CHARACTERIZATION OF ZHX1 IN AXILLARY LYMPH NODE-NEGATIVE BREAST CANCER

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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ABSTRACT

Women with breast cancer without local metastasis to the axillary lymph nodes (axillary lymph node-negative, ANN) have a good prognosis. However, 20 to 30% of patients with ANN breast cancer will still experience recurrence and distant metastases. Lymphatic invasion (LVI) is an important prognostic factor for ANN breast cancer. Zinc fingers and homeoboxes 1 (ZHX1) was identified as a candidate gene involved in LVI and associated with early recurrence of ANN breast cancer. I examined expression of ZHX1 in breast cancer cell lines and ANN breast tumour samples and discovered that it is expressed at variable levels. I also investigated ZHX1 copy number and determined that amplification does not appear to be a mechanism of its over-expression. From bioinformatic and proteomic analyses, ZHX1 was discovered to potentially be phosphorylated. Overall, these studies suggest that ZHX1 may be involved in ANN breast cancer.
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ABBREVIATIONS

ANN  axillary lymph node-negative
APPL2  adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 2
ASNS  asparagine synthetase
ATAD2  ATPase family, AAA domain containing 2
ATM  ataxia telangiectasia mutated
ATR  ATM- and Rad3-related
BSA  bovine serum albumin
C_{2}H_{2}  Cys_{2}His_{2}
CDC25C  cell division cycle 25 homolog C
CDK1  cyclin-dependent kinase 1
CDK2  cyclin-dependent kinase 2
CHEK1  checkpoint kinase 1
CHEK2  checkpoint kinase 2
ChIP  chromatin immunoprecipitation
DMEM  Dulbecco’s Modified Eagle’s Medium High Glucose with L-glutamine
DNMT3B  DNA (cytosine-5-)methyltransferase 3 beta
ECL  enhanced chemiluminescence
EGFP (eGFP)  enhanced green fluorescent protein
FAM83A  family with sequence similarity 83, member A
FBS  fetal bovine serum
FFPE  formalin-fixed paraffin-embedded
GAPDH  glyceraldehyde 3-phosphate dehydrogenase
HD  homeodomain
HDAC  histone deacetylase
HER2  human epidermal growth factor receptor 2
HPRT1  hypoxanthine ribosyltransferase
HRP  horseradish peroxidase
IHC  immunohistochemistry
IL-2  interleukin-2
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>LVI</td>
<td>lymphatic invasion</td>
</tr>
<tr>
<td>LVI+</td>
<td>ANN breast tumours with lymphatic invasion</td>
</tr>
<tr>
<td>LVI-</td>
<td>ANN breast tumours without lymphatic invasion</td>
</tr>
<tr>
<td>M-MLV</td>
<td>Moloney Murine Leukemia Virus</td>
</tr>
<tr>
<td>MSH</td>
<td>Mount Sinai Hospital</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NF-γ</td>
<td>nuclear transcription factor gamma</td>
</tr>
<tr>
<td>NF-YA</td>
<td>nuclear transcription factor gamma, alpha subunit</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide-3 kinase</td>
</tr>
<tr>
<td>PRKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PRKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>p53 (TP53)</td>
<td>tumour protein 53</td>
</tr>
<tr>
<td>P/S</td>
<td>penicillin-streptomycin</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering ribonucleic acid</td>
</tr>
<tr>
<td>SLRI</td>
<td>Samuel Lunenfeld Research Institute</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline with 0.1% TWEEN® 20</td>
</tr>
<tr>
<td>TMA</td>
<td>tissue microarray</td>
</tr>
<tr>
<td>ZHX1</td>
<td>zinc fingers and homeoboxes 1</td>
</tr>
<tr>
<td>ZHX2</td>
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1 INTRODUCTION

1.1 Cancer

Cancer is a disease in which abnormal cells grow uncontrollably and have the ability to spread to and invade other tissues [1]. Genetic and epigenetic changes in genes of important biological processes, such as those that control cell growth, division, and differentiation, accumulate to cause cancer [2]. Therefore, it is of great interest to identify genes and associated signalling pathways that may be involved in carcinogenesis.

1.1.1 The Cell Cycle and Cancer

Dividing cells progress through two major stages, M phase and interphase, which comprise the cell cycle. M phase consists of mitosis and cytokinesis, while interphase is the interval between cell divisions. Interphase is divided into three phases: G1, S, and G2. The cell grows during both G1 and G2, while S involves replication of DNA and chromosomes. [3]

Under normal conditions, cells in which their DNA is damaged during G1 are arrested to allow DNA repair and prevent the damaged template from being replicated. Unrepaired DNA damage causes the DNA damage checkpoint kinases, ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) to be activated. Activation of tumour protein 53 (p53) by ATM/ATR and checkpoint kinases 1 and 2 (CHEK1 and CHEK2, respectively) inhibits the cyclin-dependent kinase 2 (CDK2)-cyclin E complex, which is responsible for progression into S-phase. [4]

Cells that undergo DNA damage during G2 or enter G2 with damage acquired during G1 or S phase without repair are arrested at the G2 (also known as the G2/M) checkpoint [5]. Activated ATR phosphorylates CHEK1, which phosphorylates cell division cycle 25 homolog C (CDC25C) at Ser-216 to inhibit it and therefore prevent dephosphorylation of cyclin-dependent kinase 1 (CDK1) and entry into M phase [4]. Cells with errors in DNA replication or chromosome segregation are also arrested at this checkpoint [4].

In normal tissues, cell number is sustained by careful control of the production and release of growth-promoting signals that direct entry and progression through the cell growth-and-division cycle [6]. However, cancer cells deregulate these signals, allowing sustained proliferation, which is the most fundamental trait of cancer cells [6]. The chance of malignant transformation
is enhanced by mutations in mitotic-checkpoint pathways, as this can allow cells with genomic abnormalities to survive or continue to grow [5].

1.1.2 Oncogenes and Tumour Suppressors

Oncogenes are often amplified, over-expressed, or mutated in tumours [7, 8]. Many oncogenes promote growth and may provide resistance to apoptosis [8, 9]. On the other hand, tumour suppressors regulate a wide variety of cellular processes, including cell cycle checkpoint responses and migration, and become inactivated in numerous sporadic cancers [10].

1.1.2.1 Tumour Suppressor Inactivation

Knudson hypothesized that two mutational events are required for retinoblastoma [11]. In inherited forms of cancer, one of these ‘hits’ is acquired in the germ line, while the second ‘hit’ is acquired somatically during tumour development [11, 12]. In haploinsufficient cases, a single ‘hit’ leads to accelerated tumourigenesis [12]. Both alleles of a tumour suppressor gene are often mutated in cancer [13].

1.2 Breast Cancer

Breast cancer is the second most frequent cause of cancer death in North American women. Approximately 12% of women are estimated to develop breast cancer in their lifetime and about 3% are expected to die from it. [14, 15]

The number of involved axillary lymph nodes is the most important prognostic factor for early breast cancer [16]. The majority of breast cancer patients in North America present without local metastatic spread to the axillary lymph nodes [16]. Women with ANN breast cancer have a good prognosis [17]. However, 20 to 30% of these patients will still experience recurrence and distant metastases [18].

1.2.1 Studies on Molecular Alterations Involved in Prognosis

Age, tumour size, lymph node status, histologic type of the tumour, and pathological grade are the main prognostic factors in breast cancer [19]. Another important independent prognostic factor for ANN breast cancer is lymphatic invasion (LVI), which refers to the presence of tumour cells within lymphatic and/or vascular capillaries around the tumour [20]. LVI may increase the
probability of occult systemic metastasis and is associated with an increased risk of recurrence [20].

In addition to the above-mentioned characteristics, there are many molecular alterations associated with breast cancer prognosis, such as hormone receptor and TP53 status [19, 21]. Amplification and/or over-expression of the growth factor receptor and proto-oncogene, neu/erbB-2 (also known as human epidermal growth factor receptor 2, HER2), is correlated with poor prognosis [16]. van’t Veer and colleagues developed a 70-gene poor prognosis signature via expression microarray analysis [19, 22, 23]. It was generated from 78 sporadic tumours from ANN patients under 55 years at diagnosis. They determined that several genes involved in cell cycle, invasion, metastasis, and angiogenesis were up-regulated.

1.2.1.1 TP53

p53 is a transcription factor that regulates many essential cellular processes, including cell cycle arrest [24]. Overcoming cell cycle arrest without repair is an important feature of cancer progression [24]. Numerous cellular stresses, such as DNA damage and oncogene activation, can cause mutations and other errors that can result in cancer [24]. p53 is involved in checkpoint control, as it responds to these stresses by arresting the cell cycle at both the G1 and G2 phases [24, 25]. For example, p53 represses expression of the cell cycle progression genes, CDC25C and CDK1, preventing cells from entering mitosis [26].

TP53 is a tumour suppressor gene and is found to be mutated in 50% of cancers, making it one of the most commonly mutated genes in human malignancies [27]. Most somatic TP53 mutations are missense changes [21]. These include transitions or transversions due to DNA damage, or deletions or insertions that may be caused by DNA damage or errors during replication [28]. These mutations have been found to be the major mechanism of TP53 inactivation [29], as they may cause a loss of p53’s tumour suppressor function and gain of oncogenic activity [28].

In breast cancer, some studies have found TP53 mutations to be an important predictor of early recurrence and death [21]. Twenty-five percent of breast cancers contain TP53 mutations, of which 15% are G:C → T:A transversions, 16% are G:C → A:T transitions at non-CpG sites, and 11% are A:T → G:C transitions [28].
1.2.1.2 Characterization of a Toronto Cohort

To identify genes and pathways that may be associated with prognosis of ANN breast cancer patients, a cohort of 1902 ANN patients from nine hospitals in Toronto was assembled by Dr. Andrulis and colleagues [16, 30], with 250 of these patients eventually experiencing a distant recurrence. Fresh frozen tumour samples were acquired from 977 ANN patients with their informed consent and approval from the Mount Sinai Hospital (MSH) Research Ethics Board. RNA was extracted from 135 of these tumours based on their LVI, recurrence, HER2, or TP53 status and used for gene expression microarray studies. To further validate differential expression, tissue microarrays (TMAs) were constructed from 888 patients from the original cohort, of which tumours from 815 of these patients were independent of those used for the expression arrays.

The gene expression microarray studies were performed and analyzed statistically in collaboration with Dr. Shelley B. Bull’s group, to examine the expression of genes that may be associated with ANN breast cancer and their correlation with clinical characteristics. Human 19K cDNA microarrays (18 981 cDNAs/EST clones) manufactured by the University Health Network Microarray Centre at the Ontario Cancer Institute (http://microarrays.ca) were used to analyze RNA from the tumour specimens.

Supervised statistical analyses and hierarchical clustering were performed on gene clone IDs using BRB ArrayTools software (http://linus.nci.nih.gov/BRB-ArrayTools.html). Student’s t-test, prediction analysis of microarrays, significance analysis of microarrays, and several other statistical tests were performed to rank genes based on the relative amount of differential expression between groups, based on clinical or molecular characteristics.

From these analyses, Andrulis and colleagues determined that neu/erbB-2 amplification increases the risk of recurrence of ANN breast cancer by more than two-fold [16]. They also concluded that TP53 mutation may aid in identifying patients with neu/erbB-2 amplification at greater risk of recurrence and death [30]. More specifically, they discovered that the presence of missense mutations of TP53, particularly in combination with neu/erbB-2 amplification, is a strong prognostic indicator for disease-free survival, compared to truncating mutations or wild-type TP53 [29]. However, identifying multiple genes that are predictive of recurrence may aid in developing improved prognostic indicators.
Since LVI is an important independent prognostic factor for ANN breast cancer [20], to identify associated genes, tumours were chosen to have similar distributions of age at diagnosis, estrogen and progesterone receptor proportions, tumour size and grade, HER2 amplification, and TP53 status, between the LVI- and LVI+ groups. Sample size calculations by Dr. Bull’s group suggested that 15 to 40 subjects would be required per group to provide statistical power to detect significant differences between samples from the groups in each comparison of clinical or molecular characteristics. In comparison to the LVI- group, the LVI+ group was over-sampled to improve the statistical power of differential expression. To identify genes independent of LVI and associated with early recurrence (within four years), tumours were selected to have similar numbers of LVI- tumours between the early recurrence and disease-free (for at least 10 years) groups.

Since LVI is associated with an increased risk of recurrence [20], genes were also ranked based on the amount of differential expression between 1) LVI- and LVI+ tumours and 2) tumours from patients who experienced an early recurrence of ANN breast cancer versus those who were free of disease for at least 10 years. A list of candidate genes from both comparisons was compiled. Genes involved in transcription, transport or signalling, DNA repair, metabolism, protein degradation, oxidative stress, RNA processing or translation, and cell structure were significantly up-regulated in LVI+ tumours and those from patients who experienced an early recurrence. I selected ZHX1 for further study, as it was significantly up-regulated in LVI+ tumours (p = 6x10^{-7}, t-test) and those from patients who experienced an early recurrence (p = 4.47x10^{-3}, t-test).

1.2.2 ZHX1 is a Transcription Factor

ZHX1 is one of three members of the ZHX gene family. It contains two zinc fingers and five homeobox DNA-binding domains (HDs) and is believed to be a transcription factor that can repress gene expression. [31-34]

Transcription factors can be divided into two functional classes: general transcription factors that associate with RNA polymerase and bind at core promoter sites, and sequence-specific transcription factors that bind to regulatory sites of specific genes. The expression of several different genes may be controlled by a single transcription factor, as the latter may bind to
Numerous sites. Numerous possible combinations of interactions between transcription factors are possible, which may also affect transcription of multiple genes. [3]

Two domains are usually found in transcription factors: a DNA-binding domain that binds to a specific DNA sequence and an activation domain that interacts with other proteins to regulate transcription. Many transcription factors bind other proteins of identical or similar structure to form dimers, a property which is common to several different types of transcription factors and is believed to be important in regulating gene expression. [3]

Proteins of the zinc finger class of transcription factors contain HDs as well as Cys2His2 (C2H2)-type zinc fingers [35]. The zinc finger motif is one of the most common DNA-binding proteins in eukaryotes [3, 36, 37]. The distinct combination of zinc fingers in a transcription factor allows it to recognize a specific DNA sequence [37]. Binding to a specific region of DNA and regulating gene expression is most likely the primary role of C2H2 zinc finger proteins [38]. It is common for these proteins to form homo- or hetero-dimers to bind to proteins via their fingers [38].

The HD family of transcription factors is one of several major superfamilies of proteins [39]. This family regulates cellular commitment and differentiation in plant, fungal, invertebrate, and vertebrate systems [39]. Members contain a sixty amino acid sequence conserved during evolution, which is known as a HD [40]. Although the HD was originally reported to bind DNA [41], it is known as an interaction domain with other transcription factors or molecules involved in signal transduction pathways [42]. Homeobox genes can be considered a family of nuclear proto-oncogenes, since several have been found to mutate to oncogenes and induce transformation in vitro [43].
1.2.2.1 Subcellular Localization of ZHX1

Amino acids 734 to 768 of ZHX1 encode a nuclear localization signal (NLS; Figure 1) [31]. Nuclear localization was confirmed in HEK293 cells via use of a green fluorescent protein fused to the C-terminus of full-length ZHX1 [42]. The N-terminal region of ZHX1 (containing the zinc finger domains) diffusely localized to the cytoplasm and nuclei, while the C-terminal region (containing the HDs and NLS) mostly localized to the nuclei of HEK293 cells [34].

In a podocyte disease state, over-expression of a ZHX protein or knock down of its binding partner caused an increase in its nuclear localization. It has been hypothesized that this is due to a loss of heterodimerization, which causes individual ZHX proteins to migrate into the nucleus, as each contains a NLS. [44]

![Figure 1](image.png)

**Figure 1.** Schematic representation of the structure and functional domains of ZHX1. Znf: zinc finger motif, HD: homeodomain, R: arginine-rich region, Ac: acidic region, ID: interaction domain with the alpha subunit of nuclear transcription factor gamma, RD: repressor domain, DD: dimerization domain, NLS: nuclear localization signal. The acidic region is responsible for repressive activity. From [31].
1.2.2.2 Candidate Pathways Involving ZHX1

To determine potential biological functions of ZHX1, I used the software program, Cytoscape, designed by our collaborator, Dr. Gary Bader, and his colleagues, which visualizes published interactions [45]. I discovered that ZHX1 has several potential interactions (Figure 2). It forms homodimers and has also been found to bind to both ZHX2 and ZHX3 [32, 33, 42]. The latter complex is required for ZHX3 to repress transcription [32, 33, 42]. Dimerization of ZHX1, via amino acids 272 to 432, is required for its full repressive activity [31] (Figure 1). Amino acids 831 to 873 encoding an acidic region are required for its repressor activity [31] (Figure 1).

All three members of the ZHX gene family bind to the alpha subunit of nuclear transcription factor gamma (NF-YA) [32, 33]. Amino acids 272 to 564, which encode the HD1 and HD2 regions of ZHX1 (Figure 1), are required for interaction with amino acids 31 to 140 of the activation domain of NF-YA [46]. Nuclear factor gamma (NF-γ) activates transcription of several genes, including CDC25C [32]. However, it was discovered that ZHX2 represses promoter activity of CDC25C, which would prevent cells from progressing into M phase [32]. Since ZHX1 binds to both ZHX2 and NF-γ, this suggests that ZHX1 may be involved in the cell cycle.

In podocytes, over-expression of ZHX1 decreased expression of collagen type IV α3 (a matrix gene), aminopeptidase A and dystroglycan (podocyte surface protein genes), and paired box 2 (a transcription factor) [44]. This may indicate that ZHX1 is involved in invasion or oncogenesis.

ZHX1 expression is induced by interleukin-2 (IL-2) in lymphoid cells [47]. IL-2 regulates growth and/or differentiation of lymphocytes, as well as monocytes and some hemopoietic cells [47]. It activates three major signalling pathways: the Ras and mitogen-activated protein kinase cascade, which is involved in mitogenesis; the phosphoinositide-3 kinase (PI3K) pathway, which is involved in cytoskeleton reorganization, and the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway [47].

The JAK/STAT and PI3K pathways were discovered to be involved and de novo RNA and protein synthesis required for induction by IL-2 of mouse ZHX1 gene expression in the cytotoxic T cell line, CTLL-2 [48]. IL-2 increased the half-life of ZHX1, which may be involved in initiating cell proliferation [48].
Figure 2. A model of the potential role of ZHX1 in the cell cycle. 1) ZHX1 forms homodimers. 2) ZHX1 has also been found to bind to the other two members of the ZHX family, ZHX2 and ZHX3, which is required for ZHX3 to repress transcription. 3) All three members bind to the activation domain of the alpha subunit of nuclear transcription factor γ (NF-γ). 4) NF-γ activates transcription of several genes, including CDC25C. 5) ZHX2 represses promoter activity of CDC25C, which would prevent cells from progressing into M phase (6).
It is likely that the HDs of ZHX1 also interact with other transcription factors or molecules involved in signal transduction pathways [31]. For example, the HDs of ZHX1 interact with DNA (cytosine-5-)methyltransferase 3 beta (DNMT3B) [34], which adds methyl groups to DNA and is strongly correlated with gene repression [3]. This interaction enhances the repressive activity of DNMT3B, but does not further contribute to that of ZHX1 [34]. In some cancers, DNMT3B may be targeted to DNA regions that normally are not involved with transcriptionally repressive heterochromatin formation [49].

There are two forms of chromatin: highly condensed heterochromatin and less condensed euchromatin [50]. The N-terminal tails of histones can be covalently modified to affect transcription [50]. Gene repression occurs when proteins that bind methylated DNA interact with chromatin remodelling complexes and histone deacetylases (HDACs) that condense chromatin. HDACs, which are subunits of larger co-repressor complexes, remove acetyl groups from histones [3]. Deacetylation accompanies methylation of the lysine residue at position 9 of histone H3 molecules [3]. It is believed that deacetylation allows nucleosomes to pack more tightly together, which results in gene repression [50].

There is a possibility that transcriptional repression could be enhanced via ZHX1 recruiting other co-repressors, such as HDACs, through protein-protein interactions [34]. Therefore, ZHX1 may be a component of multiple transcription factor networks and as a result, be involved in a variety of cellular processes [34].
1.2.2.2.1 CDC25C, A Downstream Candidate Target of ZHX1

CDC25C is one of three members of the CDC25 family [51]. This protein is negatively regulated via phosphorylation at Ser-216 by CHEK1, which is phosphorylated by ATR. ATM activates CHEK2, which also phosphorylates CDC25C [4].

CDC25C becomes activated at the onset of M phase (after being inactive during most of the cell cycle) via dephosphoryation of Ser-216 and phosphorylation of Ser-285 and other activating serine/threonine sites [52, 53]. CDC25C then dephosphorylates the cyclin B/CDK1 complex, which is a key step of M-phase entry [51].

The cell cycle can be regulated at the G1/S or G2/M checkpoints [5]. The mitosis-promoting activity of the cyclin B/CDK1 complex is a critical target of the G2 checkpoint [5]. Cells are arrested in G2 when the CDC25 family of dual-specificity phosphatases is inhibited, as they can no longer activate CDK1 at the G2/M transition via dephosphorylation [5, 51].

1.2.2.2 Protein Kinases A and C (PRKA and PRKC), Candidate Regulators of ZHX1

Since ZHX1 may be involved in the cell cycle, where regulation frequently involves numerous kinases and phosphatases, I used reported algorithms to predict potential sites of phosphorylation and the kinases that phosphorylate them. PRKA and PRKC were strongly predicted to phosphorylate ZHX1 at Ser-7 and 869 and at Thr-617 and Ser-763, respectively. [54, 55]

1.2.2.2.1 PRKA

PRKA is a serine/threonine kinase that transduces signals from many hormones and G protein-coupled receptors to regulate physiological responses, such as transcription [56, 57]. The cellular localization of PRKA determines which substrates are phosphorylated [57]. PRKA also promotes mitosis via destruction of mitotic cyclins and separation of sister chromatids during the anaphase-metaphase transition [58].
1.2.2.2.2 PRKC

PRKC is a family of closely related isoenzymes, which are products of distinct genes [59]. Like PRKA, PRKC is a serine/threonine kinase [56]. PRKC transduces many mitogenic signals sent by growth factors such as platelet-derived growth factor and epidermal growth factor, and phosphorylates several substrates [59]. Alterations in PRKC activity in the epithelium lead to growth in melanocytes or transformation [59]. *PRKC* is over-expressed in breast cancer [59]. An increase in PRKC activity has been suggested to correlate with enhanced proliferation and oncogenicity [59]. Activated PRKC also protects against apoptosis [59], which could allow cancer cells to be maintained.

1.2.2.3 Amplification in Breast Cancer

Whole or portions of chromosomes may be gained or lost in cancer or pre-cancerous tissues. Aberrations often contain multiple genes, of which more than one may be important. For example, highly amplified regions containing known oncogenes may span a few megabases but consist of multiple genes that contribute to tumour progression. Alterations may also span tens to hundreds of megabases and may affect hundreds to thousands of genes. [60]

*ZHXI* is located on 8q, for which gains and amplifications are frequently observed in a number of cancers, such as breast, colon, and bladder [61]. Changes in copy number explain a significant proportion of variation in gene expression, and integrating copy number changes and gene expression may improve prediction of prognosis [62].

Interestingly, previous work in our laboratory identified gains of 8q24.13, where *ZHXI* is located, in two breast cancer cell lines, which is consistent with published literature [63, 64], and some ANN tumour samples. Gains of this region have been observed in 53% of breast cancer tumours [63]. Genes in 8q24.13 have also been found to be over-expressed [62, 63], which significantly correlates with amplification [62]. Since *ZHXI* is over-expressed and may be amplified, it may be oncogenic and therefore, an important prognostic factor in ANN breast cancer.
1.3 Rationale and Objectives

1.3.1 Rationale

Since LVI is an important prognostic factor within ANN breast cancer [20], I incorporated LVI status in my study to provide a unique approach to identify novel genes related to outcome in ANN breast cancer. This may aid in determining which patients benefit from systemic therapy and identifying novel targets for cancer therapeutics.

1.3.2 Hypothesis

ZHX1 may be over-expressed and related to altered cellular growth in ANN breast cancer.

1.3.3 Specific Aims

1) To examine expression of ZHX1 in breast cancer cell lines and primary ANN breast tumour samples.

2) To investigate the role of ZHX1 and its associated pathway(s) in breast cancer through in vitro functional studies.
2 MATERIALS AND METHODS

2.1 Tumour Samples

ANN breast tumours were tissue sampled and snap frozen at -80°C by pathologists. Tumours were stored in liquid nitrogen until DNA and RNA isolation. DNA was extracted by conventional techniques [65]. Total RNA was extracted from tumour tissue using TRIzol® Reagent (Invitrogen Corporation, Burlington, ON). The amount and quality of RNA were assessed using the UltraSpec™ 2100 pro (GE Healthcare Life Sciences, Buckinghamshire, England), NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE), and 1% agarose gels.

2.2 Quantitative Real-time Polymerase Chain Reaction (qPCR)

Genomic DNA from the tumour samples was used to determine copy number via qPCR. Singleplex qPCR using the Power SYBR® Green PCR System (Applied Biosystems, Foster City, CA) was performed with the standard curve method to determine the copy number ratios of the target gene, ZHX1, and its flanking genes, family with sequence similarity 83, member A (FAM83A), and ATPase family, AAA domain containing 2 (ATAD2), to the control genes, asparagine synthetase (ASNS) and adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 2 (APPL2), using the 7900HT Sequence Detection System (Applied Biosystems). ASNS and APPL2 are located on different chromosomes, in regions not commonly altered in breast cancers [16, 66]. Placental DNA from a pool of human females (Sigma-Aldrich Corporation, St. Louis, MO) was used to generate the standard curves and genomic DNA from MCF7 and T47D breast cancer cell lines were used as controls.

mRNA expression levels in the tumour samples were also quantified via qPCR. ZHX1 mRNA expression levels were also quantified in a human mammary epithelial cell line, MCF-10A, and breast cancer cell lines, Hs 578T, MCF7, MDA-MB-231, SK-BR-3, and T47D, to select a cell line for functional studies. The MOLT-4 cell line was used to generate the standard curves. Singleplex qPCR using the Power SYBR® Green PCR and the 7900HT Sequence Detection Systems was performed and the standard curve method used to determine the mRNA expression levels of ZHX1 and the housekeeping gene, hypoxanthine ribosyltransferase (HPRT1). Approximately 1 µg of total RNA from each tumour or cell line was used to generate cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) or M-MLV
(Moloney Murine Leukemia Virus) Reverse Transcriptase (200U/µL; Invitrogen Corporation), respectively, according to the manufacturer’s instructions.

Primers were designed with the aid of Primer3 Input (http://frodo.wi.mit.edu/primer3/) and PrimerExpress (Applied Biosystems) (Tables 1 and 2). SDS version 2.2.2 (Applied Biosystems) was used for analysis of signal intensities.

**Table 1.** Primer sequences for examining copy number in genomic DNA.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Primer Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ZHX1</strong></td>
<td>Forward: 5’-TGACACCAGCAGCAGTTCTT-3’&lt;br&gt;Reverse: 5’-CAGCATTTGAGGTGGGAATG-3’</td>
</tr>
<tr>
<td><strong>FAM83A</strong></td>
<td>Forward: 5’-ACCTGGCCTCTGCGGACAC-3’&lt;br&gt;Reverse: 5’-GGACTTCAGGCCCCATCACAG-3’</td>
</tr>
<tr>
<td><strong>ATAD2</strong></td>
<td>Forward: 5’-TGCAATGATGGCAAATGCA-3’&lt;br&gt;Reverse: 5’-TGACGGTAGTAATTCC TTCTTTGGTT-3’</td>
</tr>
</tbody>
</table>

**Table 2.** Primer sequences for examining mRNA expression levels.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Primer Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ZHX1</strong></td>
<td>Forward “A”: 5’-GTGGCAAGTGATTGGAGTG-3’&lt;br&gt;Reverse “A”: 5’-GCAGCTTTTCTCATGGTGCAA-3’</td>
</tr>
<tr>
<td><strong>HPRT1</strong></td>
<td>Forward: 5’-GACCAGTCAACAGGGGACATA-3’&lt;br&gt;Reverse: 5’-CCAAGGAAAGCAAGTCTGC-3’</td>
</tr>
</tbody>
</table>
2.3 Immunohistochemistry (IHC) Using TMAs

In collaboration with pathologists, Drs. Anna Marie Mulligan and Linda Feeley, ZHX1 protein levels and location were determined by IHC using a 1:200 dilution of rabbit polyclonal anti-ZHX1 antibody (Abcam Inc, Cambridge, MA). IHC was performed on breast cancer tumours that were formalin-fixed paraffin-embedded (FFPE) and arrayed on TMAs (MSH Special Histology Department) as described in [67]. All antibody optimization and tissue staining was performed by Gordana Luruzar (MSH Special Histology Department) after consultation with Drs. Alina Bocicariu and Mulligan.

2.4 Cell Culture

The SK-BR-3 breast cancer cell line was cultured in McCoy’s 5A Medium with L-glutamine supplemented with 10% fetal bovine serum (FBS) and a 1:100 dilution of penicillin-streptomycin (P/S; Gibco Products, Grand Island, NY). HEK293, HeLa, and murine NIH/3T3 cells were grown in Dulbecco’s Modified Eagle’s Medium High Glucose with L-glutamine (DMEM; Gibco) supplemented with 10% FBS and a 1:100 dilution of P/S. The lung carcinoma cell line, A549, was cultured in RPMI 1640 (Samuel Lunenfeld Research Institute (SLRI) Media Preparation, Toronto, ON) supplemented with 10% FBS and a 1:100 dilution of P/S. The A549, NIH/3T3, and SK-BR-3 cell lines were purchased from American Type Culture Collection (Manassas, VA). The HEK293 and HeLa cell lines were kindly provided by Dr. Jeffrey Wrana (SLRI). All cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

2.5 Western Blot Analysis

Protein extracts were prepared as follows. Cells were washed three times with ice-cold phosphate-buffered saline (SLRI Media Preparation). They were then lysed with NETN lysis buffer (Nonidet® P40 (0.5%; BioShop Canada Inc., Burlington, ON), ethylenediaminetetraacetic acid (1 mM, pH 8.0; SLRI Media Preparation), Tris (20 mM, pH 7.5; SLRI Media Preparation), sodium chloride (150 mM; VWR International LLC, Mississauga, ON), protease inhibitor (1:100 dilution; Sigma-Aldrich Corporation), Phosphatase Inhibitor Cocktail 2 (1:100 dilution; Sigma-Aldrich Canada Ltd, Oakville, ON), Phosphatase Inhibitor Cocktail 3 (1:100 dilution; Sigma-Aldrich Corporation), phenylmethanesulfonyl fluoride (1 mM; Sigma-Aldrich Corporation)) for 9 minutes on ice. The cell lysates were centrifuged for 8 minutes at 12 000 rpm at 4°C in the Centrifuge 5415R (Eppendorf North America Inc., Westbury, NY). Protein concentrations were
determined with the Pierce® BCA Protein Assay Kit according to the manufacturer’s instructions (Pierce Biotechnology Inc., Rockford, IL). Twenty-five microgram of protein were loaded into each lane and separated via 7% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Proteins were transferred to Amersham Hybond™ ECL™ nitrocellulose membranes (GE Healthcare UK Ltd, Buckinghamshire, England) at 30V at 4°C overnight. All washes were performed with Tris-buffered saline (SLRI Media Preparation) with 0.1% TWEEN® 20 (Merck KGaA, Darmstadt, Germany; TBS-T) at room temperature on an orbital shaker. Membranes were blocked in 5% skim milk powder in TBS-T for 1 hour at room temperature on the orbital shaker.

Antibodies raised in rabbit against human ZHX1 (Bethyl Laboratories, Inc., Montgomery, TX; Sigma-Aldrich Canada, Ltd) were optimized as follows. Protein from HeLa cells were used as a positive control. Protein from A549, HEK293, and NIH/3T3 cells were used as negative controls, as they should express no or very low levels of ZHX1. Membranes were incubated with 1:2000 or 1:10 000 dilutions of polyclonal rabbit anti-ZHX1 antibodies in 5% skim milk powder in TBS-T at 4°C overnight on an orbital shaker. The membranes were then incubated in a 1:10 000 dilution of goat anti-rabbit IgG (H+L) HRP conjugate (Zymed Laboratories Inc., South San Francisco, CA) in 5% bovine serum albumin (BSA) in TBS-T for 1 hour at room temperature. SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology Inc.) and Hyperfilm™ ECL (GE Healthcare UK Ltd) were used for detection.

β-actin was used as a loading control (1:10 000 monoclonal anti-β-actin from mouse clone AC-74 (Sigma-Aldrich Canada Ltd) in 3% BSA in TBS-T; 1:10 000 HRP-conjugated affinity-purified donkey anti-mouse in 3% BSA in TBS-T (Jackson Immunoresearch Laboratories, Inc., West Grove, PA)).
2.6 Short Interfering RNA (siRNA) Transfection

SK-BR-3 cells were seeded in 6-well plates at a density of 80,000 cells/well and allowed to adhere for approximately 24 hours. They were then transfected with pre-designed siRNA oligonucleotides against different regions of exon three of ZHX1 (25 nM; J-018011-09-0005 and J-018011-10-0005, “ZHX1 siRNA 09” and “ZHX1 siRNA 10”, respectively) using Transfection Reagent 2 (1:500 dilution; Thermo Fisher Scientific, Lafayette, CO). Silencer® Select Negative Control #1 siRNA and Silencer® Select GAPDH Positive Control siRNA (25 nM each; Ambion, Inc., Austin, TX) were used as the scrambled siRNA negative and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) siRNA positive controls, respectively. To quantify knock down of GAPDH and ZHX1, RNA was isolated 24, 48, 72, and 96 hours after transfection, while protein was isolated 72 hours after transfection.

2.7 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Proliferation Assay

SK-BR-3 cells were simultaneously seeded at a density of 10,000 cells/well of 96-well plates and transfected with siRNA as for 6-well plates (see section 2.6 above), with a final volume of 100 µL. They were then incubated with 10 µL of MTT labelling reagent from the Cell Proliferation Kit I-MTT (Roche Diagnostics Corporation, Indianapolis, IN) 24, 48, 72, and 96 hours after transfection, for approximately 4 hours. One hundred microlitres of solubilization solution were added to each well. The Infinite® 200 reader (Tecan Group Ltd., Mannédorf, Switzerland) was then used to spectrophotometrically quantify the solubilized formazan products at 570 nm (with a reference wavelength of 690 nm).
3 RESULTS

3.1 Patient and Clinical Characteristics of a Subset of the Cohort

Patient and tumour characteristics of a 45-sample cohort subset that were used in copy number and expression analyses are illustrated in Table 3. 53.3% of the tumours were wild-type for TP53. 53.3% of the tumours were LVI-, which differs from the over-sampling of LVI+ tumours from the cohort used in the initial microarray analyses described in section 1.2.1.2. 66.7% of the tumours were from patients who did not experience a recurrence, in comparison to similar numbers of samples used in the microarray studies. Characteristics differed from those of the entire cohort, since the samples used for my analyses are only a small subset of the cohort for which DNA and RNA were available.

3.2 ZHX1 Copy Number in vitro and in vivo

As discussed in section 1.2.2.3, the genomic region at 8q24.13 containing ZHX1 exhibits copy number gains in some breast cancer cell lines and tumour samples, and genes in this region have been found to be over-expressed and significantly correlated with amplification [62-64]. Since changes in copy number is one mechanism of variation in gene expression, and integrating copy number changes and gene expression may improve prediction of prognosis [62], copy number of ZHX1 was first examined in established human breast cancer cell lines, MCF7 and T47D. These cell lines had previously been shown to have ZHX1 copy numbers that are unchanged or modestly amplified, respectively (Table 4). To confirm ZHX1 copy number, I used ASNS and APPL2 for normalization, since they are located on different chromosomes (Table 4) and are not commonly altered in breast cancer [16, 66].

I then determined the copy number ratio of ZHX1 to ASNS in a subset of tumour samples used for the microarray analyses. Most of the tumours did not exhibit a change in copy number of ZHX1, although a few appeared to have higher (tumours 2160 and 2544) or lower (tumours 1070, 1131, 1706, and 2253) copy number ratios (Figure 3).

To confirm ZHX1 copy number, I also used APPL2 for normalization. Most of the tumour samples still did not show any changes in copy number ratio (Figure 4). However, the few samples with higher (tumours 2160 and 2544) or lower (tumours 1131 and 2253) copy number ratios were consistent, regardless of whether ASNS or APPL2 were used for normalization.
During the process of gene amplification, genes in addition to those providing a selective growth advantage are amplified. Therefore, to investigate the size of any amplification unit, I examined copy numbers of *FAM83A* and *ATAD2*, which are centromeric and telomeric (Figure 5), respectively, to *ZHX1*, and may occur in the same amplicon [63]. A representative tumour sample with lower copy number ratio of *ZHX1* (tumour 2253) also had lower copy number ratios of *FAM83A* and *ATAD2* and a representative sample with higher ratio of *ZHX1* (tumour 2544) also had higher copy number ratios of these flanking genes (Figure 6).
Table 3. Patient and tumour characteristics of the 45 samples used in copy number and expression analyses. DNA from 25 tumour samples was used, while RNA was available from 43 tumour samples.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of Patients</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Menopausal status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-menopause</td>
<td>19</td>
<td>42.2</td>
</tr>
<tr>
<td>Peri-menopause</td>
<td>2</td>
<td>4.4</td>
</tr>
<tr>
<td>Post-menopause</td>
<td>23</td>
<td>51.1</td>
</tr>
<tr>
<td>Missing or not done</td>
<td>1</td>
<td>2.2</td>
</tr>
<tr>
<td><strong>Maximum size of tumour, cm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-2</td>
<td>12</td>
<td>26.7</td>
</tr>
<tr>
<td>2-5</td>
<td>27</td>
<td>60.0</td>
</tr>
<tr>
<td>&gt; 5</td>
<td>5</td>
<td>11.1</td>
</tr>
<tr>
<td>Missing or not done</td>
<td>1</td>
<td>2.2</td>
</tr>
<tr>
<td><strong>Estrogen receptor status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>26</td>
<td>57.8</td>
</tr>
<tr>
<td>Negative</td>
<td>16</td>
<td>35.6</td>
</tr>
<tr>
<td>Equivocal</td>
<td>1</td>
<td>2.2</td>
</tr>
<tr>
<td>Missing or not done</td>
<td>2</td>
<td>4.4</td>
</tr>
<tr>
<td><strong>Progesterone receptor status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>17</td>
<td>37.8</td>
</tr>
<tr>
<td>Negative</td>
<td>23</td>
<td>51.1</td>
</tr>
<tr>
<td>Equivocal</td>
<td>3</td>
<td>6.7</td>
</tr>
<tr>
<td>Missing or not done</td>
<td>2</td>
<td>4.4</td>
</tr>
<tr>
<td><strong>TP53 mutation status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>24</td>
<td>53.3</td>
</tr>
<tr>
<td>Mutated</td>
<td>18</td>
<td>40.0</td>
</tr>
<tr>
<td>Missing or not done</td>
<td>3</td>
<td>6.7</td>
</tr>
<tr>
<td><strong>LVI status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>24</td>
<td>53.3</td>
</tr>
<tr>
<td>Positive</td>
<td>20</td>
<td>44.4</td>
</tr>
<tr>
<td>Missing or not done</td>
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<td>2.2</td>
</tr>
<tr>
<td><strong>Histologic grade</strong></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>6.7</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>3</td>
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</tr>
<tr>
<td>Missing or not done</td>
<td>9</td>
<td>20.0</td>
</tr>
<tr>
<td><strong>Recurrence status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>14</td>
<td>31.1</td>
</tr>
<tr>
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<td>66.7</td>
</tr>
<tr>
<td>Missing or not done</td>
<td>1</td>
<td>2.2</td>
</tr>
</tbody>
</table>
**Table 4.** Copy numbers of *ZHX1*, *ASNS*, and *APPL2* and ratios in breast cancer cell lines. Adapted from [68].

<table>
<thead>
<tr>
<th>Cell Line (Chromosomal Location)</th>
<th><em>ZHX1</em> (8q24.13)</th>
<th><em>ASNS</em> (7q21.3)</th>
<th><em>ZHX1</em>/<em>ASNS</em></th>
<th><em>APPL2</em> (12q24.1)</th>
<th><em>ZHX1</em>/<em>APPL2</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7</td>
<td>5-6</td>
<td>4</td>
<td>1.25-1.5</td>
<td>4</td>
<td>1.25-1.5</td>
</tr>
<tr>
<td>T47D</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>
**Figure 3.** Determination of copy number of \(ZHX1\) in tumour samples from 25 ANN breast cancer patients. Genomic DNA from a pool of human females was used to construct the standard curves. Breast cancer cell lines, MCF7 and T47D, were used as controls. Copy numbers are reported as ratios to \(ASNS\). Bars indicate standard deviation (\(n = 2-6\)).
Figure 4. Confirmation of copy number of $ZHX1$ in tumour samples from six representative ANN breast cancer patients. Genomic DNA from a pool of human females was used to construct the standard curves. Breast cancer cell lines, MCF7 and T47D, were used as controls. Copy numbers are reported as ratios to $ASNS$ or $APPL2$. Bars indicate standard deviation ($n = 2-6$).
Figure 5. The locations of ZHX1 and FAM83A and ATAD2, which are centromeric and telomeric, respectively, to ZHX1, on 8q24.13. From [69].
Figure 6. Determination of copy numbers of \textit{ZHXL}, \textit{FAM83A}, and \textit{ATAD2} in tumour samples from four representative ANN breast cancer patients. Genomic DNA from a pool of females was used to construct the standard curves. Breast cancer cell lines, MCF7 and T47D, were used as controls. Copy numbers are reported as ratios to \textit{ASNS}. Bars indicate standard deviation (n = 2-6).
3.3 ZHX1 mRNA Expression in vitro and in vivo

To determine the level of ZHX1 expression in ANN breast tumour samples, expression of ZHX1 was first quantified, relative to HPRT1, in established human normal mammary epithelial and breast cancer cell lines. The level of ZHX1 expression varied over a wide range, with about a 12-fold difference between the MDA-MB-231 and SK-BR-3 cell lines, which expressed ZHX1 at the lowest and highest levels, respectively (Figure 7). Since SK-BR-3 cells highly expressed ZHX1, this cell line was chosen for functional studies (see section 3.7).

I then quantified ZHX1 mRNA expression in a subset of tumour samples used for the microarray analyses. Most of the tumours expressed ZHX1 at intermediate levels, while some expressed ZHX1 at levels as low as or lower than in MCF7 (in which ZHX1 copy number is unchanged) and some expressed ZHX1 at levels as high as or higher than in T47D (in which ZHX1 is modestly amplified) (Figure 8).

There did not appear to be a direct correlation between copy number and expression in the representative tumour samples examined, therefore, amplification is not likely a mechanism of over-expression of ZHX1.
Figure 7. Quantification of ZHX1 expression in a subset of human mammary epithelial and breast cancer cell lines. Expression was normalized to the housekeeping gene, HPRT1. Bars indicate standard deviation (n = 2-4).
Figure 8. Quantification of ZHX1 expression in tumour samples from 43 ANN breast cancer patients. Breast cancer cell lines, MCF7 and T47D, were used as controls. Expression was normalized to the housekeeping gene, HPRT1. Tumours for which ZHX1 copy number data is shown in previous figures are indicated by G (gain), L (loss), or NC (no change). Bars indicate standard deviation (n = 2-4).
3.4 ZHX1 Protein Levels and Localization in vivo

Since a limited number of tumours were available for validation of ZHX1 expression at the transcript level, IHC was performed by histopathologists on FFPE ANN breast cancer tumours arrayed on TMAs (described in section 2.3) to validate the expression data at the protein level and provide an independent dataset. This would allow protein levels, localization, and correlation with gene expression to be determined. The TMAs were scored by Dr. Feeley, who unfortunately observed that the staining appeared to be non-specific. Therefore, a better antibody would be required to quantify protein expression of the FFPE material.

3.5 ZHX1 and TP53 Status

As discussed in sections 1.2.1.1 and 1.2.2.2, ZHX1 may be involved in the cell cycle (through its interactions with ZHX2 and NF-γ [32, 33, 42, 46]), in which p53 is prominently involved and frequently mutated [24-27]. Since amplification of ZHX1 did not appear to be a mechanism of its over-expression, I hypothesized that TP53 mutation in ANN breast cancer tumours may be associated with high levels of ZHX1 expression.

Our lab previously identified TP53 mutations in ANN breast tumour samples [30]. Therefore, in collaboration with biostatistician Dr. Dushanthi Pinnaduwage from the Bull group, we examined whether ZHX1 expression is correlated with TP53 status. Tumours with a missense mutation of TP53 tend to have higher ZHX1 expression than those with wild-type TP53 (Figure 9). Since the distribution is not normal, the t-test is not an accurate measure of differential expression and a non-parametric test was therefore used. The difference in ZHX1 expression is borderline significant (p = 0.0813, Wilcoxon rank sum test), which suggests that ZHX1 expression may be associated with missense mutation of TP53.

![Figure 9. Distribution of tumours from 135 ANN breast cancer patients, based on TP53 status and expression of ZHX1.](image-url)
3.6 Optimization of Primary Antibody Conditions for Examining ZHX1 Protein Levels in vitro

To determine whether protein levels of ZHX1 correlate with gene expression, I first optimized two antibodies raised in rabbit against human ZHX1. Twenty-five micrograms of protein extracts from a subset of cell and breast cancer cell lines were separated by SDS-PAGE on a 7% polyacrylamide gel.

Many non-specific bands were observed using either antibody (Figure 10). Although the predicted size of ZHX1 is 98 kDa [70], there appeared to be two to three bands of about 130 kDa in size, of which one or two may represent ZHX1 and/or post-translationally modified forms of ZHX1. This slower migration of ZHX1 is in concordance with previous findings [71, 72]. Since the anti-ZHX1 antibody from Bethyl Laboratories seemed to be more promising (Figure 10a), I also tested a 1:10 000 dilution in 5% skim milk powder in TBS-T to reduce non-specific binding. Although loading was not equal, protein levels of ZHX1 appear to correlate with transcript levels (Figure 7).

![Figure 10](image)

**Figure 10.** Determination of protein levels of ZHX1 in a subset of breast cancer cell lines (BT474, MCF7, MDA-MB-231, SK-BR-3, and T47D). Twenty-five micrograms of protein from each cell line were loaded on 7% sodium dodecyl sulphate-polyacrylamide gels and run at 70V for about 3.5 hours. Extract from HeLa cells was used as a positive control, while extracts from A549, HEK293, and NIH/3T3 were used as negative controls. β-actin was used as a loading control. 1:2000 dilutions of rabbit anti-ZHX1 antibodies from a) Bethyl Laboratories, Inc., and b) Sigma-Aldrich Co., in TBS-T with 5% skim milk powder were used to block non-specific binding to the membranes and detect ZHX1.
3.7 Knock Down of ZHX1 in vitro

To confirm the identity of the correct band representing ZHX1 and to examine the potential role of ZHX1 in the cell cycle, I knocked down the expression of ZHX1 in the breast cancer cell line, SK-BR-3, which expresses ZHX1 at high levels (Figure 7). After optimization of transfection protocols, seeding densities, and culture vessels, I seeded each well of 6-well plates with 80,000 cells and transfected them with two siRNA oligonucleotides targeting different coding regions of exon three of ZHX1.

At least 80% and 60% knock down of the positive control gene, GAPDH, and ZHX1, respectively, was confirmed via qPCR (Figures 11 and 12, respectively). Knock down of GAPDH appeared to transiently decrease ZHX1 expression at 24 and 48 hours post-transfection for unknown reasons (Figure 12). All bands around 130 kDa decreased in intensity in the SK-BR-3 cells transfected with the two siRNA oligonucleotides targeting ZHX1 (Figure 13).

Since phosphorylation by kinases is important in the cell cycle and DNA damage repair [4], I added or omitted phosphatase inhibitors when lysing the cells to isolate protein, of which the latter condition would allow phosphatases to remove any potential phosphate groups that would be represented by larger bands. The latter treatment resulted in the largest bands around 130 kDa decreasing in intensity (Figure 14). Therefore, ZHX1 was knocked down at both the transcript and protein level, is about 130 kDa in size, and appears to be phosphorylated.

To examine downstream effects of ZHX1 in the cell cycle, Western blot analysis was performed to observe protein levels of CDC25C. However, CDC25C levels were too low to be detected. Therefore, I did not pursue further investigation of downstream effects on CDC25C, as it was not possible to assess expression and would be difficult to interpret.

To determine if ZHX1 has a role in proliferation, I wished to perform an MTT proliferation assay (described in section 2.7) on siRNA-transfected SK-BR-3 cells in 96-well plates. I was encouraged that one siRNA affected proliferation (Figure 15), however, an inadequate amount of RNA was available to confirm knock down of ZHX1. Therefore, the experiment must be repeated before any definitive conclusions can be made.
Figure 11. Confirmation of knock down of the positive control gene, \textit{GAPDH}, in SK-BR-3 cells transfected with siRNA. Expression was normalized to the housekeeping gene, \textit{HPRT1}. One representative experiment is shown. Similar results were acquired in two independent experiments. Bars indicate standard deviation (n = 2).
Figure 12. Confirmation of knock down of ZHX1, in SK-BR-3 cells transfected with siRNA targeting different regions of exon three. Expression was normalized to the housekeeping gene, HPRT1. One representative experiment is shown. Similar results were acquired in two independent experiments. Bars indicate standard deviation (n = 2-6).
Figure 13. Confirmation of knock down of ZHX1 in SK-BR-3 cells transfected with siRNA targeting different regions of exon three for 72 hours. Twenty-five micrograms of protein extracts from each condition were loaded on 7% sodium dodecyl sulphate-polyacrylamide gels and run at 70V for about 3.5 hours. β-actin was used as a loading control. A 1:10 000 dilution of rabbit anti-ZHX1 antibody (Bethyl Laboratories, Inc.) in TBS-T with 5% skim milk powder was used. One representative experiment is shown. Similar results were acquired in two independent experiments.
Figure 14. ZHX1 may be phosphorylated in SK-BR-3 cells. Twenty-five micrograms of protein from each cell line were loaded on 7% sodium dodecyl sulphate-polyacrylamide gels and run at 70V for about 3.5 hours. β-actin was used as a loading control. A 1:10 000 dilution of rabbit anti-ZHX1 antibody (Bethyl Laboratories, Inc.) in TBS-T with 5% skim milk powder was used. n = 2.
Figure 15. ZHX1 may be involved in proliferation. SK-BR-3 cells were transfected with siRNA targeting different regions of exon three of ZHX1 and an MTT proliferation assay was performed. n = 1.
4 DISCUSSION

From the microarray analyses, ZHX1 is significantly up-regulated in LVI+ tumours and those from patients who experienced an early recurrence (within four years). Since 8q24.13, where ZHX1 is located, can exhibit gains and genes in this region have been found to be over-expressed and significantly correlated with amplification [62-64] (which may improve prediction of prognosis [62]), I examined copy number of ZHX1 in a subset of ANN breast tumour samples.

Tumour samples with lower (e.g. 1131 and 2253) or higher (e.g. 2160 and 2544) copy number ratios were consistent regardless of whether ASNS or APPL2 was used for normalization (Figure 4). Therefore, ZHX1 may be lost or gained, respectively, in these samples. Tumour samples with lower copy number ratios of ZHX1 (e.g. 2253) also had lower copy number ratios of FAM83A and ATAD2 and samples with higher ratios of ZHX1 (e.g. 2544) also had higher copy number ratios of these flanking genes (Figure 6). This is consistent with the work of Chin and colleagues [63].

Since tumours with high ZHX1 expression did not appear to have gains or amplification of ZHX1 and those with low ZHX1 expression did not appear to have losses or deletions of ZHX1 (Figure 8), amplification may not be a mechanism of its over-expression. This may have been the result of differences between breast cancer cell lines used by other groups and tumours, since the former carry more aberrations and have a higher frequency of high-level amplification [73]. In addition, cell lines with high-level amplification may have been preferentially chosen, since this characteristic may provide a selective advantage for growth in vitro [74]. Unlike cell lines, “contamination” of breast cancer tumours with normal epithelial or stromal cells [75, 76] may have also affected copy number analyses. There is also a possibility that over-expression of ZHX1 is due to its acting as a passenger in an amplicon in which C8orf32 (telomeric to ATAD2) or ATAD2 is the driver [64, 77].

I ideally wished to validate the results from the gene expression microarray studies using qPCR, however, tumour resources were limited, a small subset of samples were therefore used, and ZHX1 mRNA levels within samples were variable (Figure 8). Therefore, IHC was performed on ANN breast TMAs to corroborate the expression data at the protein level and provide a larger and independent dataset. This would also allow ZHX1 protein levels, localization, and correlation with gene expression to be determined.
I hypothesized that ZHX1 would be located in the nucleus, since it has been found that over-expression of a ZHX protein or knock down of its binding partner in a disease state causes an increase in its nuclear localization. It has been hypothesized that this is due to a loss of heterodimerization, which causes individual ZHX proteins to migrate into the nucleus, as each contains a NLS. [44]

However, background was observed after staining of the TMAs, which may have been due to non-specific staining. Therefore, if further investigation is desired, a more specific antibody must be generated for FFPE samples.

Since amplification of ZHX1 did not appear to be a mechanism of its over-expression and ZHX1 may be involved in the cell cycle and p53 is involved in this important biological process, I hypothesized that ANN breast cancer tumours with TP53 mutation may be associated with high levels of ZHX1 expression. Our initial gene expression microarray analyses revealed that ZHX1 is significantly up-regulated in LVI+ tumours and those from patients who experienced an early recurrence. Our statistical analyses of ZHX1 mRNA expression and TP53 status indicated that ZHX1 expression may be associated with a missense mutation of TP53 (Figure 9). These findings lend support to my hypothesis that mutated TP53 may be prevented from repressing the CDK1 promoter [78] and CDC25C [26], and act in concert with ZHX1 to increase proliferation.

To characterize ZHX1 in vitro, its transcript levels were first examined in a subset of human normal mammary epithelial and breast cancer cell lines. ZHX1 was expressed at different levels in these cell lines, with about a 12-fold difference between the lowest (MDA-MB-231) and highest (SK-BR-3) levels (Figure 7). I selected the SK-BR-3 cell line for siRNA transfection experiments, since these cells expressed ZHX1 at high levels, so alterations in gene expression would be more easily detected.

To confirm that SK-BR-3 cells could be used for functional studies, ZHX1 protein levels were examined in a subset of breast cancer cell lines (Figure 10) and compared to transcript levels. To confirm the identity of the correct band representing ZHX1, its expression was knocked down via siRNA transfection. All bands around 130 kDa decreased in intensity in the SK-BR-3 cells transfected with the two siRNA targeting ZHX1 (Figure 13). Therefore, ZHX1 is about 130 kDa in size, which is in concordance with previous findings where ZHX1 appeared to be about 120 to 130 kDa in size and correlate with transcript levels [71, 72].
To determine whether ZHX1 is involved in the cell cycle and further confirm the identity of the correct band, I added or omitted phosphatase inhibitors when lysing the cells to isolate protein. The latter treatment resulted in decreased intensity of the largest bands around 130 kDa (Figure 14). This confirmed that these bands represent phosphorylated forms of ZHX1.

This finding supports that ZHX1 is phosphorylated, and possibly by PRKA and PRKC at Ser-7 and 869 and Thr-617 and Ser-763, respectively, given the identification of consensus motifs in ZHX1 for these kinases [54, 55]. As mentioned in section 1.2.2.2.2, PRKC isoforms are tumour-promoting [79] and phosphorylation of ZHX1 by PRKA may promote mitosis [58].

As discussed in sections 1.1.1, 1.2.2.1, and 1.2.2.2, phosphorylation by kinases is important in regulation of the cell cycle, of which deregulation leads to tumourigenesis [6]. Under normal conditions, unrepaired DNA damage activates the ATM, ATR, CHEK1, and CHEK2 kinases, which phosphorylate p53, activating it and therefore inhibiting the CDK2-cyclin E complex, which is responsible for progression into S-phase [4]. ATR also phosphorylates CHEK1, which phosphorylates CDC25C at Ser-216 to inhibit it [4]. ATM activates CHEK2, which also phosphorylates CDC25C [4]. These lead to G2 arrest, since the CDC25C phosphatase cannot dephosphorylate the cyclin B/CDK1 complex to activate it at the G2/M transition and initiate entry into M-phase [5, 51].

To investigate whether ZHX1 is involved in proliferation, an MTT proliferation assay was performed after knock down of ZHX1 expression in SK-BR-3 cells. One siRNA appeared to affect proliferation (Figure 15), however, an inadequate amount of RNA was available to confirm knock down of ZHX1. Therefore, the experiment must be repeated before any definitive conclusions can be made.

These results support my hypothesis that ZHX1 is involved in the cell cycle, which may contribute to LVI and recurrence in ANN breast cancer patients.
5 CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Summary and Implications of Thesis Findings

In summary, I selected ZHX1 as a candidate gene involved in lymphatic invasion and associated with early recurrence (within 4 years) of axillary lymph node-negative breast cancer.

Although most of the tumour samples have normal ZHX1 copy number, a few appear to have gains or losses. Copy numbers of ZHX1 and its flanking genes, FAM83A and ATAD2, seem to correlate in breast tumour samples, which is in concordance with previous findings [63]. From my expression analysis, ZHX1 is expressed at different levels in a subset of tumour samples used in the microarray analyses. However, amplification of ZHX1 does not appear to be a mechanism of its altered expression.

ZHX1 is also expressed at different levels in mammary epithelial cell lines, with about a 12-fold difference in expression across the cell lines. In addition, transcript and protein levels appear to correlate. Through Western blot analysis, I confirmed that ZHX1 is about 130 kDa in size and may likely be phosphorylated by PRKA and PRKC. Furthermore, ZHX1 may be associated with a missense mutation of TP53. Use of an MTT proliferation assay revealed that ZHX1 may be involved in proliferation. These results support my hypothesis that ZHX1 may act in concert with p53 (mutated) and other candidates to affect cell cycle progression.

Additional studies are required to further investigate the role of ZHX1 in LVI and recurrence of ANN breast cancer.
5.2 Future Directions

Since sustained proliferation is the most fundamental trait of cancer cells [6], siRNA transfection conditions for SK-BR-3 cells seeded in 96-well plates should be optimized and the MTT proliferation assay performed to determine whether ZHX1 is involved in proliferation. If so, I expect that a decrease in ZHX1 expression will result in decreased proliferation, indicated by a decrease in absorbance (solubilized formazan produced by metabolically active cells [80]).

Since invasion and migration are important hallmarks of cancer [8], assays can be performed on SK-BR-3 cells that are untransfected versus those transfected with siRNA targeting ZHX1. Scratch wound and/or transwell migration assays, and Matrigel assays can be utilized to investigate potential effects of ZHX1 on migration and invasion, respectively. Since ZHX1 is over-expressed in LVI+ tumours and those from patients who experienced an early recurrence, I expect that a decrease in ZHX1 expression will result in decreased migration and invasion.

To identify and confirm potential interactors of ZHX1, immunoprecipitation and/or chromatin immunoprecipitation (ChIP) experiments can be executed using commercially available antibodies. Antibodies or tagged ZHX1 can be generated if commercial antibodies are unavailable. ChIP can be followed by qPCR to identify potential transcriptional targets of ZHX1. Effects of ZHX1 on candidate interactors, such as p53, or transcriptional targets can initially be examined via siRNA knockdown of ZHX1 expression followed by qPCR and Western blotting. Results should reveal the potential role(s) and signalling pathway(s) that ZHX1 is involved in.

Since much background staining of the TMAs was present, IHC can be performed with a more specific antibody that is commercially available (or generated in-house) and/or different or higher concentrations of blocking agents. Immunofluorescence could also be applied in vitro, with and without siRNA knockdown of ZHX1, to investigate subcellular localization of ZHX1.

To confirm that PRKA and/or PRKC are responsible for phosphorylating ZHX1, inhibitors specific to these kinases can be used. In addition, serine and threonine sites predicted to be phosphorylated by these kinases can be mutated to prevent these phosphorylation events. Protein extracts from cells treated with the above-mentioned inhibitors or transfected with mutated ZHX1 should not have phosphorylated ZHX1, while extracts from cells with wild-type ZHX1 should contain these larger bands on a polyacrylamide gel.
If changes in phenotype are absent after siRNA knockdown of ZHX1, ZHX1 can be over-expressed in a cell line that expresses this gene at low levels, such as MCF7 (Figure 7). I have already acquired a non-expression pBluescript plasmid containing the full-length open reading frame of ZHX1 (Open Biosystems Products, Huntsville, AL). A green fluorescent-protein-tagged expression plasmid (for confirmation via visualization under a microscope) containing ZHX1 can be constructed to transfect MCF7 cells. This can be accomplished via PCR amplification of the ZHX1 insert, double-digestion and gel purification of the insert and pEGFP-C2 expression vector, ligation, transformation of DH5α Escherichia coli with the ligated plasmid, and isolation and purification of the plasmid.

Once ZHX1 has been successfully cloned and the presence of its full length open reading frame confirmed, MCF7 cells can be transfected with the eGFP-ZHX1 plasmid through use of the FuGENE® 6 Transfection Reagent (Roche Diagnostics Corporation) and downstream effects observed via proliferation and migration and/or invasion assays. I expect that over-expression of ZHX1 will result in increased proliferation, migration, and invasion. If over-expression results in cell death, a cell line that has a TP53 mutation may be a desirable model.
REFERENCES CITED


