccaga	59_5
14		Forward: cctagagcacggaagcaaaa Reverse: ttgcagtagaaatcactgtggaa	60
15	648	Forward: gcatagcatgtccttgatgc Reverse: atggcattcattcagcatgt	59
Exon 2 – 102A>G Digest	197	Forward: gcaacaaagcgagaccatct Reverse: TGGAGAAGAAGGATGTGGAAACT*	60
M353T SSCP	203	Forward: AAGAGCCTGAAAATGGCTCA* Reverse: TTTGAGTGCCAGTCAGATGC*	60

*Capital letters indicates Exonic nucleotides.

Table 2.2 – *Abraxas* Primers and Details

Exon	Size (bp)	Primers (5' - 3')	Annealing Temp (°C)
1	305	Forward: ctgccaccacagggtctt Reverse: agggggagagaaggcagag	60
2	263	Forward: actggtagcacatattgtatacatag Reverse: cagcataactatcaaatataggag	54
3	256	Forward: cttcctggcgtgaggtaaag Reverse: tttccattctactcagtaccacca	60
4	263	Forward: gctttggtagttgggttaggaataac Reverse: aacactgcttaaaaattctgtcaaag	60
5	406	Forward: aagaaagccattttaaggttgtt Reverse: gtgacaatctgatgcgacaa	58
6	360	Forward: gggacaagtaatctattccagca Reverse: cagcctagtttacttgagtaatgg	58
7	350	Forward: ttggtccttgacaatgaataagtt Reverse: tgtttgcacaatgataaaactgc	58
8	340	Forward: aaaggcaaatagttttgggtatt Reverse: cacctttgcactccaaccta	58
9	675	Forward: acaactgttaaaatctttttgacttaattt Reverse: gtgtattactgcaaacaggtgaacatag	54

Table 2.3 – Identified *RAP80* Variants

EXON	Variant	Amino Acid Change	Total	Ashkenazi Jewish	Mixed Canadian	Swiss	Grantham Score
1	UTR	N/A	5/94	3/34	2/34	0/26	N/A
2	102A>G	N/A	1/95	1/35	0/34	0/26	N/A
2	158C>T	R15W	4/95	1/35	1/34	2/26	Arginine to Tryptophan: 101
3	304G>C	S65S	3/95	1/35	2/34	0/26	N/A
3	337C>T	I76I	C/C=15/95 C/T=50/95 T/T=30/95	C/C=6/35 C/T=21/35 T/T=8/35	C/C=3/34 C/T=15/34 T/T=16/34	C/C=6/26 C/T=14/26 T/T=6/26	N/A
5	490C>T	S127S	2/95	1/35	0/34	1/26	N/A
6	1067T>C	M353T	1/95	0/35	0/34	1/26	Methionine to Threonine: 81
6	IVS6-9C>T	N/A	1/95	1/35	0/34	0/26	N/A
7	IVS7-45G>A	N/A	6/94	4/35	2/33	0/26	N/A
8	1413C>T	P435L	23/95	6/35	12/34	5/26	Proline to Leucine: 98
9	1453C>A	T448T	C/C=13/95 C/A=50/95 A/A=30/95	C/C=6/35 C/A=21/35 A/A=8/35	C/C=2/34 C/A=15/34 A/A=17/34	C/C=5/26 C/A=15/26 A/A=6/26	N/A
9	IVS9-37C>T	N/A	11/95	5/35	5/34	1/26	N/A
10	1640T>C	C511R	T/T= 76/95 T/C= 14/95 C/C= 1/95	T/T=27/35 T/C=8/35 C/C=0/35	T/T=27/34 T/C=5/34 C/C= 1/34	T/T= 22/26 T/C=1/26 C/C=0/26	Cysteine to Arginine: 180
11	IVS11+17A>T	N/A	4/94	2/35	2/34	0/25	N/A
11	IVS11-18A>C	N/A	1/94	0/35	1/34	0/25	N/A
11	IVS11-24G>A	N/A	1/94	0/25	1/34	0/25	N/A
13	IVS13+17G>A	N/A	G/G= 14/95 G/A= 52/95 A/A= 28/95	G/G= 6/35 G/A= 22/35 A/A= 7/35	G/G= 3/34 G/A= 15/34 A/A= 16/34	G/G= 5/26 G/A= 15/26 A/A= 5/25	N/A
15	2198A>C UTR	N/A	1/95	1/35	0/33	0/25	N/A

Table 2.4 – *RAP80* 102A>G

Ethnicity	Cases	Controls	P-Value
French Canadian	3/114	4/82	0.46
Ashkenazi Jewish	1/67	10/269	0.70
total	4/181	14/351	0.32

Table 2.5 – Identified *Abraxas* variants

Exon	Variant	Amino Acid Change	Total	Ashkenazi Jewish	Mixed Canadian	Geneva	Grantham Score
1	IVS1+48insC	N/A	1/95	0/36	0/34	1/25	N/A
1	IVS1-95insC	N/A	56/95	21/36	20/34	15/25	N/A
2	IVS2-34A>G	N/A	1/95	0/36	1/34	0/25	N/A
2	IVS2-41delTGAAT	N/A	1/95	0/36	1/34	0/25	N/A
3	IVS3-34G>A	N/A	G/G=22/95 G/A=56/95 A/A=17/95	G/G=7/36 G/A=22/36 A/A=7/36	G/G=8/34 G/A=19/34 A/A=07/34	G/G=7/25 G/A=15/25 A/A=3/25	N/A
3	IVS3-44T>C	N/A	T/T=69/95 T/C=25/95 C/C=1/95	T/T=24/36 T/C=11/36 C/C=1/36	T/T=26/34 T/C=8/34 C/C=0/34	T/T=19/25 T/C=6/25 C/C=0/25	N/A
5	IVS5+18A>T	N/A	1/95	1/36	0/34	0/25	N/A
6	IVS6+96T>C	N/A	22/95	10/36	6/34	6/25	N/A
7	IVS7+138G>A	N/A	1/95	1/36	0/34	0/25	N/A
9	1042G>A	A348T	G/G=44/95 G/A=42/95 A/A=9/95	G/G=18/36 G/A=15/36 A/A=3/36	G/G=15/34 G/A=15/34 A/A=4/34	G/G=11/25 G/A=12/25 A/A=2/25	71
9	1117G>A	D373N	G/G=76/95 G/A=17/95 A/A=2/95	G/G=28/36 G/A=7/36 A/A=1/36	G/G=27/34 G/A=6/34 A/A=1/34	G/G=21/25 G/A=4/25 A/A=0/25	23
9	1291A>G UTR	N/A	1/95	0/36	1/34	0/25	N/A
9	1294insCTATT UTR	N/A	1/95	0/36	1/34	0/25	N/A

Figures

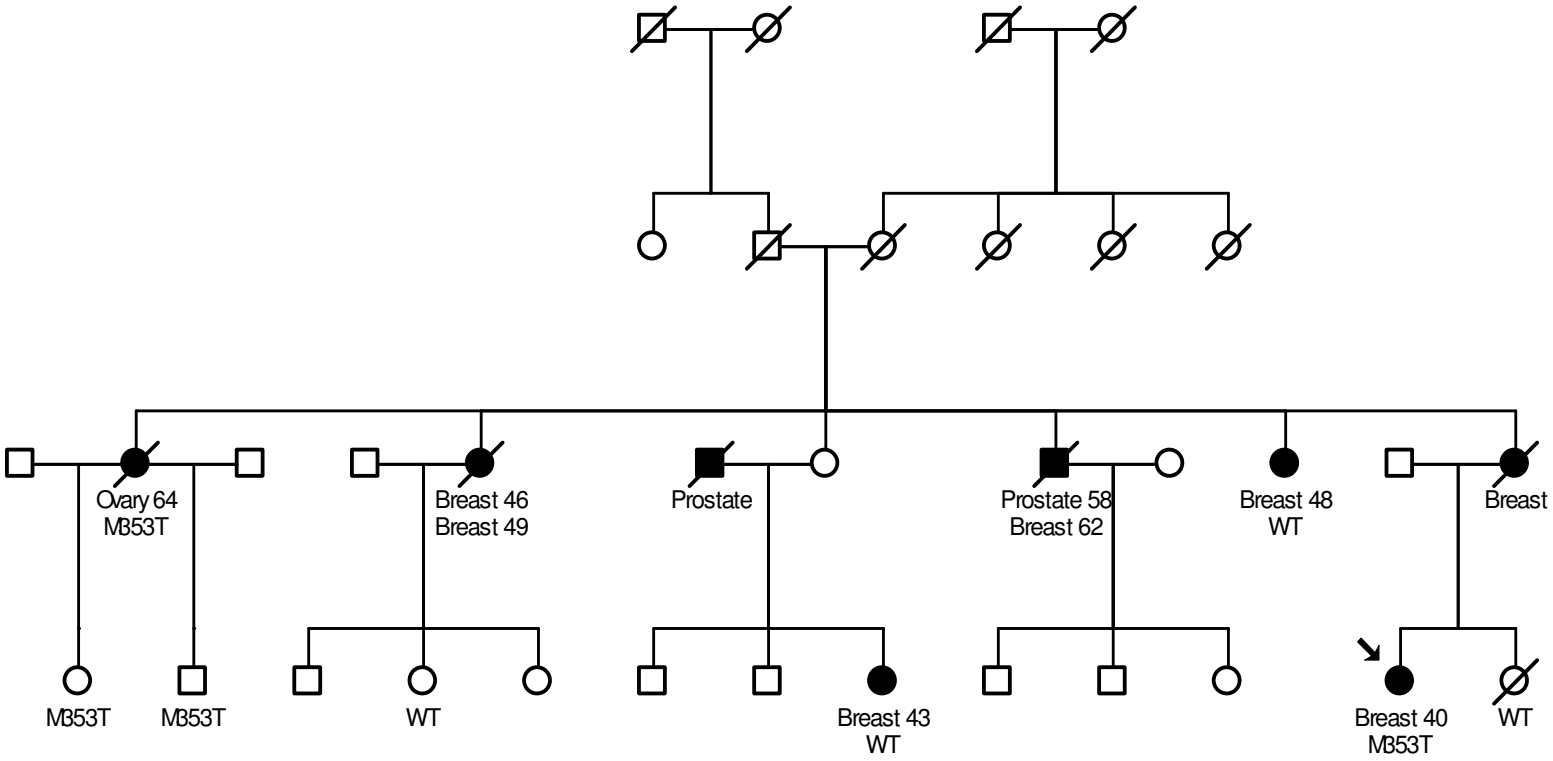

Figure 2.1: M353T Segregation analysis. The *RAP80* M353T allele was further genotyped in the family of the identified carrier to determine if there was segregation with the breast cancer phenotype. Two women diagnosed with breast cancer under 50 years did not carry the allele (denoted by WT, wildtype) suggesting that M353T is not a high penetrance allele.

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CHAPTER THREE

Checkpoint Kinase 2 (CHEK2) Related Breast Cancer Susceptability in the French Canadian Population

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Identification of a novel CHEK2 variant and assessment of its contribution to the risk of breast cancer in French Canadian women

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PREFACE

In an attempt to elucidate a proportion of familial breast cancer cases unlinked to BRCA1 or BRCA2, other candidate genes involved in DNA repair have arisen as appealing candidates. One such example involved a collection of genome wide linkage analysis which proposed linkage with familial breast cancer to a specific region on chromosome 22 [1], a region which encompasses the *EP300* and *CHEK2* genes. EP300, which codes for a histone acetyltransferase, has been extensively studied and shown to yield insignificant results, thus leaving *CHEK2* as a prime candidate gene for further analysis in this region.

Evidence demonstrating the function of checkpoint kinase 2 (*CHEK2*) in tumor suppression via its role in cell-cycle regulation [2] has been critical in stimulating the current interest in *CHEK2* as a potential contributing factor to breast cancer. *CHEK2* encodes a multifunctional kinase enzyme involved in the induction of cell cycle arrest and apoptosis, activated by the detection of damaged DNA [2]. Upon detection of DNA damage and replication blocks, *CHEK2* is activated by rapid phosphorylation of Thr68, in an ataxia telangiectasia mutated (ATM) dependant manner in addition to *CHEK2* autophosphorylation on Thr387 [3]. Once activated, *CHEK2* targets *p53* for phosphorylation on Ser20, resulting in the dissociation of *p53* from *MDM2*, a protein that targets *p53* for degradation. The result is the stabilization of the tumor suppressor *p53*, allowing transcriptional activation of genes responsible for cell cycle arrest in G1, as well as the initiation of apoptosis [4]. The *CDC25A* phosphatase activates *CDK2*, which is required for DNA synthesis. However, further regulation of the cell cycle occurs with *ATM* and *CHEK2* mediated phosphorylation of *CDC25A* on Ser123, targeting it for

ubiquitin-mediated degradation. Thus, *CDC25A* is unable to activate *CDK2*, inhibiting the advance of S phase [5]. Final regulation of the cell cycle occurs with the arrest in the G2 phase: *CHEK2* phosphorylates Ser216 of *CDC25C*, blocking entry into mitosis [3].

In addition to cell cycle regulation, *CHEK2* also participates in DNA repair and apoptosis. Under normal conditions, *CHEK2* is known to bind and regulate *BRCA1*. However, analysis has shown that as a result of irradiation, *CHEK2* phosphorylates Ser988 of *BRCA1*, resulting in the disassociation of *BRCA1* from *CHEK2*. The newly liberated *BRCA1* is then able to participate in DNA repair and further regulation of the cell cycle [6]. As a final means of regulation, *CHEK2* has been shown to mediate p52-independent apoptosis by phosphorylating *PML* [7]. Not surprisingly, *CHEK2* has recently been identified as a low-penetrance breast cancer susceptibility allele [8].

In the following study, we examined DNA from French Canadian patients who have been screened and found not to carry *BRCA1/2* mutations. Prior to the following study, the French Canadian population remained one of the known founder populations yet to be screened for such variants. The purpose of the study was to determine if *CHEK2* alleles could account, in part, for the yet unattributed breast cancer risk, and further to determine the value of offering *CHEK2* screening in a clinical setting for the French Canadian population.

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Abstract

Background

BRCA1 and *BRCA2* account for the majority of the known familial breast cancer risk, however, the impact of other cancer susceptibility genes largely remains to be elucidated. Checkpoint Kinase 2 (*CHEK2*) is an important signal transducer of cellular responses to DNA damage, whose defects have been associated with an increase in breast cancer risk. Previous studies have identified low penetrance *CHEK2* alleles such as 1100delC and I157T, as well as variants such as S428F in the Ashkenazi Jewish population and IVS2 + 1G>A in the Polish population. No founder allele has been specifically identified in the French Canadian population.

Methods

The 14 coding exons of *CHEK2* were fully sequenced for variant alleles in a panel of 25 affected French Canadian women and 25 healthy controls. Two variants were identified of which one novel variant was further screened for in an additional panel of 667 breast cancer patients and 6548 healthy controls. Additional genotyping was conducted using allele specific PCR and a restriction digest assay. Significance of amino acid substitutions were deduced by employing comparative analysis techniques.

Results

Two variants were identified: the previously reported silent substitution 252A>G (E84E) and the novel missense variant, 1217G>A (R406H). No significant difference in allele

distribution between French Canadian women with breast cancer and healthy controls was observed (3/692, 0.43% vs. 22/6573, 0.33%, respectively, P = 0.73).

Conclusion

The novel *CHEK2* missense variant identified in this study, R406H, is unlikely to contribute to breast cancer risk in French Canadian women.

Background

Breast cancer is the most common form of malignancy amongst females in the western world. Specifically, one in ten of all new diagnosed cancer cases are of the female breast [1]. Typically, less than five percent of these cases are inherited in a mendelian fashion, specifically from the segregation of highly penetrant alleles, such as mutations in *BRCA1* and *BRCA2* [2]. The existence of a large number of breast cancer families who lack linkage to either *BRCA1* or *BRCA2 [3]* suggested that other breast cancer susceptibility genes remained undiscovered. One such candidate gene, *CHEK*2, encodes a multifunctional kinase enzyme involved in the induction of cell cycle arrest, DNA repair and apoptosis [4-6]. Several large-scale studies have characterized known variants of the *CHEK2* gene [7-9], conclusively proving that *CHEK2* is a breast cancer susceptibility gene.

One *CHEK2* mutation present in the general population, 1100delC, occurs independently of *BRCA1/2* mutations [7,8]. The 1100delC variant results in a premature stop codon within exon 10, impairing the kinase ability of the enzyme and resulting in a two-fold increase in breast cancer risk [7,8,10]. In general, the population frequency of 1100delC has been reported to be ~1.9% in individuals with breast cancer, compared to ~0.7% in those without [10]. There is, however, variation in the observed frequency of 1100delC [10-13] suggesting that the prevalence of this mutation varies amongst populations.

Population isolates, also known as founder populations, have reduced genetic heterogeneity and are valuable tools for genetic analysis involving cancer susceptibility.

A recent example of such an approach has been seen with the identification of the *CHEK2* S428F mutation in the Ashkenazi Jewish population, which has been associated with a relative breast cancer risk of 2.0 amongst Ashkenazi Jewish women [14]. Similarly, a splice site mutation, IVS2 + 1G>A, originally identified in a US patient with familial prostate cancer [15], has been identified as a founder mutation in the Polish population with a population frequency of 0.3% [16]. The allele is associated with a two- to four-fold elevated risk for prostate, as well as a moderate increase in risk for breast cancer [16,17]. Most recently, Walsh et al. [18] discovered a novel 5.4Kb deletion, leading to a loss of exons 9 and 10, in two families of Central European ancestry. This mutation was found in 1.3% of 631 patients and in none of the 367 healthy controls. Further analysis of *CHEK2* may reveal additional founder mutations in other populations. One such population yet to be investigated, and the focus of this study, is the French Canadian population.

Established in Quebec between 1608 and 1760, the population now includes approximately 6 million French Canadians, who are descendants of an estimated 8000-10000 migrants from France [19]. Altogether, approximately 80% of these founders still have descendants in Quebec today, and they account for the major part of the French Canadian gene pool [20]. Many of the hereditary disorders in the French Canadian population show evidence of founder effects (for review, see [19]). In particular, French Canadian founder mutations have been identified in *BRCA1*, *BRCA2* and *PALB2* [21-24].

In the current study, we examined a panel of 25 *BRCA1/2* negative, affected French Canadian women alongside 25 healthy controls, to investigate the impact of *CHEK2* variants on breast cancer susceptibility in the French Canadian population.

Methods

Study Population

French Canadian women, previously affected by breast cancer, and determined through sequencing to be negative for all exonic *BRCA1* and *BRCA2* mutations, were used for SNP discovery (n = 25). Cases had a family history of breast cancer with at least three cases of either breast cancer diagnosed before 65 years of age, male breast cancer, or ovarian cancer within three degrees from the index case [21]. Healthy French Canadian women with unknown *BRCA1/2* mutation status were used as controls (n = 25). Controls were requited either through random dialing or as spouses of cases ascertained for previous studies of cancer, in the French Canadian population (**Group 1**, n = 50).

Variants identified in the initial case/control group were further screened for in extended groups of breast cancer cases and unaffected controls, using the original carrier samples as a positive control. **Group 2** consists of cases (n = 124) which were tested, and found negative, for French Canadian *BRCA1/2* mutations reported by Tonin et al. [21]. Women included in this group were diagnosed at a mean age of 54 (range = 26-76) years old and were referred to cancer genetics clinics at McGill University hospitals. Patients included in Group 2 were selected for either a high risk family history of at least three cases of breast and/or ovarian cancer within three degrees from the index case, or for presentation of multiple consecutive breast cancer cases prior to the age of 76. Cases included in this panel were genotyped alongside a subset of healthy French Canadian women, recruited through random dialing, in the clinic or as spouses of cases from previous investigations, as controls (n = 116). **Group 3** includes an extended group of

French Canadian women (n = 543) previously diagnosed with breast cancer at Hotel-Dieu Hospital, Montreal, at a mean age of 47 (range = 26-65) years old. All women in this group had previously been tested and found negative for French Canadian *BRCA1/2* founder mutations. Recruited patients were either under 50 years of age at diagnosis, or were diagnosed between 50 and 65 and had a first degree relative with breast cancer. **Group 4** consists of a panel of French Canadian neonatal controls (n = 6432), which have been previously tested for several known *PALB2* variants [24] as well as the known *BRCA1* and *BRCA2* French Canadian founder mutations.

All patients have provided written consent to participate in current research based investigations. The study is in compliance with the Helsinki declaration, and has been granted ethical approval by the institutional review boards of McGill University and the University of Toronto.

Molecular methods

Genotyping: SNP discovery was performed on Group 1 by direct PCR and sequencing (sequencing was conducted by the *McGill University and Genome Quebec Innovation Center* in both the forward and reverse directions). Sequencing was performed on all of the 14 coding exons of *CHEK2* as well as at the intron/exon boundaries. Primers used for PCR were designed using the online Primer3 program (Primer3). All primers used, annealing temperatures and amplicon sizes are summarized in **Table 3.1**.

Long Range PCR: Any variants found within exons 10-14, which are known to be duplicated wholly or in part on various chromosomes, were reamplified via long range

PCR; a ~9.2 Kb fragment encompassing exons 10-14 was generated using primers F5'-CGACGGCCAGTCTCAAGAAGAGGACTGTCTT-3' and R5'-GCTATGACCATGCACAAAGCCCAGGTTCCATC-3' as previously described [14]. PCR was conducted using the Expand Long Template PCR system (Roche Applied Science, Cat No. 1-681-834) with an annealing temperature of 58°C.

Products obtained from Long-range PCR were then used as a template in a second round of amplification, using appropriate primers to isolate individual exons for sequencing.

Allele-Specific PCR: To determine the frequency of 1217G>A in Group 2, a forward primer with the last nucleotide specific to the variant was designed and used in conjunction with the exon 10 primers designed for sequencing. PCR was conducted at an annealing temperature of 54°C and the product was visualized by gel electrophoresis.

Allele-specific amplification was preformed as above for Group 4 which was followed by fluorometric detection of the PCR product using SybrGreen. A scatter plot was derived from the raw fluorescence of both alleles which was then analyzed to compute the genotype as previously described [25]. The accuracy of this method is 99.0% and the average rate of data rejection is below 1.00%.

Restriction Assay: Samples from Group 3 were genotyped via a restriction digest assay. Samples were amplified by PCR twice: the first to isolate *CHEK2* exon 10, and the second using nested primers to obtain a smaller fragment of 202bp, encompassing 1217G>A. Products obtained from the second round of amplification were incubated overnight at

37°C with +*Nla*III (1U/sample, New England BioLabs, USA). NlaIII digests after the consensus sequence of CATG, and thus cut the variant (A) allele, resulting in three fragments of 4, 76 and 122 bp, respectively. After digest, the wildtype *CHEK2* allele results in two fragments of 4 and 198bp, respectively. A sample mutant for R406H (confirmed by sequencing) and a wild-type sample were randomly seeded on each 96-well plate and used as positive and negative controls respectively in the screening process. Digested products were visualized by gel electrophoresis. The presence of 1217G>A was confirmed by direct sequencing using the BigDye® Terminator v1.1 Cycle Sequencing Kit and 3130xl Genetic Analyzer (Applied Biosystems, USA).

1100delC mutation Analysis: The presence of 1100delC within samples encompassing Group 2 was determined by generating S-35 labeled PCR products. PCR product was denatured for 15 min at 95°C prior to loading in a 5% denaturing polyacrylamide gel. PCR products were separated for 2 hours at 80W and visualized by audioradiography.

Amino Acid Stability, Conservation and Severity

To estimate the impact of amino acid substitutions on phenotype, mean chemical distance between the wild type amino acid and its substitute was evaluated using the Grantham matrix score (Grantham, 1974), Grantham variation (GV) and Grantham deviation (GD). Conservation of the wild type amino acid was analyzed using the multiple sequence alignment program ClustalW. Substitution tolerance was estimated using the SIFT algorithm (Sorting Intolerant From Tolerant).

Statistical Analysis

Allele and genotype frequency is expressed as a proportion of the entire sample set. Fisher's exact test was used to test for significance. In the circumstance where a sample would not amplify, it was excluded from all calculations. Two-tailed p values are presented.

Results

SNP discovery in *CHEK2* coding regions was conducted by sequencing 25 cases and 25 controls simultaneously. This approach provides an 80% power to detect an allele with a frequency of 1% or more [26]. Furthermore, this eliminates the potential biases inherent when studying cases first and then searching for only those variants identified, in the control set. From this, we have identified two variants: the previously reported silent variant, 252A>G (E84E), observed in 2/25 cases versus 2/25 controls, in addition to the novel missense variant 1217G>A, which results in an amino acid substitution at position 406, of an arginine for a histidine (R406H, **Figure 3.1**) observed in 1/25 cases.

The missense mutation, R406H was further screened for in extended groups of cases and controls. Through allele-specific PCR, we identified one additional affected case (1/124, 0.81%) from Group 2. Group 3 was genotyped by a restriction assay and was found to contain one affected case (1/543, 0.18%). Within our neonatal set of controls, Group 4, R406H was observed in 22 samples (22/6432, 0.34%). Overall, the frequency of the R406H allele was not significantly elevated in total breast cancer cases (3/692, 0.43%) compared with healthy controls (22/6573, 0.33%) P = 0.73 (**Table 3.2**).

To predict the significance of the R406H substitution, sequence alignment of *CHEK2* exon 10 was analyzed across ten species, revealing a modest conservation of the arginine residue amongst higher eukaryotes, with 6/10 species displaying homology (**Table 3.3**). When comparing the mean chemical difference between arginine and histidine, a Grantham score of 29, GV of 124.29 and a GD of 0.0 is obtained, suggesting the neutrality of this substitution. Furthermore, tolerance of this substitution is indicated via analysis by the SIFT algorithm (SIFT score of 0.10).

Additionally, patients included in Group 2 were further genotyped for 1100delC. Including the fully sequenced 25 cases and controls, 1100delC was observed in 2.01% (3/149) of cases versus 0.7% (1/141) of controls.

Discussion

Inherited breast cancer has been associated with germline mutations in more than ten different genes, most of which are involved in the maintenance of genomic integrity. A large proportion of such cases can be accounted for by mutations in the tumor suppressor genes *BRCA1* and *BRCA2*. Additionally, *TP53*, *PTEN*, *CDH1* and *STK11* are considered high-risk breast cancer susceptibility genes. Mutations in *ATM, BRIP1, PALB2, CHEK2* and possibly *NBS1, RAD50* are also associated with a moderately increased risk for breast cancer, and many low penetrance genes have recently been identified. However, roughly 50% of familial breast cancers remain to be elucidated [27,28].

In the current study, 25 French Canadian breast cancer patients and 25 healthy controls were fully screened for variants within the *CHEK2* gene. Two variants were identified: the silent variant E84E and the novel R406H missense variant. E84E, which has been reported in several other *CHEK2* screens, is likely a neutral allele with no association to breast cancer [14,29,30]. In addition, given that the primary structure of *CHEK2* is unaltered by the E84E mutation, and further, that it was observed at a similar frequency in cases and controls suggests against the possibility that this variant may affect an exonic splicing enhancer or aberrantly affect protein translation rates. Thus, no further investigation of this variant was conducted. R406H, however, was genotyped for in an extended panel of breast cancer cases and healthy controls. Neither variant was observed at a significantly high frequency in breast cancer cases when compared with controls.

To further characterize any potential impact of R406H, bioinformatic tools were employed. In short, conservation analysis, substitution evaluation and a tolerance test lack any indication of a pathogenic contribution from this allele.

Large international studies [10,31-33] have shown that 1100delC is associated with increased breast cancer risk in many, but by no means all, world populations. Our findings in cases (Table 2) when combined with previous data on controls [32] suggest that this allele is also associated with breast cancer risk in the French Canadian population. The evidence that other *CHEK2* alleles are associated with an increased risk in the general population is less convincing [34,35]. However, some founder alleles that do seem to be associated with an increased risk in specific populations have been identified.

To date, five interesting *CHEK2* founder alleles have been identified, all of which are associated with an elevated risk for breast: 1100delC, I157T, IVS2 + 1G>A, S428F and del5395. All five variants have been shown to contribute to breast cancer risk provided they are present in the population of interest, with the latter three particularly being observed with high degree of ethnic specificity. The IVS2 + 1G>A splicing mutation has been observed in the Polish population as a founder mutation with a 0.3% population frequency [36] and associates with approximately a two-fold elevated risk for breast cancer. In the Ashkenazi Jewish population, Shaag et al. [14] discovered the novel missense mutation S428F (1283C>T) at a frequency of 2.88% amongst 1632 breast cancer patients compared to 1.37% of 1673 controls, thus suggesting S428F is associated with breast cancer risk; a yeast complementation assay supported the hypothesis that this variant aberrantly affects *CHEK2* protein function. The most recently identified founder mutation, del5395, resulting in a loss of exons 9 and 10, was originally identified in two families of Czech or Slovak origin [18]. This founder mutation has twice been studied in detail; the first observing the deletion in 1.3% of 631 breast cancer cases and 0.0% of 367 healthy controls from the Czech and Slovak Republics. In agreement with the first study, Cybulski et al. [37] investigated the 5,395bp deletion in Poland, observing the frequency to be 0.9% of 4,454 breast cancer cases versus 0.4% of 5,496 healthy controls (OR = 2.0; 95% CI = 1.2-3.4). It is likely other *CHEK2* founder mutations are yet to be discovered, as to date, *CHEK2* has not been thoroughly investigated in many ethnic groups.

One such group, the French Canadian population has proved to be valuable in investigations of other breast cancer susceptibility genes. For example, several common pathogenic *BRCA1/2* founder mutations are recognized in the French Canadian

population [21-23]. Moreover, the proposition that additional French Canadian founder mutations have yet to be revealed is supported by the recent identification of a *PALB2* truncating mutation, Q775X [24].

The results presented here represent the first systematic analysis of *CHEK2* in the French Canadian population. The novel variant we identified, R406H, is almost certainly not associated with increased risk for breast cancer and *CHEK2* alleles other than 1100delC are unlikely to contribute to breast cancer risk in this population. However, the possibility that *CHEK2*, due to its role in cell cycle regulation, may influence the risk of other familial cancers in the French Canadian population, such as prostate, colon, ovarian or colorectal cancer, and would thus be an informative population for such future investigations. Interestingly, some of the well known variants, such as I157T have been associated with colon cancer [38], whereas the truncating variants 1100delC and IVS2 + 1G>A have been associated with an elevated risk for familial prostate cancer in both the Polish and Finish population [16]. Most recently, all three variants in addition to the del5395 have been associated with an increased susceptibility to bladder cancer in Poland [39].

The emerging picture suggests that some functionally significant variants in *CHEK2* are able to predispose cells from a wide distribution of organs to an elevated risk of cancer. Thus, much remains to be studied with respect to *CHEK2* alleles in the French Canadians, but it seems unlikely that a specific, common founder mutation for breast cancer exists in this population.

Conclusions

Sequencing of the *CHEK2* gene in 25 breast cancer patients and 25 healthy controls, from the French Canadian population did not reveal any pathogenic mutations. The one novel missense variant identified in this study, R406H, does not appear to be associated with breast cancer risk. Additional investigations of *CHEK2* and French Canadian breast cancer, utilizing large panels of familial and/or sporadic cases, would be necessary to refute the notion that additional *CHEK2* susceptibility alleles exist in the French Canadian population. However, it is unlikely that *CHEK2* alleles other than 1100delC significantly influence familial breast cancer risk within our study group.

Note added in Proof: We have recently completed MLPA (MRC-Holland, kit P190) analysis on 41 French Canadian women with a personal and familial history breast cancer. Cases had previously been screened for all known founder *BRCA1* and *BRCA2* mutations, as well as *CHEK2* 1100delC. No genomic deletions or insertions were identified.

Competing interests

The author(s) declare that they have no competing interests

Authors' contributions

Experimental design was conceived by DJN, LQC, NH and WDF. Data acquisition was conducted by DJN under the supervision of WDF. Initial technical optimizations were conducted by VR and NH. Sample recruitment and implementation was carried out in collaboration with PG, PT and AR. Neonatal genotyping was performed by GC and FR. Additional French Canadian R406H genotyping was carried out by SAN and PZ. DJN drafted the manuscript, which was revised by WDF. All authors have given their final approval of the version to be published.

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Tables

Table 3.1 – *CHEK2* Primers and Details

Fragment	Size (bp)	Exon	Amino Acid	Primers (5'->3')	Annealing Temp.(°C)
CHEK2EX01	565	1	1-106	Forward: gaactataggtctgggctgttagg Reverse: tccacctggtaatacaactttctg	57
CHEK2EX02	582	2&3	107-197	Forward: tgccttcttaggctattttcctac Reverse: aaccatattctgtaaggacaggac	56
CHEK2EX04	354	4	198-228	Forward: ctcaagggctttacaatatg Reverse: gaaatgagaaaccaccaatc	54
CHEK2EX05	499	5	229-264	Forward: gaatttcacaatccagggctac Reverse: ctcacaaattcatccatctaagcag	56
CHEK2EX06	632	6	265-282	Forward: tagagctgggtttggaactcag Reverse: agctaggcatgtgtgtgaatg	68
CHEK2EX07	434	7	283-304	Forward: aagaagactgggaagagacctagc Reverse: gcaagcctacattagattcttgg	56
CHEK2EX08	365	8	305-336	Forward: catctcatttcttagtttccaactg Reverse: tctgcctaatttcagggagtaattc	56
CHEK2EX09	331	9	337-365	Forward: ctgtgagatgtgtgtgttggtaac Reverse: tctggataagagcagtatcacctg	58
CHEK2EX10	546	10	366-420	Forward: ttaatttaagcaaaattaaatgtcc Reverse: ggcatggtggtgtgcatc	54
CHEK2EX11	353	11	421-458	Forward: gctgggattacaagcctaagg Reverse: gaagaaactcccaccacagc	69
CHEK2EX12	541	12	459-487	Forward: ggcctgttaatttctggcatactc Reverse: aaaggttgtagcctggccag	67
CHEK2EX13	488	13	488-514	Forward: cctctgggaaggtagaggc Reverse: caatccctagctgtgcttatcg	66
CHEK2EX14	585	14	515-543	Forward: cccccacttttactggaagc Reverse: gcaaaaccctgtctctacaaaat	64
CHEK2 R406H Allele Specific	N/A	10	N/A	Forward: ggactgctgggtataacca	54
CHEK2 Long Range	~9,200	10-14	366-543	Forward : cgacggccagtctcaagaagaggactgtctt Reverse : gctatgaccatgcacaaagcccaggttccatc	58
CHEK2 Restriction	546	10	366-420	Forward : ttaatttaagcaaaattaaatgtc Reverse : ggcatggtggtgtgcatc	57
CHEK2 Restriction Nested	202	10	380-420	Forward : catgagaaccttatgtggaaccc Reverse : cctggacaacagagcaagacacat	58
CHEK2 1100delC Sizing	196	10	366-396	Forward :aatagaaactgatctagcctacgtgt Reverse : gaacttcaggcgccaagt	60

Summary of primers, annealing termperatures and PCR amplicon sizes for the 14 coding exons of CHEK2. Additional details are listed for primers used for Long Range PCR, R406H and 1100delC genotyping.

Table 3.2 – *CHEK2* 1217G>A Frequency

Group	BRCA	CTRL	P-Value
1	4.00% (1/25)*	0.00% (0/25)*	1.00
2	0.81% (1/124)*	0.00% (0/116)*	1.00
3	0.18% (1/543)	N/A	N/A
4	N/A	0.34% (22/6432)	N/A
Total	**0.43% (3/692)**	**0.33% (22/6573)**	**0.73**

*Genotyped for 1100delC which was observed in 2.01% (3/149) of cases vs 0.7% (1/141) controls. If we compare the frequency in cases with that seen in the same neonatal controls used in this study, that were also tested for 1100delC by Zhang et al. [32] (19 1100delC carriers among 6460 controls), then the difference between cases and controls is statistically significant ($P = 0.01$).

Table 3.3 – Sequence Alignment of *CHEK2* Exon 10

Mosquito	VSDFGSSKFLDHTIFMRTICGTPEYVAPEVLESNGQKPYT**R**QVDVWSLGVVLYTM --256
Fruit Fly	VSDFGLSKFVQKDSVMRTLCGTPLYVAPEVLITGGREAYTKKVDIWSLGVVLFTC --376
Homo Sapiens	ITDFGHSKILGETSLMRTLCGTPTYLAPEVLVSVGTAGYN**R**AVDCWSLGVILFIC --420
Chimpanzee	
Dog	ITDFGQSKILGETSLMRTLCGTPTYLAPEVLNSFGTAGYN**R**AVDCWSLGVILFIC --421
Mouse	ITDFGQSKILGETSLMRTLCGTPTYLAPEVLVSNGTAGYS**R**AVDCWSLGVILFIC --424
Rat	ITDFGQSKILGETSLMRTLCGTPTYLAPEVLISNGTAGYS**R**AVDCWSLGVILFIC --423
Chicken	-TYFGQSKILGETSLMKTLCGTPTYLAPEVLNSFGTAGYS**R**AVDCWSLGVILFVC --391
Fugu	VTDFNQSRILEETMLMRTLCGTPSYLAPEVFTQASTTGYSLAVDAWSLGVLLFVC --396
Tetraodon	VTDFNQSRILEETMLMRTLCGTPSYLAPEVFTQASTSGYGLAVDAWSLGVLLFVC --430
C. Elegans	LTDFGMAKNSVN—RMKTHCGTPSYCAPEIVANQG-VEYTPKVDIWSLGCVLFIT --370

Figures

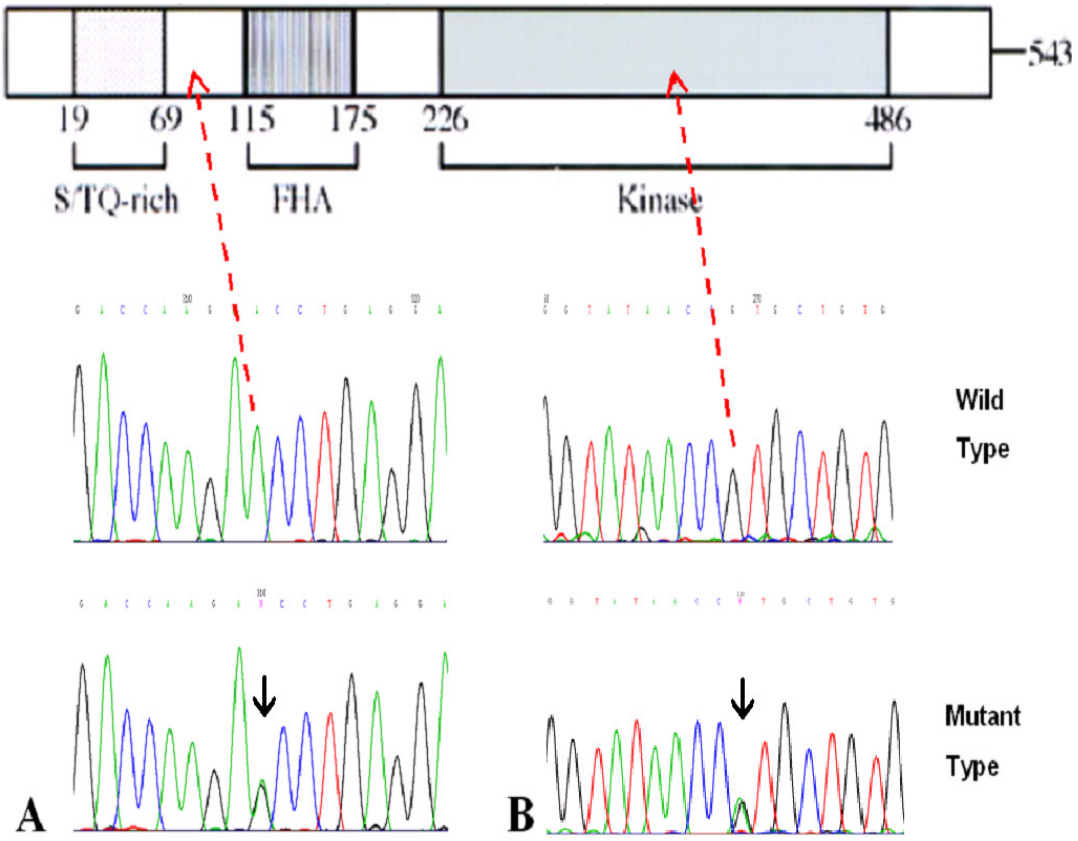

Figure 3.1: Functional Domains Associated with the *CHEK2* Variants. (**A**) Chromatogram of the silent E84E with arrow illustrating its location N' Terminal to the CHEK2 fork-head association domain. (**B**) Chromatogram of R406H and its location within the CHEK2 Kinase domain.

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CHAPTER 4

Partner and Localizer of *BRCA2* (*PALB2*) Heterozygous Mutations and Breast Cancer Susceptibility

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Manuscript in preparation as:

The Characterization of Genomic Instability in Lymphoblastoid Cell Lines Derived from Heterozygous PALB2 Mutation Carriers

Novak DJ, Amrein L, Reddy J, Wark L, Aloyz R, Mai S and Tischkowitz M

PREFACE

The focus of our investigations up to this point has been on the identification and assessment of breast cancer risk arising from variant alleles of genes associated with one of the major breast cancer susceptibility genes, *BRCA1*. One of the other majorly penetrant breast cancer genes, *BRCA2*, plays a significant role in controlling the localization and function of RAD51, a vital protein which interacts with the single-stranded DNA overhangs of DSBs and promotes homologous pairing and strand invasion of these regions during HR [1]. The interaction with BRCA2 is required for the mobilization of RAD51 after DNA damage [2] in addition to the formation of DNA damage-induced RAD51 nuclear foci [3].

The molecular picture of BRCA2 was further revealed in 2006 when Xia et al. [4] identified the Partner and localizer of BRCA2, PALB2, through the use of mass spectrometric analysis to identify proteins that immunoprecipitated with BRCA2 from HeLa cell extracts. *PALB2*, located on chromosome 16p12, is comprised of 13 exons coding for a 1,186 amino acid protein with a molecular mass of 130KD [5,6]. Immunohistochemical staining of a human osteosarcoma cell line illustrated the localization of PALB2 with BRCA2.

The function of PALB2 was further elucidated when Xia et al. [4] showed that immunodepletion of BRCA2 codepleated a significant abundance of PALB2, whereas immunodepletion of PALB2 codepleted almost all of BRCA2. Furthermore, the refocusing of both PALB2 and BRCA2, after exposure to ionizing radiation, suggested that like BRCA2, PALB2 participates in the DNA damage response. However, upon

depletion of PALB2 by siRNA, BRCA2 foci formation was largely absent, even after exposure to ionizing radiation. Thus, PALB2 appeared to promote the stable interaction of BRCA2 with nuclear structures. Finally, specific germline mutations in *BRCA2* identified in breast cancer patients, such as W31R, W31C and G25R, appeared to disrupt PALB2 binding and abolish the HR based DNA repair function of BRCA2 [4]. Most recently, the first functional link between BRCA1 and BRCA2 has been illustrated by Zhang et al. [7], by showing that PALB2 also physically interacts with BRCA1, linking BRCA1 and BRCA2 in a DNA damage response network through interaction with its NH2 and COOH terminal ends.

Due to the essential function of PALB2 in DNA repair and tumor suppression, Reid et al. [6] suggested that monoallelic *PALB2* mutations could confer susceptibility to adult cancer. Following this hypothesis, Rahman et al. [8] fully sequenced the *PALB2* gene in affected individuals from breast cancer families with no mutations in *BRCA1* or *BRCA2*. They identified monoallelic truncating mutations in 10 of 923 individuals with familial breast cancer in comparison to none in their control set, suggesting that such mutations confer a 2.3-fold relative risk of breast cancer (95%CI 1.4-3.9). Of the four variants identified in this study, the heterozygous mutation 3549C>G and the frameshift mutation, 3116delA, were both identified in three separate sets of three unrelated women with breast cancer, all of whom had a family history of multiple breast cancer cases. Further examples of *PALB2* conferring an increase in breast cancer susceptibility come from Erkko et al. [9] who identified a 1-bp deletion, 1592delT, in 3/113 *BRCA1/BRCA2* negative, breast and breast-ovarian cancer families from northern Finland versus the presence of this mutation in 6/2,501 controls. This mutation was further identified in

18/1918 unselected breast cancer cases (odds ratio of 3.92, 95%CI 1.5-12.1). Additional *PALB2* mutations have been identified in families with Fanconi anemia, complementation group N, as discussed in the next chapter. These results solidify the position of *PALB2* as a moderately penetrant breast cancer susceptibility gene.

In addition to breast cancer susceptibility, *PALB2* has recently been associated with an elevated risk of pancreatic and possibly even prostate cancer. For example, Jones et al. [10] identified a germline deletion of 4-bp, 172delTTGT, resulting in a frameshift at codon 58, in addition to an acquired somatic mutation affecting splicing of *PALB2* exon 10, IVS10+2C>T, in one pancreatic patent. Additionally, a patient with familial pancreatic cancer was identified with the splicing mutation IVS5-1G>T. Overall, Jones et al. [10] identified *PALB2* truncating mutations in 3 of 96 patients with familial history of both breast and pancreatic cancer. Most recently, the role of *PALB2* as a modest contributor to pancreatic cancer has been supported by the recent discovery of a 6.7Kb germline deletion, resulting in the loss of exons 12 and 13 in 1/254 individuals with prostate cancer [11]. Evidence suggesting at the role of *PALB2* in prostate cancer has also recently arisen, as Erkko et al. [9] identified one *PALB2* truncating mutation, 1592delT which segregated in 1 multigenerational prostate cancer family. Further analysis will subsequently determine if indeed *PALB2* is involved in prostate cancer pathogenesis.

Rather than attempting to identify additional susceptibility alleles, as has been the primary focus of the previous two chapters, the following study attempts to establish both a molecular and cellular link between *PALB2* heterozygous mutations and genomic instability, an important precursor to carcinogenesis.

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ABSTRACT

Background

PALB2 is estimated to confer a moderately increased risk for breast cancer, likely due to a disruption of genomic regulation. Fanconi anemia proteins may play a critical role in the telomere maintenance pathway, due to their core function in genetic recombination. In the current study, we attempt to establish both a molecular and cellular link between *PALB2* heterozygous mutations and genomic instability, an important precursor to carcinogenesis.

Methods

Nine lymphoblastoid cell lines (LCLs) were analyzed in the current study: one carrier of the *PALB2* 229delT allele, one carrier of 2523delA, one of 3323delA, three carriers of Q775X, and three wild-type for *PALB2*. Genomic integrity was assessed by telomeric Q-FISH, centromeric FISH and spectral karyotyping. The response to cellular cytotoxicity was examined in the presence of Mitomycin C and Cisplatin through the metabolism of the tetrazolium salt, WST-1.

Results

No significant difference was observed in telomere number or median telomeric intensity between the two *PALB2* control lines, control 1 and control 2 (P = 0.287). The *PALB2* 3323delA LCL did not significantly differ in telomere count and mean fluorescence intensity (P = 0.386 and P = 0.786). A slight reduction in mean fluorescence probe

intensity was observed in the 2521delA LCL (P = 0.078 vs control 1). The 229delT LCL was observed with an increase in observable telomere signals associated with a reduction in mean intensity (P = 2.22E-12 and P = 8.52E-15). The Q775X LCL was observed with a reduced telomere count associated with an increase in mean probe intensity (P = 2022E-12 and P = 8.52E-15). Results were confirmed through a second Q-FISH analysis including two additional Q775X carriers, a new 229delT LCL from the same patient and three fibroblast cell lines. No significant differences were observed in total centromere number between carrier LCLs and controls. No duplications, translocations, deletions or rearrangements were observed to be consistent with the pathogenicity of the *PALB2* variants. *PALB2* heterozygous LCLs do not appear to display elevated sensitivity to MMC and cisplatin.

Conclusion

Our results are suggestive of a possible telomere instability mechanism which may be an important first step in setting the stage for the development of *PALB2* related breast cancers, predisposing heterozygous carriers to an increased susceptibility to additional tumourgenic mechanisms.

INTRODUCTION

The proposal that familial breast cancer is, in part, associated with impaired control of genomic stability has been supported by the growing number of genes linked to familial breast cancer that participate in DNA damage response pathways [1]. One such gene, *BRCA2*, codes for one of the two major breast cancer susceptibility proteins and is involved in homologous recombination (HR) and HR-based DNA double strand break repair (DSBR). Monoallelic *BRCA2* mutations have been associated with high risks of breast and ovarian cancer (for review see [2]. Furthermore, biallelic *BRCA2* mutations are known to cause a subtype of Fanconi anemia (FA-D1) [3]. However, individuals lacking *BRCA2* mutations, presenting with a similar FA phenotype (FA-N) have been identified, suggesting deficiencies in other proteins functionally related to *BRCA2*.

PALB2 has recently been identified as a nuclear partner of BRCA2, promoting localization and stability upon interaction [4]. Given the functional and phenotypic links between PALB2 and BRCA2, it is reasonable to assume that monoallelic *PALB2* mutations also confer susceptibility to adult cancer; to date, a select group of such mutations has been identified [5-10]. In addition to enriching the database of *PALB2* pathogenic mutations, it is critical to determine how heterozygous *PALB2* mutations promote breast cancer development.

A recent analysis of PALB2 in Spanish breast cancer families has presented the first and only case of a loss of heterozygosity (LOH) [11], a common mechanism in BRCA2-related tumor progression, in a PALB2-associated tumor. However, the apparent lack of LOH observed through the investigation of multiple heterozygous *PALB2*-related

tumors .[6,12], suggests that although possible, LOH is not likely the major mechanism driving PALB2 tumorgenesis. Recently, A study conducted by Potapova et al. [13] has suggested the presence of *PALB2* hypermethylation as a method of *PALB2* inactivation in sporadic primary *BRCA2* breast and ovarian tumors. However, similar analysis within our lab has failed to detect methylation in the putative CpG island region of the *PALB2* promoter through bisulphate sequencing of blood and tumor samples taken from *PALB2* carriers, thus arguing against methylation-mediated transcription suppression in our patient group [Hamel et al. unpublished data]. Therefore, alternative mechanisms including haploinsufficiency, the rarely demonstrated dominant-negative effect and allele silencing via promoter methylation have yet to provide conclusive insights into how truncating *PALB2* mutations promote breast cancer development. An alternative perspective in the progression towards cancer development, currently under intense investigation, is the role of telomeres in genomic instability; specifically, telomere dysfunctions, which when occurring concurrently with cell cycle checkpoint/regulator abnormalities can potentially lead to tumor initiation and/or progression.

Telomeres are composed of TTAGGG repeats that associate with telomere-specific binding proteins allowing for one to measure the number and length of telomeres in single cells by capturing individual telomere fluorescence signals, an assay known as PNA-FISH. Telomeres are known to provide three essential functions: (1) protecting chromosomal DNA ends so they are not recognized as DSBs, (2) protecting chromosomal ends from enzymatic degradation and (3) preventing chromosomal aggregates [14]. In humans, telomeres shorten with each somatic cell division, due to a down-regulation of the enzyme telomerase. Normally, this telomere shortening would lead to replicative cell

senescence due to the inability of telomerase to maintain the protective cap, and thus becoming recognized as a DSB, targeted ultimately by TP53 [15] Rare cells that have a compromised checkpoint pathway may continue to shorten their telomeres, resulting in the initiation of a phase termed crisis, characterized by chromosomal fusions and non-reciprocal translocations [16].

In addition to chromosome structural abnormalities, cytotoxic agents are commonly used to identify varying responses associated with DNA damage response deficiencies. For example, FA cells are commonly characterized by a hypersensitivity to DNA cross-linking agents, such as mitomycin C, a phenotype also observed in both *BRCA1* and *BRCA2* deficient cells [17]. In concordance with a *FA/BRCA2* deficient phenotype, cells lines harbouring biallelic *PALB2* mutations and those deficient of *PALB2* via siRNA have been shown to exhibit a similar sensitivity when exposed to mitomycin C [4,18].

In an effort to demonstrate biological differences between heterozygous disease causing mutations of *PALB2*, wildtype alleles, we investigated the effect of *PALB2* heterozygous mutations on genomic control as a potential contributing factor in the pathogenesis of *PALB2*-related breast cancers. In the current study we investigated the chromosome integrity and cellular phenotype of *PALB2* heterozygotes by analyzing lymphoblastoid cell lines harbouring four distinct *PALB2* monoallelic mutations: 2521delA, 3323delA, Q775X and 229delT, in addition to two cell lines from related individuals that were wild type for PALB2.

METHODS

Patient Derived Cell Lines

Patients: Lymphoblastoid Cell lines were obtained from nine patients referred to the cancer genetic clinics at McGill University Hospitals: one carrier of the *PALB2* 229delT allele, one carrier of 2523delA, one of 3323delA, three carriers of Q775X, and three wild-type for *PALB2*. At the time of this study, only three fibroblast cell lines were available for analysis; those of the *PALB2* 229delT mutation carrier affected with breast cancer and two of her unaffected children, Control 1 and Control 2, who did not inherit the mutation. Cell lines, patient information, disease status and treatment information are listed in **Table 4.1**.

Molecular Methods

Genotyping: The mutation status of all patient derived cell lines were confirmed through direct sequencing. DNA was extracted from LCLs using the Gentra systems PUREGENE DNA Purification KIT (Gentra). DNA aliquots were made at a concentration of 50ng/μL and were used to amplify regions encompassing the variants of interest via direct PCR and sequencing (Sequencing was conducted by the McGill University and Genome Quebec Innovation Center). Primers used for PCR were designed using the online PRIMER3 algorithm (http://frodo.wi.mit.edu/primer3/). All primers used and associated conditions are summarized in **Table 4.2**.

Cellular Methods

Cell Cultures: All LCLs were started from their frozen state in Isocovs Modified Dulbecc's Eagles Medium (IMD(E)M) supplemented with 20% fetal bovine serum (FBS), 1% penicillin/Streptomycin and 0.5% fungizone. Culture media was immediately changed after 24 hours of start-up, being replaced with culture media consisting of IMDM supplemented with 10% FBS and 1% penicillin/Streptomycin. Cell aliquots were frozen down in a preparation of 70% IMDM, 20% FBS and 10% DMSO. Cells were placed in cryogenic preservation by immediate exposure to -80°C for 3 days, followed by permanent storage in liquid Nitrogen.

Cytotoxicity Assay: WST-1: Cells in culture for at least five days were cultured to 60-70% confluency and seeded into 96-well plates at a concentration of 50,000 cells/well at a volume of 200µL. The first column of wells was seeded with media and no cells as a blank control. A row consisting of serial dilutions for each cell line was plated in order to test for plating efficiency. Cells were then incubated at 37°C for 3 hours, after which, a predetermined concentration of the cytotoxic agent was seeded into the corresponding wells at 10 concentration points and in triplicate (cisplatin and mitomycin C: 0µg-10ng/mL resulting in a working concentration of 0ug-1000µg/mL at a 1:10 dilution factor). Incubation time of the four PALB2 LCLs was determined to be 72 hours based on cell line doubling time. After incubation with the cytotoxic agent, WST-1 was seeded into wells at a 1:10 dilution factor, and further incubated at 37°C. Changes in absorbance were determined to be at highest sensitivity 3.5 hours after addition of WST-1. Absorbance was recorded by a photospectrophotometer at 450nm with 650nm of

reference wavelength. In all experiments absorbance of the media alone with WST-1 was used to negate any interference within the assay.

Cytogenetic Methods

Q-FISH Slide Preparation: Of the readily available assays applicable for use in telomere length measurement, such as flow-FISH or southern blot analysis, Q-FISH is the most sensitive and informative, and was thus utilized in this study. Cell lines were cultured to 80% confluency, of which 5-8 million cells were then harvested and submitted to hypotonic shock by the addition of 5mL 0.075M KCl for 10min at room temperature, following a protocol that preserves the shape of the 3D nuclei [19]. 5mL of freshly prepared cellular fixation solution (methanol/acetic acid 3:1) was gently added to overlay the cell/KCl suspension, inverted and spun at room temperature for 10 minutes at 800rpm. The supernatant was removed and replaced with 5mL of fresh fixative to resuspend the cell pellet. Cell solution was spun for 10min at 800rpm at room temperature, and supernatant was once again removed. If slides were to be immediately prepared, a working concentration of fixative solution was added to obtain a concentration of approximately 10000 cells per hybridization area per slide. Alternatively, cell pellets were resuspended in 1-2mL of fresh fixative solution for storage at -20°C.

Peptide-nuclei-acid (PNA)-Q-FISH: After fixation, slides are dehydrated with 100% Ethanol followed by a 15min wash in 3.7% formaldehyde to ensure maximum fixation. The formaldehyde prefixation is followed by a 3x5min wash in 1X PBS. Slides were then

subjected to a pepsin treatment, where slides were incubated for 10min in 0.01M HCL/1mg/ml pepsin at 37^{o}C, followed by a 1x5min wash in PBS and 2min incubation in 3.7% formaldehyde/1xPBS. A 3x5min PSB wash was once again preformed followed by dehydration in 70%, 90% and 100% ethanol for 2min each, respectively, at which point slides were left to dry. 8µl of the CY3 PNA probe was added to the dried slides. Coverslips and rubber cement were used to seal the slides after probe application, readying the slides for a 3min denaturation at 80^{o}C. After denaturing, slides were hybridized for 2h at 30ºC in using the Hybrite^{TM} system.

Rubber cement and coverslips were carefully removed, and slides were then washed (2x15min) in 70% formamide/10mM Tris. Slides were subjected to a 1x1min wash in 1x PBS, 1x5min wash in 0.1XSSC and a final 2x5min wash in 1xPBS/0.05% Tween-20, prior to applying 50µl of 0.2mg/ml DAPI stain. Slides were then incubated for 3min, rinsed with ddH2O, dehydrated in 70%, 90% and 100% ETOH prior to the addition of 20uL Vectasheild and the application of a coverslip.

Slides were stored at 4^{o}C, in a light proof slide folder. All slides were imaged ~24 hours after hybridization.

Imaging: Image acquisition was conducted on fluorescent sections using the AxioImager Z1 microscope and an AxioCam HR CCD (Carl Zeiss Canada Ltd). Imaging of LCLs was conducted using a 63x/1.4 oil objective with an acquisition time of 450ms for Cy3 staining (telomere) and 100-300ms for DAPI staining (nuclei) and imaging of Fibroblasts occurred under similar conditions. A minimum of 40 cells were captured for each patient in each group. For the LCLs and fibroblasts, eighty z-stacks were acquired at a sampling

distance of *xy*: 107nm and *z:* 200nm, respectively. The Axiovision 4.6 software (Carl Zeiss Inc. Canada) and constrained iterative algorithm [20] are utilized for the deconvolution of the acquired Z-stack images into one 3D image.

Teloview Analysis: For each cell line, 30 single cells were analyzed using TeloView, a program written using a Matlab language and DipImage [21]. Through teloview, each 3D image was specifically processed to quantify the number, location and intensity of all telomeres within the nucleus (see [21,22] for details). Raw data were loaded into Microsoft Excel which was then used for all further statistical and image processing.

Metaphase Slide Preparation*:* For metaphase chromosome preparation, cells were fixed by the drop fixation method [23]. In brief, media was changed for the culture of interest 12 hours prior to harvesting the cells. 0.1µg/mL of colcimide is added to an approximately 65% confluent culture 2 hours prior to collection of the cells. Pelleted cells are subjected to hypotonic shock in 5mL 0.075M KCl for 30min at room temperature. The shocked cells are collected and the supernatant removed immediately followed by the drop fixation in freshly prepared fixation solution. Fixative solution was used to fix the cells in the following 13 step method: using a pasture pipette 1 drop of fixative was added to the cell pellet followed by a 1min incubation which was repeated 5 times, followed by 2x2, 5x2, 7x2, 10x2, 15x2, 20x2, 30x2, 60 drops for 2min. Upon completion, the solution was centrifuged for 10min at 800 RPM. Supernatant was discarded and the cell pellet was resuspended in 5mL fixative for 10min at room temperature. The previous step was repeated two additional times, increasing the incubation time by 10mins each

subsequent run through. Cells are resuspended in 2-5mL fixative solution pending on storage or immediate usage. Care was always taken to change the fixation solution before usage. Metaphase chromosomes are prepared from cells following the standard drop method on super cooled slides.

Spectral Karyotyping: Human spectral karyotyping (SKY) was carried out using the ASI kit for human chromosomes (Applied Spectral Imaging) following the suppliers protocol. Imaging was performed using the Spectra Cube on a Carl Zeiss Axioplan 2 microscope. A 63x/1.4 oil objective and the Spectral Imaging 4.5 software was used for image acquisition followed by analysis with the HiSKY 5.5 software for PC. For each cell line 20 metaphases were analyzed.

Statistical Analysis

The statistical significance of the mean probe intensity was derived by calculating the mean, number of observations and variance within each cell line analyzed. A two-tailed P-value is presented, derived from a two independent sample t-test assuming unequal variances. All calculations were conducted in Microsoft Excel 2007.

RESULTS

Cytogenetics

Telomere Measurements after PNA-Q-FISH

To determine whether *PALB2* heterozygoues cell lines are prone to telomere abnormalities such as accelerated shortening, telomeres were directly visualized through quantitative fluorescence in-situ hybridization (results summarized in **Table 4.3**). Control1 was observed to emit a maximum intensity of 190,000 arbitrary fluorescent units (a.u.) and a maxima of 68 telomeres at a modal value of 10,000 a.u. (**Figure 4.1A**). Similarly, Control2 was observed to emit a maximum intensity of 190,000 a.u., a maxima of 83 telomeres at a modal value of 10,000 a.u. (**Figure 4.1B**). No statistical difference was observed in telomere number or median telomeric intensity between the two control lines ($\bar{x}$ = 24305.75 vs 23529.79, P=0.287).

The *PALB2* 229delT LCL was observed to emit a maximum intensity significantly less than either control cell line, at 120,000 a.u. Furthermore, the maxima was calculated to occur at 99 telomeres at a modal value of 10,000 a.u. (**Figure 4.1C**). The mean intensity of the *PALB2* 229delT LCL was observed to be significantly different from both Control1 and Control2 ($\bar{x}$ = 19025.12 vs 24305.75, P=6.05E-17 and $\bar{x}$ = 19025.12 vs 23529.79, P=1.77E-12, respectively. Similarly, an increased number of telomeres was observed when the *PALB2* 229delT fibroblast cell line was analyzed alongside the two control fibroblasts; however, it is interesting to note the suggested length of the telomeres is not significantly shorter than those of the controls, and may in

fact be slight larger, as indicated by the mean probe intensity displayed by the PALB2 229delT fibroblast compared to the two controls (results presented in **Table 4.4**).

Probe intensity was markedly reduced in the *PALB2* 2521delA LCL when compared to either control, at an intensity of 140,000 a.u. Additionally, the maxima was calculated to occur at 81 telomeres at a modal value of 10,000 a.u (**Figure 4.1D**). No significant differences were observed when comparing the mean intensity of the *PALB2* 2521delA LCL with either Control1 or Control2 ($\bar{x}$ = 23096.23 vs 24305.75, P=0.078 and $\bar{x}$ = 23096.23 vs 23529.79, P=0.532).

The *PALB2* Q775X LCL emitted a maximum intensity of 310,000 a.u., and a reduced maxima of 58 telomeres at a modal value of ~15,000 a.u (**Figure 4.1E**). The mean fluorescent intensity of the *PALB2* Q775X cell line differed significantly from both Control1 and Control2 ($\bar{x}$ = 31187.24 vs 24305.75, P=2.22E-12 and $\bar{x}$ = 31187.24 vs 23529.79, P=8.52E-15).

The *PALB2* 3323delA mutation carrier was observed to be quite similar to both controls, emitting a maximum intensity of 190,000 a.u. Further, the maxima was calculated to be 79 telomeres at a modal value of ~17,000 a.u (**Figure 4.1F**). No statistically significant differences are observed when the mean teleomere intensity is compared with that of Control1 and Control2 ($\bar{x}$ = 23716.49 vs 24305.75, P=0.386 and $\bar{x}$ = 23716.49 vs 23529.79, P=0.786).

Centromere FISH

In an effort to demonstrate if the abnormal number of telomeres detected was a result of aberrant cellular division and thus an abnormal cell ploidy both centromere

FISH and spectral karyotyping was conducted. No significant differences were observed, in the mean number of centromeres, between and amongst any of the mutant or controls cell lines (data not shown).

Spectral karyotyping

Culture dependant abnormalities including polyclonal chromosomal rearrangements, translocations and isolated incidents of aneuplody were observed with no specific consistencies amongst all four *PALB2* LCLs analysed: 229delT, Q775X and two the two Control lines. However, overall there was no indication of a genome wide abnormal polyploid karyotype in the mutant cell lines versus controls (**Table 4.5**).

Cell Survival/Toxicity

In order to investigate *PALB2* related sensitivity to DNA damaging agents, the metabolic WST-1 assay was employed. No indication of increased sensitivity to mitomycin c was observed between heterozygous *PALB2* LCL mutation carriers and controls when treated in the range of 0-1000ng/mL (**Figure 4.2A**).

Similarly, no significant sensitivity difference between mutation carriers and controls is observed in the presence of cisplatin. Although there appears to be a slight indication of a mild heterozygous effect when treated in the range of 0-1000ng/mL (**Figure 4.2B**), we cannot yet confidently conclude upon this due to the range of sensitivity between our two control LCLs.

DISCUSSION

In the current study we have utilized the PNA-FISH assay and 3D fluorescence microscopy to observe differences in the number and size of telomeres in nine separate lymphoblastiod cell lines; three wildtype for *PALB2* and one for each of the following truncating *PALB2* mutations: 229delT, 2521delA, 3323delA and two for the *PALB2* Q775X mutation. Four of these cell lines were further utilized to investigate a possible cellular phenotype resulting from monoallelic *PALB2* mutations.

Two of the control LCL's, Control1 and Control2, were derived from two unaffected immediate relatives of the 229delT mutation carrier. Due to the close genetic background of these two LCL's, the similarity in telomere maximum intensity, maxima and modal value they have served as excellent controls, specifically in the analysis and comparison with the 229delT cell line. Control3 was included as an unrelated, population control.

Relative to both controls the 229delT cell line displayed a significantly reduced maximum telomere intensity suggesting greatly reduced telomere length. Furthermore, the maximum at the modal value is greatly increased in the 229delT cell line, suggesting an increased number of telomeres. These results are indicative of changes in nuclear architecture that are likely to promote chromosome instability from telomere loss, as chromosome aggregates are known to form when telomeres become critically short during crisis [16]. Finally, these aggregates can then lead to dynamic chromosomal rearrangements and polyploidy due to nondisjunction during cell division.

Chromosomal end-to end fusions can also occur without critically shortened telomeres, namely through the formation of telomeric aggregates [22,24,25]. Telomeric aggregates are generated in interphase nuclei and represent a close association or even a fusion of telomeres, likely due to telomere uncapping. As a result, telomeric signals in the PNA-Q-FISH assay will appear with to emit a much greater maximum intensity, likely in conjunction with a reduced number of counted signals at the maxima as a result of the multiple end to end fusions which have occurred, resulting in the aforementioned increased signal intensity. Such an explanation can readily account for the significant differences observed in both of our *PALB2* Q775X mutation carriers when directly compared to any of the three wild type controls.

Similar to the profile observed with the *PALB2* 229delT LCL, shorter telomeres were also observed in the *PALB2* 2521delA LCL. This could potentially be indicative of a mechanism resulting in telomere instability parallel to that observed in the Q775X LCL. Specifically, that the telomeres are shortening (perhaps due to rapid cell division and downregulation of telomerase) to such an extent that the protective cap is almost depleted, at which point aggregates would begin to form.

The observation that the telomeric profile of the 3323delA LCL did not significantly differ from the controls may be attributable to the fact that this LCL was the only one derived from an unaffected male individual, thus suggesting that different *PALB2* mutations may in fact confer varying levels of risk. Another possibility is that the instability observed in the other mutation carriers may not yet have progressed to a significant state in the 3323delA carrier, and thus a subtle developing phenotype cannot be excluded as a possibility.

The formation of chromosomal end-to-end fusions can lead to the evolution of complex karyotypes; it has been shown that dicentric chromosomes as well as Robertsonian translocation chromosomes form as a result of the discussed telomeric changes [22,26]. Dicentric chromosomes usually break at anaphase and initiate the breakage/fusion/bridge (B/F/B) cycle, first described in maize by McClintock [27]. In short, the unrelated chromosomes or fused sister chromatids form a bridge during anaphase which will break as the two centromeres are pulled to their respective ends of the cell. The result is unequal breakage; one chromosome with a duplication on its end and the other with a terminal deletion. This process will continue to repeat itself as neither chromosome has a sufficiently protected chromosome end due to the disrupted telomere. Such a mechanism has been associated alone with a modest increase of cancer in mice [28], and a high incidence of human-like carcinomas when seen in conjunction with a deficiency of P53, a cell-cycle and apoptotic regulator [16], indicating this mechanism as a viable pathway to many human tumors [29].

In order to demonstrate whether the increased telomeric signals observed in the 229delT carrier, and the reduced telomeric signals observed in the Q775X carrier, when compared to the three control cell lines, could be interpreted as an indication of an abnormal number of chromosomes in the respective cell lines, cells were analysed using centromere specific FISH and SKY. Results indicated no significant deviations in centromere number between all cell lines analysed, and similarly, all cells were observed to be comprised of the expected 46 chromosome range. Therefore, these results can be taken to indicate that the telomere degregation we observed, excess telomere signals and indications of chromosomal aggregates likely do not result in abnormal polyploidy. Any

slight deviations observed in chromosomes count can likely be attributed to culture dependant and/or EBV mediated changes [30].

An accelerated shortening of telomeres has previously been reported in FA patients [31-33], although the molecular mechanism of such an effect remains unknown. In a similar investigation to our own, Callen et al. [34] demonstrated a reduction in telomere length in addition to a generation of extra-chromosomal telomeric DNA signals, in primary lymphocytes derived from FA patients, independent of proliferative shortening and the telomere binding factor TRF2. Similarly, the results presented herein seem to portray a subtle heterozygous effect resulting from the same mechanism. It is important to note that although not discussed in depth here, an indication of a PALB2 heterozygous effect was deemed replicable upon a second undertaking of the Q-FISH analysis. Additionally, we were interested in excluding a possible telomere erosion effect induced by the paclitaxel treatment undergone by our 229delC carrier, and thus a second LCL was acquired and investigated from the 229delT (229delT-2) carrier, established from blood lymphocytes drawn after a prolonged period post tamoxifen and paclitaxel treatment, alongside two, recently acquired Q775X LCLs (results summerized in **Table 4.6**). The reoccurring indication of less intense and more abundant telomere signals or more intense and less abundant signals is suggestive of two distinct phases of the same instability mechanism. Namely, that the reduction in telomere length and extra telomeric signals observed in the *PALB2* 229delT, 2521delA and Q775X-2 carrier likely results from telomere erosion leading to end-to-end fusions ultimately resulting in direct telomeric breakages and extra-telomeric TTAGGG signals. Similarly, the phenotype displayed by the Q775X and Q775X-3 carriers, specifically the reduction in observable

TTAGGG signals and an increased signal in a portion of those observable may have arisen as a result of subtelomeric breaks following the formation of telomere aggregates, resulting in un-probed chromosome ends and an increased signal arising from the newly formed and potentially, broken off fusions. It is possible that upon further cell division, these telomere aggregate may undergo further direct telomeric breaks resulting in the formation of multiple telomeric peices rather than one large aggregate. Following this line of hypothesis, the results presented by the *PALB2* 229delT fibroblast cell line, specifically an abundance of telomere signals, as observed in the LCLs, with no apparent reduction in size, may represent an intermediate phase of instability where chromosomal end-to–end fusions still persist (**Figure 4.3**). Although some of these fusions will have undergone direct telomeric breakages accounting for the increase in telomere signals, some fusions may still exist, or have undergone direct telomeric breaks, resulting in increased probe hybridization amongst these telomere fragments, increasing the overall mean probe intensity to normal, or slightly above normal levels.

It is well known that FA patients show a high sensitivity to the effect of cross linking agents such as mitomycin C [35,36], a phenotype also common amongst *BRCA1* [37] and *BRCA2* [17] deficient cells. Therefore, we were interested to determine if there was an observable correlation between the hypothesized increase in telomeric breakages observed and a cellular phenotype of sensitivity to cross-linking agent's, eventually resulting in DSBs. Treating the two *PALB2* cell lines with the most pronounced telomere instability, 229delT and Q775X, with mitomycin C and cisplatin, no increased sensitivity was observed in comparison to two control cell lines. Although a subtle heterozygous effect is suggested with the treatment of cisplatin, the range in sensitivity between our

two control lines goes against the difference arising from a *PALB2* mutation, but rather an artefact of genetic background, age of cell donor and variables alike. These results are in agreement with a wide array of investigations showing a lack of sensitivity to such cytotoxic agents in Fanconi anemia and *BRCA2* heterozygous cell lines [17,38,39], likely a result of there being sufficient wild type protein to negate any quantifiable effect.

Currently, the pathogenic mechanisms of *PALB2* related carcinomas remain unclear. However, due to the function of PALB2 in DNA damage response, valuable insight may be gained by assessing a progression of genomic instability in relation to *PALB2* mutation carriers. Accordingly, through the analysis of patient derived *PALB2* heterozygous cell lines we suggest that telomere integrity may be compromised as a result of *PALB2* deficiency; specifically, through rapid telomere degradation, chromosomal fusions and breakages. Due to the infancy of *PALB2* research and lack of *PALB2* related resources, it will be of great value to revisit the investigations discussed in this study, specifically by utilizing non-EBV transformed cell lines and most importantly a *PALB2* null tumour cell line. However, the findings discussed herein suggest at a potential gateway mechanism underlying *PALB2*-related disease development.

Tables

Table 4.1 – *PALB2* Patient and Cell Line Information

PALB2 mutation	Gender	Date of Birth	Date of Sample Acquisition	Cell Type	Clinical Diagnosis	Treatment
229delT	Female	01-Jun-45	30-Oct-06	LCL	three infiltrating ductal carcinomas at 39, 42 and 60	—Lumpectomy in 1984 followed by radio and chemo therapy 5-FU for 15 months —Lumpectomy in 1988 followed radio and Methotrexate chemotherapy for 6 months —Tamoxifen and paclitaxel in 1995
229delT-2	Female	02-Jun-45	04-Dec-07	LCL	As above	As above
229delT	Female	03-Jun-45	30-Oct-06	Fibroblast	As above	As above
2521delA	Female	16-Jun-55	04-Mar-04	LCL	Right side infiltrating ductal carcinoma at 29 left side lobular carcinoma at 46	—Radio therapy followed by 4xAC —6x5-FU chemotherapy, epirubicin and cyclophosphamide in 2002 followed by 5 years on tamoxifen
3323delA	Male	09-Apr-55	04-Mar-04	LCL	Unaffected	N/A
Q775X-1	Female	22-Sep-62	13-Jul-07	LCL	infiltrating ductal carcinoma at 36	—4xAC starting July 8, 1999 —Radio therapy November 19-22, 1999
Q775X-2	Female	12-Nov-54	27-Jul-07	LCL	left side DCIS at 49 Right side infiltrating ductal carcinoma and DCIS at 50	—Left breast lumpectomy in 2004 —Left breast Mastectomy in 2005 —Right Breast Mastectomy in 2005 —Chemotherapy with AC and Taxol in 2006
Q775X-3	Female	11-Apr-48	27-Jul-07	LCL	infiltrating ductal carcinoma and DCIS	—Radio therapy followed by 4xAC tamoxifen in 2005
wild type (Control 1)	Male	14-Aug-74	Jan-07	LCL	Unaffected	N/A
wild type (Control 2)	Female	07-Apr-76	Jan-07	LCL	Unaffected	N/A
wild type (Control 1)	Male	14-Aug-74	Jan-07	Fibroblast	Unaffected	N/A
wild type (Control 2)	Female	07-Apr-76	Jan-07	Fibroblast	Unaffected	N/A
wild type (Control 3)	Female	14-Jan-63	23-Apr-08	LCL	Unaffected	N/A

Table 4.2 – Primers used to Amplify Regions Encompassing *PALB2* Mutations

PALB2 Mutation	Size (bp)	Primers (5'-3')	Annealing Temp (°C)
229delT	594	Forward: tgtcactgattctttcttaaataaatgtt Reverse: tgggcagttggtggaatta	61
2521delA	275	Forward: atttggagctttgctgctgt Reverse: tgactgaattcttttcagttcatt	58
3323delA	259	Forward: ttgtttggtttttgtctctgc Reverse: tgtgtttgcacagtgccttt	58
Q775X	569	Forward: acatcccaaaaggccaaact Reverse: taaacgtggaaggcccaat	60

Table 4.3 – Telomere Specific Q-FISH Results

Cell Line	Mean Probe Intensity [A.U.]	P-value vs Control 1	P-Value vs Control 2	Modal Value	Model Intensity	Maximum Intensity
Control 1	24305.75	N/A	0.287	68	10,000	190,000
Control 2	23529.79	0.287	N/A	83	10,000	190,000
229delT	19025.12	6.05x10-17	1.77x10-12	99	10,000	120,000
Q775X	31187.24	2.22x10-12	8.52x10-15	58	15,000	310,000
2521delA	23096.23	0.078	0.532	81	10,000	140,000
3323delA	23716.49	0.386	0.786	79	17,000	190,000

Table 4.4 – Telomere Specific Q-FISH Results from Three Fibroblast Cell Lines

Cell Line	Mean Probe Intensity [A.U.]	P-value vs Control 1	P-Value vs Control 2	Modal Value	Model Intensity	Maximum Intensity
229delT – Fibroblast	24034.04	0.646	0.013	139	11000	303000
Control 1 – Fibroblast	23743.49	N/A	0.059	93	10000	212000
Control 2 – Fibroblast	22494.07	0.059	N/A	90	12000	217000

Table 4.5 – Overview of SKY Results

	CONTROL 1		CONTROL 2		229delT-2		Q775X	
Metaphase Number	*Karyotype*	*Structural Aberrations*	*Karyotype*	*Structural Aberrations*	*Karyotype*	*Structural Aberrations*	*Karyotype*	*Structural Aberrations*
1	46,XY		44,XX	m	36,X, -X		45,XX	rb(6;14)
2	46,XY		40,X, -X		45,XX	rb(14;19), rb(15;3)	46,XX	
3	45,XY	t(10;11), t(11;10;18)	44,X, -X	rb(22;21),t(8;18)	46,XX		46,XX	
4	46,XY, +XX	t(6;22)	42,X, -X	rb(21;21)	38,XX	rb(14;22)	39,X, -X	
5	37,XY	rb(22;9)	44,XX	t(7;6),t(18;12)	44,XX	rb(11;21)	46,XX	
6	46,XY		46,-XX	t(2;1),t(3;1), t(22;21)	46,XX		45,XX	
7	45,XY		45,X, -X		47,XX		44,XX	i(11;14)
8	42,XY	rb(22;21), t(5;11)	41,XX	t(1;7),t(2;21)	39,XX	t(6;5), rb(14;22)	46,XX	
9	36,XY	rb(22;Y)	45,XX	t(3;4),t(15;22)	46,XX		45,XX	rb(15;15)
10	44,XY		42,XX	t(15;22),t(20;11)	46,XX		45,XX	
11	45,XY		42,XX	rb(11;21), t(20;18)	47,XX		46,XX	
12	42,XY	m	42,XX		46,XX	rb(13;13), t(X;9)	42,XX	
13	47,XY	rb(17;22)	37,XX	rb(14;16), rb(13;20)	46,XX	t(16;9)	40,XX	t(11;19)
14	34,X, -Y		44,XX	t(9;2)	46,XX		46,XX	
15	45,XY	rb(15;14)	46,XX	rb(14;14)	46,XX		44,XX	
16	43,X, -Y	rb(14;13), rb(18;14)	45,XX		46,XX		42,XX	
17	45,XY		45,XX		46,XX	rb(15;13), t(X;2)	45,XX	t(11;120)
18	43,XY		44,XX	t(7;X)	47,XX		41,X, -X	
19	44,XY	rb(15;22), t(5;18)	44,XX	rb(13;22), dic(8;9)	47,XX		46,XX	
20	51,XY	rb(15;21)	46,XX	t(9;1)	46,XX		46,XX	

Table 4.6 – Telomere Specific Q-FISH Including a New 229delT and Control LCL in Addition to two Additional Q775X Carriers

Cell Line	Mean Probe Intensity [A.U.]	P-value vs C1	P-Value vs C2	P-Value vs C3	Modal Value	Model Intensity	Maximum Intensity
Control 1	12975.51	N/A	2.63x10-5	0.371	116	6000	106000
Control 2	14539.92	2.63x10-5	N/A	2.76E-07	112	8000	102000
Control 3	12658.23	0.371	2.76E-07	N/A	127	5000	75000
229delT-2	9005.32	6.02x10-35	1.39E-60	2.66x10-31	150	4000	43000
Q775X-2	10889.25	2.35x10-8	2.93x10-21	1.44x10-6	144	3000	149000
Q775X-3	15941	4.95x10-12	0.001	1.03x10-14	93	8000	124000

Figures

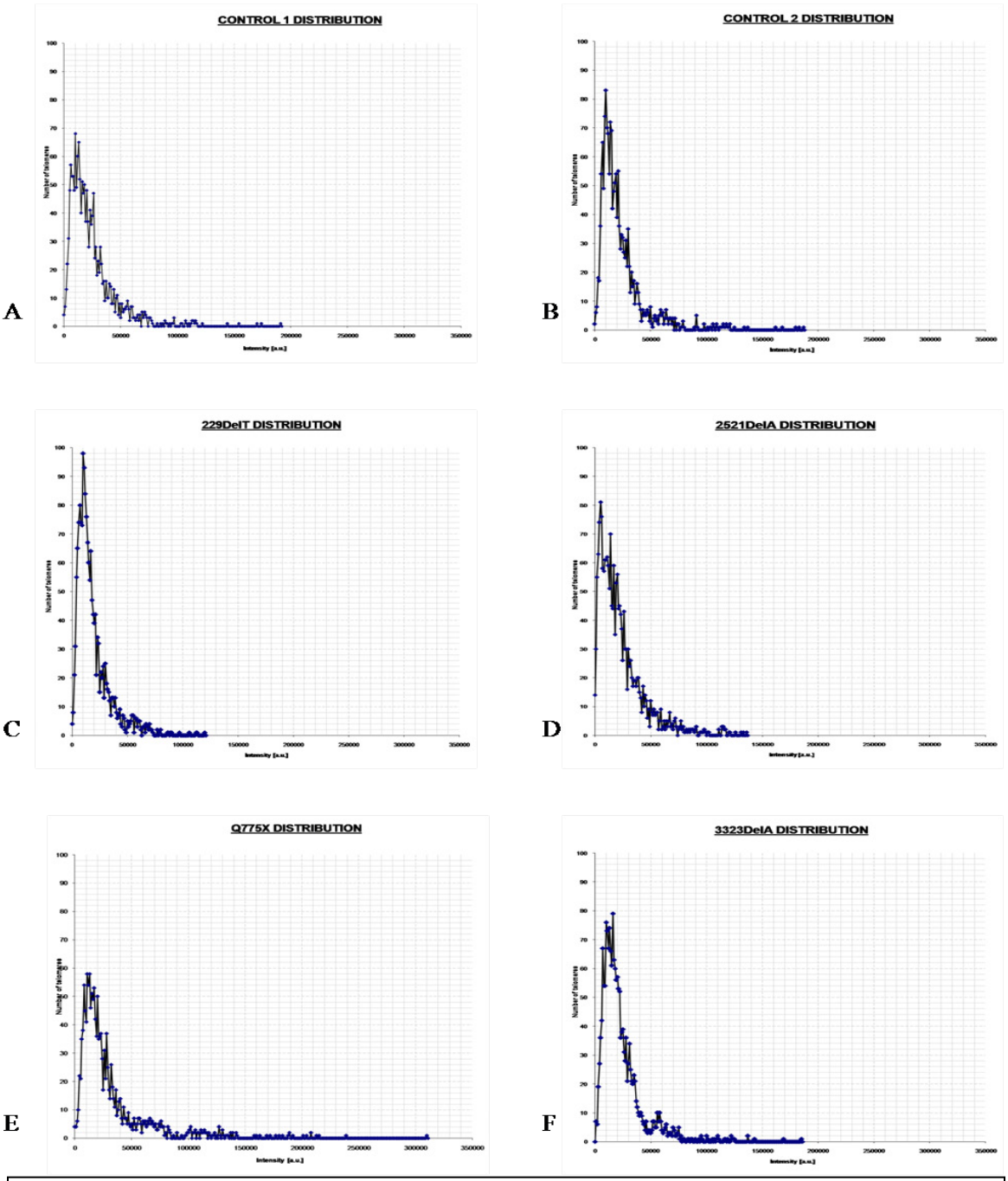
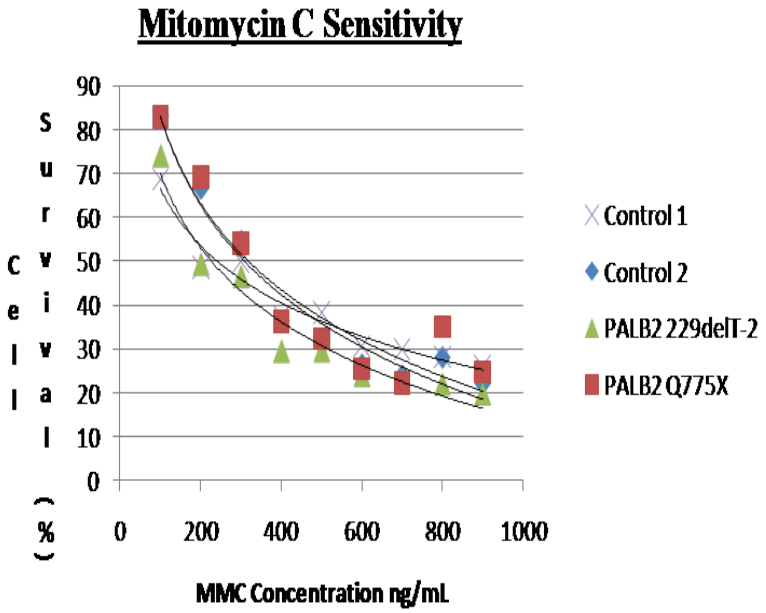


Figure 4.1: Q-FISH Telomere Distribution Plots. (**A-F**) Telomere frequency and intensity plots indicating the number of telomeres and their flourescent intensity distribution expressed as relative fluorescent intensity. The distribution of heterozygous PALB2 cell lines were directly compared with two cell lines wild type for PALB2, (**A**) Control 1 and (**B**) Control two. (**C**) The PALB2 229delT LCL. Note the reduced maximum telomere count and strong maximum fluorescence intensity compared to the two controls. Similarly, (**D**) The 2521delA LCL was observed with a slightly increased maximum telomere count associated with a reduced maximum fluorescence intensity. (**E**) The Q775X LCL. Note the largely reduced telomere count associated with a large increase in maximum fluorescence intensity. (**F**) The 3323delA LCL. Both the maximum telomere count and fluorescence intensity do not deviate significantly from the range observed between the two controls.

A

cell line	IC50
Control 1	247
Control 2	312
PALB2 229delT-2	232
PALB2 Q775X	324



B

cell line	IC50
Control 1	1178
Control 2	1813
PALB2 229delT-2	428
PALB2 Q775X	763

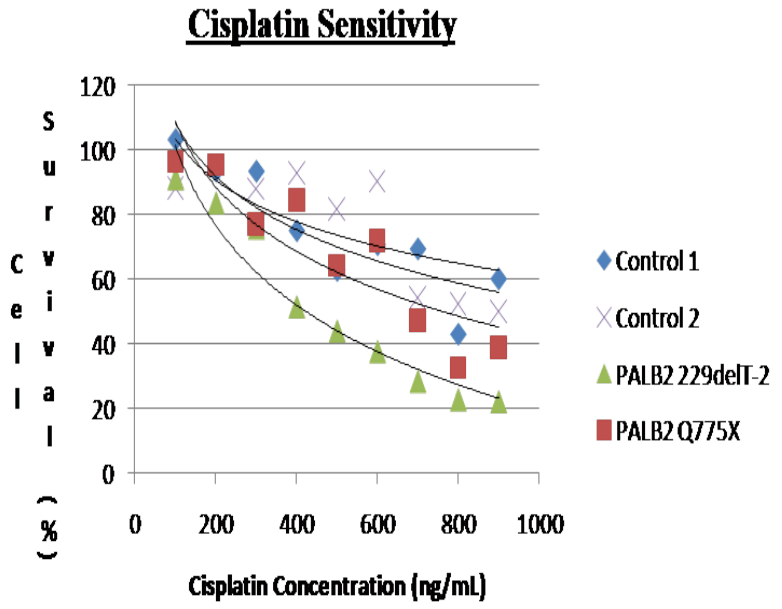


Figure 4.2: WST-1 Metabolic Cytotoxicity Assay. Cells were treated in the presence of (**A**) mitomycin C and (**B**) cisplatin. From these results it does not appear that heterozygous PALB2 mutations sensitize carrier cell lines to these DNA damaging agents.

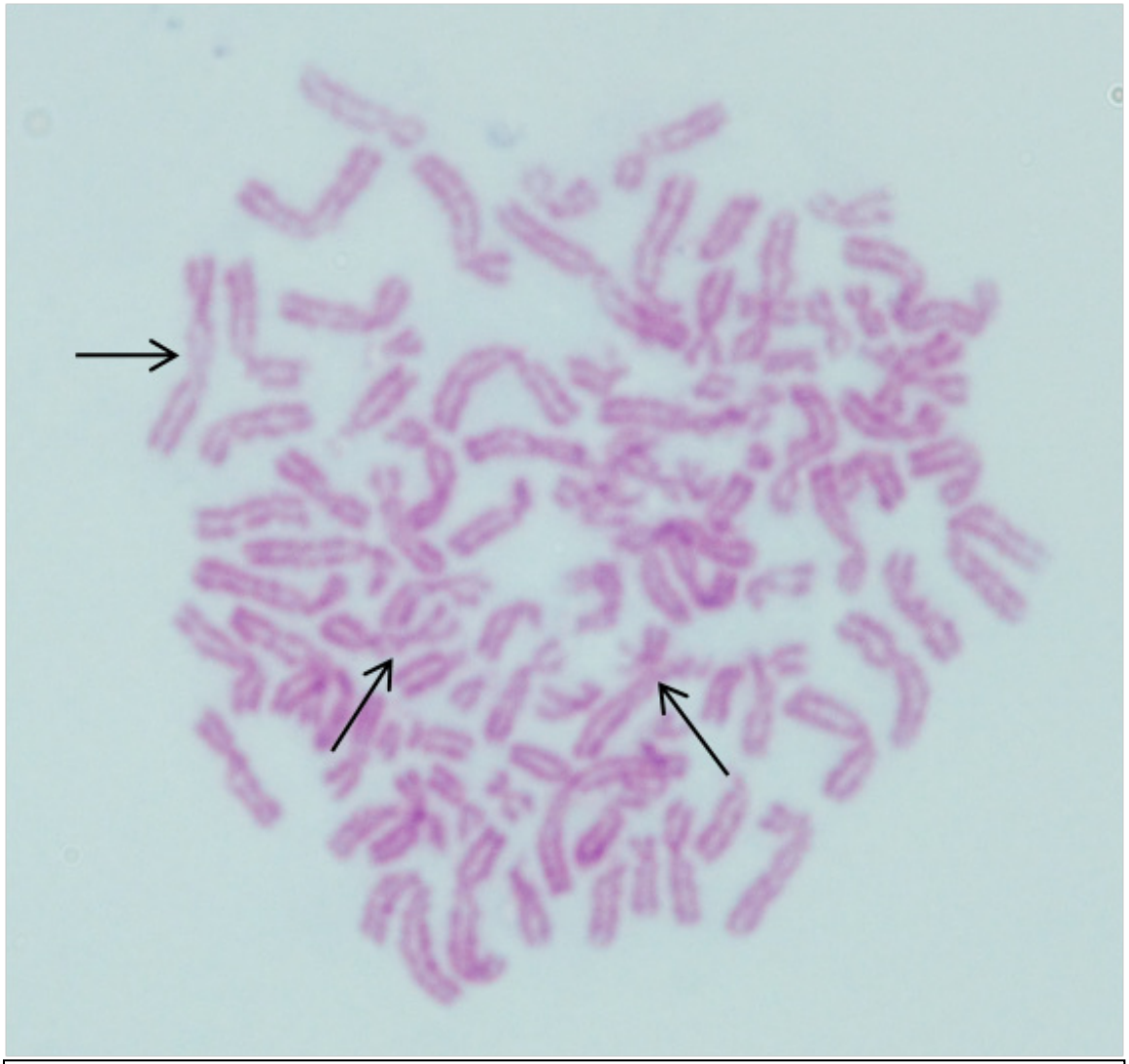


Figure 4.3: Metaphase Spread. A metaphase spread of a cell from the PALB2 229delT fibroblast line. As indicated by 3D imaging and analysis, the 229delT cell line displays telomeric fusions (black arrows indicating chromosomal end-to-end fusions). Fusions are likely to undergo both direct and subtelomeric breaks resulting in the observed maximum telomere count and varying mean fluorescence intensity, depending on whether fusions remain (increases mean signal), undergo direct breaks (decreases mean signal), or undergo subtelomeric breaks (increases mean signal).

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CHAPTER FIVE: DISCUSSION AND CONCLUSION

Summary of Results and Discussion

Taken as a whole, the investigations presented throughout this thesis have attempted to further elucidate hereditary breast cancer by employing a multifaceted approach: through the examination of two recently identified *BRCA1* interacting genes, *RAP80* and *Abraxas*, by assessing the presence and contribution of pathogenic *CHEK2* alleles within the previously un-evaluated French Canadian population, and finally by the exploration of the molecular and cellular phenotype associated with *PALB2* heterozygous breast cancer susceptibility alleles. The connecting theme evident throughout each chapter is that each independent analysis focuses on genes which directly interact with either *BRCA1* or *BRCA2*, a commonality shared amongst many of the breast cancer genes implicated in the maintenance of genomic stability.

In Chapter two we screened the entire coding regions of the *BRCA1* associating genes *Abraxas* and *RAP80*, in 95 high-risk, *BRCA1/2* negative breast cancer cases, derived from those of Askenazi Jewish, mixed Canadian and Swiss descent. BRCA1 directly binds in complex with Abraxas, which is the critical mediator allowing RAP80 to recruit BRCA1 to DNA damage foci [1,2]. Furthermore, RAP80 has been implicated in the recruitment of the BRCA1-CtIP complex [2] , thus providing a compelling argument that these two genes may contribute to breast cancer susceptibility, as mutations which

alter the function of either protein may ultimately result in a disruption to the *BRCA1* mediated DNA damage response.

In our analysis, multiple variants were identified in both *RAP80* and *Abraxas*, none of which appeared to be functionally significant. One previously unreported missense variant, *RAP80* M353T, was further investigated in the extended family of the proband, although no significant segregation with the allele and the breast cancer phenotype was observed. However, since the publication of our results, a study by Nikkila et al. [3] identified the *RAP80* delE81 mutation, located within one of the ubiquitin interaction motifs of *RAP80*. By screening a less selected cohort compared to our own, Nikkila et al. identified the *RAP80* delE81 mutation in 1/112 *BRCA1/2* negative affected Finnish familial breast cancer index cases, in 1/323 healthy controls (P-value = 0.45, OR = 2.92; 95% CI of 0.18-47.1), and in 1/503 unselected breast cancer cases [3]. Furthermore, this mutation was shown to significantly reduce RAP80 ubiquitin binding, DSB localization and impair BRCA1-Abraxas DBS recruitment through a hypothesized dominant negative interaction which was associated with an significant increase in chromosomal aberrations, particularly chromosomal breaks [3]. Although the difference in frequency of this mutation between cases and controls was not statistically significant, further investigation of *RAP80*, within larger cohorts of diverse geographical origin is warranted. Specifically, it will be interesting to determine whether the *RAP80* delE81 mutation is specific to the Finnish population, if this allele segregates with familial breast cancer, and whether or not this suggests at the presence of a *RAP80* mutational hot-spot.

In addition to the reignited interest in *RAP80* as a potential breast cancer susceptibility allele, other key players in the BRCA1-Abraxas complex may reveal genetic alterations associated with hereditary breast cancer in future investigations. For example, three additional proteins involved in the BRCA1-Abraxas-Rap80 complex have been identified as the deubiquitinating enzyme BRCC36, the adaptor protein BRCC45/BRE and most recently, the mediator protein MERIT40. Specifically, MERIT40 is thought to regulate BRCA1 retention at DNA breaks and though cell cycle regulation [4,5]. In order to carry out its function, MERIT40 directly binds to Abraxas in addition to both BRCC36 and BRCC45, two proteins which directly interact with the BRCA1-Abraxas complex and appear to be involved in the BRCA1 DNA damage response via activation and relocation [6,7]. In fact, a study conducted recently by Solyom et al. [8] screened for *MERIT40* mutations in 125 hereditary breast and breast-ovarian cases, 110 of which were negative for mutations in *BRCA1/2*, *TP53* and *PALB2*. Although several new mutations were identified, many seemed unlikely to be pathogenic, with the exception of two, *MERIT40* 87G>A and *MERIT40* L274R, both of which, upon further investigation, could potentially represent low penetrance susceptibility alleles of unknown functional significance [8]. Additional studies such as this will be extremely valuable in determining if any of the BRCA1-Abraxas complex genes contribute to breast cancer susceptibility.

In Chapter three we maintained our focus on the *BRCA1* DNA damage response pathway by screening for *CHEK2* mutations in 25 *BRCA1/2* negative, French Canadian breast cancer patients, all of whom had a strong family history of breast cancer. *CHEK2* is a well recognized moderately penetrant breast cancer susceptibility gene

which, upon activation by ATM, is involved in cell cycle control, apoptosis and DNA repair through the direct interaction with TP53 and BRCA1 [9,10]. In our analysis, one previously unidentified coding variant was observed, *CHEK2* R406H, which upon further investigation was determined unlikely to be associated with breast cancer risk (observed in 3/692 cases vs 22/6573 controls, P = 0.73).

It is reasonable to suggest, however, that due to the limited size of our fully genotyped cohort, low to moderately penetrant *CHEK2* alleles may have been missed. For example, the well defined *CHEK2* 1100delC allele, which is typically associated with a two-fold increase in breast cancer risk is generally observed at a frequency of 1.9% in breast cancer patients compared to 0.7% in those without [11,12]. Interestingly, although this variant was not identified in our fully sequenced cohort, we did identify it in a larger sample of the French Canadian population (3/149, 2.01% of cases vs 1/141, 0.7% of controls or 20/6601, 0.3% when including the neonatal control group analyzed both our study and in the study by Zhang et al. [13], P = 0.6 and P = 0.01, respectively). Furthermore, Ashkenazi Jewish *CHEK2* founder alleles such as the *CHEK2* Y424H and the *CHEK2* S428F appear to exists at frequencies of 1.2% (2/172 cases) [14] and 2.88% [15] amongst individuals with breast cancer (47/1632 cases vs 23/1673 controls; odds ratio 2.13, 95% CI 1.26-3.69; P = 0.004), respectively. In light of this, it seems reasonable to suggest that a larger group of affected French Canadian women would be required to definitively conclude the existence, or lack thereof, of a *CHEK2* contribution to breast cancer amongst the French Canadian population. Furthermore, as mentioned in Chapter three, the emerging picture of a *CHEK2* contribution to familial prostate, colon,

ovarian or colorectal cancer acts to highlight the importance the French Canadian population may serve in future investigations.

The final study presented in this thesis differs radically from the first two, in that the focus of Chapter four was on the characterization of known breast cancer susceptibility alleles rather than their identification. In chapter four, we focus on the BRCA2 interacting protein PALB2 which has recently been shown to also interact with BRCA1. In short, PALB2, through an association with the BRCA2 N-terminal region, helps recruit BRCA2 to DNA damage foci and stabilizes this interaction by protecting BRCA2 from the effects of proteosome mediated degredation [16]. Furthermore, PALB2 is thought to form a complex with BRCA1 via the BRCA1-BRCT domain, recruiting BRCA1 to sites of DNA damage, functionally linking BRCA1 to BRCA2 [17].

The clinical significance of *PALB2* first arose when Xia et al. [18] identified a patient from an uncharacterized Fanconi anemia complementation group, designated subtype N. This individual appeared to have no pathogenic alterations in *BRCA2* and *BRIP1/FANCJ*, and the reduced amount of BRCA2 suggested the existence of alterations in the BRCA2 binding partner *PALB2*. Through multiple techniques such as sequence analysis of genomic and cDNA and MLPA, Xia et al. [18] identified compound heterozygosity for two mutations in the *PALB2* gene, the *PALB2* 1802T-A transversion resulting in a premature stop codon (Y551X), in addition to an intragenic deletion inherited on the paternal allele. This discovery was simultaneously supported by the analysis of Reid et al. [19], which identified pathogenic biallelic mutations in *PALB2* within seven Fanconi anemia families.

Due to the critical function of PALB2 in BRCA2-mediated DNA repair and tumor suppression, and the similarity in phenotype associated with biallelic mutations in both *PALB2* and *BRCA2*, the proposition that monoallelic *PALB2* mutations may confer to breast cancer susceptibility seemed reasonable. The existence of such susceptibility alleles has since been identified and associated with a breast cancer risk two to three times greater than those without these alleles, as discussed in Chapter four [19-22].

A common phenotype amongst both *BRCA1* and *BRCA2* tumor cells is that both are known to contain a high degree of genomic instability, characterized by chromosomal gains and losses, rearrangements and the progressive loss of function in associated tumor suppressor genes, which in turn enable unchecked proliferation and tumourigenesis [23,24]. A similar instability phenotype is also commonly associated with Fanconi anemia cell lines, which due to the integral role of the Fanconi proteins in DNA repair, tend to be prone to aneuploidy, chromosomal rearrangements and breakages (reviewed in [25-27]). The instability inherent to FA cells, and *BRCA1/2*-deficient cells alike typically result in a high sensitivity to DNA cross-linking agents such as mitomycin C [28-30].

In chapter four, we investigated whether heterozygous *PALB2* cell lines could be distinguished from wildtype controls due to their sensitivity to two cross-linking agents: mitomycin C and bleomycin. No significantly distinguishable phenotype was observed between the mutation carriers versus the control cell lines, which is in line with previously reported analysis failing to observe a distinguishable cytotoxic heterozygous effect in FA and *BRCA2* heterozygous cell lines [30,31]. It is interesting to note, however, that a few studies in the past have reported observing a distinguishable

heterozygous phenotype in FA cells in response to DNA damage. For example, Djuzenova et al. [32] reported an increase in fragmented DNA released by FA heterozygotes, as measured by the comet assay, following x-ray exposure. Two points are of interest when considering this study, the first being that although the authors detected a measurable response as a result of X-ray exposure, no detectable heterozygous effect was associated with exposure to mitomycin C, the agent used in our investigation. Secondly, the results of this investigation have been called into question by a more recent analysis by Mohseni-Meybodi et al. [33], who by utilizing the comet assay with an extended incubation window failed to detect a measurable heterozygous phenotype induced by X-ray exposure. Furthermore, a study conducted by Pearson et al. [34], reported a slight increase in DEB induced aberrations within FA heterozygotes, however, upon inclusion of the 95% CI, the aberrations observed between heterozygotes and wild-type controls largely overlap, a caveat identified by the authors. In light of the fact that heterozygous individuals from FA families are largely free from clinical symptoms supports the lack of phenotype observed in our cellular cytotoxicity assays. However, one could hypothesize that subtle aberrant mechanisms are likely to exist, which would account for the increased breast cancer susceptibility of heterozygous carriers.

Telomeres play important roles in genome stability and in maintaining the individuality of linear chromosomes [35]. Interestingly, an accelerated erosion of telomeres is becoming a common phenotype observed amongst FA patients [36,37], and this erosion may serve as an important precursor to the genomic instability and aberrant replication observed in FA cells. As such, in chapter four we investigated the telomeric profile of our heterozygous *PALB2* cell lines through the use of quantitative fluorescence

in-situ hybridization. Surprisingly, telomere length differed significantly from our wild-type control lines in all our *PALB2* cell lines (or approached significance as is the case with the 2521delA carrier), with the exception of one cell line with the *PALB2* 3323delA mutation. It may be of importance to point out however, that the *PALB2* 3323delA cell line is the only cell line in our cell bank derived from an *unaffected male* carrier. These results were shown to be replicable as the *PALB2* 229delT phenotype of more abundant telomeric signals remained consistent upon analysis of a fibroblast cell line and a second LCL derived from the same patient. Furthermore, two additional *PALB2* Q775X cell lines from two newly identified carriers were included in our second Q-FISH analysis: one carrier clearly demonstrating a phenotype similar to that observed in the *PALB2* 229delT LCLs with less intense (indicating shorter telomeres) and more abundant signals, and the second remaining consistent with the first *PALB2* Q775X carrier analyzed, with more intense and less abundant signals. It is an exciting proposition that these two telomeric profiles observed could be an indication of two distinct, but related phases of the same instability pathway: a pathway being driven by two important concepts, direct telomeric and subtelomeric breakages (**Figure 1**). For example, it is possible that *PALB2* heterozygous mutations induce accelerated telomere erosion through an as of yet undetermined mechanism, thereby leading to the formation of end-to-end fusions, which upon formation of these fusions may undergo multiple, direct telomeric breaks, subtelomeric breaks, or a combination of both. In the event of multiple direct telomeric breaks, the phenotype observed through Q-FISH would be expected to represent precisely what was observed in our *PALB2* 229delT, *PALB2* 2521delA and one of the *PALB2* Q775X LCLs. Furthermore, in the event of subtelomeric breaks, one would expect the

newly uncapped (or critically short) chromosome ends to lack probe hybridization, whereas the newly broken off telomeric fusion would appear as an abnormally long telomere, a phenotype observed in our two *PALB2* Q775X carriers. It is important to note that these two mechanisms are not mutaually exclusive, as suggested by the intermediate phenotype observed in the PALB2 229delT fibroblast, where telomere aggregate likely still persist, some of which may have undergone subtelomeric breaks in addition to direct telomeric breaks, accounting for the normal or slightly elevated probe intensity, associated with an excess in telomere signals.

Much remains to be uncovered with respect to *PALB2* related susceptibility, the fruits of which will undoubtedly contribute to the development of effective therapeutic avenues. One such avenue relates to Poly (ADP-ribose) polymerase (PARP), an enzyme involved in base excision repair. The inhibition of PARP leads to an increase in DNA lesions, typically repaired through HR, an activity dependant on *PALB2* in addition to both *BRCA1/2*. *BRCA1/2*-null cell lines are profoundly sensitive to PARP inhibition, resulting in cell cycle arrest, chromosome instability and cell death, thus demonstrating lethality to tumor cells, with no observable toxicity to normal or heterozygous cell lines [38]. It will be incredibly interesting to determine the effect of PARP inhibitors on *PALB2*-related breast cancer cell lines, an experiment we were unable to conduct as to our knowledge no such lines exists at present.

Until the opportunity to acquire *PALB2* tumor cells presents itself clinically, two important directions of focus should be on the development of a *PALB2* mouse tumor cell line in addition to investigating commercially available breast cancer cell lines,

specifically those prone to chromosomal instability or those currently under-characterized, for *PALB2* related mutations. In fact, preliminary results obtained during the duration of this studentship suggested that two cell lines in particular, MDA.MB.436 and the HCC1954 may in fact harbor *PALB2* deficiencies. For the MDA.MB.436 this deficiency is hypothesized to be post-translational, as the sequencing of the entire PALB2 coding region revealed no clearly pathogenic alleles. Further investigation of these cell lines was confounded by the inability to cleanly purify the PALB2 protein by currently available PALB2 antibodies; an obstacle which may be overcome by the future development of a PALB2 monoclonal antibody specific to one epitope, or alternatively by employing different techniques compared to the ones used throughout our investigation, such as immunoprecipitation prior to western blot analysis.

Conclusions

Overall, this thesis has presented a multifaceted approach at elucidating breast cancer associated with *BRCA1* and *BRCA2* interacting genes; specifically, by determining if an association exists between two previously unscreened *BRCA1*-associating genes, *RAP80* and *Abraxas*, and breast cancer, assessing the contribution and probing for the existence of *CHEK2* alleles within the previously uninvestigated French Canadian population, and finally by offering a possible mechanism contributing for the increased breast cancer risk incurred upon by heterozygous *PALB2* mutation carriers.

Our analysis suggests that *RAP80* and *Abraxas* are unlikely to be major contributors to breast cancer susceptibility, although future studies may indeed identify low penetrance alleles within these genes. Similarly, no frequent *CHEK2* founder allele is likely to exist within the French Canadian population, although 1100delC and potentially other moderate to low penetrant alleles may be identified. Finally, the distinguishable *PALB2* heterozygous phenotype revealed by telomere Q-FISH may prove to be an important first step in setting the stage for the development of *PALB2* related breast cancers, predisposing heterozygous carriers to an increased susceptibility to additional tumourgenic mechanisms.

Figures

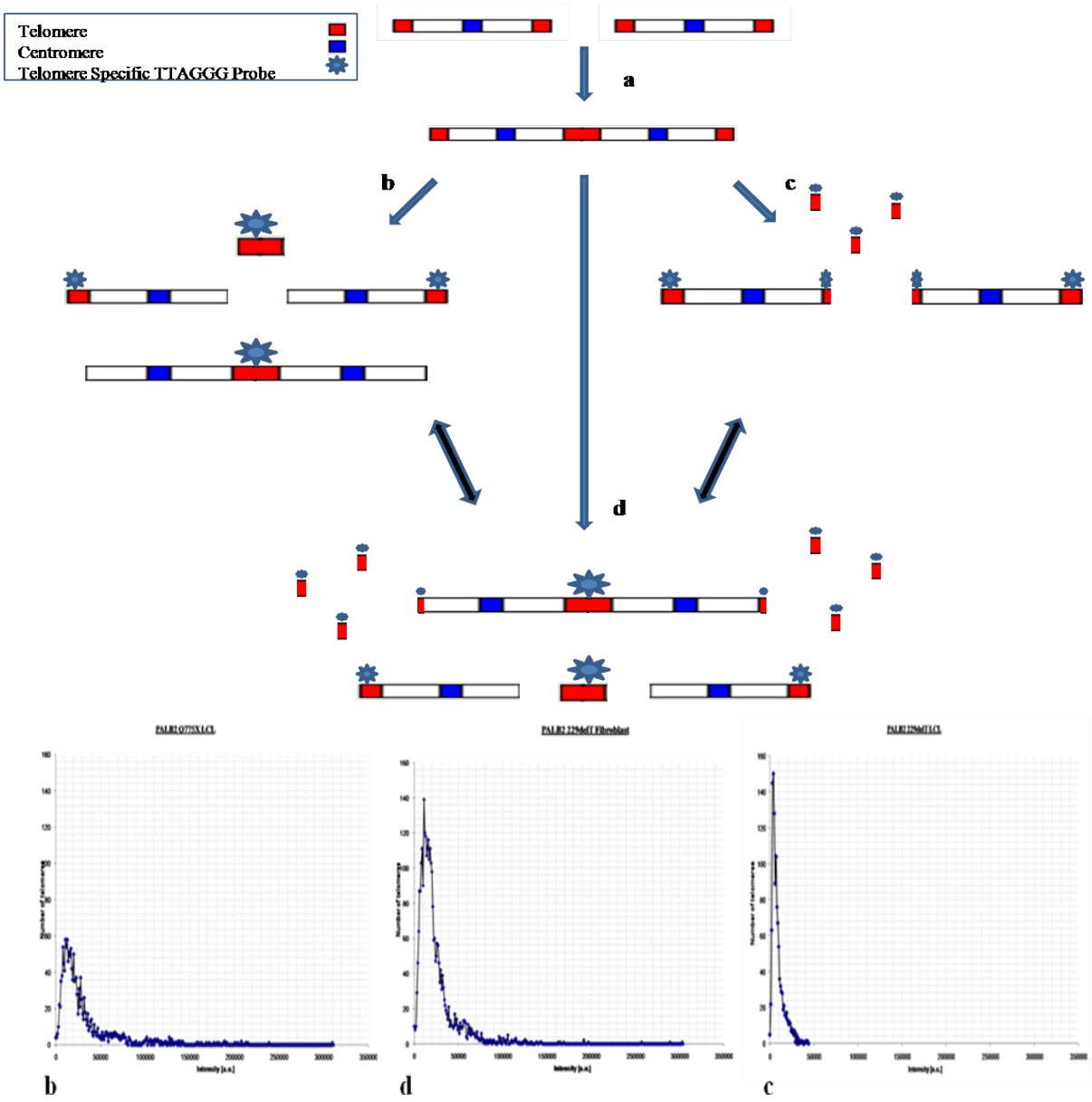

Figure 1: Hypothesized Mechanisms Underlying PALB2 Breast Cancer Susceptibility. PALB2 heterozygous mutations induce, through an as of yet undefined mechanism, accelerated telomere erosion leading to telomeric fusions (a). Upon fusions, a portion may undergo subtelomeric breaks (b) liberating large telomeric fragments which will undergo hybridization with an increased portion of the telomere specific probe. This will result in an overall increase in the fluorescence intensity observed through Q-FISH (telomeric probe intensity indicated by the relative size of the "Telomere Specific TTAGGG Probe" graphic in the figure above; for example, larger indicating a higher fluorescence intensity emitted). Furthermore, critically uncapped ends will be unable to hybridize with the probe resulting in an overall decrease in the observed number of telomere signals. Upon subtelomeric breaks (c) multiple smaller telomere fragments will be liberated, hybridizing less telomere specific probe, resulting in an increase in telomere signals observed associated with a lower overall mean intensity. These two phases are likely to be linked through an intermediate phase of instability (d) where telomeric aggregates still persist, some of which may have undergone both direct and subtelomeric breaks accounting for an observed excess in telomere signals associated with an overall mean intensity closer to that observed in the control cell lines. The plots below the figure, illustrate the observed telomere distribution within three of our analyzed cell lines. The letter next to the plot indicates which phase of the proposed instability pathway these cells associate with.

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APPENDIX

List of Abbreviations

5-FU – 5-Fluorouracil

AC – Adriamycin and Cyclophosphamide

BRCT – BRCA1 COOH-terminal repeats

B/F/B – Bridge/Fusion/Breakage Cycle

DEB – Diepoxybutane

DNA – Deoxyribonucleic acid

DSB – Double Strand Break

EBV – Epstein Barr Virus

FA – Fanconi Anemia

FISH - Fluorescence In-Situ Hybridization

HR – Homologous Recombination

IVS – Intronic Variation Sequence

LCL – Lymphoblastoid Cell Line

LFL – Li-Fraumeni-Like Syndrome

LFS – Li-Fraumeni Syndrome

MMC – Mitomycin C

PCR – Polymerase Chain Reaction

PNA-Q-FISH – Peptide-Nucleic-Acid Quantitative Fluorescence In-Situ Hybridization

Q-FISH – Quantitative Fluorescence In-Situ Hybridization

SKY – Spectral Karyotyping

SNP – Single Nucleotide Polymorphism

SSCP – Single Stranded Conformational Polymorphism

UIM – Ubiquitin Interacting Motif

UTR – Untranslated Region

WST-1 - 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate / tetrazolium salt