VALIDATION OF A GENE-EXPRESSION BASED ASSAY FOR 
BRCA1 FUNCTION

By

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Abstract

Breast cancer is a disease that afflicts a significant proportion of women globally. 5-10% of breast cancer cases are linked to inherited polymorphisms in critical genes such as \textit{BRCA1}, a tumour suppressor essential for genomic stability. A dysfunctional \textit{BRCA1} gene can increase breast cancer risk by 60-80%. Previous microarray analysis established that differential gene expression in unperturbed Epstein-Barr virus transformed lymphocyte cell lines (EBV-LCL) was able to distinguish \textit{BRCA1} mutation carriers from controls with a high degree of accuracy. A 43-gene radiation-independent classifier for \textit{BRCA1} status was constructed.

We hypothesize that this differential gene expression can be observed in a subset of these genes using quantitative PCR (qPCR) in both EBV-LCL and B-lymphocytes isolated from patients with known \textit{BRCA1} mutation carrier status.

The 43-gene classifier was analyzed using gene ontology analysis and 4 target genes selected based on predictive value, expression intensity and gene ontology similarity. Genes selected were \textit{CXCR3, TBX21, MX2, and IFIT1}, with \textit{GusB} as an endogenous reference gene. EBV-LCL established from known \textit{BRCA1} mutation carriers and from \textit{BRCA1} wildtype individuals were obtained and RT-qPCR (reverse transcriptase qPCR) performed on isolated RNA. Our results showed significant downregulation of \textit{CXCR3} and \textit{TBX21} in \textit{BRCA1} mutation carriers (p=0.018 and p=0.003, respectively), as expected from previous microarray results. \textit{IFIT1}, while showing a non-significant upregulation (p=0.183), agreed with the expected trend. \textit{MX2} did not show significant differential expression. These results indicate that differential gene expression has the
potential to accurately distinguish pathogenic variants, even if it may require EBV immortalization of B-lymphocytes.

To determine whether the assay could be extended to fresh blood samples, B-lymphocytes were isolated from patients with known \textit{BRCA1} mutation carrier status from North York General Hospital in Toronto, ON. An optimized protocol to enrich the B-lymphocyte population using magnetic separation was developed for this purpose. RT-qPCR using RNA isolated from these lymphocytes showed no significant differential gene expression in \textit{CXCR3} and \textit{TBX21}. However, a low sample size, use of non-sequenced controls and a need for further qPCR optimization may call these results into question. In addition, problems with blood sample transportation from off-site sources resulted in an unacceptable drop in RNA integrity.

While this gene expression assay may be limited to screening a small number of blood samples, results indicate that may still have clinical relevance that can be explored. This would necessitate further optimization of the qPCR methodology and resolution of the issues surrounding RNA integrity and sample transport.
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<td>ER</td>
<td>Estrogen Receptor</td>
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<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
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<tr>
<td>BRCT</td>
<td>BRCA1 Carboxy-Terminal</td>
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<tr>
<td>IR</td>
<td>Ionizing Radiation</td>
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<td>G1</td>
<td>Gap1</td>
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<td>G2</td>
<td>Gap2</td>
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<td>S</td>
<td>Synthesis Phase</td>
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<td>M</td>
<td>Mitosis</td>
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<td>NHEJ</td>
<td>Non-Homologous End Rejoining</td>
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<td>HR</td>
<td>Homologous Recombination</td>
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<td>VUS</td>
<td>Variants of Unknown Significance</td>
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<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
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<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
</tr>
<tr>
<td>EBV-LCL</td>
<td>Epstein-Barr virus Transformed Lymphocyte Cell Line</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>RIN</td>
<td>RNA Integrity Number</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<tr>
<td>TAC</td>
<td>Tetrameric Antibody Complex</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
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Chapter 1
Introduction and Literature Review

1.1 Breast Cancer as a Disease

1.1.1 Breast Cancer in Canada

Breast cancer remains one of the most prevalent cancers in women, despite intensive research. According to the Canadian Cancer Society, breast cancer is second only to non-melanoma skin cancer as the most prevalent type of cancer in Canada [1]. Canadian Cancer Statistics 2012 reported an estimate of 22,700 newly diagnosed cases of breast cancer in females, resulting in an age-standardized incidence rate of 96 per 100,000 individuals [1]. This represents 26% of all new cancer cases in women for 2012. For the same time period, it was estimated that there would be 5,100 deaths in women due to breast cancer, giving an age-standardized death rate of 19 per 100,000 individuals [1].

1.1.2 Breast Cancer Physiology and Classification

Breast cancer is broadly defined as neoplasms in the breast originating from ductal or lobular tissue. Breast cancer is a varied disease, with subtypes dependent on tissue origin and location. This variety in tumour types has given rise to a comprehensive system of classification utilizing morphological factors such as tumour grade (based on histology and tumour size) [2], tumour stage (incorporating lymph node involvement and metastasis) [3], and immunohistochemical factors such as the increased presence of
biomarkers including but not limited to human epidermal growth factor 2 (HER2), estrogen receptor (ER), and progesterone receptor (PR) [4], [5]. More recent classifiers that are being studied are based on transcriptome analysis using genomic classifiers such as copy number alterations [6] and whole genome profiling [7], [8], [9].

Though the direct causes of breast cancer are unclear, there is a consensus that it arises from a combination of environmental and genetic factors. Environmental risk factors that have been identified include age [10], gender [11], alcohol intake [12], and the lack of hormone therapy at menopause [13]. Genetic risk factors include mutations in genes critical to cell processes such as DNA damage response and repair such as \textit{RAD51C} [14], \textit{ATM}/\textit{ATR}, [15], and \textit{BRCA1} and \textit{BRCA2} [16]. Inactivation or disruption of these critical pathways will negatively impact genomic fidelity and is shown to be responsible for the development of uncontrolled, invasive neoplasms from normal differentiated tissue [17]. In general, the variety of factors that can cause breast cancer, its polymorphic nature and diverse risk factors create unique challenges for its treatment and study.

\textbf{1.1.3 Hereditary Breast Cancer}

While breast cancer can be classified in different ways, it can be broadly categorized as either sporadic or hereditary in origin. This classification is dependent on whether there is prior genetic predisposition towards breast cancer development [18]. Approximately 10\% of breast cancer cases are classified as hereditary breast cancers, which occur in individuals with genetic alterations in critical tumour suppressors [19].
These mutations have a clear pathogenic impact as they negatively affect the DNA replication and repair mechanisms that maintain the genome [19]. Mutations in the gene \textit{BRCA1} (and similarly in \textit{BRCA2}) are highly linked to hereditary breast cancer, as inheritance of an inactivating mutation of \textit{BRCA1} greatly increases the chances of developing early-onset breast and ovarian cancer, resulting in a 60\% to 80\% chance of cancer development by the age of 70 [20], [21]. While \textit{BRCA1} polymorphisms account for 40-45\% of all hereditary breast cancer cases, in families with multiple cases of breast and ovarian cancer, \textit{BRCA1} polymorphisms are responsible for 80\% of cases [16].

\textbf{1.2 \textit{BRCA1}}

\textbf{1.2.1 Discovery and Characterization}  

The discovery of \textit{BRCA1} began when a hereditary breast cancer syndrome highly prevalent in certain families was described [22]. This familial inheritance led to the theory that there could be an inherited factor that could lead to a predisposition towards breast cancer and that a certain proportion of breast cancer cases could be directly linked to this inherited factor [23]. The location of this factor was first determined to be within chromosome 17q in 1992 [23], [24]. A high density map was created of a specific region within the chromosome [25] and multiple studies identified the gene in 1994 through linkage studies [26] and positional cloning methods [27].

\textbf{1.2.2 \textit{BRCA1} Structure indicates Protein Function}  

The \textit{BRCA1} gene encodes a protein that is 1863 amino-acids in length. This protein is involved in a wide variety of cellular processes, and while the primary function
Figure 1: Schematic Diagram of the BRCA1 Protein

The BRCA1 protein: 1653 amino acids long with well characterized domains that include the N-terminal RING finger [28], C-terminal tandem BRCT domains [34], Nuclear Localization Signal (NLS) domains [36] and Serine Cluster Domain (SCD) [40].
for tumour suppression still unclear, insight may be gained from structure of the protein itself. The protein contains very specific, highly-conserved regions that provide strong indications for protein function. The N-terminus contains a RING finger domain from amino acids 1-109, a zinc-binding motif that is essential in ubiquitination pathways [28]. It has been shown that BRCA1 acts as an E3 ubiquitinligase through interaction with BRCA1 Associated RING Domain protein 1 (BARD1) and the E2-ubiquitinating conjugating enzyme UbcH5 [29], [30]. Targets that have been identified include histone protein H2A, ER-alpha and PR [31], [32], [33]. The C-terminus of the protein contains two tandem globular domains identified as BRCA1 Carboxy-Terinal (BRCT) domains, a conserved domain found in proteins involved in cell-cycle control in response to DNA damage [34]. In BRCA1, the BRCT domain manages interactions between BRCA1 and proteins phosphorylated by ATM/ATR, along with DNA binding and other protein interactions [35]. The protein also contains nuclear localization signal (NLS) domains at amino acids 503-604, allowing the completed protein to travel through the nuclear membrane, with binding sites for a variety of proteins such as Rad50/Rad51, RB and PALB2 in close proximity [36], [37], [38], [39]. Finally, a Serine Cluster domain (SCD) located near the C-terminus (at amino acids 1280-1524) interacts with the ATM/ATR kinases involved in cell cycle arrest and DNA damage repair [40].

The presence of these structures implicates BRCA1 in processes such as the DNA damage response [41] and in cell cycle arrest in response to ionizing radiation (IR) [42]. In addition, it has been observed that BRCA1 plays a role in chromatin structure
regulation such as through interaction with histone protein H2A [31], [43]. Other studies have indicated that BRCA1 is also involved in other cellular processes such as sex chromosome inactivation [44], [45], [46], spindle pole body duplication [47], transcriptional regulation [48], and global DNA methylation control [49]. The protein domains within BRCA1 suggest that it plays an important role in the maintenance of genomic integrity as an important part of both the cell cycle checkpoint mechanism and in DNA damage response.

1.3 BRCA1 as Caretaker and Gatekeeper

1.3.1 Caretakers versus Gatekeepers

Vogelstein and Kinzler first classified cancer susceptibility genes either as a caretaker gene or a gatekeeper gene [50]. Caretaker genes are defined as genes that are responsible for maintenance of genomic integrity and reduction of gene mutation rate, while gatekeeper genes are tissue-specific genes that regulate cell growth. [50], [51], [52]. Loss of a caretaker gene will result in deregulation of critical DNA repair mechanisms, leading to accumulation of errors in the genome. Similarly, loss of a gatekeeper gene disrupts normal cell progression, allowing for uncontrolled cell growth. While an inactivating mutation of a caretaker or a gatekeeper may be enough to cause cancer by itself [53], [54], the synergistic effect of losing both results in cancer through accumulation of genomic damage and rapid cell proliferation.

The categories of caretakers and gatekeepers are not absolute classifications, as certain genes can act as either a caretaker or a gatekeeper dependent on tumour type and
stage of development [50], [51]. BRCA1 is one such gene in that it has functions allowing it to act both as a gatekeeper and as a caretaker.

1.3.2 BRCA1 as a Gatekeeper

BRCA1 as a gatekeeper gene is responsible for influencing cell growth and limiting proliferation through its contribution in the cell cycle checkpoint system [42], [47]. The checkpoint system is vital to maintaining genomic integrity as it arrests the progression of the cell cycle upon detection of genomic damage, allowing for DNA repair mechanisms to attempt any repairs and to initiate programmed cell death, if appropriate. Within the cell cycle checkpoint, the BRCA1 protein has a variety of functions. In the G1/S checkpoint, it has been seen that BRCA1 stimulates transcription of p21 both in a p53-independent manner as well as through phosphorylation of CHEK2 and p53 [55], [56]. This maintains G1-phase arrest by binding to and inactivating both Cdk2/cyclin E and Cdk4/cyclinD [57], [58]. In the G2/S checkpoint, BRCA1 regulates expression, phosphorylation, and localization of CHEK1, a regulator of the G2/M checkpoint [59]. In addition, Shabbeer et al. have seen that BRCA1 is responsible for the ubiquitination of cyclin B and Cdc25C, leading to their degradation and maintenance of G2 arrest [60].

Given this, BRCA1 acts as a mediator protein in the cell cycle checkpoint, relaying signals from the upstream sensor proteins that detect DNA damage to the transducers immediately downstream.
1.3.3 The Sensor-Mediator-Transducer-Effector Cascade

To further understand the gatekeeper functions of BRCA1, the signal cascade it is an essential part of must be considered. The proteins involved in the checkpoint system can be broadly classified into 4 categories which work in sequential fashion to detect DNA damage and create an appropriate response. It bears mentioning that these definitions are not absolute as certain proteins may act in different capacities, but these definitions may serve as a framework for understanding checkpoint regulation.

The first class is the DNA damage sensor proteins that initially detect genomic damage and initiate the signal cascade. Receiving the signal from the sensor proteins are mediator proteins that pass the signal down to protein kinases and phosphatases termed transducer proteins, which in turn interact with effector proteins that either arrest the cell cycle or allow it to proceed [61]. DNA sensor proteins can be further subdivided based on protein structure, such as the Phosphoinositide-3-kinase-like-kinase proteins ATM and ATR, and the 911-Rad17-RDC complex [62], [63]. Mediators include proteins such as BRCA1, MDC1 [64], TOPBP1 [65], and TP53BP1 [66]. Their targets are the transducer proteins such as CHEK1 and CHEK2 [67], [68] that handle signals relayed from ATR and ATM respectively. Finally, effector proteins include the Cyclin/Cdk complexes and are the end-targets of the entire cascade, as they directly regulate cell cycle progression [61].

1.3.4 The Cell Cycle and Checkpoint Control
The cell cycle is a complex process that mitotic cells undergo in which genetic material is duplicated and cellular material is equally partitioned into two daughter cells. This process can be divided into 4 phases: Gap1 (G1), Synthesis (S), Gap2 (G2), and Mitosis (M). Cell-cycle checkpoints are cellular mechanisms that determine whether a mitotic cell should go into the next phase of the cell cycle, or whether it must remain at a particular phase. These mechanisms are found between G1 and the S-phase, between G2 and the M-phase, and within the M-phase, the metaphase portion of mitosis. The G1/S checkpoint serves as a safeguard against propagation of any genomic errors during the S-phase [69], [70]. Genomic damage that is detected by sensor proteins activates pathways that culminate in cell-cycle arrest and prevents progression into the S-phase [71]. The effectors of this checkpoint are the cyclin D/Cdk 4,6 and the cyclin E,A/Cdk2 complexes [72]. In a similar fashion, the G2/M checkpoint prevents any errors within the genome from being propagated, though in this case it ensures that mitosis does not occur in cells with existing DNA damage post-DNA replication [73]. This checkpoint has the cyclin B/Cdk1 (also known as Cdc2) complex as effectors of cell cycle arrest [74].

Once a cell encounters one of these checkpoints, it must repair any detected genomic damage to continue with the cell cycle. Otherwise, if the damage is too severe and repair unsuccessful, the checkpoint will activate mechanisms that lead to programmed cell death [42], [69].

1.3.5 BRCA1 as a Caretaker
BRCA1 can also function as a caretaker protein due to its role in DNA damage repair. There are a variety of agents that cause DNA damage such as DNA intercalating agents [75], reactive oxygen compounds, IR and ultraviolet radiation [76]. BRCA1 has multiple functions in DNA damage repair, specifically in non-homologous end joining (NHEJ), homologous recombination (HR), and Nucleotide Excision Repair. [41], [77], [78], [79]. In addition, BRCA1 acts as a mediator in the signal cascade, shuttling damage signals from sensor proteins to downstream transducers and effectors [77], [80]. Finally, BRCA1 directly interacts with repair proteins and acts as a transcriptional regulator influencing the expression of genes involved in DNA damage repair [81], [82], [83].

Homologous recombination is a method of DNA repair using sister chromatids as the basis for replacing lost nucleotide sequences, specifically in double-stranded breaks. It is a nucleolytic process involving proteins like BRCA1, BRCA2, the MRN complex, RPA, Rad51 and Rad52 [84]. Specifically, BRCA1 recruits BRCA2 during HR, which in turn localizes Rad51 to allow Rad51 to bind to the site of DNA damage, initiating strand invasion and nucleofilament formation [85], [86]. In addition, BRCA1 co-localizes with Rad50 following DNA damage, but directly binds to DNA, inhibiting Mre11 [87]. This inhibition allows BRCA1 to regulate the length of 3’ overhang present since Mre1 recruits nucleases that resects flush ends of DNA at sites of DSB [88].

NHEJ is another mechanism by which cells repair DSB. This method has little to no homologous sequence to base any repairs upon. Therefore, NHEJ is error-prone and frequently results in deletions and mutations as well as chromosomal translocations and
aberrations [79]. It has been suggested that BRCA1 contributes to NHEJ through interaction with the MRN complex and through suppression of the nuclease activity of NBS1, in a Ku80-independent NHEJ pathway [37], [79], [89]. However, the exact role of BRCA1 in NHEJ remains unclear, as studies conflict on whether NHEJ is impaired given BRCA1 deficiency [41], [90].

1.3.6 BRCA1 in Cellular Proliferation and Differentiation

Another cellular function that BRCA1 plays an important part in is the determination of the cellular fate of mammary epithelial cell progenitors. Studies such as ones conducted by Furuta et al. in 2005 showed that reduction of BRCA1 in mammary epithelial cells had a negative impact on cell differentiation while having little effect on proliferation [91]. These findings were reinforced by a similar study conducted by Liu et al. in 2007 using a humanized mouse model [92].

This regulation of differentiation combined with the vital functions BRCA1 has in maintaining genomic integrity means that elimination or dysfunction will result in tumourigenesis characterized by an increase of non-differentiated progenitor cells. Indeed, BRCA1-deficient breast cancers display a basal-like phenotype with little expression of surface receptors such as ER and PR [93].

1.3.7 BRCA1 Haploinsufficiency versus Loss of Heterozygosity

A variety of functions that BRCA1 is responsible for has been characterized, however it is still unclear which function is critical for tumour suppression. This is a significant challenge in the diagnosis and treatment of BRCA1-related hereditary breast
cancer. It has been seen that individuals with germline \textit{BRCA1} mutations will display loss of heterozygosity and show inactivation of the remaining wildtype allele at tumourigenesis [94], [95]. This would be sufficient evidence to classify \textit{BRCA1} as a classic, two hit tumour suppressor. However, it has also been observed that \textit{BRCA1} haploinsufficiency is enough to predispose an individual towards cancer development through genomic instability [96], [97]. Therefore, whether to classify \textit{BRCA1} as a classical two-hit suppressor or whether haploinsufficiency is sufficient for cancer predisposition remains unclear.

Research by Konishi et al. showed that \textit{BRCA1} haploinsufficiency allows alterations in vital tumour suppressor genes such as p53 to accumulate, allowing cancer progenitor cells that lose wildtype \textit{BRCA1} to avoid cell death [94]. This study used two immortalized breast epithelial cell lines into which a heterozygous \textit{BRCA1} mutation was introduced through gene targeting. They observed that epithelial cells with heterozygous \textit{BRCA1} mutations resulted in a significant decrease in both HR and homology-mediated repair. In addition, it was also observed that these cells had similar properties to \textit{BRCA1}-null cells, albeit to a lesser degree. Furthermore, they also showed that \textit{BRCA1} heterozygosity resulted in genomic instability both \textit{in-vitro} and \textit{in-vivo} using non-cancerous breast tissue samples from known \textit{BRCA1} mutation carriers [94].

Another proposed theory states that \textit{BRCA1} heterozygosity negatively affects cellular differentiation. This results in an increase in expression of basal cell markers and an increase of cells displaying a basal-like phenotype [98], [99], [100]. While this
promotes an environment conducive to breast cancer formation, a second “hit” is required to inactivate the 2nd functional allele, derailing the DNA damage response and checkpoint control resulting in proliferation deficiencies. The synergy between the loss of both caretaker and gatekeeper functions, resulting in an increased basal phenotype and loss of DNA damage response, greatly increases the chance of tumour formation and cancer development.

1.4 BRCA1 Polymorphisms and Variants of Unknown Significance

1.4.1 Pathogenic Mutations of BRCA1

BRCA1 holds a vital role in the maintenance of genomic integrity through its functions in cell-cycle control and in DNA damage repair. Consequently, any mutation in the BRCA1 gene that inactivates the resulting protein product or impedes its normal function has negative consequences. Current methods of classifying BRCA1 mutations stem from whole exon sequencing and direct identification of variations within the coding regions of the gene [101]. Screening can reveal normal gene expression, a benign polymorphism that will not affect protein function, or a complete inactivating mutation. These mutations may simply result in a truncated protein or contribute to a pathogenic state through disruption of normal BRCA1 protein function. Detection of an established pathogenic mutation, while indicating a worse prognosis, gives patients and healthcare providers an opportunity to plan preventative measures with the knowledge of an increased risk of disease.

1.4.2 Variants of Unknown Significance
In addition to BRCA1 variants that have clearly defined clinical impact, there are also variants that are of uncertain clinical significance termed Variants of Unknown Significance (VUS). It is unclear whether a detected VUS is benign or has pathogenic consequences, therefore posing a significant challenge in the treatment of breast cancer. This uncertainty complicates both the creation of treatment plans and patient monitoring. Identifying the pathogenic impact of these VUS and determining whether certain variants contribute to increased cancer risk is a priority.

VUS are mutations that either happen in an intronic or regulatory site of BRCA1, or are single-base deletions/insertions that maintain the reading frame [102]. The nature of VUS alterations does not allow for immediate classification; their pathogenic impact must be determined by experimental data. There are many VUS that have been identified; research has shown that there are at least 1,200 different variants for BRCA1 currently present in the population [102]. Of these variants, missense variants represent almost 30% of all reported mutations and of these variants, more than 50% have only been reported once [103]. VUS pose a significant challenge to the utility of genetic screening in hereditary breast cancer as it may be difficult to determine if a detected VUS will have pathological significance. This lack of certainty gives healthcare providers and patients limited options on how to proceed with treatment if confronted with a VUS.

1.4.3 Methods of VUS Classification

There have been many attempts to develop a method to identify and classify such variants. Initially, evaluation of the clinical significance of VUS was dependent on the
availability of family data like cancer history and cosegregation with the disease, and on clinical data such as tumour features and loss of heterozygosity [104]. However, for rare variants that only occur in a single family, this method of evaluation is not effective. To address this deficiency, both multifactorial classification models that combine multiple independent features to determine VUS pathogenicity, and BRCA1 functional assays have been developed [105], [106].

BRCA1 functional assays attempt to independently classify VUS based on their effect on protein formation and function, and whose end results can be integrated with other independent classifiers (such as familial and epidemiological data) in order to show a clearer picture of VUS effects [107]. While there have been a large variety of assays developed, they can be classified into very broad categories, depending upon the assay’s focus.

Some functional assays are based on the effect of mutations in certain areas of the gene, and the subsequent effects on protein transcription [108] and other phenotypes such as cellular sensitivity to DNA damage [109]. Specific examples include assays focusing on the ubiquitin ligase function of BRCA1 that aim to determine whether the amino-terminal RING domain retains its function as an ubiquitin ligase with VUS present [110]. Similarly, the transcription activation assay observes the function of the carboxy-terminal BRCT domains, as these domains are responsible for interaction with a number of transcription factors and any pathogenic VUS will inhibit transcriptional activation [111]. While potentially effective, these approaches have drawbacks stemming from the
polyvalent nature of BRCA1 itself and the current lack of a defined function that is critical to its tumour suppression function. It has been shown that the ubiquitin ligase ability of Brca1 is not required for cell viability, chromosomal stability and resistance to genotoxic stress in mouse embryonic stem cells [112], and for tumour suppression in a mouse model [113]. This suggests that there is no one underlying mechanism of carcinogenesis that can be linked to loss of BRCA1 domain function. Given this and the overall lack of understanding of the tumour suppressor function of BRCA1, the accuracy and utility of an assay that specifically focuses on one aspect of the protein can be questionable.

Another approach to developing functional assays is to look at the function of the protein as a whole, based on experimental observations regarding BRCA1. For example, the small colony phenotype assay was developed based upon the observation that BRCA1 expression results in inhibition of yeast colony growth, leading to small colonies on a plate culture given a functional BRCA1 protein [114], [115]. There is no negative effect on colony size with known pathogenic variants. Another assay is the centrosome amplification assay, based upon the observation that BRCA1 depletion results in centrosome amplification [116], [117]. It has been shown that certain variants located in residues responsible for zinc binding (such as the RING finger at the amino terminus) cause centrosome amplification, perhaps indicating that these variants will disrupt BRCA1 function [116].
Other novel methodologies have also been explored. An approach suggested by Fleming et al. and Abkevich et al. uses an evolutionary approach coupled with sequence comparisons to examine VUS [118], [119]. However, it has been seen that certain regions of the BRCA1 gene have been undergoing rapid evolutionary changes powered by positive selection, thus sequence comparisons cannot be completely considered as a predictor without corroborating evidence. A particularly notable methodology was developed by Chang et al. that uses Brca1-deficient mouse embryonic stem cells along with the variant’s location on the gene and its effect on normal cellular processes to classify variants [120]. However the fact that the assay uses cell survival as a determinant to whether a variant is pathogenic or not limits its usefulness, again given the pleiotropic nature of BRCA1.

A commonality shared by these classification methods is that, given that they focus on specific functions of the BRCA1 protein, the multi-functional nature of BRCA1 and its involvement in multiple cell processes make it difficult to assess the clinical relevance of these assays. This is a major caveat to functional classification assays for BRCA1, as observations resulting from these assays may not necessarily indicate that BRCA1 variants are pathogenic.

1.5 Gene Expression Assays

1.5.1 Gene Expression Assays as a Diagnostic Tool

Classification assays that focus on a specific BRCA1 function may have uncertain clinical relevance. To address this, indirect approaches have been developed that do not
rely upon specific BRCA1 protein function in order to classify VUS. These approaches rely on observing patterns of gene expression through microarray analysis or quantitative polymerase chain reaction (qPCR) and using differences of expression between wildtype BRCA1 and BRCA1 VUS to classify a variant as pathogenic or not.

Gene expression assays rely on the hypothesis that BRCA1 mutation will significantly affect the expression of certain genes or the regulation of certain pathways in comparison to wildtype BRCA1, and that these differences are significant enough to distinguish between a BRCA1 wildtype and a BRCA1 heterozygous mutation. The use of gene expression as a susceptibility assessment and prognostic tool has been implemented before in other diseases, with varied degrees of success. In cancer research, gene expression profiling is an extensively studied area with applications as a method of predicting cancer susceptibility and patient survival in different cancers such as basal cell carcinoma [121], gastric cancer [122], [123], and ovarian cancer [124], [125].

In terms of hereditary breast cancer, microarrays have been used with cultured fibroblasts exposed to DNA damage to predict BRCA1 mutation carrier status with a high degree of accuracy [126]. In a similar example, Waddell et al. have used microarrays with lymphoblastoid cell lines to accurately classify truncating and missense mutations in BRCA1 [127]. While previously the ability to study the expression levels of a large number of genes was limited by the high expense required and the limited technology available, significant advances over the past few years have brought
technologies such as microarray analysis and qPCR to the fore, providing a relatively inexpensive and efficient method of observing large numbers of genes at a time.

1.5.2 Whole Genome Microarray Profiling can Predict BRCA1 Carrier Status

Studies previously undertaken by Dr. Scott Davey and Dr. Harriet Feilotter have shown that it is possible to determine BRCA1 and BRCA2 carrier status through gene expression profiling of unperturbed Epstein-Barr Virus transformed B-lymphocyte cell lines (EBV-LCL) [128]. These lymphocyte cell lines were derived from known carriers of BRCA1 and BRCA2 VUS and have known mutations in those genes, primarily frameshift and nonsense mutations with some missense splice mutations. These cell lines along with wildtype controls were either exposed to 2Gy IR or mock-irradiated. RNA from these cell lines were hybridized to Agilent whole genome microarrays with 43,376 features. Gene expression levels were normalized and analyzed using Nearest Shrunken Centroid Analysis. It was discovered that in BRCA1 mutation carriers, gene expression in mock-irradiated EBV-LCLs was able to differentiate carriers from controls with a high degree of accuracy. This finding is important as the ability of unperturbed cells to predict BRCA1 carrier status allows a functional assay for BRCA1 status to be built around it. A 43-gene classifier was constructed that included genes that had high predicative value for BRCA1 status independent of IR exposure. In addition to this, the genes which were
determined as good predictors for BRCA1 status were analyzed with Ingenuity Pathway Analysis in order to further understand the underlying biology of this classification scheme. It was discovered that these genes had roles in Hematological System Development and Function, Cell-to-Cell Signaling and interaction, and Cellular Growth and Proliferation. In addition, certain genes are involved in the immune response as cytokines or cell surface receptors.

1.5.3 Gene Expression Profiling for VUS Classification

Since its characterization in 1994, BRCA1 has been extensively studied and has been conclusively linked to hereditary cancer. However, its polyvalent nature has made it difficult to determine the function critical for tumour suppression. While BRCA1 has shown gatekeeper qualities due to its actions as a vital mediator in the cell cycle checkpoint cascade, its contribution to DNA DSB repair through HR allows it to be classified as a caretaker gene as well. In addition, it is still unclear whether loss of heterozygosity of BRCA1 is required for tumourigenesis, or whether haploinsufficiency is sufficient to predispose an individual towards cancer development. This lack of understanding adds to the difficulty in developing functional assays for BRCA1 variant classification as assays that focus on a single BRCA1 function may not necessarily reflect the pathogenicity of a BRCA1 variant as it may cause carcinogenesis through a different method of action. Therefore, indirect assays that use changes in gene expression as indicators of BRCA1 variant pathogenicity hold great potential as a classification tool.
1.6 Hypothesis and Experimental Aims

1.6.1 Hypothesis

The previous microarray-based study by our laboratory hypothesized that heterozygous $BRCA1$ mutation carriers displayed changes in gene expression in comparison to wildtype controls, and that this differential gene expression could be utilized to accurately classify $BRCA1$ mutation carriers from $BRCA1$ wildtype controls. Using microarray analysis on EBV-LCLs, this study was able to accurately predict $BRCA1$ mutation carriers from controls without exposure to radiation, indicating that baseline gene expression was sufficient to show differential gene expression.

Given this, we hypothesize that this same difference in gene expression between $BRCA1$ mutation carriers and wildtype controls can be observed using RT-qPCR (Reverse transcriptase qPCR) with a smaller set of genes in both EBV-LCLs and whole blood from known $BRCA1$ mutation carriers. Therefore, the overall goal of this project is to validate previous microarray results in EBV-LCLs using RT-qPCR and to observe whether the same differential gene expression is present in whole blood samples from known $BRCA1$ mutation carriers. This will require development of a method to extract high quality RNA from whole blood B-lymphocytes for qPCR analysis.

1.6.2 Experimental Aims

- Identification of key genes indicative of $BRCA1$ mutation carrier status must be identified. Target genes ideally have a variety of expression intensities and have similar gene ontology functions.
• Validation of observed differential gene expression in EBV-LCL through RT-qPCR. This requires RT-qPCR optimization for gene targets and isolation of high-quality RNA from EBV-LCL.

• Development of a reproducible protocol for RNA extraction from B-lymphocytes isolated from whole patient blood. The small cell population will necessitate a method of enrichment and optimization of RNA extraction protocols for small cell numbers.

• Validation of differential gene expression in B-lymphocytes from patients with known BRCA1 mutation carrier status through RT-qPCR with target genes. A source of blood samples must be found and B-lymphocytes isolated using the protocol developed in the second aim.
Chapter 2

Materials and Methods

2.1 EBV-Transformed B-Lymphocyte Cell Culture

EBV-transformed lymphocyte cell lines were obtained from the NIH Breast Cancer Family Registries. There were a total of 25 cell lines included in this study, 12 wildtype BRCA1 controls \( (+/+ \) \) and 13 heterozygous for BRCA1 mutation \( (+/-) \). An exact listing of the lines used and their specific mutations can be found in Table 1. All LCLs were stored in liquid nitrogen before culture. Cell lines were cultured in RPMI-1640 media (Sigma-Aldrich, St. Louis, MO) supplemented with 10% Fetal Bovine Serum (FBS) (Life Technologies, Burlington, ON) and 5% Penicillin/Streptomycin (Life Technologies). Cell culture was carried out in 25cm\(^2\) flasks at 37\(^{\circ}\)C in a 5% CO\(_2\) atmosphere. Cells were split in a 1:2 ratio and allowed to grow for 24 hours before harvest and lysis.

2.2 RNA Extraction though Column-Based Purification

EBV-LCL RNA extraction was performed with the Sigma-Aldrich GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) following the recommended protocol. RNA quality was evaluated using a spectrometer (Eppendorf, Mississauga, ON). Samples with a concentration lower than 50ng/\(\mu\)L or an absorbency ratio (26nm/280nm) less than 1.8 were discarded and re-extracted. RNA quality was also
<table>
<thead>
<tr>
<th>Cell Line ID</th>
<th>Mutation</th>
<th>Designation DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>B14023</td>
<td>F</td>
<td>2190delA</td>
</tr>
<tr>
<td>B14643</td>
<td>F</td>
<td>1294del40</td>
</tr>
<tr>
<td>B14663</td>
<td>N</td>
<td>4446C-T</td>
</tr>
<tr>
<td>B14832</td>
<td>F</td>
<td>2594delC</td>
</tr>
<tr>
<td>B14834</td>
<td>F</td>
<td>1135insA</td>
</tr>
<tr>
<td>B15268</td>
<td>F</td>
<td>5382insC</td>
</tr>
<tr>
<td>B15736</td>
<td>M</td>
<td>310G-A</td>
</tr>
<tr>
<td>B18318</td>
<td>F</td>
<td>3875delGTCT</td>
</tr>
<tr>
<td>B22893</td>
<td>F</td>
<td>1294del40</td>
</tr>
<tr>
<td>B26842</td>
<td>N</td>
<td>446C-T</td>
</tr>
<tr>
<td>B27129</td>
<td>F</td>
<td>Dup Exon 13</td>
</tr>
<tr>
<td>B27131</td>
<td>M</td>
<td>4603G-T</td>
</tr>
<tr>
<td>B27636</td>
<td>N</td>
<td>3276C-T</td>
</tr>
</tbody>
</table>

Note: F=Frameshift, N=Nonsense, M=Missense
assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) at a concentration of 250ng/µL with a volume of 5µL. The resulting RNA Integrity Number (RIN) is an indicator of RNA integrity and is the result of an algorithm that analyzes the complete electrophoretic trace of an RNA sample, including the 28S peak, 18S peak, and any degradation products. Samples with an (RIN) below 7.0 were discarded.

Samples that met these criteria were snap-frozen using liquid nitrogen and stored at -80°C for future use.

2.3 Reverse-Transcriptase Quantitative PCR

2.3.1 Selection of Targets for RT-qPCR Analysis

The 43-gene classifier for BRCA1 mutation carrier status from previously obtained microarray data [128] was analyzed to determine target genes for RT-qPCR validation. Gene ontology information was obtained from the NCBI Gene Database (http://www.ncbi.nlm.nih.gov/gene/). Genes were organized according to Function, Process, and Component. 5 target genes were selected for RT-qPCR analysis based on predictive ability to distinguish BRCA1 carriers, expression intensity, similarities in gene ontology, and expression direction. Genes selected were CXCR3, TBX21, MX2, IFIT1, and GLDC. GusB was selected as a reference gene due to evidence showing it to be an ideal reference in B-lymphocyte gene expression studies. [129], [130]

2.3.2 Selection of TaqMan Gene Expression Assays

Gene-specific TaqMan probes and primers for all genes were obtained from Applied Biosystems (Applied Biosystems, Foster City, CA) using the Gene Expression
### Table 2: Gene Expression Assays for RT-qPCR

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene Name</th>
<th>Assay ID</th>
<th>Fluorophor</th>
<th>Amplicon length (bp)</th>
<th>Reporter Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR3</td>
<td>Chemokine Receptor 3</td>
<td>Hs00171041_m1</td>
<td>FAM</td>
<td>111</td>
<td>TGGTCCTTGAGGTGAGTGACCACCA</td>
</tr>
<tr>
<td>TBX21</td>
<td>T-Box 21</td>
<td>Hs00203436_m1</td>
<td>FAM</td>
<td>62</td>
<td>CAGGGACGCGGATGTCTCCCATTCC</td>
</tr>
<tr>
<td>MX2</td>
<td>Myxovirus Resistance 2</td>
<td>Hs00159418_m1</td>
<td>FAM</td>
<td>54</td>
<td>GAGATAACACAAAGCCCAGAAACGTCAG</td>
</tr>
<tr>
<td>IFIT1</td>
<td>Interferon-induced Protein with Tetratricopeptide Repeats 1</td>
<td>Hs00356631_g1</td>
<td>FAM</td>
<td>88</td>
<td>ATTTACAGCAACCATGATACAAAT</td>
</tr>
<tr>
<td>GLDC</td>
<td>Glycine Dehydrogenase</td>
<td>Hs01580586_g1</td>
<td>FAM</td>
<td>135</td>
<td>GTCAATCCGCTGAAGATGTCTCCAC</td>
</tr>
<tr>
<td>GusB</td>
<td>Glucoronidase, Beta</td>
<td>Hs99999908_m1</td>
<td>VIC</td>
<td>81</td>
<td></td>
</tr>
</tbody>
</table>

Note: Amplicon length is size of PCR product after elongation phase. Reporter sequences are included in product information sheet, with the exception of GusB.
Forward and Reverse primer sequences are proprietary to Applied Biosystems and were unavailable.
* - GusB reporter sequence was not included with product documentation and was unavailable.
Assay Search Tool. With the exception of GLDC, primers spanned exon junctions, reducing the possibility of non-specific genomic contamination. Reporter sequences and amplicon lengths can be found in Table 2.

2.3.3 RT-qPCR using RNA from EBV-LCL

All RT-qPCR reagents were obtained from Applied Biosystems (Applied Biosystems). Each 20µL reaction contained 10µL 2X TaqMan One-Step RT-PCR Master Mix, 0.5µL 40X Multiscribe Reverse Transcriptase, 1.0µL pre-made gene expression assay containing gene-specific TaqMan probes and oligonucleotide primers, and 200ng of RNA template. This Master Mix contained AmpliTaq Gold® DNA Polymerase, dNTP with dUTP, a passive reference dye and optimized buffer components.

Reactions were plated in triplicate onto 96-well skirted plates. Plate controls comprised of a reaction without reverse transcriptase for every gene-sample combination, and a reaction with no RNA template for every gene primer used. RT-qPCR was performed using an Eppendorf Realplex MasterCycler (Eppendorf). A total of three RT-qPCR experiments were performed using RNA extracted at three different times.

The program used was as follows: 48°C for 30 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. Baseline and threshold values were established via Realplex software (Eppendorf) and mean Ct values with standard deviation across reaction triplicates obtained. Experiments were plated in monoplex; with the endogenous reference gene being amplified in a separate reaction well than target genes.
2.3.4 Creation of Standard Curves and RT-qPCR Efficiency Validation

Prior to experimental RT-qPCR, standard curves were constructed with known amounts of pooled RNA to validate reaction efficiencies and determine optimal RNA concentration. RNA dilutions were 750ng, 375ng, 187.5ng, 93.75ng, 46.88ng, and 23.43ng of template RNA per data point. Template RNA was from multiple BRCA1 control and BRCA1 mutation carrier cell lines. Reagent and reaction volumes and RT-qPCR program were identical to values stated above. One standard curve was constructed per target gene and plated in duplicate. Ct values were obtained by the Realplex software and linear regression performed. Reaction efficiencies were calculated based on the slope, where efficiency = $10^{-\frac{1}{\text{slope}}$}.

2.4 RT-qPCR Data Analysis

Fold difference for each gene as it relates to a reference gene and calibrator sample was calculated by the $\Delta\Delta C_t$ method [131]. Mean Ct for each target gene across three biological replicates was obtained and normalized to the mean Ct of GusB. The resulting $\Delta C_t$ was then normalized to an arbitrarily chosen wildtype cell line; in this case WT19998. The formula is outlined below:

$$\Delta C_t = C_t(\text{TargetGene}) - C_t(\text{GusB})$$

$$\Delta\Delta C_t = \Delta C_t(\text{Cell line}) - \Delta C_t(WT19998)$$

Fold Difference = $2^{-\Delta C_t}$

This method requires that the efficiencies of both the target gene reaction and reference gene must be similar. Otherwise, efficiency correction must be applied to the
formula. This was not required as selected gene expression primers were optimized to have a reaction efficiency of 100% under ideal conditions, and this was validated using standard curves under experimental conditions.

Statistical analysis for differences between BRCA1 mutation carriers and wildtype controls was performed using a two-tailed student’s t-test. Inter-replicate variation was assessed using ANOVA.

2.5 B-Lymphocyte Isolation from Whole Blood

2.5.1 Isolation of Peripheral Blood Mononuclear Cells Using Ficoll-Paque Separation

15mL to 20mL of whole blood was obtained from volunteers through standard phlebotomy techniques using BD butterfly needles (BD Biosciences, Mississauga, ON). Samples were stored in 6mL BD Vacutainers with Ethylenediaminetetraacetic acid (K2-EDTA) (BD Biosciences) as an anticoagulant at 4°C. Blood samples were used within 24 hours of collection. Blood was diluted at a 1:1 ratio with 1% PBS and layered onto Ficoll-Paque (GE Healthcare, Baie d’Urfe, QC) in a 50mL centrifuge tube (Sarstedt, Montreal, QC). The amount of Ficoll-Paque used varied depending upon the initial volume of blood used, with a ratio of 1mL of Ficoll for every 1mL of blood at blood volumes greater than 10mL. The layered solution was centrifuged using an Allegra X-14 benchtop centrifuge (Beckman Coulter, Mississauga, ON) at room temperature and at a speed of 400xg for 30 minutes with slow acceleration and no brake.
The resulting buffy coat was extracted using a 10mL pipette (Sarstedt) and washed twice using 1% PBS, with a pelleting spin at 800g for 5 minutes in between re-suspensions. Resulting lymphocytes were counted using a dye exclusion method, with a 10µL hemocytometer (Fisher Scientific, Ottawa ON) with a solution of trypan blue and cell suspension at a ratio of 50µL per 100µL cells. Trypan blue was used to evaluate B-lymphocyte survival and exclude dead cells from the cell count.

2.5.2 B-Lymphocyte Enrichment by Magnetic Separation

The B-lymphocyte population was enriched using the EasySep Human CD19 Positive Selection Kit (Stemcell Technologies, Vancouver, BC). This method was chosen as it has been shown to result in high purity and recovery and allows for quick processing of multiple samples [132]. CD19 was used for positive selection as B-lymphocytes display the CD19 surface receptor from the earliest lineage cells [133].

Buffy coat obtained from whole blood was pelleted at room temperature using an Allegra X-14 benchtop centrifuge (Beckman Coulter) at 800xg for 5 minutes. After removal of supernatant, pelleted cells were resuspended inside a 12x75mm 5mL polystyrene clear tube with separation medium which consisted of 1% PBS, supplemented to 2% FBS and 1mM EDTA (pH 8.0). Cells were resuspended to a concentration of 1x10⁸ cells/mL. Tetrameric Antibody Complex (TAC) was added at a concentration of 100µL per 1mL cell suspension and allowed to stand at room temperature for 15 minutes, after which the nanoparticle suspension was added at a concentration of 100µL per 1mL cell suspension and allowed to stand at room
temperature for 10 minutes. A volume of suspension buffer was then added to increase the volume of the cell suspension to 2.5mL and the suspension placed inside an EasySep magnet (Stemcell Technologies). This encloses the suspension inside a strong magnetic field and localizes the magnetically-labeled cells to the sides of the reaction vessel. The cell suspension was left within the magnet at room temperature for 5 minutes, after which both the magnet and tube were inverted, decanting the supernatant containing unwanted cells and leaving the labeled CD19+ B-lymphocytes inside the tube. The tube was removed from the magnet and 2.5mL suspension buffer added to resuspend labeled cells. The suspension was then replaced within the magnet and allowed to sit at room temperature for another 5 minutes before repeating the decanting procedure, for a total of 2 supernatant decanting steps. Finally, the resulting B-lymphocyte suspension was counted using a 10µL hemocytometer (Fisher Scientific).

This procedure contains several modifications to the recommended protocol in order to increase recovery of the B-lymphocyte population. Changes include increasing the addition of nanoparticles from a concentration of 50µL per 1mL cell suspension to 100µL per 1mL cell suspension and decreasing the number of supernatant decanting steps within the magnet from 4 suspensions to 2.

2.6 Flow Cytometry for Cell Population Analysis

Flow cytometry was utilized to quantify cell populations and ensure the quality of buffy-coat extraction and B-lymphocyte enrichment. Cells were pelleted at 4°C using a benchtop centrifuge (Beckman Coulter) at 114xg for 5 minutes. The pellet was
resuspended to a concentration of $1 \times 10^6$ cells per 100µL PBS supplemented to 10% FBS and 1% sodium azide and kept on ice. This cell suspension was split between three 5mL clear polystyrene tubes; one experimental, one isotype control and one negative control. In the experimental tube, a primary antibody for CD20 conjugated to PE-Cy5 (Abcam, Cambridge, MA) was introduced into the cell suspension at a concentration of 20µL antibody per $1 \times 10^6$ cells and incubated for 30 minutes. The cell suspension was brought up to a volume of 2.0mL and pelleted through centrifugation after which the supernatant is decanted and the pellet resuspended. This was repeated for a total of three washes after which the cells were fixed in 2% paraformaldehyde. Cells were then kept on ice before flow cytometry using an EPICS ALTRA flow cytometer (Beckman Coulter) in order to quantify cell populations. CD20+ cell populations were visualized through Pe-Cy5 staining, followed by analysis using an EPICS ALTRA flow cytometer (Beckman Coulter). Samples were gated on a forward scatter/side scatter histogram to exclude debris.

2.7 Effects of Collection Method and Transit Time on Separation Quality and RNA Integrity

The protocol for extracting B-lymphocytes from whole blood was established using locally-sourced volunteer blood samples that were processed within 24 hours of collection. Because this may not always be clinical if patient blood is sourced from a non-local healthcare facility, the effects of shipping times within a temperature-controlled container on RNA quality were determined. In order to accomplish this, blood samples
from volunteers were collected and divided into three groups- one was processed immediately and the others were stored for 24 and 48 hours at 4-8°C before processing. After the allocated storage period, blood samples were processed as per prior protocol. RNA integrity was determined for each sample through spectrometer and bioanalyzer analysis.

2.8 RT-qPCR Analysis of Whole Blood from Known BRCA1 Carriers

Prior to blood collection, this project obtained approval from the Research Ethics Board. Patients from North York General Hospital (Toronto, ON) with known BRCA1 carrier status were given information letters containing the purpose of this study and how collected blood samples would be handled and processed if they agreed to volunteer. Upon patient consent, 25mL of blood was taken using BD butterfly needles and BD Vacutainers (BD Biosciences) with K2-EDTA as an anticoagulant. Samples were bagged and sealed inside styrofoam containers with ice packs for overnight shipment to Kingston, ON. Upon receipt, patient blood samples were processed as per the established protocol within 24 hours of blood collection.

A total of 10 blood samples were obtained; 5 samples from patients who are known BRCA1 mutation carriers, 1 sample from a sequenced BRCA1 wildtype individual, and 4 samples from non-sequenced volunteers. Gene targets for this assay were limited to CXCR3, and TBX21 with GusB as an endogenous reference gene. A change from the method of RNA extraction from cultured LCLs was the use of a different kit for RNA extraction and purification, as the Sigma Mammalian RNA prep kit previously used
resulted in low yields of RNA from whole blood samples. The QIAgen RNEasy Miniprep kit (QIAgen) was used to extract and purify RNA from whole blood sourced from patients. After verifying RNA quality using a spectrometer, samples were immediately snap-frozen in liquid nitrogen and stored at -80°C.

Standard curves to validate reaction efficiency had RNA dilutions of 500ng, 250ng, 125ng, 62.5ng, and 31.3ng of pooled RNA template per data point. Standard curves were plated in duplicate with one standard curve per gene.

RT-qPCR was performed with extracted patient RNA in triplicate. There were only a total of two experiments performed as there was insufficient RNA for three complete experiments. Reaction and reagent volumes as well as the RT-qPCR program for both validation standard curves and experimental RT-qPCR were identical to prior experiments. All ΔCt’s (after normalization to GusB Ct) were normalized to ΔCt values from the sequenced BRCA1 wildtype patient. Statistical analysis for differences between BRCA1 mutation carriers and controls was performed using a two-tailed student’s t-test.
Chapter 3

Results

3.1 Gene Ontology Analysis and Selection of Target Genes

In order to choose target genes for qPCR analysis, 43 genes from previous microarray analysis identified as predictors for \textit{BRCA1} status were analyzed [128]. Using the NCBI Gene Database, the gene ontology of these genes was analyzed, noting gene function, processes which the gene is involved in, and the component of the cell wherein the protein product is located (Table 3).

Five genes were selected for RT-qPCR validation. Gene selection was based on predictive value in distinguishing \textit{BRCA1} mutation carriers and controls, expression intensity, similarities in gene ontology and expression direction (upregulation versus downregulation.) Based on these criteria, \textit{CXCR3}, \textit{TBX21}, \textit{MX2}, \textit{IFIT1}, and \textit{GLDC} were selected as target genes.

While target genes have a variety of functions, in general target genes are involved in immune response and lymphocyte movement. \textit{CXCR3} is implicated in the largest number of processes, including chemotaxis and inflammatory response. This involvement in immune response is echoed by \textit{TBX2} through its transcription regulation and immunoglobulin isotype switching function. \textit{IFIT1} is implicated in the viral defense response and \textit{MX2} is involved in interferon signaling and protein transport. While \textit{GLDC} does not have the same involvement with the immune response, it was selected as a target based on its predicative value and high expression level.
Table 3: Gene Ontology Analysis of Selected Target Genes for RT-qPCR

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Function</th>
<th>Process</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CXCR3</strong></td>
<td>Chemokine (C-X-C motif) Receptor 3</td>
<td>CXC Chemokine Receptor Activity, Chemokine Receptor Activity, Chemokine Binding</td>
<td>T-Cell Chemotaxis, Calcium-Mediated Signalling, Chemotaxis, Angiogenesis, Cell Adhesion, Integrin Activation, Apoptotic Process, Cellular Component Movement, Regulation of PI3K activity, Inflammatory Response, Regulation of MAP kinase activity, Regulation of leukocyte migration, Elevation of Cytosolic [Ca2+]</td>
<td>Plasma Membrane, Integral to Plasma Membrane, External side of Plasma Membrane, Cytoplasm</td>
</tr>
<tr>
<td><strong>TBX21</strong></td>
<td>T-box 21</td>
<td>Sequence Specific DNA Binding, Transcription Factor Activity, Transcription Regulatory Region DNA Binding</td>
<td>T-cell Differentiation, Positive Regulation of IgG switching, Lymphocyte Migration, Virus Response, DNA Dependent Transcription, Positive Regulation, Multicellular Organismal Development</td>
<td>Neuronal Cell Body, Nucleus</td>
</tr>
<tr>
<td><strong>IFIT1</strong></td>
<td>Interferon-induced Protein with Tetraprolactone Repeats 1</td>
<td>RNA Binding, Protein Binding</td>
<td>Cellular Response to exogenous dsDNA, Negative Regulation of Protein Binding, Cellular Response to Type 1 Interferon, Regulation of Viral Genome Replication, Cytokine-mediated signalling pathway, Type I Interferon-mediated signalling pathway, Defense Response to Virus, Negative Regulation of Helicase Activity, Intracellular Transport of Viral Proteins in Host</td>
<td>Cytoplasm, Cytosol</td>
</tr>
<tr>
<td><strong>MX2</strong></td>
<td>Myxovirus (influenza) resistance 2 (mouse)</td>
<td>GTPase, GTP Binding</td>
<td>GTP Catabolic Process, Regulation of Nucleocyttoplasmic Transport, mRNA transport, Cell Cycle Regulation, Defense Response, Protein Transport, Cytokine-mediated Signaling Pathway, Type I Interferon-mediated signalling pathway</td>
<td>Cytoplasm, Nucleus, Cytosol, Nuclear Pore</td>
</tr>
<tr>
<td><strong>GLDC</strong></td>
<td>Glycine Dehydrogenase (Decarboxylating)</td>
<td>Electron Carrier Activity, Lyase Activity</td>
<td>Glycine Catabolic Process</td>
<td>Mitochondrion</td>
</tr>
</tbody>
</table>
3.2 RT-qPCR with RNA Isolated from EBV-LCLs

Monoplex standard curves to determine the efficiencies of selected RT-qPCR primers resulted in efficiencies of 0.9-1.0 (Figure 2). Relative expression levels from RT-qPCR monoplex samples were calculated through the ΔΔCt method, with target gene Ct normalized to GusB Ct and the resulting ΔCt value normalized to WT19998. Relative gene expressions are plotted in Figure 3. No-template RT-qPCR controls for GLDC showed positive signals, indicating amplification of genomic contamination. This high Ct signal was consistently present throughout all cell lines and across all three biological replicates. As a result, use of this gene target was discontinued.

A significant difference in expression between BRCA1 mutation carriers and BRCA1 wildtype controls was observed for CXCR3 and TBX21 (p=0.018 and p=0.003, respectively). Both had lower relative gene expression in BRCA1 mutation carriers versus controls, as expected from previous microarray results. Expression levels for IFIT1 were on average higher in BRCA1 mutation carriers (as expected from previous microarray results) but did not reach statistical significance (p=0.183). MX2 on average did not show significant differential expression between mutation carriers and wildtype controls.

Comparison of gene expression levels observed between microarray and RT-qPCR showed CXCR3, TBX21 and IFIT1 expression decreases in BRCA1 mutation carriers in both microarray and RT-qPCR (Figure 4). MX2 expression levels did not show differential gene expression in BRCA1 mutation carriers, in contrast to microarray data, which show upregulation in mutation carriers. Two-sample t-test did not show any significant differences between microarray and RT-qPCR for CXCR3, TBX21 and IFIT1.
Figure 2: RT-qPCR Standard Curves with EBV-LCL RNA

Log-linear regressions of serial dilutions for each gene target using pooled EBV-LCL RNA. Dilutions were at 750ng, 375ng, 187.5ng, 93.75ng, 46.88ng and 23.43ng. Linear regression of mean Ct values from two experimental replicates was performed by the Realplex software. Amplification efficiency is calculated from the slope of the regression where Efficiency = $10^{-\frac{1}{\text{slope}} - 1}$. 

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Figure 3: Fold Difference in Gene Expression for CXCR3, TBX21, MX2 and IFIT1

X-Y plots of relative gene expression in EBV-LCL. Cell lines on the left are wildtype for BRCA1 and lines on the right are heterozygous for BRCA1 mutation. Values were calculated using the ΔΔCt method with GusB as an endogenous reference gene and normalized to cell line WT19998. The blue dashed line is mean gene expression across all wildtype or heterozygous cell lines. Student’s two-tailed t-test was used to determine statistical significance. (A) Relative gene expression of CXCR3. (B) Relative gene expression of TBX21. (C) Relative gene expression of MX2. (D) Relative gene expression of IFIT1. RT-qPCR shows gene expression for CXCR3 and TBX21 are significantly downregulated in BRCA1 mutation carriers. IFIT1 shows non-significant increase in gene expression, while MX2 shows no differential expression.
We conclude that based on these results, *CXCR3* and *TBX21* might be appropriate targets for RT-qPCR using patient blood samples. While *IFIT1* gene expression did not reach statistical significance, it showed an upregulation in *BRCA1* mutation carriers. Perhaps a larger sample size may show a significant difference; therefore *IFIT1* could be utilized in a future multi-gene classifier.

### 3.3 Isolation and Enrichment of B-Lymphocytes from Whole Blood Samples

Given that EBV-transformed lymphocytes are derived from whole-blood B-lymphocytes, extension of this assay to fresh blood samples necessitates isolation and enrichment of the B-lymphocyte cell population.

Ficoll-Paque separation was used to isolate the leukocyte fraction present in whole blood samples. Cell counts using trypan blue exclusion showed $3.0 \times 10^7$ cells per 10mL whole blood processed. Flow cytometry showed that the B-lymphocyte population composes about 10% of the total mixed leukocyte population. (Figure 5)
Figure 4: Comparison of Gene Expression Profiles between Microarray and RT-qPCR

Plots of relative gene expression in EBV-LCL from both microarray and RT-qPCR. Gene expression values in these plots were calculated by taking the log₂ fold expression for one gene and normalizing this value to the average log₂ fold expression across both carrier and wildtype cell lines. The red/black dashed line is mean gene expression across all wildtype or heterozygous cell lines from microarray data and RT-qPCR respectively. (A) Gene expression of CXCR3. (B) Gene expression of TBX21. (C) Gene expression of MX2. (D) Gene expression of IFIT1. RT-qPCR and microarray results agree in CXCR3, TBX21 and IFIT1.
The B-lymphocyte population was enriched through positive selection using anti-CD19 antibodies conjugated to magnetic nanoparticles. For this purpose, the EasySep Magnetic Separation System was selected after analysis of current cell-enrichment options. Primary considerations were separation purity, required equipment, reagent costs and processing time. Level of B-lymphocyte purity was validated through flow cytometry which showed an increase in B-lymphocyte purity from 8.2% to 63.8% after separation. (Figure 6)

3.4 Optimization of Magnetic Separation for Cell Recovery

A caveat of having very high B-lymphocyte purity is that there is very low recovery of usable B-lymphocytes for RNA extraction and downstream applications. Cell counts for the previous experiments were reduced from $1 \times 10^6$ cells prior to magnetic separation to $1 \times 10^4$ cells, a cell number that yields an insufficient amount of RNA for RT-qPCR.

With increasing the blood volume collected from patients being an impractical solution, blood samples must be processed as efficiently as possible. This necessitates determining a balance between cell recovery and purity. Optimization experiments were performed where the manufacturer’s protocol was modified, as it has been seen that in positive selection, increasing the concentration of reagent and lengthening incubation times increase purity while decreasing the overall cell recovery, while the reverse increases cell recovery while decreasing purity [132]. In addition, increasing the number of magnetic separations increases purity and decreases recovery. Therefore, modified variables included increasing reagent concentration (both the CD19+ TAC and
Figure 5: Flow Cytometry Histogram: Visualization of B-Lymphocyte Population

Visualization of CD-20+ B-lymphocyte population from buffy coat extracted from whole blood. Visualization is via PE-Cy5 staining (X-axis) and is indicative of CD-20+ cells in a heterogeneous cell population. (A) No antibody control. (B) αCD-20 conjugated to PE-Cy5. It can be seen that CD20+ B-lymphocytes compose only 10.3% of the heterogeneous lymphocyte population.
Figure 6: Flow Cytometry Histogram: Visualization of B-Lymphocyte Purity after Magnetic Separation

Visualization of CD20+ B-lymphocyte population before and after magnetic enrichment. A heterogeneous cell population was obtained from buffy coat and divided, half for direct staining and visualization and half for magnetic enrichment. The B-lymphocyte population was enriched through EasySep magnetic separation using manufacturer recommended protocol. Visualization is via PE-Cy5 staining (X-axis) and is indicative of CD20+ cells in a heterogeneous cell population. An increase in the B-lymphocyte population is clearly visible after magnetic enrichment.
Figure 7: Flow Cytometry Histogram: Optimization of B-Lymphocyte Magnetic Separation

Visualization of CD20+ B-lymphocyte population during optimization of the magnetic enrichment protocol. The manufacturer’s recommended protocol was modified and each modification visualized determining the effect on B-lymphocyte enrichment. Visualization is via PE-Cy5 staining (X-axis) and is indicative of CD20+ cells in a heterogeneous cell population. It can be seen that 10 minute TAC incubation followed by two 5 minute magnetic separation steps, and a 5 minute TAC incubation followed by two 5 minute magnetic separation steps result in an increase of B-lymphocyte purity as compared to an unsorted cell population.
Blank - negative control

Blank - negative control

αCD20 (PE-Cy5)

αCD20 (PE-Cy5)

5 minute TAC incubation
2x 10 minute magnetic separation

Pe-Cy5 Fluorescence (675+/20nm)

5 minute TAC incubation
2x 5 minute magnetic separation

Pe-Cy5 Fluorescence (675+/20nm)

10 minute TAC incubation
2x 5 minute magnetic separation

Pe-Cy5 Fluorescence (675+/20nm)

Unsorted lymphocytes

Pe-Cy5 Fluorescence (675+/20nm)
nanoparticle suspension), incubation times and number of magnetic separations (encompassing incubation time within magnet and decanting steps.)

Both flow cytometry with a CD20 antibody and hemocytometer cell counts were used to assess resulting purity and cell recovery. It was determined that doubling the concentration of nanoparticles added from 5µL nanoparticles per 100mL cell suspension to 10µL per 100mL and decreasing the number of separations within the magnet from 4 to 2 results in a large increase in cell recovery with a tolerable decrease in separation purity. Flow cytometry shows B-lymphocyte purity at 65.6% (75.8% if accounting for CD20 antibody underestimation) with an increase in cell recovery (Figure 7).

3.5 The Effects of Blood Collection Methods and Travel Time on RNA Integrity

Given that the validation of this assay requires whole patient blood from known \textit{BRCA1} mutation carriers, the effects of both the blood collection method and shipping time must be taken into account. There are a variety of phlebotomy collection tubes and numerous studies on how different methods affect downstream RNA integrity [134], [135], [136], [137] but choices are limited due to a specific cell population needing enrichment and isolation before RNA extraction. In addition, the effect on RNA integrity due to storage of blood samples in transit must also be determined.

During collection of volunteer blood samples for blood protocol optimization, BD Vacutainer tubes with K2 EDTA additive were used. While RNA quality from these vacutainers was acceptable, other blood collection methods such as PAXgene tubes were explored. PAXgene blood collection tubes are similar to Vacutainer tubes; however
PAXgene additives are specially formulated to stabilize RNA and ensure its integrity is maintained. Though studies have shown its effectiveness [138], [139], [140], PAXgene tubes lyse cells immediately after blood collection, making B-lymphocyte enrichment impossible and resulting in RNA coming from a mixed cell population. Therefore, it was decided to continue using K2 EDTA tubes.

We also attempted to determine how long whole blood can be stored at 4°C before RNA integrity is degraded enough to render the sample unusable. Volunteer blood was taken and processed at three different time points. It was observed that blood processed immediately after venipuncture yielded the highest RNA quality, with RIN numbers decreasing as time between collection and processing increased (Table 4). It is surprising that despite the decreasing trend in RIN, some samples exhibited an RIN number greater than 7.0, indicating that even blood stored for 48 hours may still yield usable RNA. However, at 48 hours buffy coat isolation becomes difficult due to widespread cell lysis and degradation of the sample.

3.6 RT-qPCR of RNA from Known BRCA1 Mutation Carriers

Standard curves with RNA from patient blood showed similar reaction efficiencies to prior standard curves with RNA from EBV-LCL (Figure 8). It was noted that Ct values from patient blood RNA were higher than Ct values from EBV-LCL RNA at similar concentrations, to the point that small concentrations of RNA did not have sufficient amplification to reach the threshold value at 40 cycles. Experimental RT-qPCR showed no significant difference in gene expression with two target genes between
**Table 4: Effect of Storage Time on RNA Integrity**

<table>
<thead>
<tr>
<th>Storage time</th>
<th>Sample</th>
<th>RIN</th>
<th>260/280</th>
<th>260/230</th>
</tr>
</thead>
<tbody>
<tr>
<td>t = 0</td>
<td>Sample C</td>
<td>9.2</td>
<td>2.00</td>
<td>2.15</td>
</tr>
<tr>
<td></td>
<td>Sample D</td>
<td>9.2</td>
<td>2.21</td>
<td>2.37</td>
</tr>
<tr>
<td></td>
<td>Sample E</td>
<td>4.5</td>
<td>1.47</td>
<td>0.34</td>
</tr>
<tr>
<td>t = 24 hrs</td>
<td>Sample F</td>
<td>6.3</td>
<td>1.99</td>
<td>1.89</td>
</tr>
<tr>
<td></td>
<td>Sample G</td>
<td>8.4</td>
<td>2.36</td>
<td>1.44</td>
</tr>
<tr>
<td>t = 48 hrs</td>
<td>Sample H</td>
<td>7.3</td>
<td>---*</td>
<td>---*</td>
</tr>
<tr>
<td></td>
<td>Sample I</td>
<td>5.7</td>
<td>2.06</td>
<td>1.29</td>
</tr>
</tbody>
</table>

Note: RIN = RNA Integrity Number. Absorption ratios were obtained from a spectrometer and are indicative of protein contamination and presence of RNA extraction reagents.

*- Spectrometer did not produce any values.
Figure 8: RT-qPCR Standard Curves with Patient B-lymphocyte RNA

Log-linear regressions of serial dilutions for each gene target using pooled RNA from patient blood. RNA was isolated from enriched B-lymphocytes from known *BRCA1* mutation carriers and wildtype controls. Dilutions were at 500ng, 250ng, 125ng, 62.5ng, and 31.3ng. Linear regression of mean Ct values from two experimental replicates was performed by the Realplex software. Amplification efficiency is calculated from the slope of the regression where Efficiency = $10^{-1/slope} - 1$. 
BRCA1 mutation carriers and controls (Figure 9). There was significant variability between the two biological replicates. There were a number of issues in these experiments, such as only 1 control being properly classified as BRCA1 wildtype. These issues are further considered in the discussion.
Figure 9: Fold Difference in Gene Expression for CXCR3 and TBX21 in Patient Blood

X-Y plots of relative gene expression in EBV-LCL. Samples on the left are non-sequenced BRCA1 control samples (C1-C4) and one sequenced BRCA1 wildtype (B1). Samples on the right are heterozygous for BRCA1 mutation. Values were calculated using the ΔΔCt method with GusB as an endogenous reference gene and normalized to sample B1. The blue dashed line is mean gene expression across all wildtype or heterozygous mutant cell lines. (A) Relative gene expression of CXCR3. (B) Relative gene expression of TBX21. Two-tailed student’s t-test showed no significant difference in gene expression for these genes between BRCA1 mutation carriers and BRCA1 controls. Error bars represent standard deviation between three replicates.
Chapter 4

Discussion

4.1 General Discussion

In this study, it was shown that EBV-LCLs derived from known BRCA1 mutation carriers display differential expression of certain genes. This study had four experimental aims: the first was to identify key genes indicative of BRCA1 mutation carrier status. Four target genes were chosen from a pool of 43 genes after gene ontology analysis and reflected a variety of functions, expression levels and expression directions.

The second aim was to validate predictors for BRCA1 carrier status discovered from microarray gene expression profiles through RT-qPCR. It was observed that CXCR3 and TBX21 displayed significant downregulation in BRCA1 mutation carriers, as expected from previous microarray results. IFIT1 differential expression, while not statistically significant, displayed upregulation that agreed with previous microarray data. MX2 did not show any significant differential gene expression. Data on GLDC expression was unreliable due to genomic contamination in no-template controls; therefore no further analysis was done with this gene.

We also aimed to develop a protocol to validate these findings using whole blood from known BRCA1 carriers as the third aim. We were able to develop a protocol that allows for quick and efficient processing of whole blood for downstream RT-qPCR. This protocol includes leukocyte extraction from buffy coat, B-lymphocyte enrichment through magnetic positive selection and RNA extraction; all steps included appropriate
quality control methods. Protocol optimization involved modifying the B-lymphocyte enrichment stage in order to increase cell recovery. While successful, this was at a cost to B-lymphocyte separation purity. This was required as B-lymphocytes are found in very small amounts per unit of whole blood. This reliance on a very rare cell population is a significant hurdle that has to be overcome and will require steps be taken to either ensure that the greatest possible amount of RNA per cell is extracted and that the initial cell population going into RNA isolation is as high as possible.

Finally, we attempted to discover whether the same differential gene expression observed in EBV-LCL could be found in blood samples from patients heterozygous for pathogenic \textit{BRCA1} mutations. We obtained 5 \textit{BRCA1} mutant patient blood samples utilized the optimized protocol to enrich the B-lymphocyte population and isolate RNA. RT-qPCR using this RNA showed that there were no significant differences in gene expression for selected target genes between \textit{BRCA1} mutation carriers and non-mutant controls. However, issues such as having non-sequenced controls, a small sample size and the scarcity of RNA and degradation of integrity during transport have to be addressed.

While RT-qPCR on patient blood proved inconclusive due to significant issues, data from both RT-qPCR and microarray analysis of EBV-LCL suggests that in certain genes, differential gene expression can distinguish pathogenic \textit{BRCA1} mutation carriers from \textit{BRCA1} wildtype, establishing the potential for a gene-expression based assay for \textit{BRCA1} variant classification. In addition, the effect of this differential expression profile
on cancer predisposition can be further studied by examining gene function and systemic interactions.

4.2 Differential Gene Expression in BRCA1 Mutation Carriers may contribute to a Pro-Tumourigenic Microenvironment

We have shown differential expression of CXCR3 and TBX21 in cells with pathogenic BRCA1 mutations. Therefore we can speculate that this differential gene expression may contribute to a pro-tumourigenic microenvironment conducive to cancer development. Research has shown that dysregulation of gene expression is often a contributing factor to cancer formation and metastasis through expression of pro-angiogenic and pro-inflammatory factors [141], [142], [143]. In the context of this study, we speculate that downregulation of immune-response genes CXCR3 and TBX21 contribute to cancer development through inhibition of the immune response to tumour formation, as well as through an increase in metastatic potential and tumour progression, while IFIT1 downregulation could be an indicator of chemotherapy resistance.

CXCR3 codes for a G-protein coupled receptor that initiates a signal cascade inducing cellular responses involved in leukocyte traffic. Studies have seen that aberrant expression of certain CXCR3 splice variants are implicated in breast and renal cell carcinoma [144], [145] and that aberrant expression is associated with increased metastasis, and increased tumour differentiation and size [146], [147]. It has also been seen that inhibition of CXCR3 expression may reduce the effect of chemokines CXCL9 and CXCL10 in chemotaxis of anti-tumour immune response cells [148], [149], [150].
*TBX21* is a transcription factor that contains the T-box DNA binding domain, and has been implicated in regulation of the expression of interferon-gamma, the primary cytokine for T-Helper Cells, Type 1 [151]. It has been seen that *TBX21* deficiency significantly increases prostate cancer metastasis and tumour progression in a murine mouse model [152]. Similarly, Werneck *et al.* showed that *TBX21* is a critical part of NK cell response to tumours and that knockdown of this gene disrupts NK-mediated cross-talk between innate and adaptive immune responses to metastatic disease [153]. Zhu *et al.* also observed that deficiency in T-bet coupled with deficiency in the transcription factor eomesodermin has a negative impact on adaptive immune response to tumour formation by significantly lowering CD8$^+$ T-lymphocyte infiltration to the tumour site and through disruption of T-lymphocyte function [154]. Additionally, research by Lord *et al.* indicated that *TBX21* knockdown also impairs expression and function of *CXCR3*; this may help explain the observed downregulation of *CXCR3* [155].

*IFIT1* codes for a rapidly synthesized protein that is synthesized in response to interferon and is most commonly seen as a reaction to viral exposure [156] and as a response to the cellular response to DNA damage through the Stat1/interferon pathway [157]. While *IFIT1* has rarely been studied in the context of cancer, recent research by Weichselbaum *et al.* have seen that upregulation of a subset of genes they identify as IFN-related DNA damage resistance signature (IDRS) indicates increased resistance to chemotherapy and a lower recurrence-free survival post treatment [158]. This subset includes *STAT1, ISG15* and *IFIT1*, and it was also seen that knockdown of *IFIT1* re-
sensitized the squamous cell carcinoma sub-line Nu61 to doxorubicin [158]. This indicates that IFIT1 differential expression can be a marker for treatment efficacy and recurrence-free survival.

Microarray data identified MX2 as being upregulated in BRCA1 mutation carriers. While the RT-qPCR analysis did not observe any significant differential expression, this could be attributed to the low expression level of the gene. This increases the difficulty of detecting any differential gene expression, while making it more sensitive to degraded RNA template.

4.2.1 BRCA1 Mutation may Indirectly Influence Gene Expression

It is a logical assumption that BRCA1 mutation contributes to the differential gene expression in heterozygous mutant BRCA1 cells. Since BRCA1 has DNA binding capability and functions as a transcriptional regulator, a pathogenic BRCA1 mutation may cause dysfunction in gene transcription which can lead to an observed differential gene expression.

A recent study by Gorski et al. attempted to profile the BRCA1 transcriptome and elucidate its role in transcriptional regulation through BRCA1 knockdown [159]. This study observed that, while BRCA1 inactivation has some direct effect on its transcription regulation function, the majority of differentially-expressed genes represent indirect BRCA1 transcriptional targets. This may explain why genes not directly regulated by BRCA1, such as CXCR3, TBX21, and IFIT1 show differential expression given BRCA1 mutation.
4.3 Small Cell Population and small RNA concentration is a Significant Hurdle in Assay Development

In the adaptation of any laboratory-developed assay for clinical use, it must be kept in mind that conditions in the laboratory may not always be representative of clinical conditions. In this study, low RNA amounts in B-lymphocytes isolated from patient blood presented a significant obstacle. This was not a significant problem when using EBV-LCL as these cells were immortalized cultures that had rapid growth rates, allowing access to a virtually unlimited cell population in which each cell had a large amount of RNA. In contrast, B-lymphocyte populations in whole blood are very small [160], [161] and are limited to what is available per sample. While it was attempted to solve this issue by increasing cell recovery during the magnetic enrichment stage, procedural optimization cannot fully compensate for this problem. RNA yields are limited by the absolute amount within B-lymphocytes, and by the small numbers of B-lymphocytes per unit of blood. To adapt this assay for clinical use, a process must be developed wherein the initial B-lymphocyte cell population is increased, or the qPCR assay adapted for use with very small amounts of template genomic material. The former can be accomplished by increasing the initial blood volume used in the assay. This is not feasible in the long run as collecting increased volumes of blood from patients may cause health complications. In addition, the need for large volumes of blood will increase the difficulty of finding volunteers.

Increasing the population of B-lymphocytes available, such as through cell culture, is a plausible alternative to collection of large blood volumes. B-lymphocyte
culture has been proposed as a way to expand isolated B-lymphocyte populations in order to have a steady supply of cells for downstream analysis [162], [163]. Care must be taken that the cell population consists of as pure a population of B-lymphocytes as possible in order to prevent other cell types such as T-lymphocytes and other monocytes from proliferating in culture and influencing the RNA profile. In addition, it must be determined whether cultured B-lymphocytes are still functionally identical to freshly-isolated B-lymphocytes, and to compensate for any variability that might occur.

4.3.1 Alternative qPCR Methodologies

Other than increasing the cell population available, alternative qPCR methodologies such as two-step qPCR are possible options. Two-step qPCR involves two separate steps: an independent reverse transcriptase step to create complimentary DNA (cDNA) outside the qPCR reaction vessel, and the actual qPCR reaction using the synthesized cDNA as template. This is in contrast to one-step RT-qPCR which involves having the reverse transcriptase reaction in the same vessel as the amplification and quantification reaction.

The one-step methodology was initially chosen as it would be ideal for screening multiple samples along while reducing procedural errors and cross contamination. Although it was effective with EBV-LCLs, the small cell population and correspondingly low RNA amounts in whole blood make it difficult to utilize this method effectively. It must be kept in mind, that the choice between one-step versus two-step methods is not a simple one; research is conflicting on whether one method is results in superior
quantitation than the other [164], [165]. The consensus seems to be that convenience, minimization of contamination and procedural error, and speed are the primary features of one-step qPCR, while flexibility, increased sensitivity, and the ability to stock cDNA are desirable features of two-step qPCR. While shifting our established protocol from one-step to two-step qPCR is relatively simple, some challenges have to be addressed. The ability to create cDNA stocks may be a way to bypass the low RNA amounts isolated from B-lymphocytes. However, two-step qPCR requires more optimization than one-step qPCR, given that the reverse transcriptase step must be optimized to yield high quality cDNA. Similarly, the addition of a cDNA synthesis step requires that a method for assessing the quality of any cDNA transcript be utilized, such as spectroscopic analysis and bioanalyzer assessment. In conclusion, a two-step methodology would take more time as compared to one-step qPCR, but the flexibility of available cDNA stocks and increased sensitivity may make this an option worth considering.

Another potential alternative is qPCR with extremely low template concentrations, is single-cell qPCR. While our current protocol states that 0.1ng to 1µg RNA can be used per reaction, we have observed that RNA template concentrations well above this minimum result in extremely high Ct values and high intra-replicate variation. A recent study has shown that as template concentration decreases, there is a corresponding increase in intra-assay variability and unsuccessful qPCR reactions [166].

Single-cell qPCR was first explored in a paper by Taniguchi et al. in 2009, where they were able to synthesize a cDNA library from single cells using oligo d(T) bound to
capture beads [167]. Their methodology was able to successfully perform qPCR using incredibly small amounts of RNA; however single-cell qPCR is most effectively used to assess the heterogeneity of a cell population. Adaptation of this method in our protocol would invalidate the clinical relevance of our proposed assay, as the differential gene expression that distinguishes \textit{BRCA1} mutation carrier status was observed in a mixed B-lymphocyte population. Single-cell analysis of patient samples may display expression patterns that may not be present in the entire cell population. Another factor to consider with single-cell qPCR is that it requires additional evaluation of replicates and limits of detection, and careful consideration of analysis methods. This is to ensure that the signal noise inherent in single-cell qPCR is taken into account. While a comparative study has shown that single-cell qPCR using small-cell protocols performed in a standard qPCR thermocycler has comparable efficiency and performance when compared to a specialized microfluidic qPCR system [168], the very nature of single-cell assays requires that a very high number of assays be performed for any significant results, and this high-throughput analysis requires specialized equipment and reagents [169].

Future directions may include this type of qPCR to examine the effects of \textit{BRCA1} mutations on individual cells belonging in a heterogeneous population, such as PMBC’s isolated from whole blood. In the meantime, alternate qPCR methods must be explored which are sensitive enough to result in effective quantitation given the small amounts of template available.
4.4 Validation of Differential Gene Expression in Patient Blood

We attempted to validate the differential gene expression observed with EBV-LCLs using whole blood from known BRCA1 mutation carriers. Blood samples were obtained from patients at North York General Hospital. 5 blood samples from patients heterozygous for BRCA1 mutation and 1 control blood sample from a sequenced BRCA1 wildtype were obtained. These samples were processed using the optimized protocol and resulted in a usable amount of RNA for RT-qPCR. In addition, four blood samples from non-sequenced local volunteers were also processed. This was a significant issue with this approach, as problems with patient accrual and limited time did not allow us to enough sequenced BRCA1 wildtype controls. This uncertainty regarding control samples casts doubt on the accuracy of the RT-qPCR as an undiscovered pathogenic BRCA1 mutation in a supposed wildtype control would result in false results.

RT-qPCR showed no significant difference in expression for CXCR3 and TBX21 between BRCA1 mutation carriers and controls. While this result contradicts our hypothesis, there were significant issues with these experiments that need to be addressed, such as a small sample size. There were only 10 blood samples available for RT-qPCR, in contrast to a total of 25 EBV-LCL used in the prior validation experiment. The small sample size may cause subtle differences in expression to go undetected and may contribute to the lack of significance observed. These issues can be solved by increasing the number of patients recruited and blood samples analyzed, and by obtaining sequenced BRCA1 controls. This would require the involvement of other healthcare
centres and recruitment of more BRCA1 mutation carriers, both of which could be a future direction for this project.

Finally, it was observed through both validation with standard curves and experimental RT-qPCR that the reference gene GusB Ct values were exceedingly high, surpassing Ct values observed in EBV-LCL. As this indicates either low expression or low detection of the reference gene, it casts doubts on the effectiveness of GusB as the reference gene of choice. A possible solution would be to use a panel of more than one reference gene with a wide range of expression in B-lymphocytes. This will minimize reliance on a single gene signal for normalization and will allow for wider coverage of gene expression ranges.

4.4.1 Increasing Transport Time has a Detrimental Effect on Whole Blood Quality

In preparation for receiving blood samples, the effects of transit on blood samples in K2 EDTA vacutainer tubes were analyzed. It was observed that as time between blood collection and processing increases, there was a corresponding decrease in both buffy coat separation quality and RNA integrity, though not to a degree that the RNA was unusable. An incubation period of 48 hours did not cause the RNA integrity to drop below an RIN of 7.0. This shows that, while the ideal situation would be to process whole blood immediately after collection, a transit time of no longer than 48 hours is acceptable for maintaining RNA quality. We did not choose to have a timepoint greater than 48 hours as extrapolation from our prior experiments shows that whole blood which has been in transit for greater than 48 hours would be unusable due to physical degradation of
the sample. It did not seem prudent to waste valuable blood samples to validate longer
time points as it was possible to obtain samples within that time window. In addition, the
geographic proximity of our blood source meant that transit should not take longer than
48 hours in normal circumstances. However, if other sources are to be considered, this
analysis should be extended as these locations may have longer shipping times. While
there are options for extending the shelf life of whole blood, careful analysis must be
undertaken in order to determine if these adaptations have any effect on B-lymphocyte
cell populations and on gene expression. In addition, blood storage methods must also
maintain RNA integrity, while preserving blood in a form that B-lymphocytes can be
isolated from.

4.4.2 Patient Treatment and Condition may serve as Exclusion Criteria for Study

A factor that was not initially considered was the effect of treatment patients were
undergoing at the time of blood collection. Given that this study is heavily reliant on a
rare cell population, any stimulus that reduces the number of usable cells, or alters
baseline gene expression must be anticipated and compensated for. For example, high-
dose chemotherapy has been shown to significantly reduce the numbers of circulating
lymphocytes in whole blood [170], [171]. In order to alleviate this, human granulocyte
colony-stimulating factor is often co-administered during periods of chemotherapy,
slightly reducing the immunotoxic effects of treatment [172], [173]. Given that
chemotherapy regimens for breast cancer are most often cytotoxic, patients currently
under chemotherapy may have a significantly reduced B-lymphocyte population,
rendering them ineligible for this study and reducing the availability of BRCA1 mutation carriers that we can have access to.

Other than its lymphotoxic effects, it has also been shown that neoadjuvant systemic therapy (NST) causes changes in gene expression. In a specific example, Gonzales-Angulo et al. observed depletion of immune-related signals after NST, with a corresponding upregulation of metabolism-related processes [174]. This study also found that B-lymphocyte receptor signaling factors and other immune-related genes are downregulated in tissues post-NST. Considering that the predictor genes our assay relies upon are involved in the immune system, any alteration of immune system gene expression due to exogenous factors will limit the accuracy of our assay.

This effect on immune system gene expression combined with the decrease of the lymphocyte population makes our assay difficult to implement in patients undergoing chemotherapy. Similarly, this assay will not be as effective with patients who are immunocompromised and have a reduced lymphocyte population. This should not significantly reduce the clinical relevance of our assay as individuals can submit blood before undergoing chemotherapy as part of the process of determining whether they are at a higher risk of cancer occurrence.
4.5 Future Directions

1. Of five target genes selected, *CXCR3* and *TBX21* had significant differential expression that agreed with previous microarray data, and *IFIT1* displayed an expected but non-significant downregulation. *MX2* did not show any significant differential expression, and *GLDC* was eliminated due to technical reasons. This may necessitate further qPCR with other genes that have been identified as accurate predictors for *BRCA1* status, and perhaps a larger sample size may result in significant differential expression being observed.

2. The small B-lymphocyte population in whole blood and the degradation of RNA integrity are significant issues that have to be addressed for the continuation of this study. Options include exploring methods to either increase the amount of template available (whether RNA or cDNA), increase the cell population used in the assay through short term B-lymphocyte culture, or switch to a more sensitive qPCR methodology. In addition, inter-replicate variability in RT-qPCR must be addressed. It must be determined whether this variability is due to experimental error or due to an intrinsic factor that is not consistent between biological replicates. Multiple reference genes may be used to ensure the accuracy of the analysis. We chose *GusB* as a reference gene for RT-qPCR due to the fact that was shown to be an ideal reference gene for expression studies using B-lymphocytes [129], [175]. However, this does not limit us to a single reference
gene as other genes such as ACTB (Beta-Actin) also show minimal variation and can serve as an ideal reference gene in conjunction with GusB [175].

3. A significant hurdle to the continued progress of this project was the limited availability of BRCA1 mutation carriers going through North York General. This combined with possible exclusion factors and the required informed consent creates difficulty in obtaining a sufficiently large sample population. In order to ensure a large enough sample population, it may be necessary to involve multiple healthcare facilities in different geographic locations. This would necessitate determining the effects of and compensating for longer blood shipping times for more distant locations.

4.6 Conclusions and Clinical Relevance

We have shown in a previous microarray-based study that BRCA1 mutation carriers display differential gene expression compared to BRCA1 wildtype for certain genes in unperturbed B-lymphocyte cell lines transformed with EBV. We theorized that this differential gene expression profile is also found in whole blood B-lymphocytes heterozygous for pathogenic BRCA1 mutations. If validated, this theory ensures quicker identification of pathogenic VUS and less uncertainty about their classification. In addition, this study has great potential to be extended into a clinical assay to screen for increased hereditary breast cancer risk.
In this study, we aimed to validate microarray results through RT-qPCR of selected genes that have been previously identified as predictors for *BRCA1* mutation carrier status. In addition, we aimed to develop a protocol to enrich the B-lymphocyte population from whole peripheral blood and isolate high integrity RNA for RT-qPCR. Of 4 genes tested, three genes displayed differential gene expression that agreed with previous microarray results. In addition, we were able to develop an efficient methodology with associated quality control steps that resulted in high-integrity RNA from B-lymphocytes isolated from whole blood.

The creation of this reproducible methodology using whole blood allows us to determine whether the observed differential gene expression can be found in whole blood sourced from known *BRCA1* mutation carriers. While no significant differential gene expression in patient blood samples was detected, issues including a small sample size, uncertainty of *BRCA1* wildtype in control samples, and degradation of RNA due to transport call these results into question.

Eventually, we hope that this gene-expression based assay can eventually be used as part of a complete set of screening techniques for *BRCA1* mutation carrier status. While further validation may be required using other predictor genes, we believe that there is strong indication that this differential gene expression profile is present in pathogenic mutation carriers. After further optimization, this assay has high potential to be used as a parallel screening methodology alongside currently established screening methods. In addition, predictor genes with significant differential expression can be
observed in greater detail in order to help understand the underlying molecular mechanisms governing cancer predisposition given \textit{BRCA1} mutation. It is our hope that this will allow for earlier detection of pathogenic \textit{BRCA1} variants and so result in improved patient care and disease outcome.
References


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QUEEN'S UNIVERSITY HEALTH SCIENCES AND AFFILIATED TEACHING HOSPITALS
ANNUAL RENEWAL

Queen's University, in accordance with the "Tri-Council Policy Statement, 1998" prepared by the Medical Research Council, Natural Sciences and Engineering Research Council of Canada and Social Sciences and Humanities Research Council of Canada requires that research projects involving human subjects be reviewed annually to determine their acceptability on ethical grounds.

A Research Ethics Board composed of:

Dr. A.F. Clark, Emeritus Professor, Department of Biochemistry, Faculty of Health Sciences, Queen's University (Chair)
Dr. H. Abdollah, Professor, Department of Medicine, Queen's University
Dr. R. Brison, Professor, Department of Emergency Medicine, Queen's University
Dr. M. Evans, Community Member
Dr. S. Horgan, Manager, Program Evaluation & Health Services Development, Geriatric Psychiatry Service, Providence Care, Mental Health Services Assistant Professor, Department of Psychiatry
Ms. J. Hudacin, Community Member
Dr. B. Kisilevsky, Professor, School of Nursing, Departments of Psychology and Obstetrics and Gynaecology, Queen's University
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Dr. B. Simchison, Assistant Professor, Department of Anaesthesia and Perioperative Medicine, Queen's University
Dr. A.N. Singh, WHO Professor in Psychosomatic Medicine and Psychopharmacology Professor of Psychiatry and Pharmacology Chair and Head, Division of Psychopharmacology, Queen's University

has reviewed the request for renewal of Research Ethics Board approval for the project Development of a Functional Assay for Detection of BRCA1 Carriers as proposed by Dr. Scott Davey of the Cancer Research Institute, at Queen's University. The approval is renewed for one year, effective September 16, 2012. If there are any further amendments or changes to the protocol affecting the participants in this study, it is the responsibility of the principal investigator to notify the Research Ethics Board. Any unexpected serious adverse event occurring locally must be reported within 2 working days or earlier if required by the study sponsor. All other adverse events must be reported within 15 days after becoming aware of the information.

[Signature]
Date: September 24, 2012

Chair, Research Ethics Board
Renewal [ ] Renewal 2 [X ] Extension [ ] Code# PATH-115-10 Romeo file# 6005745