X CHROMOSOME STUDIES AND BREAST AND OVARIAN CARCINOMA

by

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Abstract

Skewed somatic X inactivation (XCI), X-linked gene overexpression and abnormal X content have been associated with breast and ovarian cancer. Partial or complete reactivation of the inactive X in females may be a step in breast and ovarian cancer progression, leading to overexpression of some tumour enhancing gene. Markers of an X reactivation event were examined: X gene dosage, expression, and methylation in 8 ovarian cancer cell lines. Another marker of an X reactivation event, skewed XCI, was assayed in peripheral blood DNA from 106 breast and/or ovarian cancer patients (52 *BRCA1* mutation carriers, 24 *BRCA2* mutation carriers, 30 non-mutation carriers), 147 age-matched population controls. Combined RNA/DNA FISH was used to quantify the number of inactive Xs compared to total number of Xs. Five cell lines had increased X content. Three cell lines localized *XIST* to the presumptive inactive X; however the numbers of inactive Xs were variable. Expression levels of 8 X-linked genes were assessed by real-time PCR. Expression was inconsistent between different genes and among cell lines, ranging from a 2 to 300-fold increase compared to a control. Overall, expression was greatly increased for genes subject to inactivation but not increased in genes that escape inactivation for most ovarian cancer cell lines. Methylation at *AR* and *FMR1* was quantified by a real-time PCR based assay and SNuPE respectively. Methylation was lower than expected for 7 of 8 ovarian cancer cell lines at *AR* or *FMR1*, while three cell lines had low or no methylation for both genes. Skewed XCI was evaluated using a methylation-based PCR assay at *AR*. There was no significant increase in skewing above 90% for any cancer group assayed. In addition, we assayed two

markers of X reactivation in two low passage cultures of normal ovarian surface epithelium from *BRCA1* mutation positive breast cancer patients. One sample did not localize *XIST* to the inactive X and three of five genes subject to inactivation were overexpressed. In summary, there is evidence for loss of X silencing or gain of active X content in ovarian cancer cell lines and normal ovarian surface epithelium from *BRCA1* mutation carriers.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AR	androgen receptor
ARAF1	v-raf murine sarcoma 3611 viral oncogene homolog
BRCA1	breast cancer 1 gene
BRCA2	breast cancer 2 gene
DIG	digoxigenin
DMSO	dimethylsulfoxide
ER+	estrogen receptor positive
ER-	estrogen receptor negative
ESR1	estrogen receptor alpha
ESR2	estrogen receptor beta
FISH	fluorescence *in situ* hybridization
FMR1	fragile mental retardation 1 gene
KLF8	Kruppel-like factor 8
MIC2	CD99 molecule
OSE	ovarian surface epithelium
PCR	polymerase chain reaction
POF	premature ovarian failure
PR+	progesterone receptor positive
PR-	progesterone receptor negative
RT-PCR	reverse transcriptase PCR
SNP	single nucleotide polymorphism
TIMP1	TIMP metallopeptidase inhibitor 1
VBP1	von Hippel-Lindau binding protein 1
WAS	Wiskott-Aldrich syndrome
XIC	X inactivation centre
XIST	X (inactive)-specific transcript
ZFX	zinc finger protein

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Chapter 1: Introduction

1.1 Opening remarks

Both breast cancer and ovarian carcinoma are devastating illnesses. Breast cancer will affect one of every ten women in her lifetime. Ovarian carcinoma is less frequent, but has a poor survival rate as the early warning signs are very difficult to detect and there are no adequate diagnostic tools available to identify it at a highly treatable stage. Current research into these two cancers attempts to provide genetic clues that could lead to better treatment options. The identification of two breast and ovarian carcinomas predisposing genes, *BRCA1* and *BRCA2*, has allowed for screening of high-risk patients and successful preventative options. However, mutations in these genes only account for a small subset of the total breast and ovarian carcinomas cases.

In the past five years, a connection has been made between X chromosome inactivation, the process of silencing one of the two X chromosomes in females to maintain dosage compensation with males who only possess one X chromosome, and breast and ovarian carcinomass. Skewed X chromosome inactivation, where one X chromosome is predominantly silenced, is increased in blood samples from breast and ovarian carcinomas patients (Buller et al. 1999, Kristiansen et al. 2002). In addition, there have been reports that BRCA1 associates with the inactive X chromosome in a subset of cells (Ganesan et al. 2002), although the level of association has been disputed (Pageau et al. 2007; Xiao et al. 2007). Moreover, overexpression of some X chromosome genes occurs in *BRCA1*-associated ovarian carcinomas when compared to sporadic tumours (Jazaeri et al. 2002).

The objective of the work discussed in this thesis was to establish a connection between the X chromosome and breast and ovarian carcinomas. While exploring this topic, an assay was employed to assess X-chromosome inactivation that makes use of a hormone receptor polymorphism in the androgen receptor gene. This led to an additional investigation of different hormone receptor polymorphisms and their potential involvement as genetic modifiers in breast and ovarian carcinomass. In this Introduction, I will provide a brief description of breast cancer and ovarian carcinoma, review the unique features of the X chromosome and highlight specific hormone receptors. I will also provide a link between the X chromosome and breast and ovarian carcinomas genetics with a specific focus on the first breast cancer susceptibility gene discovered, *BRCA1*.

1.2 Breast and ovarian carcinomas

1.2.1 Breast cancer

Breast cancer is highly prevalent among all populations of women and the most common cancer in North American women. Each year in Canada, breast cancer is diagnosed in 20,500 women and is fatal for 5400 women (Public Health Agency of Canada, PHAC, 1999). Breast cancer is a highly heterogeneous disease, which may be reflective in part of capturing the same cancer at different stages of tumour progression (Gabrielson and Argani, 2002). More than 95% of breast cancers are carcinomas that arise from the epithelial elements of the breast. They are generally characterized as ductal or lobular. Breast cancers confined to the ducts or lobules (or *in situ* carcinomas) are not invasive and have a high cure rate, but may be precursors to invasive breast carcinomas, particularly ductal carcinomas (Gabrielson and Argani, 2002). Invasive breast cancers

are those that have escaped the duct or lobule into the surrounding tissue (Sainsbury et al. 2000). These can be of several subtypes, with infiltrating ductal the most frequent. Other special type breast cancers generally have a better prognosis (Sainsbury et al. 2000). Metastasis is the primary cause of breast cancer mortality and better prognostic tools for establishing which cancers will metastasize are needed (Weigelt et al. 2005).

The genetic causes of breast cancer. Family history is implicated in approximately 10% of all breast cancers, owing in part to mutations in two breast and ovarian carcinomas tumour suppressing genes, *BRCA1* and *BRCA2* (Narod and Foulkes, 2004). Risk estimates vary among studies. Two different meta-analyses of penetrance have indicated that a mutation in *BRCA1* increases the lifetime risk of developing breast cancer from 10% to 57% (CI: 47%-66%) (Chen and Parmigiani, 2007) or to 65% (CI: 44%-78%) (Antoniou et al. 2003). Similarly, a mutation in *BRCA2* increases the risk of developing breast cancer to 49% (CI: 40% to 57%)(Chen and Parmigiani, 2007) or to 45% (CI: 31%-56%) (Antoniou et al. 2003). These two genes are believed to account for approximately 30% of all familial breast cancers (Ligtenberg et al. 1999). However, the majority of familial breast cancers have an unknown etiology though genetic modifiers of risk have been identified and another high penetrance gene has been postulated to exist. To date, a putative *BRCA3* gene has not been identified and most researchers believe that the other familial cases are caused by a number of interacting genes and the environment (Narod and Foulkes 2004). There are other genes that have been implicated in breast cancer including *P53*, *BRIP1*, *RAD51*, *CHEK2*, *BARD1* and *ATM*, though they are primarily causative of other genetic syndromes including Li-Fraumeni syndrome, Cowden syndrome and Ataxia telangiectasia or have only a small effect in the development of breast cancer (Hodgson et al. 2004).

The molecular pathology of breast cancer. Breast cancer is considered to be a suite of different diseases that segregate in regards to their molecular pathology. It can be divided into four subtypes based on the expression of certain genes (Sorlie 2004). There are two estrogen receptor expressing subtypes (ER+), luminal A and luminal B. Both luminal A and B have poor growth rates and low metastatic ability which allows them to be treated more easily. Currently, a targeted therapy named Tamoxifen is available for treatment of luminal A and B types (Widakowich et al. 2007). Tamoxifen was the first targeted therapy in breast cancer, developed over thirty years ago. When Tamoxifen is combined with traditional cancer therapies such as surgery, radiation and chemotherapy, it yields a very high survival rate. Tamoxifen is an estrogen mimic with a high affinity for the estrogen receptor. Tamoxifen cannot recruit a co-activator necessary for the growth response, so, by binding to the estrogen receptor, it arrests the estrogen receptor pathway.

There are also two breast cancer subtypes that do not express the estrogen receptor gene (ER-) (Sorlie 2004). Between 63% and 90% of *BRCA1* mutation carriers develop ER- breast cancer, particularly when onset is at a young age (Honrado et al. 2006). ER- breast cancers can be further subdivided by HER-2 status. Breast cancers that are ER- and HER-2 positive can be successfully treated with trastuzumab (also known as Herceptin) combined with traditional cancer therapies (Widakowich et al. 2007). Trastuzumab is a monoclonal antibody that binds *HER-2*, a protein overexpressed in these cancers that activates the *Akt-MAPK* pathway. By binding *HER-2*, Herceptin leads to reduced cell growth and enhanced apoptosis. The final subtype is ER-, and HER-2 negative, also referred to as basal-like breast cancer. This subtype has the lowest survival rate due to the fact that currently there are no complementary, targeted therapies available. Breast cancers from *BRCA1* mutation carriers are predominantly basal-like

whereas *BRCA2* mutation carriers can have tumours belonging to any subtype (Sorlie 2004).

1.2.2 Ovarian carcinomas

Ovarian cancer is the most deadly gynaecological cancer in North America. It affects approximately 2500 Canadian women each year, and is fatal for approximately 70% of this group (PHAC, 1999). Tumours affecting the ovarian surface epithelium (OSE), or ovarian carcinomas, account for almost 90% of all ovarian cancer (Wong and Auersperg, 2003). Other ovarian cancers such as germ cell tumours and sex cord or stromal tumours occur at a far lower frequency, are often diagnosed at early stages and have a high survival rate (Cvetkovic 2003). Ovarian carcinomas can be further subdivided into seven categories: serous, mucinous, endometroid, clear cell, squamous, mixed and undifferentiated. High-grade serous tumours are the most common form of ovarian carcinomas, occurring approximately 50% of the time (Bell 2005).

There are two likely origins of ovarian carcinomas (Crum et al. 2007). The first origin of ovarian carcinomas is through epithelial inclusions from the Mullerian epithelium into the OSE. These type I ovarian carcinomas can be of any subtype and at age stage of disease progression from benign to malignant. The second type of ovarian carcinomas comprises solely of high-grade serous carcinomas involving the OSE. These type II ovarian carcinomas were originally believed to originate from the OSE. However, there has never been a precursor legion or early stage carcinoma identified in the OSE for high-grade serous tumours (Bell 2005). It appears probable that these carcinomas arise from malignancies in the distal part of the fallopian tube which subsequently metastasize to the ovarian surface, rather than arising from the OSE itself (Crum et al. 2007).

Evidence for a tubal origin of serous carcinomas includes the resemblance of these tumours to the fallopian tube mucosa by gene expression profiling and the high frequency of occult fallopian tube neoplasms in samples from prophylactic salpingo-oophorectomy surgery (ovary and fallopian tube removal) from *BRCA* mutation carriers (2.3-17%) (Callahan et al. 2007).

Ovarian carcinoma has a high mortality rate due to the difficulty of early diagnosis. The most common early warning signs of ovarian carcinomas are abdominal pain and swelling, nausea, fatigue, leg pain, lower back pain and bladder and menstrual disorders. These symptoms are often not severe or specific enough to ensure that the patient seeks treatment so ovarian carcinoma symptoms are often ignored until the tumour size increases enough to press on other organs (Webb et al. 2004). In addition, no diagnostic tools exist at this time that are both sensitive and specific enough for early cancer detection, though novel screening strategies are currently being developed (Munkarah et al. 2007). Given this, most women with ovarian carcinomas are not diagnosed until stage III or later, where the five year survival rate drops from 90% in stage I to approximately 20% (Cvetkovic 2003).

The genetic causes of ovarian carcinomas. As is true for breast cancer, family history is implicated in approximately 10% of all ovarian carcinoma cases. Of these, a *BRCA1* mutation will increase a person's lifetime risk of developing an ovarian carcinoma from 1-2% to approximately 39% (CI: 18%-54%) (Antoniou et al. 2003, Chen and Parmigiani, 2007). A *BRCA2* mutation increases the risk of developing an ovarian carcinoma to a lesser extent, to 18% (CI: 13% to 23%) or to 11% (CI: 2.4%-19%) depending on the meta-analysis (Chen and Parmigiani, 2007, Antoniou et al. 2003). Germline mutations in *BRCA1* and *BRCA2* are the only known genetic changes that lead to a significantly

increased risk of developing an ovarian carcinoma (Wong and Auersperg 2003). This may be due to the difficulty of assessing early events in ovarian carcinoma progression as most tumour samples available for study tend to be in later stages of disease progression (Cvetkovic 2003).

The molecular pathology of ovarian carcinomas. As mentioned, ovarian carcinomas can be categorized based on morphology into discrete subtypes. The genetic profiles of these subtypes are variable; however, some specific genetic mutations are known to associate with certain subtypes (for a review see Bell 2005). The genetic profile of a tumour may reflect the path in which the tumour has arisen and whether it is a type I or type II ovarian carcinoma (Christie and Oehler, 2006). Type II carcinomas or high-grade serous tumours, the most common subtype, are associated with *BRCA1* or *BRCA2* dysfunction and *TP53* mutations (Crum et al. 2007). Type I ovarian carcinomas include all the different subtypes and often contain *PTEN*, *KRAS*, *BRAF* and beta-catenin mutations (Crum et al. 2007). More specifically, low grade serous carcinomas are often characterized by *KRAS* or *BRAF* mutations, mucinous carcinomas can have *KRAS* mutations and low grade endometroid carcinomas often have *beta-catenin* and *CTNNB1* mutations and microsatellite instability (Christie and Oehler, 2006). Other subtypes have not been fully molecularly characterized.

1.2.3 Non-genetic risk modifiers of breast and ovarian carcinomas

There are several non-genetic influences that can affect breast and ovarian carcinomas risk. Most of the known risk factors can be linked in some way to the hormonal environment, usually involving estrogen (MacMahon 2006, Lukanova and Kaaks, 2005;

Table 1.1). Both a woman's reproductive history and her exogenous and endogenous hormone exposure contribute to her risk of developing breast and ovarian carcinomass.

Reproductive/endogenous hormone risks. Parity is positively associated with decreased risk of breast and ovarian carcinomas (Persson 2000). Parity has a protective effect against breast cancer, where there is increasing protection with each pregnancy (Chie et al. 2000, Albrektsen et al. 2005). Similarly, ovarian carcinoma risk is decreased in parous women when compared to nulliparous women, with an additional decrease in risk with subsequent pregnancies (Hankinson et al. 1995). Though the risk of breast cancer decreases with increased parity, there is an initial, short-term increase in breast cancer risk after giving birth. This short-term increased relative risk becomes greater with increased maternal age (Albrektsen et al. 2005).

Age at first pregnancy has also been associated with both breast and ovarian carcinomass. A woman having her first child at a younger age has a reduced risk of breast and ovarian carcinomas (Wohlfahrt and Melbye 2001). Breast cancer risk increases with a woman's age at first birth and surpasses the risk of nulliparous women after the age of 35 (MacMahon et al. 1970).

Breast feeding does not largely affect a woman's risk of breast or ovarian carcinoma. However, lactation appears to exhibit a modest protective effect against breast cancer in young women (Tryggvadottir et al. 2001; Siskind et al. 1997).

It has been well documented that women with an earlier age of menarche and/or a later age of menopause have an increased risk of breast cancer (Hsieh et al. 1990, Pathak and Whittemore 1992). The risk involved with early menarche or late menopause is less clear for ovarian carcinoma. There is some evidence for increased risk of ovarian carcinoma with late menopause (Booth et al. 1989), but most studies find no effect on

risk (Titus-Ernstoff et al. 2001). Similarly, some studies find a weak decrease in risk with later age at menarche in pre-menopausal women only (Titus-Ernstoff et al. 2001).

Perhaps the best evidence that breast cancer is associated with estrogen levels is that women undergoing oophorectomy without mastectomy, and consequently the removal of the major source of estrogen and progesterone, have a significant decrease in breast cancer risk (Trichopoulos et al. 1972). Ovarian carcinoma risk correspondingly decreases significantly when a woman has had a hysterectomy (Booth et al. 1989) or tubal ligation (Miracle-McMahill et al. 1997).

Exogenous hormone risks. Exogenous hormone exposure also influences the risk of these cancers. Long-term oral contraceptive use has been linked to a small increase in breast cancer in premenopausal women in some studies (Shantakumar et al. 2007) but not all (Hankinson et al. 1997) and also to a notable decrease in ovarian carcinoma risk (Booth et al. 1989, Hankinson et al. 1995). Hormone replacement therapy (HRT) is associated with an elevated risk of breast cancer in pre-menopausal women (Shantakumar et al. 2007) and also with an increased risk of ovarian carcinoma (Garg et al. 1997).

Lifestyle risks. Lifestyle risks, such as height, weight, exercise, diet, alcohol consumption and early radiation exposure have all been associated with either breast or ovarian carcinoma (Persson 2000; McMahon 2006). However, lifestyle risks do not exclude the possibility that estrogen is the causal element. Increased weight is positively correlated to breast cancer risk in post-menopausal women (Pathak and Whittemore 1992, Hsieh et al. 1990). Similarly, alcohol consumption increases a woman's risk of breast cancer when compared to non-drinking women (Hankinson et al. 1995). Both increased weight and alcohol consumption are associated with increased endogenous estrogen (Hankinson et al. 1995).

Table 1.1. Epidemiologic factors that affect breast and ovarian carcinomas risk. Trends that are opposite in breast and ovarian carcinomas are in blue. Trends that are opposite in *BRCA1* mutation carriers and non-carriers are in red. Not applicable (n/a) was noted when a difference between *BRCA1* carriers and non-carriers has not been studied to the author's knowledge.

Factor	**Breast Cancer**		**Ovarian carcinoma**	
	Non-carriers	*BRCA1 carriers*	*Non-carriers*	*BRCA1 carriers*
Late Menarche	⇓	-	⇓ (modest in premenopausal women only)	n/a
Early menopause	⇑	-	⇓ (modest in premenopausal women only)	n/a
Increased parity	⇓	-/⇑	⇓	n/a
Age at first birth	⇑ (with ⇑ age)	-/⇑	⇓	n/a
Breast feeding	⇓ (modest in premenopausal women only)	⇓	⇓ (modest)	n/a
Oral contraceptive use	⇑ (very modest effect in premenopausal women only)	⇑	⇓	⇓
Hormone Replacement Therapy	⇑	Insufficient data	⇑ (insufficient data)	n/a
Oophorectomy	⇓	⇓	⇓	⇓
Tubal Ligation	n/a	n/a	⇓	⇓

Adapted from table in Rosen et al. 2005

1.3 The breast cancer susceptibility genes, *BRCA1* and *BRCA2*

1.3.1 *BRCA1*

Knowing that breast and ovarian carcinomas could segregate in families provided the impetus for research geared at finding causative genes. The *BRCA1* gene was first mapped to chromosome 17q in 1990 by analysing genetic linkage in families multiple cases of breast cancer and early age of onset (Hall et al. 1990) and was cloned four years later (Miki et al. 1994). The *BRCA1* gene is large, at 24 exons and encoding 1863 amino acids in humans (Miki et al. 1994). The *BRCA1* protein has several functional domains

including an N-terminal zinc-finger domain which interacts directly or indirectly with DNA and C-terminal BRTC binding domains (for a review of protein function see Paterson 1998).

BRCA1 has been implicated in diverse cellular processes. It is involved in the regulation of transcriptional activation, DNA repair, cell-cycle checkpoint control and chromosomal remodelling. It interacts with many different molecules such as tumour suppressors, oncogenes, cell-cycle regulators and transcriptional activators and repressors (for a review see Deng and Brodie, 2000).

BRCA1 is also involved in the XY body formation during mammalian meiosis in males. The XY body is formed when the X and Y chromosomes become transcriptionally silenced during late prophase, also known as meiotic sex chromosome inactivation (Handel et al. 2004). *BRCA1* is present along unsynapsed areas of the X and Y chromosome, and its appearance closely matches the presence of the phosphorylated histone, *H2AX*, which is required for meiotic sex chromosome inactivation (Turner et al. 2004). Further, *Brca1* deficient mice fail to form an XY body (Turner et al. 2004). *BRCA1* has been linked to another type of sex chromosome inactivation in female mammals, X chromosome inactivation (Ganesan et al. 2002) (see section 1.5 for further detail). *BRCA1* has also been implicated in hormonal pathways (discussed in section 1.6). Understanding how *BRCA1* interacts with other proteins is likely important for understanding its role in tumourigenesis.

Because of its involvement in so many important processes, *BRCA1* is believed to be integral to human embryonic survival. In keeping with this notion, transgenic mice carrying homozygous *Brca1* mutations die in early development (Ludwig et al. 1997). The low prevalence of *BRCA1* mutations in the general population (approximately

0.051%) makes it difficult to observe whether this phenomenon is occurring in humans (Antoniou et al. 2002). However, in the Ashkenazi Jewish population, where a risk of having a *BRCA1* or *BRCA2* mutation is approximately 1 in 50 (Struewing et al. 1997), no individual has been identified with a homozygous *BRCA1* mutation (Gal et al. 2004), indicating that homozygous *BRCA1* mutations in humans may also be embryonic lethal.

BRCA1 is considered a tumour suppressor because loss of heterozygosity of the wild-type allele is observed in both breast and ovarian tumours from *BRCA1* mutation carriers (Smith et al. 1992). Many different causative *BRCA1* mutations have been identified in the general population. Most of these mutations result in a truncated protein or loss of protein product (Xu and Solomon, 1996). In the Ashkenazi population, two mutations are highly prevalent: 185delAG and 5382insC, which account for the higher risk of breast and ovarian carcinomas in this population (Struewing et al. 1997). These two mutations are also increased in the general population and account for approximately 10% of the mutations observed.

1.3.2 *BRCA2*

The *BRCA2* gene was linked to 13q12-q13 using genetic linkage analysis in families with male cases of breast cancer (Wooster et al. 1995). The *BRCA2* protein is even larger than *BRCA1*, at 3418 amino acids in length (Wooster et al. 1995). It is involved in homologous recombination, and interacts with *BRCA1*, though other details of its cellular functions are unknown (Narod and Foulkes, 2004). Like *BRCA1*, *BRCA2* acts as a tumour suppressor. Many mutations in *BRCA2* have been identified in families with breast cancer. The common mutation found in the Ashkenazi Jewish population, where risk of a *BRCA2* mutation is high, is 6174delT, while other mutations are rarely seen (Struewing et al. 1997). This mutation is also observed in most other populations.

1.3.3 *BRCA1* vs. *BRCA2*

There are several differences between *BRCA1* and *BRCA2* mutation carriers. Unlike *BRCA1*, homozygosity for a *BRCA2* mutation is not an embryonic lethal condition, though it can rarely cause Fanconi anaemia, medulloblastoma or Wilms' tumour in children (Narod and Foulkes, 2004). Furthermore, *BRCA1* tumours are predominantly basal-like, whereas *BRCA2* breast tumours follow the pattern of histological subtypes found among sporadic breast tumours (Sorlie 2004). In addition, gene expression arrays can distinguish between *BRCA1* and *BRCA2* tumours (Turner et al. 2004). *BRCA2* mutation families are associated with ages of onset consistent with sporadic cases and cases of male breast cancer while *BRCA1* mutation families are associated with early age of onset and cases of ovarian carcinoma (Narod and Foulkes, 2004). Based on these differences, the breast and ovarian carcinomass of *BRCA1* and *BRCA2* mutation carriers must be considered separately. This thesis will mainly focus on *BRCA1*.

1.3.4 *BRCA1* and *BRCA2* in sporadic breast and ovarian carcinomas

Though only a small subset of women with breast or ovarian carcinoma may have a mutation in the *BRCA* genes, changes in expression of these genes may still account for a larger proportion of these patients. Some sporadic cases of both breast and ovarian carcinomas are a result of decreased or absent expression of *BRCA1* or *BRCA2* (Hilton et al. 2002). Epigenetic misregulation or loss of *BRCA1* expression via promoter hypermethylation has been linked to some cases of sporadic breast and ovarian carcinomas, though only in tumours that display loss of heterozygosity of *BRCA1* (McCoy et al. 2003). Transcriptional regulation of *BRCA1* may also occur via control of the promoter by the positive regulatory region in the 5^1 end of the *BRCA1* gene, which is needed for activation of *BRCA1* transcription. The positive regulatory region is often

hypermethylated in breast and ovarian tumours (McCoy et al. 2003).

1.3.5 Non-genetic risk modifiers in *BRCA1* mutation carriers

As in the general population, risk of breast and ovarian carcinomas in *BRCA1* mutation carriers can be modified by epidemiologic factors. However, environmental risk factors can sometimes trend in the opposite direction when a *BRCA1* mutation is present (Rosen et al. 2005). Contrary to the general population, late age at first pregnancy protects against breast cancer and increased parity increases the risk of disease in *BRCA1* mutation carriers. Parous *BRCA1* and *BRCA2* mutation carriers were at a significantly higher risk of developing breast cancer by age 40 than nulliparous women (Jernstrom et al. 1999). It appears that some ovarian carcinoma risk factors affect the general population and *BRCA1* or *BRCA2* carriers in a similar fashion, such as oral contraceptive use and tubal ligation, while the specific effect of other modifiers on mutation carriers is unknown (Narod and Boyd, 2002, Table 1.1).

1.4 The X chromosome

The X chromosome is unique in several ways. It has a silencing mechanism, a history and a function that are unlike that of any autosome. Autosomal genes are generally expressed biallelically, while genes on the X chromosome are generally expressed monoallelically, since male mammals only possess one X chromosome while female mammals inactivate their second X chromosome via a process known as X chromosome inactivation (see section 1.4.1). In addition, while autosomal chromosome pairs are virtually indistinguishable, the human sex chromosomes, X and Y, share little homologous DNA. Small regions of homology do exist between the sex chromosomes because the Y chromosome is an ancestral derivative of an early X chromosome and also

because they have since partially co-evolved (Graves, 1998). Studying X chromosome genetics may enable researchers to explore the largely female specificity of breast and ovarian carcinomas.

1.4.1 X chromosome inactivation

Mammalian females have two X chromosomes while males have one, which could result in a situation where females have double the X chromosome gene products of their male counterparts. In 1961, Mary Lyon first hypothesized the phenomenon now known as X chromosome inactivation (XCI) in mice. XCI is the process that allows females to achieve X chromosome gene dosage equivalence with males who only possess one X chromosome by effectively shutting down (or inactivating) one X chromosome in each female cell (Lyon 2003). Though XCI is not fully understood, many hallmarks of the process have been identified (for a review see Chow et al. 2003).

There are three basic steps in the process of XCI (Boumil and Lee, 2001) (Figure 1.1). The first step is initiation of inactivation, which involves counting (the determination of the number of X chromosomes that are to be inactivated) and choice (the determination of which X chromosome(s) are to be inactivated). The second step is the spread of inactivation, where the inactivation is established along the X chromosome. The third step is maintenance of inactivation, so that with each cellular division the same inactive X chromosome always remains inactive.

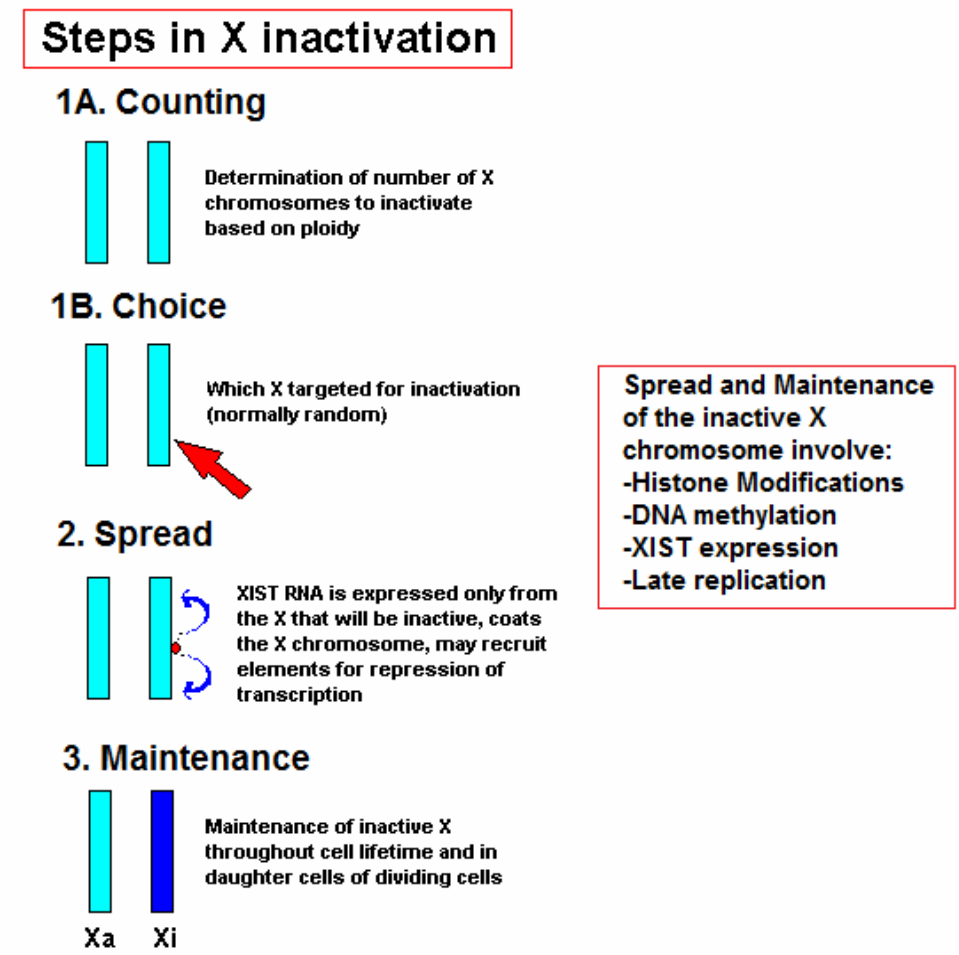

Figure 1.1. The process of X chromosome inactivation (XCI) in females involves counting, choice, spread and maintenance of the inactive X chromosome. These steps are not fully elucidated and not all interacting genes/proteins have been identified. *XIST* plays a central role in XCI as it is expressed from and subsequently coats only the inactive X chromosome.

Initiation of X inactivation begins in the early embryo. The X inactivation centre (XIC), a region of approximately 1 Mb in humans at position Xq13, is a key component of initiation (Avner and Heard, 2001). *XIST*, the X (inactive)-specific transcript, is a non-coding RNA within the XIC that is required for initiation of X inactivation (Brown et al. 1992). *XIST* is transcribed from the inactive X chromosome and subsequently coats the inactive X chromosome in *cis* (Brown et al. 1999). *XIST* is the only gene found in the XIC that has a definitive role in human XCI. Other genes within this region are involved

in mouse XCI, though their roles in the human process have yet to be established (Brown and Chow, 2003).

In mice, *Tsix* and *Xite*, non-coding functional RNAs in the XIC, are involved in choice (Shibata and Lee, 2004). *Tsix*, a non-coding RNA that is antisense to *Xist*, is only expressed from the active copy of the X chromosome and may repress the transcription of *Xist* RNA via antisense interference (Shibata and Lee, 2004). *Xite*, or X-inactivation intergenic transcription element, promotes *Tsix* activity (Ogawa and Lee, 2003). There is a *TSIX* gene in humans, but it is truncated compared to the mouse homologue, with no CpG island, a region of high CG content that is often found in promoters of housekeeping genes (Brown and Chow, 2003). In addition, human *TSIX* transcripts do not seem to interfere with normal inactivation in humans (Migeon et al. 2001). Therefore, *TSIX* may have no function in the human XCI, though a conserved role in choice cannot be ruled out at this time (Brown and Chow, 2003).

The exact mechanism for the counting process is unknown; however, there are two models by which it may occur. One is that a blocking factor must exist that protects only one X chromosome from inactivation. The other model is that in addition to a blocking factor, a competence factor that induces XCI must also exist (Avner and Heard, 2001). Recently, *Tsix* and *Xite* have been linked to the counting process in mice (Lee 2005). A reduction of these RNAs results in chaotic choice and variable numbers of inactive X chromosomes, whereas too much of these RNAs leads to the inability of cells to undergo XCI (Lee 2005).

Spread of XCI is primarily achieved by *XIST* RNA. *XIST* is expressed solely from the inactive X chromosome and promotes spreading of the inactivation signal across the whole chromosome in *cis* (Avner and Heard, 2001). *XIST* appears to recruit other

elements of XCI to the inactive X chromosome (Boumil and Lee, 2001). For example, when *Xist* is deleted in mice, cells fail to localize macroH2A, a histone variant involved in XCI spread and maintenance, to the inactive X chromosome (Csankovski et al. 1999).

Maintenance of the inactive X chromosome is achieved by preserving the X chromosome as facultative heterochromatin. Hallmarks of the inactive X chromosome as facultative heterochromatin are late replication, DNA methylation, presence of modified histones, recruitment of the histone variant macroH2A and expression of the non-coding RNA, *XIST* (Boumil and Lee, 2001). It is apparent that *XIST* is very important for process of XCI. However, *XIST* does not act alone. *XIST* has been shown to be necessary but not sufficient for XCI to occur (Brown and Willard, 1994).

XCI is established in early embryogenesis. The process of XCI is usually random, where different cells in the early embryo will have either the paternal or the maternal X chromosome inactive. Females are, therefore, mosaics for two cell populations, one having an inactive paternally inherited X chromosome and the other having an inactive maternally inherited X chromosome (Avner and Heard, 2001). Once XCI has been established, the inactive X chromosome chosen is thought to remain the inactive X chromosome regardless of subsequent cellular divisions, except in oogenesis (where both X chromosomes are active).

A feature of the X chromosome hypothesized to be important for XCI is the enrichment of long interspersed nuclear elements (LINES) (Bailey et al. 2000). Recent sequencing of the X chromosome has indicated that interspersed repeats account for 56% of the X chromosome compared to the genome average of 45% (Ross et al. 2005). Lyon has postulated that the abundance of LINEs may be involved in the process of XCI if LINES behave as waystations for the silencing machinery, allowing inactivation to

spread on the X chromosome (Lyon, 2003). However, transposable elements may accumulate in regions that have fewer recombination events, which would also explain the increased propensity for LINE elements on the X chromosome, as the X chromosome only fully pairs and undergoes recombination in females (Wichman et al. 1992).

1.4.2 Genes that escape X chromosome inactivation

Despite XCI, approximately 15% of X chromosome genes are expressed biallelically in females (Carrel and Willard, 2005). These genes are said to escape inactivation. Some of these escaping genes have exact counterparts on the Y chromosome. These genes are considered pseudoautosomal, as they are expressed from both sex chromosomes, regardless of whether an individual is male or female (Ross et al. 2005). The majority of pseudoautosomal genes are found near the short arm tip of the X and Y chromosome in a region of about 2.7 Mb, named the pseudoautosomal region 1 (PAR1) (Ross et al. 2005). A second pseudoautosomal region, PAR2, is found at the tip of the long arm of the X and Y chromosomes and is 330kb in size. PAR1 is believed to have evolved from a translocation event about 105 million years ago that added autosomal genetic material to the ancestral sex chromosomes, while PAR2 is believed to have been caused by an X chromosome duplication followed by translocation to the Y chromosome that occurred between chimpanzee and human evolution (Ross et al. 2005). There are 25 X/Y homologous genes that do not fall in PAR1 or PAR2, which are remnants of the early history of the X and Y chromosome as a pair (Ross et al. 2005).

A small number of X chromosome genes that are not pseudoautosomal and thus have no counterpart on the Y chromosome also escape XCI in humans. The effect of gender differences in gene expression is unknown but increased expression of such genes may confer some advantage to females. Few genes in mice escape inactivation, which

may explain why mice with monosomy of the X chromosome (XO) are generally asymptomatic, while 45,X humans have Turner syndrome, the phenotype of which can include short stature, broad trunk, webbed neck and primary amenorrhea (Brown and Greally, 2003). Furthermore, there is variability among females in regards to the inactivation of some X chromosome genes, such that a gene that is expressed in one female can be silenced in another. At least 10% of X chromosome genes tested were heterogeneous in regards to expression (Carrel and Willard, 2005). The consequences of XCI variability among women remain to be determined, but may be important in establishing causes for differences in sex-specific diseases such as breast and ovarian carcinomass. However, expression from two X chromosomes rather than one does not necessarily mean that there is twice the gene expression.

1.4.3 Skewed XCI

XCI is usually a random process, resulting in some cells having an active paternal X chromosome and others having an active maternal X chromosome. Skewed XCI occurs when one X chromosome is silenced more frequently than the other. Skewed XCI may arise from choice, selection or a stochastic event (for a review see Brown and Robinson 2000; Migeon, 1998; Clerc and Avner, 2006; Figure 1.2).

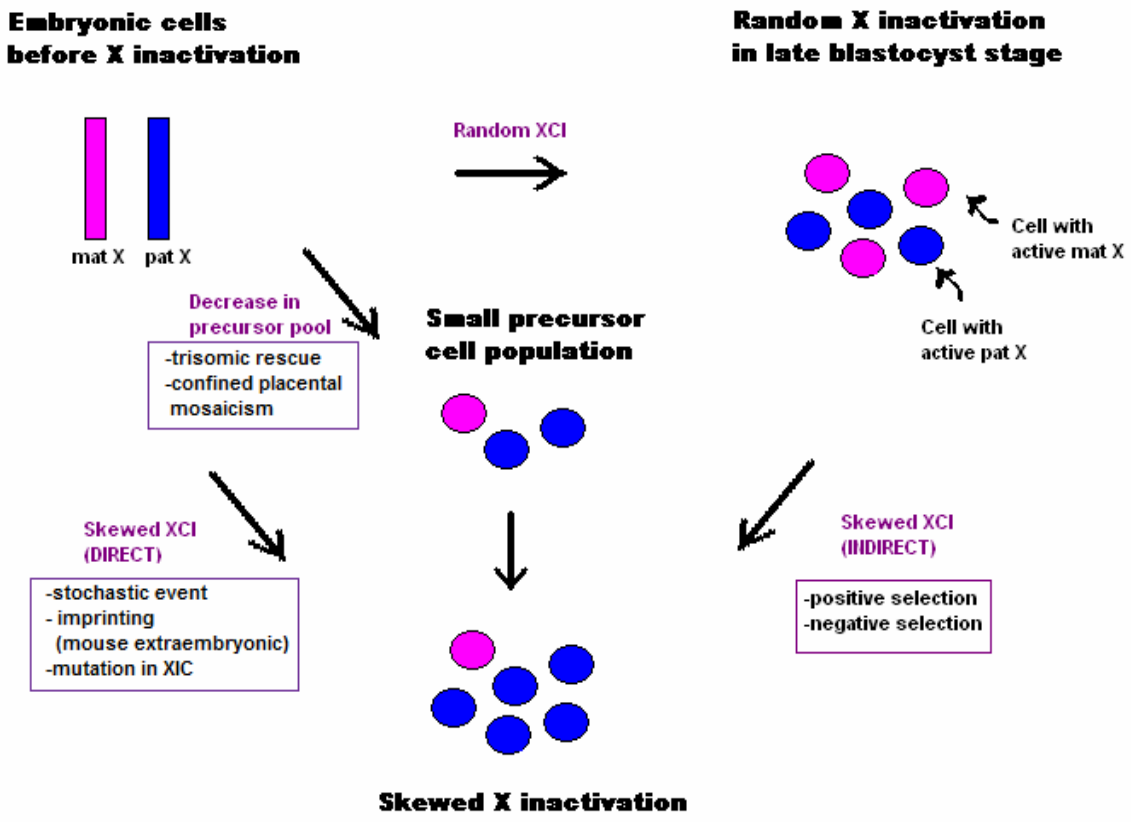

Figure 1.2. Skewed X chromosome inactivation. Skewed XCI can arise directly via a stochastic event, imprinting in some species (e.g. marsupials, mice extraembryonic tissues), indirectly via positive or negative selection (e.g. chromosomal rearrangements, mutations in X chromosome genes) or through a decreased precursor pool caused by such things as trisomic rescue *(adapted from Brown, unpublished).*

Choice. Primary skewing can be caused by a disruption of *XIST* expression. If a mutation arises that disrupts the function of *XIST* (the non-coding RNA essential for the silencing of the X chromosome from which it is expressed), the other X chromosome bearing the wild type copy of the gene will be inactivated. Skewed XCI occurs in mice with induced mutations in either *Xist* or *Tsix* (Brown and Robinson, 2000). In humans, only one report exists describing a mutation in *XIST* that leads to skewed XCI (Plenge et

al. 1997). However, skewing was not observed in all family members carrying the mutation. Primary skewing also occurs in marsupials and the extraembryonic tissues of mice, where the paternal X chromosome is always inactivated (Migeon, 1998).

Selection. Secondary skewing can arise when cells have a selective growth advantage or disadvantage depending on which X chromosome is active. In this case, it is assumed that the process of X inactivation remains random but that after XCI has been established, selection occurs.

X chromosome rearrangements such as mutations or deletions that interfere with the proliferative capabilities of a cell during early embryogenesis can cause secondary skewing (Migeon, 1998). Here, cells with an active mutated X chromosome will be at a selective disadvantage compared to cells maintaining the normal X chromosome active.

Similarly, X:autosome translocations can cause secondary skewed XCI (Migeon, 1998). The derivative autosome which is translocated to the portion of the X chromosome containing the XIC will be partially inactivated. Conversely, the derivative X chromosome lacking the XIC will be unable to undergo inactivation. Therefore, cells that have the derivative chromosomes inactive will have partial monosomy for the autosome that is silenced due to the presence of the XIC and partial X chromosome disomy for the derivative X chromosome that no longer undergoes XCI. Resultant skewing toward inactivating the normal X chromosome will occur as its inactivation will allow for correct gene dosage.

Having a small embryonic precursor cell population at the timing of XCI can also result in secondary skewing (Brown and Robinson, 2000). Confined placental mosaicism (CPM), a situation where trisomy is limited to the placenta while the fetus is predominantly diploid, can result in a decreased embryonic cell pool if the origin of the

CPM is trisomic rescue. Trisomic rescue occurs when a cell from a trisomic conceptus loses the supernumerary chromosome and returns to a state where it has a normal chromosome complement. Since diploid cells have a growth advantage over trisomic cells, it appears possible for the embryo proper to be derived from a single cell. Skewed XCI is significantly increased in CPM cases where the trisomy had a meiotic origin (Lau et al. 1997).

Stochastic event. Skewed XCI can also arise from a random event (Migeon, 1998). From mouse studies it is known that XCI is initiated after blastocyst implantation and when the embryonic precursor pool size is at least 10-20 cells in size (Lau et al. 1997). Thus, some women can have skewed XCI arising from chance due to the relatively small number of precursor cells in a chromosomally normal embryo.

Skewed XCI and age. The degree of skewed XCI in peripheral blood increases with a woman's age. Newborns have significantly less skewed XCI in peripheral blood than do adult women, at approximately 0.5% and 3.6% respectively for skewing above 90% (Amos-Landgraf et al. 2006). Skewing appears to increase at a greater rate after the age of 30 (Hatakeyama et al. 2004). Why skewing in blood increases with age is unknown but may be due to properties of the tissue in question, biases introduced with the way in which skewing is currently ascertained or stem cell depletion causing blood cells to be derived from a smaller number of precursor cells (Hatakeyama et al. 2004).

Skewed XCI and disease. The degree of skewed XCI can play a role in how an X-linked disease is manifested in females (Van den Veyver, 2001). In heterozygous women, selection that leads to silencing of the mutated X chromosome can allow an X-linked disease to be masked in that individual. For example, women with a mutated copy of *HPRT* are asymptomatic for the X-linked recessive disease, Lesch-Nyhan syndrome,

because the mutation-bearing X chromosome is predominantly silenced in blood (Migeon et al. 1988).

For some X-linked diseases, there is no early selective pressure for or against the disease causing mutation. In such cases, the level of skewing can influence the phenotype of a patient, from asymptomatic to severe, depending on whether the mutation-bearing X chromosome is more often silent or active (Van der Veyver, 2001). For example, Rett syndrome, an X-linked dominant, male lethal disease caused by a mutation in *MECP2* has a variable phenotype in females. Skewing assessed in peripheral blood leukocytes of Rett syndrome patients has indicated a relationship between disease severity and degree of skewing (Amir et al. 2000).

Recall that skewed XCI does not have to be disease causing. Different estimates of the amount of extreme skewing (above 90%) in the peripheral blood of unaffected women have been cited ranging from 3.6 to 11% (Amos-Landgraf et al. 2006; Hakayama et al. 2003).

1.4.4 Assaying skewed XCI

The most commonly used assay to measure skewed XCI exploits the fact that the CpG islands of most genes on the inactive X chromosome will be methylated while these same genes will be unmethylated on the active X chromosome (Figure 1.3, Allen et al. 1992). Methylation-sensitive restriction enzymes that only cut unmethylated DNA are used to digest a DNA sample. Primers are designed around the cut sites of a polymorphic X chromosome gene in order to quantify the amount of methylated maternal and paternal gene products (as only methylated, silent DNA will be amplified). The amounts of both alleles are quantified and compared to obtain a value for the amount of skewed XCI. An undigested sample is run in concert with each digested sample to visualize the amount of

each X chromosome present. Alleles are represented as peaks and their heights or areas can be compared to obtain a skewing percent. Random XCI would appear as two peaks of roughly the same height and area in the digested sample (Figure 1.3 d). Skewed XCI would appear as one peak height and area significantly larger than the other in the digested sample (Figure 1.3 e).

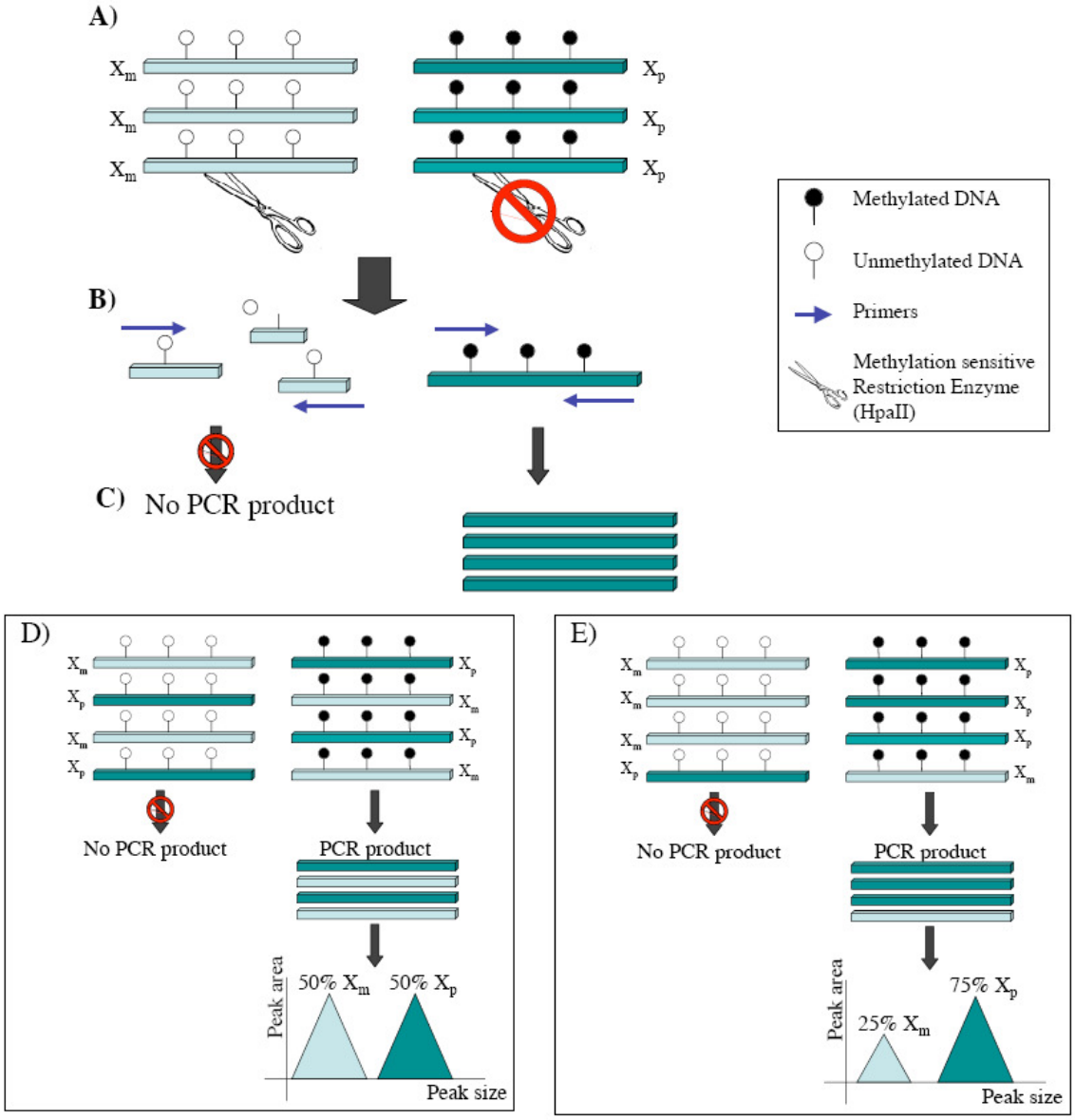

Figure 1.3. The principle of the X chromosome inactivation skewing assay. A) *HpaII*, a methylation-sensitive restriction enzyme will only cut unmethylated DNA. Most genes on the inactive X chromosome (in this case the paternal X chromosome, X_p) will have methylated CpG islands in their promoters. B) Primers can be designed around a gene that is methylated on the inactive X chromosome. C) Only the methylated, inactive X chromosome fragment will yield a product. In this case, there is 100% skewing because the paternal X chromosome is always methylated and the maternal X chromosome is always unmethylated. X_p vs. X_m inactivation can be visualized on an ABI Prism®310 Genetic Analyser using a size polymorphic X chromosome gene (D, E). D) Random XCI (50% inactive X_p, 50% inactive X_m). E) Moderate skewed XCI (75% X_p, 25% inactive X_m)

1.5 *BRCA1* and the X chromosome

1.5.1 A putative role for *BRCA1* in X chromosome inactivation (XCI)

An intriguing question in *BRCA1* research is: why does a mutation in *BRCA1*, a gene known to be involved in so many important cellular processes, lead to a greatly increased risk of female-specific cancers but not to others (Narod and Foulkes, 2004)? While estrogen effects may be involved, a putative link between *BRCA1* and XCI may partly explain the female-specificity of these cancers (Ganesan et al. 2002). *BRCA1* is present on the unsynapsed regions of the sex chromosomes and is required for the inactivation of the XY body during male meiosis (Turner et al. 2005). Since *BRCA1* is involved in one type of DNA inactivation, it is possible that it also functions in XCI, though the method of silencing is different. Ganesan et al. (2002) were first to state that *BRCA1* interacts directly or indirectly with *XIST*, a key player in XCI. They observed *BRCA1* localization with *XIST* on the inactive X chromosome in a significant subset of cells (5-10%). In addition, when *BRCA1* protein was absent, either in a *BRCA1*$^{-/-}$cell line (HCC1937) or in *Brca1* knock-down mice, they found that *XIST* failed to localize properly to the inactive X chromosome. The reintroduction of wild-type *BRCA1* allowed *XIST* foci to reform in most cells. This implied that *BRCA1* was involved in the maintenance and/or establishment of the inactive X chromosome.

The validity of this study has been called into question (see Table 1.2 for a review of recent findings). Recently, this work has been repeated by two other groups who have noted little or no complete overlap of *XIST* and *BRCA1* signals in somatic cells, though they found increased partial overlap (3-5% and ~3% respectively; Pageau et al., 2007; Xiao et al. 2007) and increased signal abutment (10-15%; Pageau et al. 2007). Other

studies note the presence of an overlapping *BRCA1* signal with the inactive X chromosome in humans and mice, but do not quantify the frequency of this association (Chadwick and Lane, 2005; Ouyang et al. 2005; Diaz-Perez et al. 2006, Silver et al. 2007). Interestingly, when *BRCA1* and *XIST* appear to be overlapping, closer study reveals that *BRCA1* fills in the gaps left by the *XIST* signal (Pageau et al., 2007; Xiao et al. 2007). Markers of the inactive X chromosome are known to occupy specific, non-overlapping domains (Chadwick and Willard, 2004). Therefore, *BRCA1* might be involved with other, *XIST*-independent markers of the inactive X chromosome or with another aspect of X chromosome gene silencing.

If *BRCA1* is involved in XCI and repression of some specific X chromosome genes, then a mutation in *BRCA1* could lead to the reactivation or partial reactivation of the silent X chromosome (Stone et al. 2003). This in turn could lead to overexpression of an X chromosome gene or suite of genes normally subject to X inactivation, kick-starting cancer progression.

Table 1.2. The main points of contention of the *BRCA1* and XCI studies can be broken down into three categories: **1)** Do *BRCA1* and *XIST* co-localize to the inactive X chromosome (Xi)? **2)** When there is no *BRCA1* protein present in a cell is there an absence of *XIST* RNA coating the Xi? **3)** When you reintroduce wild-type *BRCA1* protein into a cell line lacking *BRCA1*, do you see *XIST* RNA re-coat the Xi? (n/a: no data)

STUDY	**1. *BRCA1/XIST* co-localization**	**2. No *BRCA1*= No *XIST* staining**	**3. Add *BRCA1*, *XIST* localizes to Xi**
Studies supporting a connection between BRCA1 and XCI:			
1. Ganesan et al. 2002 (***original study***)	5-10% of cells co-localize *BRCA1* and *XIST*	No *XIST* stain in *BRCA1*-null cell line (HCC1937), murine cell lines, siRNA knock downs (KD)	Accumulation of *XIST* on Xi with introduction of *BRCA1* into BRCA1 null cells
2. Ouyang et al. 2005	*Brca1* is concentrated on the Xi in mouse cells	n/a	n/a
3. Chadwick and Lane, 2005	*BRCA1* interacts with Xi at late S phase (3 fold higher # of interactions than reported in (1))	n/a	n/a
4. Diaz-Perez et al. 2006	Same as in (2)	n/a	n/a
5. Silver et al. 2007 *[from same group as (1), comment on (8)]*	Subset of cells co-localize *Brca1* and *Xist* in mouse mammary cell line (numbers not given)	To see siRNA effect, need *BRCA1* knocked down by >90%, *Brca1* conditional knock outs (KO) lost *XIST*	No further evidence presented
Studies refuting a connection between BRCA1 and XCI:			
6. Pageau et al. 2007	3-5% abutting/ partially overlapping 10-15% adjacent	HCC1937 had no XIST in 97% of cells (no mature XIST in cell able to coat the Xi)	add *BRCA1* to *BRCA1*$^{-/-}$ cells, *XIST* transcription ↑d to 57-62%, no *XIST* localization to Xi
7. Pageau and Lawrence, 2006	BRCA1 near active X (7%), BRCA1 near inactive X (10%); same results reported as in (3)	No *XIST* lost w/ *BRCA1* KD; breast cancer cells lost *XIST* signal with or without *BRCA1*	n/a
8. Xiao, C. et al. 2007	< 3% signals abut or partially overlap (0% completely overlapping signal)	Some HCC1937 had *XIST* (focal point or diffuse cluster); no loss of *XIST* with siRNA to *Brca1*	No *XIST* stain on inactive X with intro of *BRCA1*/*Brca1* into HCC1937 or murine *Brca1* KO

1.5.2 Evidence of X reactivation in breast and ovarian carcinomas

There are several lines of evidence suggesting that reactivation may occur in breast and ovarian carcinomas. Firstly, 30 of 178 X chromosome genes assayed by microarray were overexpressed in *BRCA1* mutated ovarian carcinoma tumours when compared to sporadic ovarian carcinoma tumours (Jazaeri et al. 2004). In addition, when *BRCA1* null cells were transfected with *BRCA1* protein, expression of 21 of these overexpressed transcripts was decreased, with a median decrease of 2.4 fold (Jazaeri et al. 2004). Many of these overexpressed genes were found in areas of major cancer testis antigen gene clusters and 11 of the overexpressed genes mapped to the Xp11 region (Jazaeri et al. 2004). The cancer-testis antigens genes are normally expressed in the testis, but have been observed to be expressed in cancer such as in ovarian carcinomas (Yakirevich et al. 2003). Therefore, reactivation of these genes via *BRCA1* loss may lead to breast or ovarian carcinoma.

In addition, fifty years ago it was observed that there was a decreased number of Barr bodies (or inactive X chromosomes) present in breast carcinomas (Kimel et al. 1957). Recently, this finding has been repeated by other studies (Sirchia et al. 2005, Richardson et al. 2006). A decrease in the number of inactive X chromosomes may reflect the reactivation of the inactive X chromosome when *BRCA1* protein is absent. In basal-like breast cancer, reactivation and partial reactivation of the X chromosome have been observed in 22% of cases studied (Richardson et al. 2006). *BRCA1* null breast cancers are often basal-like (Sorlie 2004). Thus, *BRCA1* loss may be necessary for X reactivation. However, *BRCA1* mutations may not be necessary for X reactivation. In seven primary breast carcinomas with two active X chromosomes from patients with and

without *BRCA1* mutations, heterozygosity for several markers was observed (Sirchia et al. 2005). This indicates that the two different active X chromosomes observed may have resulted from an X reactivation event.

Conversely, absence of an inactive X chromosome in a numerically balanced cell may be due to the loss of the inactive X chromosome and subsequent duplication of the active X chromosome (Sirchia et al. 2005, Richardson et al. 2006). Six $BRCA1^{-/-}$ and $BRCA1^{wt}$ breast cancer cell lines with two active X chromosomes were found to be homozygous for 18 markers on the X chromosome (Sirchia et al. 2005). In addition, of 18 basal-like breast cancer samples, 61% had two identical copies of the X chromosome (Richardson et al. 2006). It seems that both situations, X reactivation and inactive X loss and subsequent duplication, can occur. The absence of an inactive X chromosome may be a necessary step in breast tumour progression.

Finally, individuals with Klinefelter syndrome who possess an extra X chromosome (47, XXY) have a 20 to 50 fold increased risk of developing breast cancer compared to normal males (Giordano et al. 2002). Klinefelter males undergo XCI to silence their second X chromosome via the same process of XCI that occurs in females. Therefore, Klinefelter males may have an increased risk of breast cancer because of the possibility of X chromosome reactivation in their cells and subsequent overexpression of a cancer causing gene(s). However, Klinefelter males also have a higher estrogen/androgen ratio than normal males and tend to develop hormone receptor positive cancers (Giordano et al. 2002).

1.6 Hormone receptors

Estrogen exposure mediates the risk of breast and ovarian carcinomas (see previous sections 1.2.3 and 1.3.5) and it has been postulated that a mutation in *BRCA1* might only lead to cancer in the presence of estrogen (Narod and Foulkes 2004). In contrast to the normal trend, women with a *BRCA1* mutation show a marked decrease in breast cancer risk once they become menopausal, a state where a woman's ovaries no longer produce hormones such as estrogen, although ovarian carcinoma risk remains the same (Rosen et al. 2005). In addition, of 4 women bearing both *BRCA1* and *BRCA2* mutations, two had subfertility (one had premature ovarian failure (POF) and the other had primary sterility) but were asymptomatic for breast and ovarian carcinomas, despite a presumed increased risk (Friedman et al. 1998). The authors postulated that decreased estrogen may have played a role in the low penetrance of the mutations. Finally, certain hormone receptors interact with *BRCA1* (Rosen et al. 2005). Though estrogen involvement is plausible, endometrial cancer is not increased in *BRCA1* mutation carriers, which might be expected to occur if estrogen alone played a role (Narod and Foulkes 2004). In addition, most *BRCA1* mutated breast cancers are estrogen receptor negative (ER-) (Sorlie 2004).

If estrogen does influence breast and ovarian carcinomas risk, then polymorphisms that decrease estrogen exposure would modify the risk of developing cancer. Thus, hormone receptor polymorphisms may alter breast and/or ovarian carcinoma risk in women carrying a *BRCA1* mutation.

1.6.1 Androgen receptor

The androgen receptor (*AR*) is a ligand-dependent nuclear receptor in the activated Class I steroid receptor family. *AR* binds its ligand (androgen) in the cytoplasm and then translocates to the nucleus where it can then activate transcription of androgen-regulated genes (for a review of *AR* function, see Gobinet et al. 2002). *AR* predominantly regulates genes involved in male sex differentiation, spermatogenesis and male gonadotropin regulation. The *AR* gene maps to Xq11-q12 and its protein is approximately 919 amino acids in length.

AR protein size is variable due to the presence of a trinucleotide (CAG) repeat polymorphism located in the N-terminal transactivation domain of exon 1. The normal repeat size ranges from 9 to 39 repeats in length, and 91-99% of all individuals regardless of race have repeat lengths between 16-29 repeats (Buchanan et al. 2004). Repeat sizes outside of this range (greater than 39 repeats) have been conclusively linked to Spinal and Bulbar Muscular Atrophy (Kennedy disease). Increased repeats have also been associated with other diseases (prostate, breast, ovarian, endometrial and colorectal cancer, polycystic ovarian syndrome and male infertility) (Gottlieb et al. 2004). Kennedy disease is a neurological disorder whose primary symptom is degeneration of motor neurons in the brain stem and the spinal cord (Greenland and Zajak, 2004). Patients with Kennedy disease can also exhibit androgen insensitivity syndrome, a disorder in XY individuals leading to variable degrees of feminization, including complete sex reversal. Mutations elsewhere in the *AR* gene can also lead to androgen insensitivity syndrome.

Though only a CAG expansion greater than 39 repeats in length is disease causing, it has been observed that increased repeat size is inversely correlated to receptor

function (Chamberlain et al. 1994). There is also a linear relationship between repeat size and the amount of *AR* mRNA transcribed (Choong et al. 1996). Recently, a relationship has been made between repeat length and interdomain communication, a necessary interaction for *AR* transactivation (Buchanan et al. 2004). It seems that only individuals with repeats in the 16-29 range have a satisfactory interdomain interaction, which may explain why diseases have been associated with the upper and lower boundaries of the normal range (Buchanan et al. 2004).

AR repeat polymorphisms have been associated with several reproductive cancers including prostate, endometrial, breast and ovarian carcinomass (Gottlieb et al. 2004). *AR* repeat length studies in breast cancers have produced conflicting results. Similar to prostate cancer, short alleles (alleles bearing short CAG repeat lengths) have been associated with both increased risk of aggressive breast cancer (Yu et al. 2000) and of early-onset cases (Dagan et al. 2002). Short alleles have also been reported to be associated with decreased breast cancer risk (Liede et al. 2003). Furthermore, long alleles have been associated with an increased risk of cancer in young patients (Suter et al. 2003, Kristiansen et al. 2002) and with an increased early risk in patients with a *BRCA1* or *BRCA2* mutation (Rebbeck et al. 1999). Other reports found no connection between *AR* repeat length and breast or ovarian carcinoma at all (Menin et al. 2001). One inherent problem in comparing different studies is that the repeat cutoffs used are variable and there may be population stratification which confounds association studies.

1.6.2 Estrogen receptor

There are two receptors that mediate estrogen action, estrogen receptor alpha (*ESR1*) and estrogen receptor beta (*ESR2*), both part of a nuclear receptor superfamily that includes *AR* (Pearce and Jordan, 2004). *ESR1* is a ligand-activated transcription factor that maps to 6q25.1. The *ESR1* gene is over 140 kb long and encompasses 8 exons. *ESR1* has a role in male and female sexual maturation, bone formation, fertility and behaviour (Herynk and Fuqua, 2004). *ESR1* also subdivides breast cancer into two categories, ER+ and ER- (see section 1.2.1). The *ESR2* gene was discovered ten years after ESR1, maps to 14q23.2 and encodes a protein that is 530 amino acids in length (Pearce and Jordan, 2004). *ESR1* and *ESR2* have very similar DNA binding domains, but differ in sequence for their hormone binding domains and their N-terminal trans-activation domain (Nilssen and Gustafsson 2000). *ESR1* and *ESR2* are also predominantly expressed in different tissues, where *ESR1* is mainly expressed in tissues important for reproduction including the pituitary, the breast and the ovaries and *ESR2* is expressed more ubiquitously (Nilssen and Gustafsson 2000). This thesis will focus on *ESR1*.

Polymorphisms in *ESR1*. There is a dinucleotide repeat (TA) present in the promoter of *ESR1*. Whether this polymorphism results in functional differences is unknown but, based on its location, it may affect which promoter is used (Gennari et al. 2005). The distribution of these repeats is bimodal in nature, with the largest peak at either 14 or 15 repeats, depending on population differences (Gennari et al. 2005). This repeat has been associated with osteoporosis, cardiovascular disease, premature ovarian failure, endometriosis and breast cancer (Gennari et al. 2005, Kunnas et al. 2000, Bretherick et al. 2007, Kim et al. 2005, Iobagiu et al. 2005).

There are two SNP polymorphisms located upstream of the TA repeat in *ESR1* and in linkage disequilibrium with it. The SNPs are referred to as -397T/C or *PvuII*, and -351A/G or *XbaI*, with the former assignation indicating the position and the base pair change and the latter indicating the restriction enzyme cut site that is disrupted by the SNP. Polymorphisms in both SNPs have also been associated with osteoporosis and breast cancer (Gennari et al. 2005, Onland-Moret et al. 2005).

The *ESR1* repeat has been associated with breast cancer risk when considered in combination with other repeat polymorphisms. There is an increased risk of breast cancer when a woman has a short *AR* CAG repeat, a long *ESR1* CA repeat and a short TA repeat (Iobagiu et al. 2005).

1.6.3 Fragile mental retardation 1 (*FMR1*)

The *FMR1* gene is not a hormone receptor; however, repeat length has a role in female reproductive health and will be discussed here. *FMR1* maps to Xq27.3 and comprises 18 exons within 38kb of sequence (for a review see Oostra and Chiurazzi, 2001). The function of *FMR1* is not fully understood, though it can interact with proteins and RNA, and may be involved in post-transcriptional control (Oostra and Chiurazzi, 2001).

FMR1 has a variable trinucleotide repeat (CGG) located in the 5^1 untranslated region of exon 1 (Oostra and Chiurazzi, 2001). A normal repeat length is between 5 and 50 repeats. Having a repeat length greater than 200 is considered a full mutation and will result in fragile X syndrome, which is characterized by moderate to severe mental retardation. Having a repeat length between 50-100 results in a premutation, where an individual does not display a disease phenotype but has an increased chance of

transmitting an expanded repeat which is classified as a full mutation (Oostra and Chiurazzi, 2001). The *FMR1* premutation has been associated with premature ovarian failure (POF), a syndrome where women undergo menopause prior to the age of 40 (Murray et al. 1998) and Fragile X-associated tremor/ataxia syndrome (Jacquemont et al. 2004).

FMR1 has never been associated with breast or ovarian carcinoma risk but has been associated with reproductive disease. Women with POF have a significantly increased chance of having *FMR1* repeat sizes in the high end of the normal spectrum or above (Bretherick et al. 2005). This premature menopause reduces the endogenous estrogen exposure earlier in these women. If having reduced fertility decreases estrogen exposure, it may also confer some protective effect against breast and ovarian carcinomas. Therefore, increased *FMR1* repeat length may occur in women who are asymptomatic for cancer but have mutations in *BRCA* genes that would typically increase their risk for breast and/or ovarian carcinoma.

1.6.4 Hormone receptors and *BRCA1*

Loss of heterozygosity of *BRCA1* often leads to breast and/or ovarian carcinoma, therefore its involvement in hormone receptor function may be integral to how disease develops. Both *ESR1* and *AR* are known to interact with *BRCA1* (Rosen 2005). *BRCA1* physically interacts with *AR* and influences its activity (Park et al. 2000). *BRCA1* also represses the activity of *ESR1* in human breast and prostate cancer cell lines, while truncated copies of *BRCA1* cannot. In addition, when the wild-type *BRCA1* protein is lost, there is spurious activation of *ESR1* (Fan et al. 2001).

1.7 Thesis objectives

Skewed XCI, X chromosome gene overexpression and abnormal X chromosome content and silencing have all been associated with breast and ovarian carcinomas (Buller et al. 1999, Kristiansen et al. 2001, Jazaeri et al. 2004, Richardson et al. 2006). Furthermore, *BRCA1*, the breast and ovarian carcinomas predisposing gene, localizes to the X chromosome in a subset of cells (Ganesan et al. 2002, Silver et al. 2007). The main hypothesis of this thesis is that reactivation of the silent X chromosome is a step in the breast and ovarian carcinomas pathway, leading to overexpression of some tumour enhancing gene. The objective of this thesis was to examine markers of an X reactivation event in ovarian carcinoma cell lines. Previous findings of skewed XCI in breast and ovarian carcinomas may be an artefact of the skewing assay if X reactivation is occurring. Therefore, the degree of skewing in the peripheral blood of breast and ovarian carcinomas patients with *BRCA1* mutations, *BRCA2* mutations or no identified mutations was also assayed.

Hormones have been linked to breast and ovarian carcinomas by epidemiological studies and the hormone receptors *AR* and *ESR1* interact with *BRCA1*. Hormone receptor polymorphisms have been studied in relation to breast and ovarian carcinomas with conflicting results. The second objective of this thesis was to assess polymorphisms in *AR* and *ESR1* in breast and ovarian carcinomas patients with *BRCA1* mutations, *BRCA2* mutations or no identified mutations. Polymorphisms of the *FMR1* gene, a gene involved in a fertility related syndrome (POF), was also assessed in this cohort.

Chapter 2: X reactivation

2.1 X reactivation in ovarian carcinoma cell lines

2.1.1. Introduction

In recent years, evidence has been mounting to suggest that the X chromosome has some role to play in the development of breast and ovarian carcinomas. Female mammals silence one of their two X chromosomes to maintain a balance of gene dosage with males who only possess one X chromosome, in a process known as X chromosome inactivation (XCI) (Lyon, 1961). It has been suggested that the inactive X chromosome is reactivated in breast and ovarian carcinomas development resulting in overexpression of an unknown cancer-causing gene (Stone et al. 2003).

As early as the 1950's it was observed that many breast cancer tumours lack an inactive X chromosome (Kimel et al. 1957), a finding confirmed by more recent studies (Sirchia et al. 2005, Richardson et al. 2006, Pageau et al. 2007). Though this may be due to the loss of the inactive X chromosome and the duplication of the active X chromosome (Sirchia et al. 2005), there may also be reactivation or partial reactivation of the inactive X chromosome (Richardson et al. 2006). Moreover, there is variability among women in regards to the inactivation of some X-linked genes, indicating possible differences in disease susceptibility among women (Carrel and Willard 2005). Finally, several X chromosome genes have been associated with breast and ovarian carcinomas development (for a review see Spatz et al. 2004).

The finding that *BRCA1*, a breast and ovarian carcinomas predisposing gene, and *XIST*, the non-coding RNA that initiates and maintains silencing of the inactive X

chromosome, co-localize to the inactive X chromosome, may support a role for reactivation as a step in breast and/or ovarian carcinoma progression (Ganesan et al. 2002). Ganesan et al. 2002 reported that *XIST* RNA and *BRCA1* protein overlapped in a significant portion of cells (5-10%) and that in a cell line lacking *BRCA1* expression, *XIST* foci failed to form while reintroduction of wild-type *BRCA1* allowed some *XIST* foci to be reconstituted. In support of this association, overexpression of 30 of 178 X-linked genes in *BRCA1*-null ovarian carcinoma tumour samples compared to sporadic ovarian carcinoma tumour samples was observed by microarray analysis (Jazaeri et al. 2004).

The co-localization of *XIST* and *BRCA1* has been called into question by two groups (Pageau et al. 2007, Xiao et al. 2007). Consistent, fully overlapping *XIST* RNA and *BRCA1* signals were never observed in either study, while partially overlapping signals occurred in approximately 2% of cells and 3-5% of cells respectively (Xiao et al. 2007, Pageau et al. 2007). Adjacent signals were also reported in 10-15% of cells across three cell lines assayed (Pageau et al. 2007). Other investigators have claimed to have reproduced the original findings of *BRCA1* and *XIST* signal overlap but have not quantified how often they observed co-localization (Chadwick and Lane 2005, Ouyang et al. 2005, Diaz-Perez et al. 2006, Silver et al. 2007).

Other aspects of a *BRCA1* and XCI connection were reported, including: 1) the absence of *XIST* RNA coating the inactive X chromosome in *BRCA1*-null cells; 2) the presence of *XIST* RNA re-coating the inactive X chromosome after reintroduction of wild-type *BRCA1* and; 3) the mislocalization of *XIST* after RNA interference to *BRCA1* (Ganesan et al. 2002). However, when these findings were studied by other researchers,

the results were not reproducible (Pageau et al. 2007, Xiao et al. 2007). Firstly, of the *BRCA1*-null cell lines studied, not all lacked *XIST* signaling (Pageau et al. 2007). In addition, while *BRCA1* overexpression in an inducible system did increase *XIST* transcription, the pattern of *XIST* localization was not consistent on the inactive X chromosome and controls had similar *XIST* localization (Pageau et al. 2007). Moreover, an siRNA knockdown system against *BRCA1* failed to alter *XIST* signaling (Xiao et al. 2007) or any other markers of XCI studied (macroH2A, UbFk2, K2mK27; Pageau et al. 2007). The original group has reproduced several of their earlier results, citing the specific methods employed as the reason for discrepancies with other studies (Silver et al. 2007).

Despite contradictory findings, it is clear that while *BRCA1* may not directly co-localize with *XIST* RNA, its presence abutting and partially overlapping the inactive X chromosome in a subset of cells may indicate a potential role in some aspect of X chromosome inactivation or maintenance. Furthermore, whether or not there is a real connection between *BRCA1* and XCI, the question remains: Why does a mutation in a ubiquitously expressed, multi-purposed gene mainly predispose to female-predominant cancers? While this may be due to an estrogen-mediated effect, other aspects of female biology may also be important.

Reactivation or partial reactivation of the inactive X chromosome, whereby a gene or suite of genes become overexpressed enabling tumourigenesis, may account for the connection between the X chromosome and breast and ovarian carcinomas, and may also shed light on the relationship between *BRCA1* and XCI. If X reactivation does occur, specific changes would mark the event. Three such markers were assessed in

ovarian carcinoma cell lines: 1) decreased or absent *XIST* signal in cells; 2) overexpression of monoallelically expressed X chromosome genes using real-time PCR and; 3) decreased or absent methylation at the promoter of two monoallelically expressed X chromosome genes (*AR* and *FMR1*). Though none of these three markers alone is direct evidence for X chromosome reactivation, each could be attributable to such a phenomenon. The presence of *XIST* and X-linked gene expression were also assessed in short term cultures from the normal ovarian surface epithelium (OSE) of women with a *BRCA1* mutation and a history of breast cancer. Changes in these markers could indicate an early role for X reactivation in the progression of ovarian carcinoma.

Finally, another mark of X reactivation was assayed, skewed XCI, in the peripheral blood of breast and ovarian carcinomas patients with and without *BRCA1* mutations (Chapter 2.2). If there is allele-specific X reactivation in these patients, this could lead to skewed XCI, where the observed skewing is actually an artifact of the skewing assay itself.

2.1.2 Materials and methods

Cell lines

Eight ovarian carcinoma cell lines, one immortalized ovarian surface epithelium cell line and two short-term ovarian surface epithelium cultures of *BRCA1* mutation carriers with a history of breast cancer were generously provided by Dr. Nelly Auersperg. A 3-X chromosome cell line was also used as a control and was obtained from Dr. Carolyn Brown. Details about these cell lines are presented in Appendix 1. Cells were grown for three purposes: DNA extraction, RNA extraction and RNA/DNA FISH.

Assessing XIST RNA signaling in ovarian carcinoma cell lines

One of the markers of the inactive X chromosome that can be visualized with fluorescence in situ hybridization (FISH) is *XIST* RNA localization to the inactive X chromosome. By hybridizing fluorescently labeled *XIST* RNA to cells, a decrease in *XIST* localization indicating potential X reactivation or partial reactivation can be observed. The cells that were studied were derived from cancer cell lines, which can have markedly different chromosome content when compared to a normal 46 XX or XY individual. It was therefore important to also assess the number of X chromosomes present within each cell.

Cell culture and fixation

All cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM), high glucose, L-glutamine (Invitrogen, Burlington,Ontario). Gibco's Fetal Bovine serum and a Penicillin and Streptomycin solution were added to the media at a concentration of 10% and 1x respectively (Invitrogen, Burlington,Ontario). When cells reached 90% confluence, the old media was aspirated from the culture dish, the cells were washed with Dulbecco's 1x distilled PBS and then trypsinized using a 0.25% solution of Trypsin-EDTA (Invitrogen, Burlington,Ontario). The subculture ratio depended on the cell line in question.

For RNA and DNA extraction, one 50 ml tissue culture flask was required (Becton Dickinson Labware, Franklin Lakes, NJ). Cells for FISH were grown directly onto 22 x 22 mm Gold Seal® Cover Glass (Ted Pella Inc., Redding, CA) in 60 x 15 mm Tissue Culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ). When cells were 70-90% confluent of the cover slips, they were fixed for use with FISH.

To fix the cells onto the slides, slides were first washed briefly in CSK buffer (0.1M Pipes pH7.8, 1M NaCl, 1M $MgCl_2$, sucrose, water) (Hall et al. 2002). The cells were then washed in a solution of 9 ml CSK buffer, 500 µl ribonucleoside vanadyl complexes (Sigma, Saint Louis, MO) and 500 µl of 10% Triton X-100 solution (Roche Diagnostics, Indianapolis, IN) for three to five minutes. Following this wash, slides were placed in 10 ml of 4% paraformaldehyde [10 ml microfiltered 16% formaldehyde (Ted Pella Inc., Redding, CA), 4mL 10X distilled PBS (Invitrogen, Burlington,Ontario), 26 ml of distilled H_2O] for eight to ten minutes. The slides were finally rinsed in 70% ethanol and stored in 70% ethanol at 4ºC until use.

RNA/DNA FISH

To assess and to simultaneously quantify the number of X chromosomes present within a cell and the presence or absence of the *XIST* RNA signal, a technique that combines RNA and DNA FISH on the same slide was employed (Hall et al. 2002, Chow et al. 2007). Slides of interphase cells were first labeled with an RNA probe. The RNA probe used was a Digoxigenin-11-dUTP-labeled *XIST* probe (Roche Diagnostics, Indianapolis, IN). This probe was labeled by nick translation with the BioNick™ Labeling System (Invitrogen, Burlington,Ontario). The slides were then labeled with a DNA probe. A commercially available probe, LSI Androgen Receptor Spectrum Orange, a directly labeled DNA probe to the androgen receptor (*AR*) gene, was used (Abbott Molecular Inc., Des Plaines, IL).

For RNA hybridization, cover slips were rinsed in cold 100% ethanol for 5 minutes and then air dried. An RNA hybridization mix was made and dried which contained 12 µg of human Cot-1 DNA (Invitrogen, Burlington, ON), 20 µg of a salmon

sperm and *E.Coli* tRNA solution [10 µg of salmon testes DNA (Sigma, Saint Louis, MO) and 0.01 g tRNA (Roche Diagnostics, Indianapolis, IN)] and 50 ng of dioxigenin-labeled, nick translated *XIST* probe. Human Cot-1 DNA was used to block hybridization of the signal to repetitive sequences. Both salmon sperm and *E. coli* tRNA were used to block non-specific hybridization. The dried probe was resuspended in 10 µl of 100% formamide (Sigma, Saint Louis, MO) and denatured at 80°C for 10 minutes. 10 µl of a 4:1 mix of 4 parts RNA hybridization buffer [1mg Albumin BSA (Roche Diagnostics, Indianapolis, IN), 1 ml 20X SSC (Invitrogen, Carlsbad, CA), 2 ml 50% Dextran Sulfate] and 1 part vanadyl ribonucleoside complexes (New England Biosystems, Pickering, ON) was added to the probe. The slides were then placed onto the probe mix on a glass plate and incubated overnight in a humid chamber at 37°C. Slides were then subjected to a series of washes to remove the probe mix: 5 ml formamide and 5 ml 4X SSC at 37°C for 20 minutes, 2X SSC at 37°C for 20 minutes, 1X SSC at room temperature for 20 minutes and 4X SSC at room temperature for 1 minute. For detection of the probe, 40 µl of a mixture of 1 µg of anti-Digoxigenin-Fluorescein Fab Fragments (Roche Diagnostics, Indianapolis, IN) and 500 µl 4X SSC with 1% BSA (Roche Diagnostics, Indianapolis, IN), was placed on a glass plate and allowed to incubate with the slides at 37°C for 1 hour. This was removed with three washes: 4X SSC, a mixture of 4X SSC and 0.1% Triton X-100 (Roche Diagnostics, Indianapolis, IN), 4X SSC and 4% paraformaldehyde. All washes were performed at room temperature, with agitation and in the dark (to protect the fluorescent signal).

For DNA hybridization, a mixture of formamide, 20X SSC buffer and water was heated to 73°C and placed in a water bath (at 70 to 75°C) in a fume hood. Slides were

placed into the formamide mixture for 5 minutes then placed into cold 70% ethanol for 5 minutes and finally into cold 100% ethanol for 5 minutes. After these washes the slides were air dried. All of these steps were done in the dark to preserve the fluorescent *XIST* RNA signal. A DNA hybridization mix was prepared using 14 µl LSI/WCP Hybridization Buffer, 1 µl LSI Androgen SO Probe probe and 5 µl water (Abbott Molecular Inc., Des Plaines, IL). The probe was denatured at 80°C for 10 minutes then placed on a glass plate with the slides. The slides and probe were incubated in a humid 37°C chamber overnight. Slides were then subjected to a series of washes to remove the probe mix: 5 ml formamide and 5 ml 4X SSC at 37°C for 20 minutes, 2X SSC at 37°C for 20 minutes, 1X SSC at room temperature for 20 minutes and 4X SSC at room temperature for 1 minute. The slides were finally incubated in 4´,6-diamidino-2-phenylindole, dilactate (DAPI) (Invitrogen, Burlington, ON) for 20 seconds and rinsed twice in 1X PBS. Cover slips were mounted onto slides using Vectashield® Mounting Medium (Vector Laboratories Inc., Burlingame, CA).

A Leica DMI 6000 B microscope was used to visualize cells and signals (Leica Microsystems, Weslar, DE). A QImaging RETIGA 4000 4 camera and Q Capture computer software was used to capture images (QImaging, Surrey, BC). A total of 300 cells were counted per cell line.

Measuring X chromosome gene expression levels

Based on four criteria, eight X chromosome genes were chosen to assess overexpression via real-time PCR (Table 2.1). The criteria were: 1) overexpression in *BRCA1* ovarian carcinomas; 2) repression with the introduction of *BRCA1*; 3) absence of a CpG island and; 4) variable expression. The first two criteria were based on previous microarray

data indicating overexpression of certain X-linked genes in *BRCA1* mutation carriers with ovarian carcinoma, compared to sporadic ovarian carcinomas, and repression of expression of certain overexpressed genes with the introduction of wild-type *BRCA1* (Jazaeri et al. 2004). The third and fourth criteria stem from a comprehensive study of X chromosome genetics and epigenetics (Carrel and Willard, 2005). This study provides a catalogue of information upon the genes of the X chromosome including the presence or absence of a CpG island and the expression of each gene from one or both X chromosomes. CpG islands are methylated in genes that undergo XCI. X chromosome genes that do not possess CpG islands may be more easily reactivated than others because they lack one form of DNA silencing. Variable expression of some X-linked genes was observed among women and may influence disease susceptibility in women (Carrel and Willard, 2005). *ZFX* and *MIC2* were used as controls since both genes escape XCI. Their expression may not increase if X reactivation occurs. *XIST* was also assayed since it is expressed solely from the inactive X chromosome in a normal cell. A decrease or absence of *XIST* expression could also indicate X reactivation.

Table 2.1. Genes studied for X gene overexpression assay.

Gene	**Location**	***Data from Jazaeri et al.*** (J Transl Med. 2004 2(1):32)		***Data from Carrel and Willard*** (Nature. 2005 434(7031):400-4)	
		Overexpressed in BRCA1$^-$ ov. can.	**Repressed with BRCA1**	**CpG island?**	**Expression from the inactive X?**
WAS	Xp11.4	Yes	No	No	vary (4 of 9 express)
TIMP1	Xp11.3	Yes	Yes (2.1 fold)	No	vary (3 of 9 express)
VBP1	Xq28	Yes	No	No	vary (1 of 9 express)
KLF8	Xp11.21	Yes	Yes (2.5 fold)	No	No (0 of 9 express)
ARAF1	Xp11.4	Yes	No	Yes	No (0 of 6 express)
ZFX	Xp22	Yes	No	Yes	Yes (9 of 9 express)
MIC2	Xp22.32	Yes	No	Yes	Yes (9 of 9 express)
XIST	Xq13.2	No	No	No	Yes (9 of 9 express)

Note: Colours sort genes by how they express from Xi. Yellow: variable expression between women; Blue: monoallelic expression; Green: biallelic expression

RNA extraction

RNA extraction was performed on cells grown in culture to 90% confluence in T25 flasks. The Sigma-Aldrich GenElute™ Mammalian Total RNA Miniprep kit was used for RNA extraction (Sigma, Saint Louis, MO). Media was aspirated from the attached cells, which were then used directly for RNA extraction. For attached cells with a surface area of 25 cm^2, 500 µL of a 100:1 solution of lysis solution and 2-mercaptoethanol was added to the flasks in order to lyse the cells and inactivate RNases. After allowing the mixture to sit with the lysis solution for 1 to 2 minutes, the lysate was rocked back and forth and filtered into a GenElute Filtration Column, then centrifuged for 2 minutes at 13 500 rpm. The resulting lysate was mixed with an equal volume of 70% ethanol and loaded into a GenElute binding column. The binding column was subsequently washed once with Wash Solution 1 and twice with Wash Solution 2. The RNA was finally eluted from the binding column with 5 µL of Elution Solution. This step was performed twice. The extracted RNA was stored at –70°C.

RT-PCR

Reverse transcriptase PCR (RT-PCR) was performed to convert the extracted RNA into cDNA using the Qiagen QuantiTect® Reverse Transcription Kit following manufacturer's instructions (Qiagen Inc., Mississauga, ON). First, genomic DNA was removed from the RNA by incubation with gDNA Wipeout Buffer at 42°C for 2 minutes. Next, the cDNA was prepared by mixing RNA with Quantiscript Reverse Transcriptase, Quantiscript RT Buffer and RT Primer mix and incubating at 42°C for 15 minutes then at 95°C for 3 minutes. Resultant cDNA was stored at -20°C for no longer than two weeks.

Real-time PCR

Using the cDNA obtained from the reverse transcriptase reaction, real-time PCR was used to quantify gene expression of the ovarian carcinoma cell lines compared to the control ovarian cell line for eight genes using the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Real-time PCR can ascertain the initial imput amount of mRNA of a given gene by measuring the strength of a fluorescent reporter signal during PCR amplification cycles. The gene of interest or target gene was normalized by a reference gene run in tandem to it. The reference gene was a housekeeping gene, b-Actin, which was expected to express in a similar fashion across all samples. Using a reference gene provided a normalization tool for the quantity and the quality of the imputed samples. The real-time data analysis method employed was the absolute quantification method. Using this method, a standard curve of known cDNA concentrations was run for both the target gene and the reference gene. A standard curve is a serial dilution of known cDNA concentrations that spans a large range. Samples of unknown concentrations will be correctly quantified if they fall within the range of the curve. Real-time PCR expression assays employed Taqman® technology (Applied Biosystems, Foster City, CA). Taqman® probes contain a 5' fluorescent reporter and a 3' quencher. When the probe is unbound and intact, no fluorescence will be emitted. When the probe binds the specific PCR product, the quencher is cleaved from the probe and a fluorescent signal is emitted.

TIMP1 and *ARAF1* genes were analyzed using ABI custom Taqman® MGB primers and probes labeled with VIC™ (Applied Biosystems, Foster City, CA). The primers and probe for *TIMP1* were 5'-TCCAGCGCCCAGAGAGAC-3' (forward), 5'-

AACAGGATGCCAGAAGCCAG -3' (reverse) and 5'-CCAGAGAACCCACCATGGCCCC-3' respectively, as described in Simi et al. 2004. The primers and probe for *ARAF1*, 5'CCTFFATGCCAAGAACATCA-3' (forward), 5'-ACCGTGAGCCCCTCATGTAG-3' (reverse) and 5'-AGATCTCAAGTCTAACAACAT-3' (probe), were designed using Primer Express® software and made to overlap exon junctions (Applied Biosystems, Foster City, CA). The genes *VBP1*, *WAS*, *KLF8*, *MIC2*, *ZFX* and *XIST* were assayed using pre-designed TaqMan® Gene Expression Assays that use FAM™ dye-labeled, TaqMan® MGB probes (Applied Biosystems, Foster City, CA). The reference gene used was b-Actin, which is expressed in ovarian tissue and was assayed using a TaqMan® Gene Expression Assay.

A PCR mix was made for each cDNA sample of 12.5 uL 2x TaqMan® Universal PCR Master Mix, 20 uM of primer, 10 uM of probe, 7 uL of distilled water and 25 ng of cDNA. Each cDNA sample was done in triplicate and specific care was given to ensuring that all samples were thoroughly mixed and that all cDNA and master mix was evenly distributed among wells. The PCR mix was loaded in to a 96 well flat plate (Applied Biosystems, Foster City, CA) sealed with a MicroAmp™ optical adhesive film (Applied Biosystems, Foster City, CA) and run on an ABI Prism® 7000 Sequence Detection System using the absolute quantification method. Analysis of real-time PCR data was performed using ABI Prism® 7000 Sequence Detection software version 1.2.3 (Applied Biosystems, Foster City, CA).

Data was accepted if the following criteria were met: the standard curves for both the target and the reference gene had an R^2 value of 0.985 or greater, the efficiency of the

standard curves ranged from 90 to 110% (3.1-3.6), all of the samples fell within the standard curve and the triplicate values did not vary greatly (coefficient of variation was less than 1).

Reproducibility

The real-time PCR assay was highly reproducible among different RT PCR reactions and different real-time PCR runs (N=7 pairs, $p<0.0001$, Linear Correlation and Regression, Figure 2.1). In addition, different passages did not affect the real-time gene expression results when tested for one cell line (OVCAR3) and one gene (*TIMP1*) (N=4, $p=0.338$, Linear Correlation and Regression, Figure 2.2).

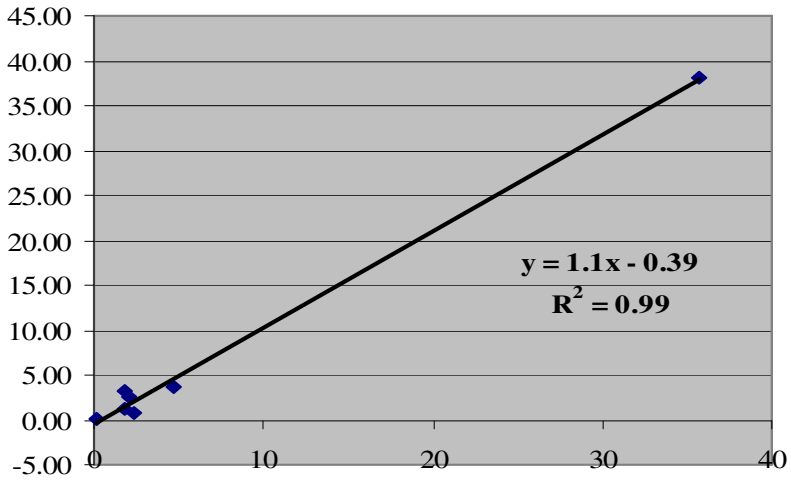

Figure 2.1. Reproducibility between real-time runs (using *TIMP1*).

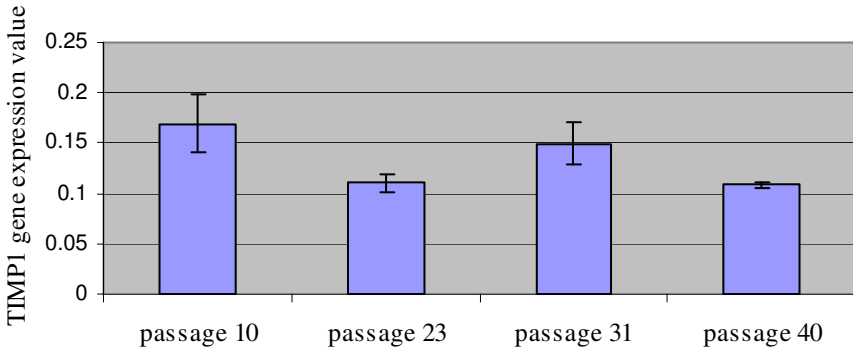

Figure 2.2. Reproducibility between different passages of a given cell line (OVCAR3) and for a given gene (*TIMP1*).

Quantifying the level of methylation of the inactive X chromosome at *AR* and *FMR1*

Another mark of the inactive X chromosome is methylation at the CpG islands of most inactive genes. If there is reactivation of the silent X chromosome there may be decreased or absent methylation at these CpG islands. To measure the level of methylation at X chromosome genes, two assays were performed. The first was a quantitative assay that measured the amount of methylation at the androgen receptor (*AR*) using real-time PCR technology and the ABI Prism® 7000 Sequence Detection System (developed by Bretherick, unpublished results). Methylation was also quantified at a second gene, *FMR1* using a SnuPE assay (described below).

AR Methylation Assay

The *AR* methylation assay makes use of the same principle as the skewed XCI assay (Chapter 2.2). The first step was to digest the DNA with a methylation-sensitive enzyme. Each digested sample contained 150 ng of DNA, 2.5U *HpaII*, 1U *RsaI* in 1x buffer I (10mM Bis Tris Propane-HCl, 10mM MgCl2, 1 mM dithiothreiotol, ph 7.0), for a total volume of 10µL (New England Biolabs, Pickering, ON). The undigested sample contained everything in the digested sample, except *HpaII*. Samples were assessed for

complete digestion by a *MIC2* PCR.

Real-time PCR quantification of the amount of DNA present in the digested sample compared to the amount of DNA present in the undigested sample was performed to assess the amount of methylation at *AR*. If there was normal methylation (50%), then the digested sample would have half the amount of *AR* PCR product amplify compared to the undigested sample, since only the methylated DNA would amplify from the digested sample. After normalization with a reference gene, the amount of *AR* from the digested sample could be divided by the amount from the undigested sample yielding a percent value. A region of *XIST* lacking a cut site was used as a reference. The primers and FAM-labeled probe for the target gene (*AR*) were 5'-TGCGCGAAGTGATCCAGAA -3' (forward), 5'-CTGCAGCAGCAGCAAACTG -3' (reverse) and 5'-AGGCACCCAGAG-3' respectively. The primers and VIC-labeled probe for the reference gene (*XIST*) used were 5'-TCTCAAGGCTTGAGTTAGAAGTCTTAAG -3' (forward), 5'-CGTTGGCCTTGTGTCACAAGT -3' (reverse) and 5'- CTGGGACAGGACACA -3' respectively (Applied Biosystems, Foster City, CA).

The standard curve used for this assay had a DNA concentration range from 1000 to 0.1. The curve required a large range in order for poorly methylated cell lines to fall on the curve.

Reproducibility

The correlation between different digests and real-time runs for the *AR* methylation assay was good (N=8 pairs, p=0.004, Linear regression and correlation, Figure 2.3).

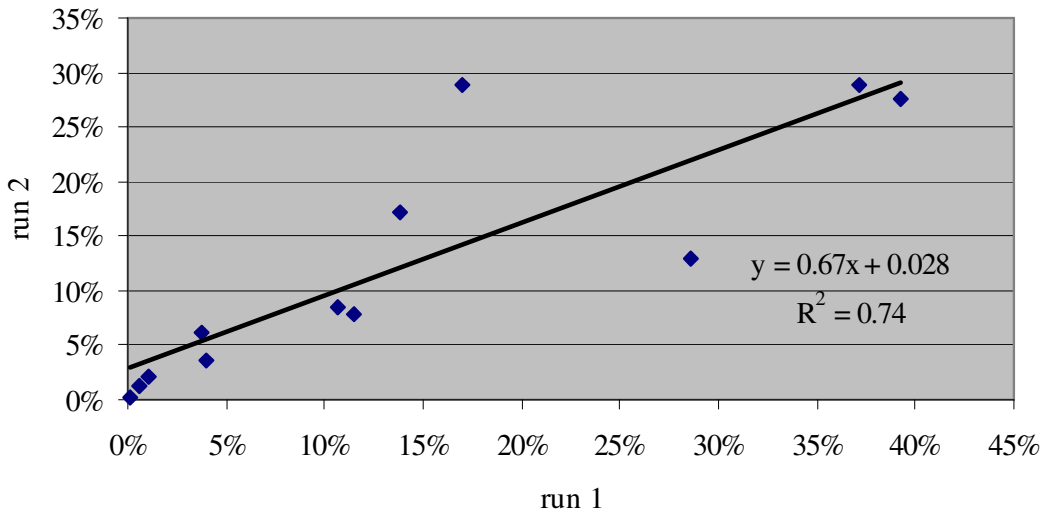

Figure 2.3. Reproducibility between real-time PCR runs of the *AR* Methylation Assay.

FMR1 Ms-SNuPE Methylation Asssay

To assess methylation at *FMR1* a Single Nucleotide Extension Assay (SNuPE) was performed. Using bisulfite treatment, unmethylated cytosines became converted to uracils, while methylated cytosines remained the same. A region of interest was then PCR amplified. Note that converted, unmethylated cytosines become thymines with replication. Strand-specific SNuPE primers will anneal to a sequence directly adjacent to a specific CpG. A single fluorescently labeled base, a TAMRA labeled C (black) or a dROX labeled T (red) will be added at the cytosine position of the CpG depending on whether the DNA was converted or not. The respective amounts of black and red fluorescence can then be compared. For *FMR1*, a gene that is monoallelically expressed and methylated on one X chromosome, the expected ratio of black peak to red peak is 50%.

DNA was first bisulfite converted with the Zymo Research EZ DNA Methylation-Gold Kit (Cedarlane Laboratories Limited, Burlington, ON). Then, the bisulfite-treated

DNA was PCR amplified at two cytosines (position C5 and C6) within a CpG island of the *FMR1* promoter using the primers 5'-TGTAAAACGACGGCCAGTTGA (-21 M13 tail) GTGTATTTTTGTAGAAATGGG -3' (forward), 5'-GCAGGAAACAGCTATGACC (-21 M13 tail) TCTCTCTTCAAATAACCTAAAAAC-3' (reverse) (Applied Biosystems, Foster City, CA, Boyd et al. 2006). The PCR reaction was performed using the ABI Amplitaq GOLD system. The PCR mix included Amplitaq 10X buffer, 25mM $MgCl_2$, 3.2 µl dNTPs, 20 µM of each primer, 0.2 µl AmpliTaq GOLD and 0.5µl bisulfite-treated DNA per sample (Applied Biosystems, Foster City, CA). The PCR cycling conditions were: 95°C for 5 minutes; 95°C for 30 seconds, 60°C for 2 minutes, 72°C for 3 minutes (4 times); 95°C for 30 seconds, 65°C for 1 minute, 72°C for 3 minutes (29 times), 60°C for 1 hour and 25 minutes. Next, 10µl of the PCR product was mixed with 3 µL of loading buffer (50% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol, 25 mM EDTA, 50 mM Tris (pH7.4)), run on a 1% agarose, 1% ethidium bromide gel at 6V/cm for 45 minutes, then visualized with UV light. If the bisulfite blank and the PCR blank were clean, the samples were treated with 6.67 U Shrimp Alkaline Phosphatase (*SAP*) and 2.6 U Exonuclease 1 (*EXO1*) (usb®, Cleveland, OH) incubated at 37°C for 1 hour, 75°C for 15 minutes, then held at 4°C.

The PCR products were then cleaned using the Zymo Research DNA Clean and Concentrator 5 (Cedarlane Laboratories Limited, Burlington, ON). The bisulfite-treated, cleaned PCR products were then used in a SNuPE reaction using the SNAPSHOT Multiplex kit (Applied Biosystems, Foster City, CA) using the primer 5'-GAGGTAGTGC/TGATTTGTTAT -3' (Bretherick, unpublished). Each reaction included 0.4 µM of primer, 5 µl of SNAPshot mix and 3µL of PCR product. The cycling

conditions were: 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 30 seconds, for 25 cycles. Finally, 5 µL of a Calf Intestinal Alkaline Phosphatase (CIAP) mixture containing 10X dephosphorylation buffer, dH2O and 1 U CIAP was added to each sample (Invitrogen, Burlington, ON). The mixture was incubated at 37°C for 1 hour, 75°C for 15 minutes, and then held at 4°C. The samples were run by capillary electrophoresis on an ABI Prism® 310 Genetic Analyzer, peaks detected by ABI Prism® Data Collection software and analyzed using ABI Prism® Genescan software.

Reproducibility of the FMR1 SNuPE assay

The correlation between different bisulfite treatments and SNuPE assay runs for the *FMR1* methylation was high (N=7 paired samples, $p<0.0001$, Linear regression and correlation, Figure 2.4). There was also a significant correlation between the two CpG sites tested, C5 and C6 (N=7 paired samples, $p<0.0001$, Linear regression and correlation, Figure 2.5).

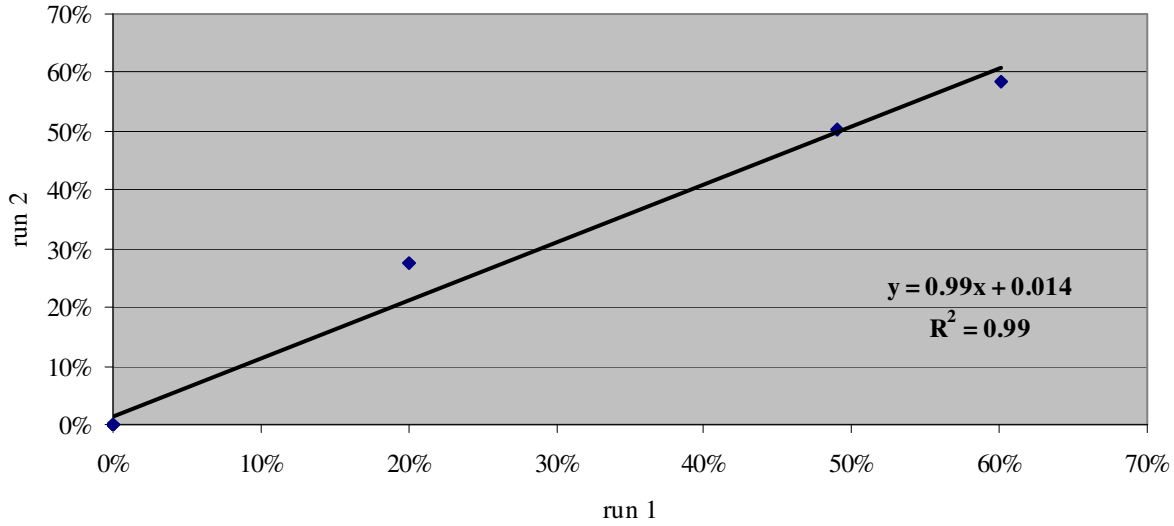

Figure 2.4. Reproducibility of the *FMR1* SNuPE assay.

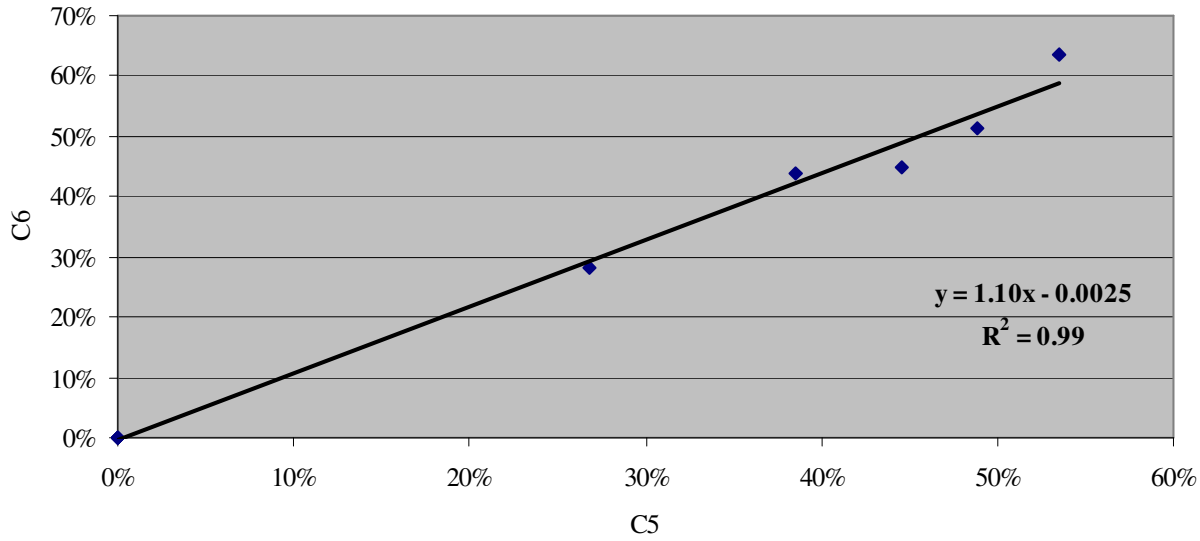

Figure 2.5. The correlation between two CpGs assayed within a CpG island (C5 and C6).

Assaying heterozygosity in ovarian carcinoma cell lines

Evidence for X reactivation by the presence of an *XIST* signal, methylation or gene expression levels cannot distinguish between duplication of an active X chromosome versus true reactivation of an inactive X chromosome. Therefore, DNA extracted from ovarian carcinoma cell lines was assessed for heterozygosity at 4 markers: *AR*, *FMR1*, DXYS122 and DXYS233. Information on *AR* and *FMR1* heterozygosity was obtained via the XCI skewing assay (Chapter 2.2). DXYS233 and DXYS122 heterozygosity were assayed by a conventional PCR around the repeating sequence followed by analysis on the ABI Prism® 310 Genetic Analyzer (see Chapter 3.2 for method). The primers for DXYS233 were Fam-labeled 5'-TGGGAATTCGAGGCTG-3' (forward) and 5'-TGATTTCCATCCTGGGGT-3' (reverse) (Dib et al. 1996). The primers for DXYS122 were Fam-labeled 5'-GCAAAAATCCCCAGCC-3' (forward) and 5'-TTCATTGCCATCCAGATTC-3' (reverse) (Dib et al. 1996).

2.1.3 Results

Lack of *XIST* RNA signaling in some ovarian carcinoma cell lines and short-term OSE cultures.

Of eight ovarian carcinoma cell lines, three lacked any *XIST* RNA signals, which may indicate that there was either reactivation of the inactive X chromosome or loss of the inactive X chromosome and duplication of the active X chromosome (Figure 2.6 a,b,c). Five cell lines had at least one *XIST* signal, however the number of *XIST* signals did not correspond to the expected N-1 (where N is the number of X chromosomes present within a cell as determined by the *AR* FISH signal) (Figure 2.6 d,e,f,g,h).

The two short term cultures were from normal ovarian surface epithelium and not expected to have a change in X chromosome content or *XIST* signaling. However, though both samples had the normal number of X chromosomes, FAM2 did not localize an *XIST* signal (Figure 2.6 i,j). This may indicate that loss of an *XIST* signal is an early event in ovarian carcinoma.

There was great variability in the X chromosome content of ovarian carcinoma cell lines. Five of eight cell lines consistently had greater than two X chromosomes, while two cell lines were remarkably variable in X chromosome content among cells (OVCAR2, OVCAR10, Figure 2.6 g,h). This indicates X chromosome instability in ovarian carcinoma.

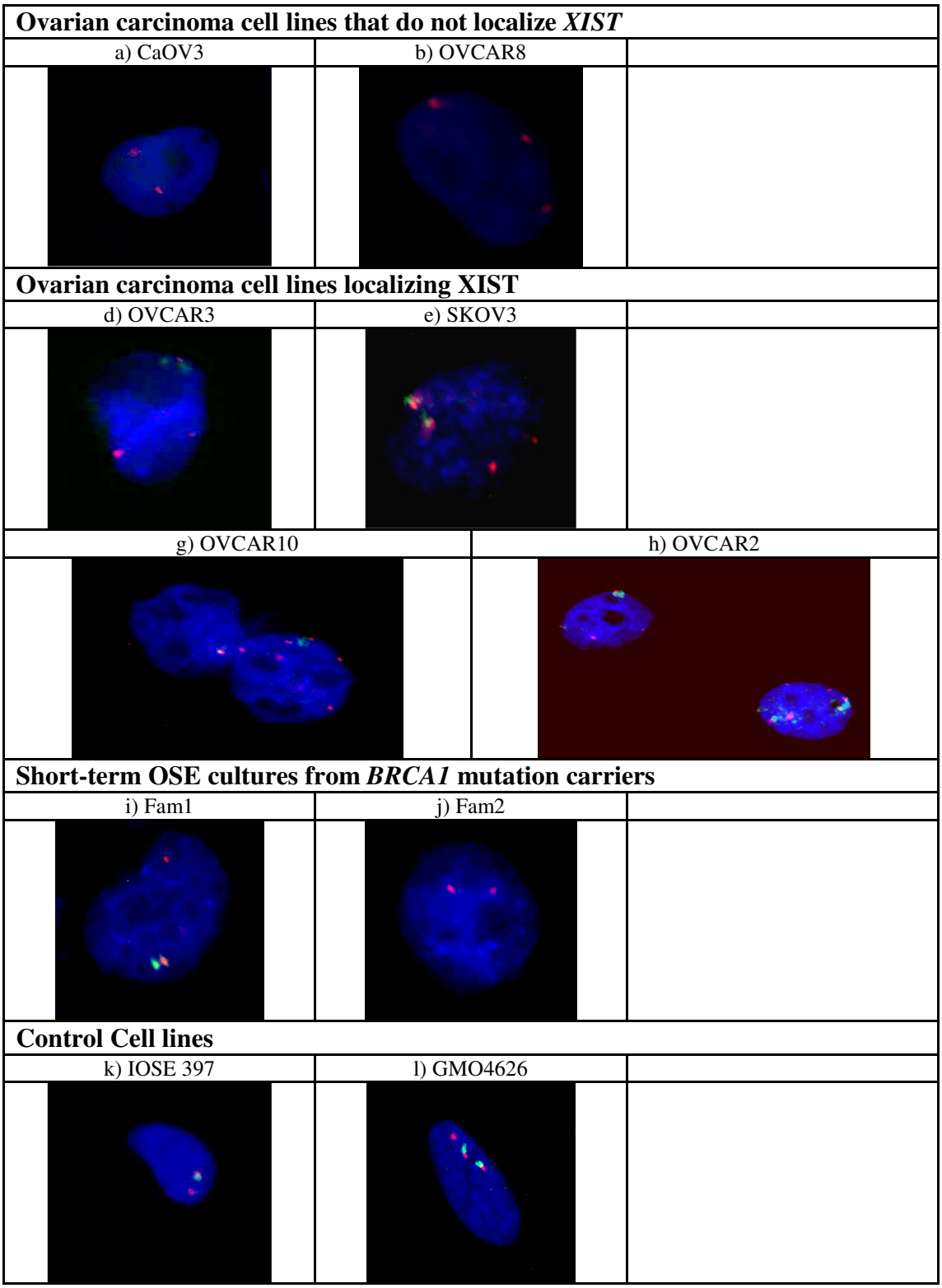

Figure 2.6. DNA/RNA FISH results for ovarian carcinoma cell lines. A representative image of the most commonly observed pattern for each cell line is shown. The number of X chromosomes is indicated by the # of *AR* signals (red) and the number of inactive X chromosomes is indicated by the # of XIST signals (green).

Increased expression of X-linked genes in ovarian carcinoma

If there is X reactivation, overexpression of X chromosome genes that are normally silenced on the inactive X chromosome would be expected (Table 2.1, Figure 2.7; yellow and blue), while genes that escape XCI and are expressed from both copies would not show a change (Table 2.1; Figure 2.7; green). When all ovarian carcinoma cell lines were considered together, this trend was observed (Figure 2.7). Following the theory of X reactivation, absent or low levels of *XIST* was expected. However, there was an overall increase in *XIST* expression, which was inconsistent with the theory of X reactivation (Figure 2.7; pink). Genes that are variably expressed among females (yellow) were overexpressed, but did not show a greater increase in expression compared to genes that are always silenced (blue) (Figure 2.7). Gene expression changes were not expected in XCI escaping genes if X reactivation occurred. Consistent with this hypothesis, genes that escape XCI and are normally expressed biallelically (green) were not overexpressed in ovarian carcinoma cell lines.

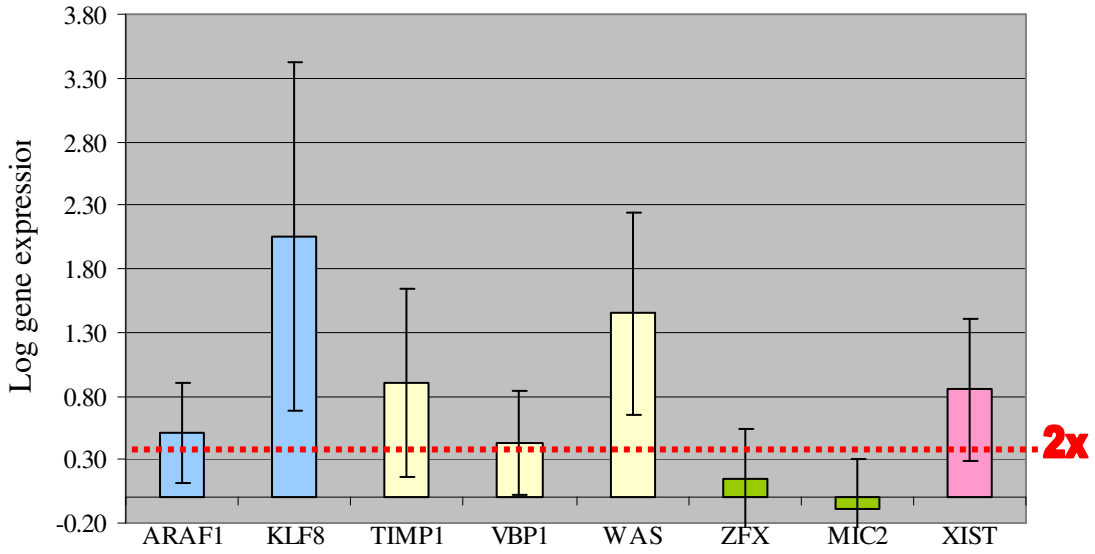

Figure 2.7. Log differences in expression of X-linked genes when considering all ovarian cell lines together (compared to an ovarian surface epithelium control cell line, IOSE 397). Blue: genes that undergo XCI, yellow: genes that undergo XCI in some women, green: genes that escape XCI, pink: *XIST*.

Despite a general increase in X chromosome gene expression from genes that undergo XCI, the patterns of increase were inconsistent between different genes and among cell lines, ranging from a 2-fold to a 300-fold increase of expression compared to a control cell line (IOSE 397). (Figure 2.8). In addition, two cell lines (CAOV3 and OVCAR5) do not have any *XIST* expression, indicating that there may be no inactive X chromosome in these cell lines. Short-term cultures from the OSE of *BRCA1* mutation carriers (FAM1 and FAM2) also had increased gene expression from certain X chromosome genes. Both FAM1 and FAM2 had highly increased expression of TIMP1 and KLF8 when compared to an OSE control cell line.

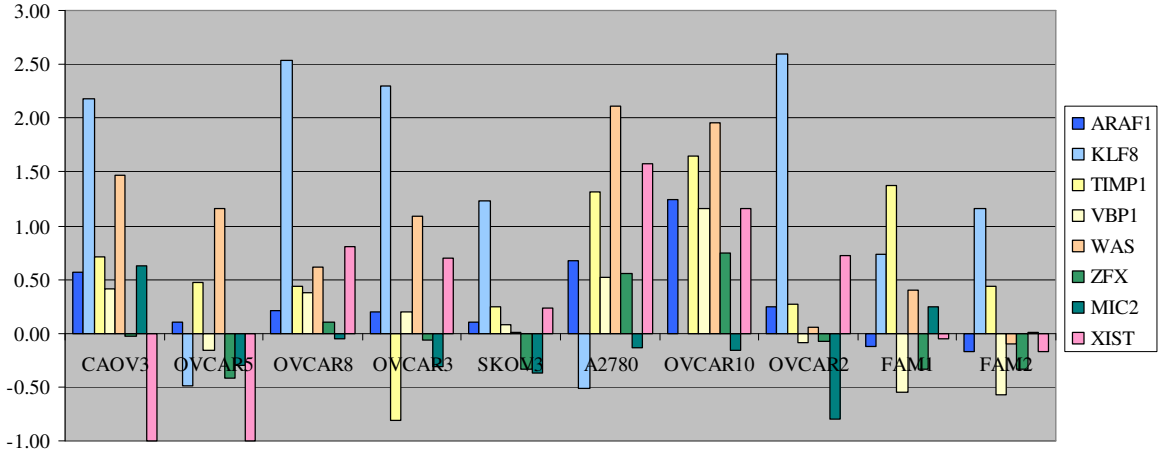

Figure 2.8. Log values X linked gene expression when compared to IOSE 397 (control cell line). Overexpression is shown here as a positive bar while underexpression is shown as a negative bar. (*Note*: CaOV3 and OVCAR5 had no measurable expression of *XIST*. Graphically, this was demonstrated as -1.00). Blue: genes that undergo XCI, yellow: genes that undergo XCI in some women, green: genes that escape XCI, pink: *XIST*.

Ovarian carcinoma cell line gene expression was compared to normal OSE gene expression from patients with a *BRCA1* mutation and history of breast cancer. Patients with a *BRCA1* mutation are predisposed to develop breast and ovarian carcinomas. Overall, expression in *BRCA1* mutation carriers was similar to the control (IOSE 397) for most genes. However, overexpression was similar for *KLF8* and *TIMP1* in ovarian carcinoma cell lines and short term cultures from *BRCA1* mutation carriers.

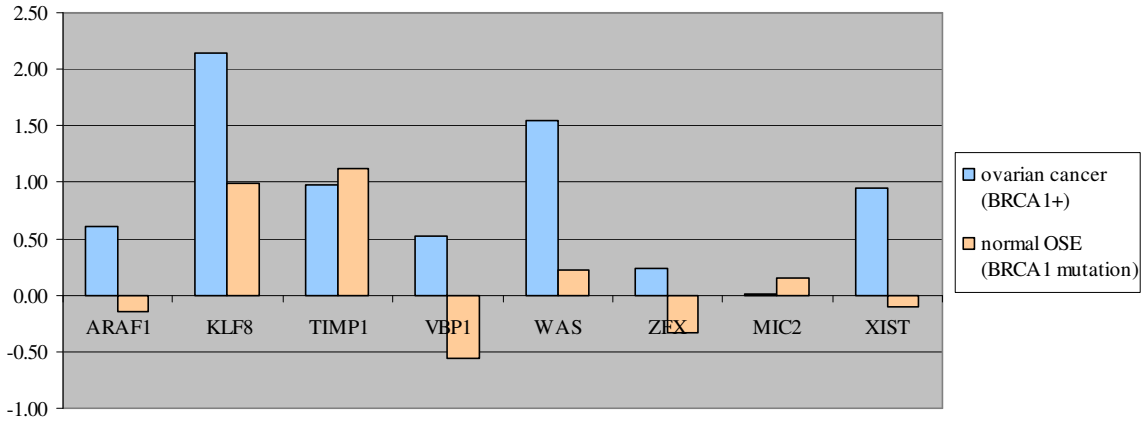

Figure 2.9. Log expression differences between *BRCA1* positive ovarian carcinoma cell lines and short term ovarian surface epithelium cultures from *BRCA1* mutation carriers.

Decreased methylation for some cell lines at two genes on the X chromosome

There was an overall decrease in methylation at *AR* and at *FMR1* in the DNA from most ovarian carcinoma cell lines when compared to controls (Table 2.2). However, methylation across both genes did not always correlate. CaOV3, OVCAR10 and OVCAR3 cell lines had decreased or absent methylation across both genes while OVCAR5, OVCAR8 and SKOV3 had decreased or absent methylation at one gene only. Methylation at *AR* tended to be lower than the expected 50% for the control used.

Table 2.2. Methylation at two X chromosome genes, *AR* and *FMR1*.

Sample	**AR ME**	**ME FMR1**
CaOV3	0%	0%
OVCAR5	40%	0%
OVCAR8	6%	41%
OVCAR3	1%	0%
SKOV3	2%	27%
A2780	23%	45%
OVCAR10	5%	0%
OVCAR2	10%	58%
Control (IOSE397)	21%	50%

**methylation values are run in duplicate and averaged*

Most cell lines heterozygous for X chromosome genes

Of 8 cell lines assayed, OVCAR5 and CaOV3 were homozygous at all markers assayed (Table 2.3). This may indicate a loss of inactive X and doubling of the active X scenario for these lines. The other cell lines were heterozygous for at least one marker assayed.

Table 2.3. Heterozygosity testing using X chromosome markers in ovarian carcinoma cell lines (values indicate the number of different alleles present for each marker).

Sample	AR	FMR1	DXYS1222	DXYS233
CaOV3	1	1	1	1
OVCAR5	1	1	1	1
OVCAR8	2	1	1	1
OVCAR3	2	1	1	1
SKOV3	≥3	1	≥3	2
A2780	2	1	≥3	≥3
OVCAR10	≥3	1	2	1
OVCAR2	2	2	2	2
Control (IOSE397)	2	1	2	2

Evaluating X reactivation potential in a cell line by comparing all markers together

The results from all assays were assessed together to attempt to observe an X reactivation event (Table 2.4). However, a clear pattern was not observed when assessing the data in this way. The two cell lines that did not have an *XIST* signal by FISH or by real-time PCR were homozygous for all X chromosome markers tested, indicating that these cell lines had possibly lost their inactive X chromosome and doubled their active X chromosome. The overexpression of several X chromosome genes and the variability of X chromosomes and inactive X chromosomes within and among ovarian carcinoma cell lines indicates a complex relationship between the X chromosome and ovarian carcinoma development.

Table 2.4. Combined data from all X reactivation markers. FISH results are presented as the number of Xs (*AR* signals)/ the number of Xi (*XIST* signals). Gene expression is presented in comparison to a control cell line. Overexpression was denoted as "over", underexpression was denoted as "under", comparable expression was denoted as "=". If a cell line was heterozygous at >1 markers, it was considered heterozygous. n/a: no data

Cell line	**FISH**		**Real-time expression**		**Methylation**		**Hetero -zygosity**
	X/XIST	*XIST*	*XCI genes*	*Escape genes*	*AR*	*FMR1*	*across 4 markers*
Control cell lines							
IOSE397	2/1 (49%)	n/a	n/a	n/a	21%	50%	hetero
GM04626	3/2 (80%)	n/a	n/a	n/a	47%	n/a	hetero
Ovarian carcinoma cell lines							
CAOV3	2/0 (70%)	no XIST	5/5 over	1/2 over 1/2 =	0%	0%	homo
OVCAR5	1/0 (95%)	no XIST	2/5 over 3/5 =	2/2 =	40%	0%	homo
OVCAR8	3/0 (76%)	over	2/5 over 3/5 =	2/2 =	6%	41%	hetero
OVCAR3	3/1 (77%)	over	4/5 over 1/5 under	2/2 =	1%	0%	hetero
SKOV3	4/2 (65%)	=	1/5 over 4/5 =	2/2 =	2%	27%	hetero
A2780	*2/1 (40%) (varies)	over	4/5 over 1/5 =	1/2 over 1/2 =	23%	45%	hetero
OVCAR10	*3/1 (40%) (varies)	over	4/5 over 1/5 =	1/2 over 1/2 =	5%	0%	hetero
OVCAR2	*2/1 (12%) (varies)	over	1/5 over 4/5 =	1/2 under 1/2 =	10%	58%	hetero
Short-term OSE cultures (from BRCA1 mutation carriers)							
FAM1	2/1 (71%)	=	3/5 over 2/5 =	2/2 =	n/a	n/a	n/a
FAM2	2/0 (92%)	under	2/5 over 3/5 =	2/2 =	n/a	n/a	n/a

2.1.4 Discussion

The results of this chapter demonstrate that many features suggestive of an XCI abnormality are present in ovarian carcinoma; however the mechanism and specific effects appear to be complex. In every cell line assayed there were at least two active X chromosomes in a majority of cells. Of eight ovarian carcinoma cell lines, three lacked any *XIST* RNA signaling. The remaining five cell lines had at least one *XIST* signal; however the number of *XIST* signals did not correspond to the number of X chromosomes present within a cell.

In addition, gross overexpression of X-linked genes was found in all ovarian carcinoma cell lines. Only genes that are usually inactive or variably inactive had increased expression. Genes known to escape inactivation did not have increased expression, suggesting that the overexpression was due to either reactivation of the inactive X chromosome or duplication of the active X chromosome with the loss of the inactive X chromosome. In a few instances, expression increases were in the magnitude of 100 to 200 fold. The cause for such extreme misregulation of gene expression cannot be accounted for simply by X chromosome reactivation.

It is worth noting that a 2 fold overexpression of all X chromosome genes may not occur with X reactivation since gene dosage does not necessarily correlate to gene expression. Studies of trisomy 13 and 21 suggest that the presence of a supernumerary chromosome leads to global expression variability (Fitzpatrick et al. 2002). Furthermore, while the presence of a third chromosome 21 in Down Syndrome derived cell lines corresponds to an overall 1.5 fold increase in chromosome 21 gene expression as expected, expression differences vary greatly among genes (Prandini et al. 2007).

Finally, methylation at two genes subject to XCI, *AR* and *FMR1*, was decreased in several cell lines. Three cell lines had decreased or absent methylation across both genes, while three others had decreased or absent methylation at one gene only. Loss of methylation of the promoters of multiple genes subject to XCI may indicate X reactivation or the presence of two active X chromosomes. That some cell lines have lost methylation at one gene only may indicate partial or random reactivation or simply gene specific effects on methylation which may or may not be related to expression.

Overall, no clear trend emerges when studying the three X reactivation markers collectively. Only one cell line, CaOV3, had no *XIST* signal via FISH or real-time PCR, increased X chromosome gene expression and no methylation at the two genes assayed. However, CaOV3 was also homozygous at four X chromosome markers assayed, indicating that it has possibly lost its inactive X chromosome and duplicated its active X chromosome. The other cell lines do not have a cohesive pattern of reactivation using the X chromosome markers assayed, indicating a complex relationship between the X chromosome and ovarian carcinoma.

Two normal OSE samples from *BRCA1* mutation carriers were also assayed for X chromosome content and gene expression. While both samples had two X chromosomes in the majority of cells, one cell line lacked an *XIST* signal. In addition, both samples had an approximately 10 fold gene expression increase for two X chromosome genes, *KLF8* and *TIMP1*. Thus, these samples may represent precursor stages in ovarian carcinoma progression. Interestingly, the trend of increased X chromosome abnormalities was found in both ovarian carcinoma cell lines and in normal OSE from *BRCA1* mutation carriers. If X chromosome abnormalities are important for the progression of all ovarian

tumours, *BRCA1* mutations may predispose women to X chromosome abnormalities via earlier X reactivation.

Overexpression of *Vbp1* in *Brca1*-null murine mammary tumours has previously been reported (Xiao et al. 2007). However, when normal tissue was compared from *Brca1*-null mice and *Brca1* positive mice, there was no increase in expression. Increased expression of *VBP1* was observed in the ovarian carcinoma cell lines assayed here but was not observed in normal ovarian tissue from *BRCA1* mutation carriers. *VBP1* overexpression may be a later event in ovarian tumourigenesis.

The loss of maintenance of the inactive X chromosome via partial or complete reactivation could lead to overexpression of some unknown growth-enhancing gene on the X chromosome. Since not all X chromosome genes are overexpressed in *BRCA1* mutated tumours (Jazaeri et al. 2004) and *BRCA1* and *XIST* occupy different spaces on the inactive X chromosome in a subset of cells (Xiao et al. 2007, Pageau et al. 2007), *BRCA1* may participate in a secondary control of silencing X chromosome genes. Certain genes may not be strictly under the control of known components of XCI, such as the subset of genes that are heterogeneously inactivated among women (Carrel and Willard, 2005). Such genes may be good candidates for X reactivation mediated tumourigenesis if their silencing involves *BRCA1*. *TIMP1* is an X chromosome gene that is variably inactivated among women (Anderson and Brown, 1999). In this study, its expression was increased in all ovarian carcinoma cell lines as well as normal OSE cultures from *BRCA1* carriers, consistent with a previous study that found increased expression of *TIMP1* in *BRCA1*-null ovarian tumours and subsequent repression with the addition of wild-type *BRCA1* (Jazaeri et al. 2004). *TIMP1*, located at Xp11.3-11.23, is a

metalloproteinase inhibitor that stimulates erythropoeisis and regulates interstitial collagenase (OMIM 305370). *TIMP1* has previously been linked to several cancers including breast cancer (Spatz et al 2004). Increased *TIMP1* mRNA and protein levels have been associated with negative outcomes in breast cancer (Ree et al. 1997, Schrohl et al. 2003). Further study upon *TIMP1* and its relationship to breast and ovarian carcinomas is merited.

Conversely, X reactivation may contribute to breast and ovarian carcinomas by creating an atmosphere of global misregulation within the cell. Early studies of embryonic stem cells were found to have normal autosome content but required either a critical deletion in the distal region or complete loss of one X chromosome in order to persist in culture (Robertson et al. 1983). These ES cells harbouring X chromosome deletions led to the identification of the X inactivation centre, a key player in X chromosome inactivation (Rastan, 1985). In mouse ES cells, if two active X chromosomes are present, there is global hypomethylation at both repetitive and genic sequences (Zvetkova et al. 2005). Aberrant methylation has been observed in several cancers (Jones and Laird, 1999). If X reactivation in turn caused global hypomethylation, this could lead to breast and ovarian carcinomas development. This may also explain why there are patients with a known family history, but no known mutations in *BRCA1* or *BRCA2* and why no group has successfully identified another major breast and ovarian carcinomas predisposing gene.

An X reactivation event is difficult to assess since many markers of such an event could also be explained by inactive X chromosome loss and subsequent duplication of the active X chromosome. Six of eight ovarian carcinoma cell lines were heterozygous at at

least one X chromosome marker. Therefore, these cell lines have likely maintained two distinct X chromosomes. Passaging cells, especially in lines that are already unstable such as cancer cell lines, can lead to changes in chromosome content and gene expression. Gene expression differences were assessed across 30 passages of one cell line (OVCAR3) at one gene (*TIMP1*). The gene expression values did not vary significantly among passages, though the possibility of changes occurring in culture cannot be excluded. An *XIST* RNA probe was used to mark the inactive X chromosome. However, it is possible that an X chromosome could lose its *XIST* RNA (which normally coats the inactive X chromosome) and remain inactive. Although *XIST* induces XCI in the early embryo and subsequently maintains inactivation with cellular divisions, *XIST* may not be strictly required for maintenance of the inactive state (Brown and Willard, 1994). An *AR* DNA probe was chosen to mark the total number of X chromosomes; however a deletion of 1 cM that encompasses *AR* has been associated with borderline and invasive epithelial ovarian carcinoma (Edelson et al. 1998). Finally, only one control cell line was used for the gene expression assay and may not be representative of normal ovarian surface epithelium. There may also be a large variation in gene expression levels among normal women which cannot be accounted for when studying only one control.

X chromosome abnormalities are present in ovarian carcinoma cell lines and in normal ovarian tissue predisposed to ovarian carcinoma development. These observations are consistent with the theory of X reactivation in breast and ovarian carcinomas; however the relationship between these cancers and the X chromosome appears complex.

2.2 Skewed XCI in breast and ovarian carcinoma patients

2.2.1 Introduction

X chromosome inactivation (XCI), the process of silencing X-linked genes for dosage compensation with males, typically occurs in a random fashion such that most females will be mosaic for two cell populations, with either X chromosome inactivated (Lyon 1961, Monk and Harper, 1979). Non-random or skewed XCI has been observed in the blood of various clinical populations including those with breast and ovarian carcinomas (Van den Veyver 2001, Kristiansen et al. 2002, Buller et al. 1999). There are a number of factors which can contribute to a non-random pattern of XCI, such as chance, selection or embryonic cell pool size (Brown and Robinson, 2000). Since *BRCA1* has been implicated in the process of XCI (Ganesan et al. 2002), X chromosome reactivation in *BRCA1* mutation carriers may contribute to the apparent skewed XCI observed in breast and ovarian carcinomas due to the use of methylation-based assays.

Non-random XCI has been associated with both breast and ovarian carcinomas (Table 2.4). Skewing was reported to be increased in the peripheral blood of invasive epithelial ovarian carcinoma patients when compared to either controls or patients with borderline epithelial ovarian carcinoma (Buller et al. 1999). Several invasive epithelial ovarian carcinoma patients with skewed XCI also had a family history of breast and ovarian carcinomas. A higher rate of skewed XCI has also been observed in the blood of young, pre-menopausal breast cancer patients (ages 27-45) when compared to controls, whereas there was no increase in skewed XCI for middle-aged (55-72) or old (73-90) breast cancer patients (Kristiansen et al. 2002). Note that the assay and the cut-off for

skewing differed in this study compared to the previous study, contributing to the discrepancies in skewing percentages in the patient and control populations.

According to Knudson's two-hit hypothesis, cancer progression is initiated by a mutation, or hit, in a tumour suppressor gene, followed by a second hit in the same gene (Knudson 2001). A hit may be a mutation or an epigenetic alteration, but must result in the loss or the significant decrease in protein or protein function. XCI presents a unique situation for cancer development. Skewed XCI can expose a mutation in a tumour suppressor gene and thus act as one hit via loss of functional heterozygosity (LOH) (Spatz et al. 2004). LOH of areas of Xq and Xp have been implicated in breast and ovarian carcinomas respectively (Piao and Malkhosyan 2002, Buekers et al. 2000). Therefore, skewed XCI in the blood of both breast and ovarian carcinomas patients may act as a hit in cancer progression for some unknown X-linked tumour suppressor gene.

Conversely, skewed XCI in breast and ovarian carcinomas patients may be due to another mechanism involving the process of XCI itself. Increased cases of invasive epithelial ovarian carcinoma and early age at diagnosis characterize the *BRCA1* mutation carrier population, so the observed skewing was hypothesized to be limited to such carriers (Buller et al. 1999). In 11 cases where the *BRCA1* mutation status was known, 9 patients had skewed XCI in peripheral blood cells (Buller et al. 1999). Moreover, associations have been made between *BRCA1* and the process of XCI (Ganesan et al. 2002), though their true relationship remains to be established (Pageau et al. 2007; Xiao et al. 2007, Silver et al. 2007). If *BRCA1* is directly involved in XCI, the loss of *BRCA1* protein in breast and ovarian carcinomas patients may lead to full or partial X reactivation

(Stone et al. 2003). If X reactivation occurs, there would be an increase in perceived skewed XCI using methylation-based assays if the reactivation was allele-specific.

The most commonly used assay to measure XCI skewing exploits the fact that the CpG islands of most X-linked genes will be methylated on the inactive X chromosome and unmethylated on the active X chromosome (Allen et al. 1992, see section 1.4.4 and figure 1.3 for more details on this assay). By digesting a sample with a methylation-sensitive restriction enzyme, then assaying a polymorphic X-linked gene that contains cut sites, a value for skewing can be calculated based on the relative amounts of maternal and paternal amplified gene products. While this assay is highly reproducible (Figure 2.10), it cannot account for a situation in which allele-specific reactivation has occurred. If only one of the two inherited X chromosomes is reactivated in breast and ovarian carcinomas patients, only one allele would lose its methylation and the measured peak of this allele would decrease. This would erroneously be assayed as skewed XCI.

Skewed XCI was assessed in *BRCA1* mutation carriers with breast and/or ovarian carcinoma using a methylation-based assay. Skewing was also assessed in breast and ovarian carcinomas patients with *BRCA2* mutations or no known mutations.

Table 2.5. Reports on skewed XCI in breast and/or ovarian carcinoma found in the literature.

Patients	Cell Type	Assay	Detection	Formula	Skewing Result (%)
Buller et al. 1999 (Iowa) - Significant association in ovarian carcinoma patients					
Invasive and borderline epithelial ovarian carcinoma patients	Peripheral blood cells	Androgen receptor (*AR*) with *HpaII* and *HhaI* restriction enzymes (Allen et al. 1992).	Analysis of ^{32}P labelled PCR products with 8% polyacrila-mide sequencing gels (Mutter et al. 1995)	Cleavage ratio (CR) = [lower band / upper band] (random = 1.0)	**≥ 3:1 band intensity difference or CR<0.33 and CR>3.0 (corresponds to ~ ≥ 70%) in invasive epithelial ovarian carcinoma** • 93 (53%) cases (N=174) vs. 15 (33%) of controls (not age-matched) (N=45) (χ^2 = 11.3, P= 0.0007) • 10 (28%) borderline epithelial ovarian carcinoma (N=36) • 9 of 11 *BRCA1* mutation carriers were skewed
Kristiansen et al. 2002 (Norway) - Significant association in young breast cancer patients					
Breast cancer patients	Peripheral blood cells	*AR* with *HpaII*	Analysis of PCR products with ABI 373 automated sequencer and GeneScan software	[relative amount of small allele]	**≥90% skew in pre-menopausal breast cancer patients (ages 27-45)** • 5 (13%) of cases (N= 40) • 1 (1%) controls (ages 19-45) (N=95) • OR=13, P=0.009
Kristiansen et al. 2005 (Sweden) - Significant association in young breast cancer patients with a family history of cancer but no BRCA mutations					
Non-*BRCA* mutation carriers with familial breast cancer, *BRCA1* and *BRCA2* carriers with breast cancer and patients with sporadic breast cancer.	Peripheral blood cells	*Same as above* *MIC2* PCR performed (to assess complete digestion)	*Same as above*	*Same as above*	**≥90% skew in young (age <55) non-carrier patients** • 12 (11.2%) of cases (N=107) vs. 7 (2.7%) of age-matched controls (N=259) (χ^2 = 11.15 , P= 0.001) **≥90% skew in BRCA1 or 2 mutation carriers** • 2 (5.7%) of cases (N=35) vs. 11 (3.1%) of controls (N=350) *(continued on following page)*

Patients	Cell Type	Assay	Detection	Formula	Skewing Result (%)
Kristiansen et al. 2005 (Sweden) - Significant association in young breast cancer patients with a family history of cancer but no BRCA mutations (continued)					
					≥90% skew in middle aged (55-72) sporadic breast cancer patients • 18 (13.6%) of cases (N=132) vs. 4 (4.4%) of age-matched controls (N=91) ($\chi^2 = 5.17$, P=0.02)
Struewing et al. 2006 (Poland) - No increased skewing in breast cancer patients					
Pre-menopausal, invasive breast cancer patients (no chemotherapy, mutation status/family history unknown)	Peripheral blood DNA from buffy coats	*AR* with *HpaII* and *RsaI*	Analysis of PCR products with ABI 3730 automated sequencer and GeneMap-per software	(unadjust) (using peak height) [allele 1 / (allele 1 + allele 2)] **Also**: adjusted for stutter bands, for undigested peak heights	**Adjusted skewing (≥75%) in pre-menopausal breast cancer patients without chemotherapy** • 23 (13.9%) of cases (N=166) vs. 22(11.3%) of age and location matched controls (N=194) (OR=1.2, P=0.3) **Unadjusted skewing (≥90%) in pre-menopausal breast cancer patients without chemotherapy** • 14(8.4%) of cases (N=166) vs. 17 (8.8%) of age and location matched controls (N=194) (OR=0.9, P=0.7)
Helbling-Leclere et al. 2007 (N=16 France) (N=22 Nebraska) - No increased skewing in *BRCA1* mutation carriers with breast cancer					
BRCA1 mutation carriers with breast cancer	EBV-immortal B lympho-cytes	*AR with HpaII*	Analysis of PCR products with ABI 3100 automated sequencer and analyzed with GeneScan software	(corrected) Corrected ratio (CR) = [Allele`s ratio of digested sample (ratio between two peaks) / allele`s ratio of undigested sample]	**CR≥10 and CR≤0.1 or ≥90% in BRCA1 mutation carriers** • 9 (23.68 %) of cases (N=38) vs. 10 (26.68%) of age-matched controls (N=41)

2.2.2 Materials and methods

Patient Samples

Two sets of peripheral blood DNA were generously provided by Dr. Doug Horsman and Dr. David Huntsman respectively. The first group of patients were ascertained by the Hereditary Cancer Program at the BC Cancer Agency and patient peripheral blood samples were collected and DNA was extracted. XCI skewing was evaluated in individuals from 41 families with at least one family member with breast and/or ovarian carcinoma and a *BRCA1* mutation (Table 2.5). In these families, 47 *BRCA1* mutation carriers with breast and/or ovarian carcinoma, 17 *BRCA1* mutation carriers without breast and/or ovarian carcinoma and 27 non-carriers without breast or ovarian carcinoma were identified. Skewing was also determined for individuals who did not have a known *BRCA1* mutation. Of these samples, 25 were *BRCA2* mutation carriers and 10 had no known *BRCA1* or *BRCA2* mutation but had a suggestive family history of breast and/or ovarian carcinoma.

The second group of patients were ascertained based on the presence of ovarian carcinoma. Peripheral blood and tumour DNA was obtained from patients with ovarian carcinoma who were subsequently tested for *BRCA1* or *BRCA2* mutation status. There were 8 patients with a *BRCA1* mutation, 3 patients with a *BRCA2* mutation, 9 patients with epigenetic alteration leading to loss of expression of *BRCA1* and 21 patients with no known mutation in this cohort (Table 2.5). Mutation screening was performed at the BC Cancer Agency by denaturing high performance liquid chromatography and multiplex ligation-dependent probe amplification analysis while epigenetic mediated loss of expression was assessed using bisulfite conversion and methylation-specific PCR.

Table 2.6. Patient Samples and their BRCA mutation status.

Samples obtained from Dr. Doug Horsman			
Total number of samples	***BRCA1* mutation carriers**	***BRCA2* mutation carriers**	**Non-carriers**
126 peripheral blood samples	• 33 breast • 12 ovarian • 17 without cancer	• 14 breast • 7 ovarian	• 27 controls (from families of *BRCA1* mutation carriers) • 10 with hereditary breast/ ovarian carcinoma (no BRCA mutations)
Samples obtained from Dr. David Huntsman			
Total number of samples	***BRCA1* mutation carriers**	***BRCA2* mutation carriers**	**Non-carriers**
41 samples peripheral blood and paired tumour samples	• 8 ovarian	• 3 ovarian	• 21 ovarian, no BRCA mutations • 9 ovarian, epigenetic *BRCA1* loss

Assaying Skewed X Inactivation

The protocol used for skewed XCI was developed by Allen et al. 1992 with adaptations reported in Beever 2002 (see 1.4.4. for further information).

Step 1: Restriction Enzyme Digest (Figure 1.3A)

DNA samples were first digested with the methylation-sensitive enzyme *HpaII*, which will only cut unmethylated DNA. Each digested sample contained 150 ng of DNA, 2.5U HpaII, 1U *RsaI* in 1x buffer I (10mM Bis Tris Propane-HCl, 10mM MgCl2, 1 mM dithiothreiotol, ph 7.0), for a total volume of 10µL (New England Biolabs, Pickering, ON). *RsaI* is used to promote efficiency and decrease the variability of the assay by cutting the genomic DNA into smaller pieces to increase accessibility of *HpaII* to its target sequence (Beever 2002). The undigested sample contained everything in the digested sample, except *HpaII* and was used to test the efficiency of the restriction digest

and correct the final skewing results for any allele amplification bias. The digested and undigested samples were then placed in a 37°C water bath overnight.

Step 2: Testing the efficacy of the digest.

A region of the *MIC2* locus that includes *HpaII* cut sites and unmethylated CpG dinucleotides was PCR amplified. *MIC2* is a gene that escapes XCI and is unmethylated on both the active and the inactive X chromosome. In a sample digested with *HpaII*, the inactive X chromosome and the active X chromosome will be cut at the *MIC2* promoter, the primers will not be able to extend past the cut site and the PCR will yield no product. The primer sequences used were 5'-AGAGGTGCGTCCGATTCTT-3' (forward) and 5'CGCCGCAGATGGACAATTT-3' (reverse). One microlitre of digested or undigested DNA was combined with 1x Rose Taq buffer (20 mM Tris HCl (pH 8.0), 10 mM KCl, 0.1% Triton X 100, 50 µgmL nuclease free BSA, 2 mM $MgCl_2$), 4% DMSO, 125 µM dNTP, 400 ng of each primer and 0.2U Taq, then PCR amplified (Rose Scientific, Edmonton, AB). The PCR cycled 35 times with conditions of 95°C for 3 minutes, 52°C for 1 minute and 72°C for 2 minutes, followed by a 7 minute final extension at 72°C.

The 15 µL PCR product was mixed with 3 µL of loading buffer [50% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol, 25 mM EDTA, 50 mM Tris (pH7.4)], run on a 1% agarose, 1% ethidium bromide gel at 6Vcm for 45 minutes, then visualized with UV light. The PCR product from the undigested sample was run in tandem with that of the digested sample in order to observe the ~400 base pair expected band and its intensity. A sample with no template DNA and a male sample were run concurrently as controls. If no band was present for the digested sample, digestion was said to be complete and the experiment proceeded to step three. If a band was present, additional

HpaII (0.025 µL) was added to the sample, and then the sample was placed in a 37°C water bath overnight. Step two was repeated until there was no band present in the digested sample.

Step 3: PCR at a polymorphic locus subject to X inactivation (Figure 1.3B)

The androgen receptor (*AR*) gene is subject to XCI and contains a variable trinucleotide CAG repeat in its first exon that is heterozygous in approximately 80% of individuals. By amplifying this region of *AR*, only uncut, methylated DNA will yield a product. The primer sequences were 5'GCTGTGAAGGTTGCTGTTCCTCAT-3' (forward) and 5'-TCCAGAATCTGTTCCAGAGCGTGC-3' (reverse). The forward primer was fluorescently labelled with an ABI Prism® dye, Hex, for visualization (Applied Biosystems, Foster City, CA). PCR amplification of 10 µL containing 1 µL of digested or undigested DNA, 1x Rose Taq buffer, 200µM dNTP, 500µM of each primer and 0.2 U Rose Taq was performed (Rose Scientific, Edmonton, AB). The PCR cycled 35 times with conditions of 95°C for 4 minutes, 95°C for 1 minute, 54°C for 1 minute and 72°C for 2 minutes, followed by a 7 minute final extension at 72°C.

Fluorescently labelled PCR products were separated and visualized on an ABI Prism® 310 Genetic Analyzer. A mixture of 1 µL of the AR PCR product, 10.8 µL deionized formamide and 0.2 µL of ROX 500 size standard (Applied Biosystems, Foster City, CA) was heat inactivated at 95°C for 5 minutes then placed in an ice water bath. The samples were run by capillary electrophoresis on an ABI Prism® 310 Genetic Analyzer, peaks detected by ABI Prism® Data Collection software and analysed using ABI Prism® Genescan software. Different sized alleles can be distinguished as different peaks and their areas can be used as a measure of the quantity of each allele present.

Step 4: Data Analysis (Figure 1.3 C-E)

In a situation of random XCI, about half of the inactive X chromosomes present will be maternal and half will be paternal in origin. This would correspond to two peaks of roughly the same area as long as the maternal and the paternal alleles are distinct in size (Figure 1.3D). If there is skewed XCI, either the paternal or the maternal X chromosome will be more prevalent and one peak will be far larger in area than the other (Figure 1.3E). The undigested sample was run in parallel to the digested sample to correct for allele specific amplification differences in PCR efficiency. Based on area comparison between the two peaks, skewing was calculated. Secondary peaks near the main peak, or stutter peaks, are a result of polymerase slippage during PCR amplification and were included in the total peak area. To calculate skewing the formula used was:

(d1/u1)/(d1/u1 + d2/u2),

where d1 and u1 are the base pair lengths of the first peak of the digested sample and undigested sample respectively and d2 and u2 are the base pair lengths of the second peak of the digested sample and undigested sample respectively.

Step 5: Assessing skewed XCI in samples uninformative at AR

If *AR* was uninformative (the maternal and the paternal allele were the same base pair length), a different polymorphic locus, *FMR1*, was used to ascertain skewing. *FMR1* has a variable CGG repeat length in its coding sequence, is subject to XCI and has 2 *HpaII* cut sites within 100 base pairs of the repeat. The primers used were 5'-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3' (forward) and 5'AGCCCCGCACTTCCACCACCAGCTCCTCCA-3' (reverse). The forward primer was labelled with ABI Prism® 310 dye 6-FAM. PCR amplification of 10 µL containing

1µL of digested or undigested DNA was combined with 1x PCR buffer, 1% DMSO, 260 µM dATP , 260 µM dTTP, 260 µM dCTP, 100 µM dGTP, 250 µM 7-deaxa GTP, 1 µM of each primer and 0.1 µM Taq enzyme mix (Expand Long Template PCR System Kit, Roche Diagnostics, Indianapolis, IN). The PCR cycled 25 times with conditions of 97°C for 30 seconds (initial denaturation), 97°C for 30 seconds, 65°C for 45 seconds (additional 20 seconds per cycle) and 68°C for 4 minutes.

Reproducibility

To confirm the accuracy of the *AR* skewing assay, twenty samples were repeated for the complete assay. This assay was highly reproducible between repeats ($p < 0.0001$, Linear Correlation and Regression, Figure 2.10).

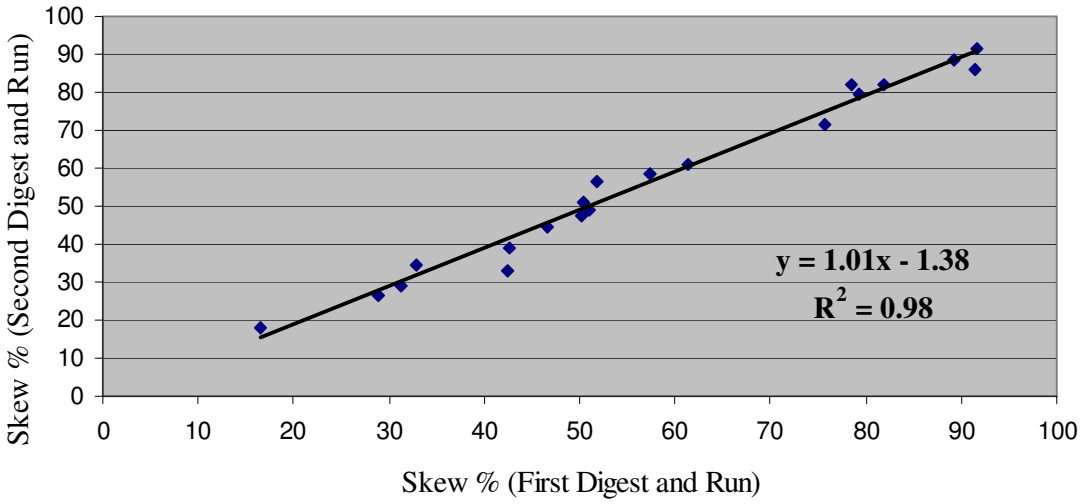

Figure 2.10. Reproducibility of the skewed XCI assay with *AR* between different digests and different runs on the ABI 310 genetic analyzer.

Statistical methods

In all cases, significance for the comparisons was determined using the Fisher Exact Probability test. For all comparisons, one-tailed tests were used, based on the hypothesis

that skewing would be greater in patients affected with breast and/or ovarian carcinoma compared to controls. Values were considered significant if the p value was less than 0.05.

2.2.3 Results

The number of individuals with skewed XCI in the patient and control groups is shown in Table 2.6. There was no evidence of an increase in extreme skewing (≥90%) in the *BRCA1* mutation carrier group when compared to family controls or age-matched controls (8% vs. 5% and 11% respectively). Similarly there was no increase in moderate skewing (≥75%) in the *BRCA1* mutation carrier group compared to either set of controls (33% vs. 24% and 49% respectively).

There was a trend towards increased extreme skewing in the non-*BRCA1* cancer patients (comprised of affected *BRCA2* mutation carriers and affected individuals with no identified mutation) when compared to the *BRCA1* mutation carrier patients, although it was not statistically significant (19% vs. 8%; P=0.09, Fisher's Test). This trend was weakly observed considering moderate skewing as well (39% vs. 33%).

Taken individually, the *BRCA2* mutation carriers and the affected uninformatives also had higher amounts of skewing (13% and 23% respectively), when compared to family controls, age-matched controls or to the *BRCA1* mutation carrier group (5%, 11% and 8% respectively) but were not significant, though sample size was small (n=24 and n=30 respectively).

Table 2.7. Extreme and moderate skewing results in breast and/or ovarian carcinoma patients and controls.

Type	**Skewed (≥90)**		**Skewed (≥75)**	
	n	%	n	%
Breast and ovarian carcinomas patients				
BRCA1$^-$ breast cancer (N=28)	2	7%	8	29%
BRCA1$^-$ ovarian carcinoma (N=17)	1	6%	6	35%
Epigenetic BRCA1 loss, ovarian carcinoma (N=7)	1	14%	3	43%
All BRCA1$^-$ and cancer (N=52)	4	8%	17	33%
BRCA2$^-$ breast cancer (N=14)*	3	21%	5	36%
BRCA2$^-$ ovarian carcinoma (N=10)	0	0%	2	20%
All BRCA2$^-$ and cancer (N=24)	3	13%	7	29%
Family history, no mutation, breast cancer (N=9)*	2	22%	3	33%
No mutation, ovarian carcinoma (N=21)*	5	24%	11	52%
All non-BRCA and cancer (N=30)*	7	23%	14	47%
All non-BRCA1 and cancer (N=54)*	10	19%	21	39%
Controls				
Age-matched lab controls (N=109)	12	11%	53	49%
BRCA1 mutation and no cancer (N=13)	1	8%	3	23%
No BRCA mutation, no cancer (N=24)	1	4%	6	25%
All BRCA1 family controls (N=37)	2	5%	9	24%

Note: no group is significant.
** indicates a trend towards increased skewed XCI*

2.2.4 Discussion

The present data do not support the hypothesis that skewed XCI in peripheral blood is associated with breast or ovarian carcinoma in *BRCA1* mutation carriers. A non-significant increase in skewed XCI in *BRCA1* mutation-negative patients was observed which cannot be explained by the original hypothesis. As with previous studies of skewed XCI and breast and ovarian carcinomas, there are a number of limitations.

No increase in skewed XCI was found in the *BRCA1* mutation carrier group when compared to controls, contrary to a finding of increased skewed XCI in ovarian carcinoma patients with *BRCA1* mutations (Buller et al. 1999). However, other investigators also failed to find an association between *BRCA1* mutations and skewed XCI above 90% in breast cancer patients (Table 1, Kristiansen et al. 2005 and Hebling-Leclere et al. 2007). In the reports on *BRCA1* mutation status and skewed XCI in breast and/or ovarian carcinoma patients including the present findings, the sample sizes of *BRCA1* mutation carriers were relatively small (N of 11, 27, 38 and 52 respectively). Though it is possible that the effect is limited to *BRCA1* mutation carriers with ovarian carcinoma, no increase in skewed XCI was found in the present study when only considering ovarian carcinoma patients with *BRCA1* mutations.

Interestingly, a trend toward increased skewed XCI was observed in patients where no *BRCA1* mutation had been identified. The non-*BRCA1* carrier group was comprised of *BRCA2* mutation carriers, hereditary breast cancer patients with no known *BRCA1* or *BRCA2* mutation and non-carrier ovarian carcinoma patients with an unknown family history. Taken separately, both *BRCA2* mutation carriers and hereditary cancer patients with no known *BRCA1* or *BRCA2* mutation had increased skewing though sample sizes were small. Increased skewed XCI in young patients with familial non-

BRCA1/*BRCA2* breast cancer was previously reported (Kristiansen et al. 2005). Unfortunately, in the present study the small sample size prohibited further analysis by patient age.

A trend toward increased skewing was also observed in *BRCA2* mutation carriers with breast cancer. This is contrary to a previous report that cited no increase in skewing in this group, although their study is also restricted by sample size (Kristiansen et al. 2005). Although the present sample size was small and findings were not statistically significant, more study on *BRCA2* mutation carriers and skewed XCI is warranted.

There are several limitations in studying skewed XCI in breast and ovarian carcinomas patients. Neither this study nor any other study reporting increased skewed XCI looked at chemotherapy status of their patient population. Chemotherapy can cause neutropenia and lymphocytopenia, which reduces the blood precursor pool size and can increase the incidence of skewed XCI (Gale et al. 1991). Previously, no observation of increased skewed XCI was reported in young breast cancer patients compared to controls when patients who had undergone chemotherapy were excluded (Struewing et al. 2006). As the timing of the blood draw in regards to chemotherapy was not known in this case, the possibility that chemotherapy affects the results cannot be ruled out. However, when a subset of samples was assessed for skewed XCI in matching blood and non-hematologic samples, the results were consistent (Buller et al. 1999). In addition, if chemotherapy does affect skewing, an increase in skewed XCI in all breast and ovarian carcinomas patient groups would be expected. However, *BRCA1* mutation carriers with cancer did not have an increased rate of skewing when compared to controls.

Another issue to consider is whether the study of blood is a good assessment of skewing in an individual. As a woman ages there is an increase in skewed XCI in her

blood (Hatakeyama et al. 2004, Amos-Landgraf et al. 2006). Since breast cancer tends to be diagnosed later in life, this would confound the results if only blood is studied and also if the patients and the controls were not age-matched (Brown, 1999). Presently, controls were age-matched to the entire patient population in order to avoid this issue. Some patient groups (e.g. *BRCA1* mutation carriers with cancer) were younger than others (e.g ovarian carcinoma patients with no *BRCA* mutations), but the controls were age-matched to the whole patient cohort. Therefore, the control group chosen likely overestimates or underestimates skewing in certain patient groups.

It is of note that the percent of controls with skewed XCI varies greatly among studies (Table 2.5). Why different groups obtain drastically different skewing results with similar assays remains to be resolved. It may be due to population, assay or blood collection differences. Until the skewing results for controls among different researchers are similar, it will be difficult to compare results between different studies.

The trend towards increased skewed XCI in patients with familial breast and/or ovarian carcinoma with no known *BRCA* mutation suggests that there may be a gene causative of breast and ovarian carcinomas located on the X chromosome (Kristiansen et al. 2005). A mutation in such a gene may either influence XCI and cause skewing or be influenced by skewed XCI itself if the mutated gene is located on the preferentially active X chromosome. For example, a mutation in the X-linked gene, *Foxp3*, leads to mammary tumours in mice, while mutations leading to *FOXP3* downregulation are overrepresented in human breast cancer samples (Zuo et al. 2007). All mammary cancer cells studied from heterozygous mice bearing a *Foxp3* mutation had silenced the wild type allele (Zuo et al. 2007). Alternatively, allele-specific reactivation may occur in non-*BRCA* breast and ovarian carcinomass and cause erroneous ascertainment of skewing.

Overall, no breast and ovarian carcinomas patient group assayed had significantly increased skewed XCI. Consistency among different researchers studying skewed XCI with methylation-based assays must be established in order to assess whether there is a real increase in non-random XCI in breast and ovarian carcinomas.

Chapter 3: Hormone receptor variants in breast and ovarian carcinomas patients

3.1 Introduction

Hormonal exposure is well established to affect breast and ovarian carcinomas, and hormone receptor activity has been linked to *BRCA1*, a breast and ovarian carcinomas predisposing gene (Rosen et al. 2005). Therefore, polymorphisms in hormone receptors that have functional effects on gene expression and/or activity may act as genetic modifiers of breast and ovarian carcinomas risk.

The androgen receptor (*AR*) at Xq11-q12 is a ligand-dependent nuclear receptor that binds testosterone or dihydrotestosterone and acts as a transcription factor for genes involved in male sex differentiation, spermatogenesis and male gonadotrophin regulation (Gobinet et al. 2002). A variable CAG trinucleotide repeat is present in the N-terminal transactivation domain of exon 1 of *AR*, where 9 to 39 repeats in length is the normal range (Buchanan et al. 2004). Repeat lengths outside of this range have been associated with diseases such as Kennedy disease, prostate, breast, ovarian, endometrial and colorectal cancer and male infertility (Gottlieb et al. 2004). Increased repeat size is inversely correlated to receptor function (Chamberlain et al. 1994) and mRNA transcription (Choong et al. 1996).

Results have been contradictory about the association between *AR* repeat length and breast and/or ovarian carcinoma. Short alleles (alleles bearing short CAG repeat lengths) have been associated with both increased risk of aggressive breast cancer (Yu et al. 2000) and early-onset cases (Dagan et al. 2002) as well as decreased breast cancer risk (Liede et al. 2003). Long alleles have been associated with an increased risk of cancer in young patients (Suter et al. 2003, Kristiansen et al. 2002) and with an increased early risk in patients with a *BRCA1* or *BRCA2* mutation (Rebbeck et al. 1999). Other reports found

no connection between *AR* repeat length and breast or ovarian carcinoma (Menin et al. 2001, Spurdle et al. 2000).

The estrogen receptor alpha gene (*ESR1*) is an estrogen-activated transcription factor mapping to 6q25.1 which is abundant in reproductive tissues and has role in male and female sexual maturation, bone formation, fertility and behaviour (Herynk and Fuqua, 2004). A dinucleotide TA repeat present in the *ESR1* promoter has been associated with osteoporosis, cardiovascular disease, premature ovarian failure, endometriosis and breast cancer (Gennari et al. 2005, Kunnas et al. 2000, Bretherick et al. 2007, Kim et al. 2005, Iobagiu et al. 2005). Two SNP polymorphisms, -397T/C and -351A/G, located upstream of and in linkage disequilibrium with the TA repeat have also been associated with osteoporosis, premature ovarian failure (POF), cardiovascular disease and breast cancer (Gennari et al. 2005, Shearman et al. 2003, Onland-Moret et al. 2005). Furthermore, the *ESR1* repeat has been associated with breast cancer risk when considered with other repeat polymorphisms. There is an increased risk of breast cancer when a woman has a short *AR* CAG repeat, a long *ESR1* CA repeat and a short TA repeat (Iobagiu et al. 2005).

BRCA1 is believed to interact with both *ESR1* and *AR* and thus may modify the risk of breast and/or ovarian carcinoma in *BRCA1* mutation carriers (Rosen 2005). *BRCA1* directly interacted with *AR* and enhanced *AR* transactivation of a reporter gene *in vitro* (Park et al. 2000). In addition, in breast and prostate cell lines, *BRCA1* protein repressed the activity of *ESR1*, while truncated *BRCA1* protein could not (Fan et al. 2001). Therefore, polymorphisms in *AR* and *ESR1* may increase the risk of breast and ovarian carcinomas and this effect may be limited to *BRCA1* mutation carriers.

Though not a hormone receptor, the *FMR1* gene contains a repeat length polymorphism that is implicated in female reproductive health. The variable CGG trinucleotide repeat (CGG), located in the 5^1 untranslated region of exon 1, ranges from 5 and 50 repeats in the general population (Oostra and Chiurazzi, 2001). Repeat lengths greater than 200, considered a full mutation, result in fragile X syndrome, which is characterized by moderate to severe mental retardation. Having an *FMR1* premutation sized allele with a length of 50-100 repeats has been associated with POF, a syndrome where women undergo menopause before the age of 40 (Murray et al. 1998) and Fragile X associated tremor/ataxia syndrome (Jacquemont et al.2004).

FMR1 has not been associated with breast or ovarian carcinoma risk. However, out of a study of four double heterozygous carriers of *BRCA1* and *BRCA2* mutations, two of the women had subfertility; one had primary sterility while the other had POF (Friedman et al. 1998). These women were asymptomatic for both breast and ovarian carcinomas despite carrying both a *BRCA1* and a *BRCA2* mutation. The authors postulated that low estrogen levels may have played a role in their decreased penetrance. Women with premutation sized alleles or repeat sizes in the high end of the normal spectrum, but not full mutations are at significantly increased risk of POF (Murray et al. 1998, Bretherick et al. 2005). Therefore, if having reduced fertility provides some protective effect against breast and ovarian carcinomas, women bearing large *FMR1* repeat alleles may confer protection against breast and ovarian carcinomas risk.

AR, *ESR1* and *FMR1* polymorphisms were assessed in breast and/or ovarian carcinoma patients with *BRCA1* mutations, *BRCA2* mutations, patients with no known mutation and unaffected relatives to determine whether any polymorphism corresponded to incidence of breast and/or ovarian carcinoma.

3.2 Materials and methods

Study group

See chapter 2.2.2 (Table 2.6).

AR repeat polymorphism

The *AR* gene contains a variable trinucleotide CAG repeat in its first exon that has approximately 80% heterozygosity. PCR amplification of this region was performed using the following primers, 5'GCTGTGAAGGTTGCTGTTCCTCAT-3' (forward) and 5'-TCCAGAATCTGTTCCAGAGCGTGC-3' (reverse). The forward primer was fluorescently labeled with an ABI Prism® dye, Hex, for visualization (Applied Biosystems, Foster City, CA). The reaction consisted of 10 µL containing 1 µL of digested or undigested DNA, 1x buffer, 200µM dNTP, 500µM of each primer and 0.2 U Taq (Rose Scientific LTD, Edmonton, AB). The PCR cycled 35 times with conditions of 95°C for 4 minutes, 95°C for 1 minute, 54°C for 1 minute and 72°C for 2 minutes, followed by a 7 minute final extension at 72°C.

A mixture was made of 1 µL of the *AR* PCR product, 10.8 µL deionized formamide and 0.2 µL of ROX 500 size standard (Applied Biosystems, Foster City, CA), denatured at 95°C for 5 minutes then placed in an ice water bath. The samples were run by capillary electrophoresis on an ABI Prism® 310 Genetic Analyzer, peaks detected by ABI Prism® Data Collection software and analysed using ABI Prism® Genescan software. Repeat size was calculated by subtracting the number of constant bases from the number of bases in the PCR amplified region and dividing the variable number of bases by the repeat size. From the base pair peak size reported by the ABI Prism® 310

Genetic Analyzer, *AR* repeat size was calculated using the formula:

(X base pairs – 216 base pairs) ÷ 3, where x = base pair size of PCR product.

AR statistical analysis

All CAG repeat sizes were compared between patients and controls. Repeat sizes were analyzed as a continuum and also using cut-offs. Chi Square and Fisher Exact tests were used to compare the distribution of allele size between groups.

ESR1 polymorphisms

ESR1 is located at 6q25.1, has a TA repeat in its promoter which has a high heterozygosity. The distribution of *ESR1* repeats is bimodal in nature, with the largest peak at either 14 or 15 repeats, depending on population differences (Gennari et al. 2005). *ESR1* repeat length was calculated by a conventional PCR around the repeating sequence followed by analysis on the ABI Prism® 310 Genetic Analyzer. The primers used were 5'GACGCATGATATACTTCACC-3' (forward) and 5'-GCAGAATCAAATATCCAGATG-3' (reverse) (Sano et al. 1995). The forward primer was fluorescently labeled with an ABI Prism® dye, Hex, for visualization. The 15 µL PCR reaction contained 50 ng of DNA, 1x buffer, 200µM dNTP, 500µM of each primer and 0.2 U Taq. The PCR cycled 35 times with conditions of 95°C for 2 minutes, 95°C for 30 seconds, 55°C for 45 seconds, 95°C for 30 seconds, 55°C for 45 seconds and 72°C for 1 minute and 30 seconds, followed by a 7 minute final extension at 72°C. From the base pair peak size given by the ABI Prism® 310 Genetic Analyzer, *ESR1* repeat size was calculated using the formula:

(X base pairs – 140 base pairs) ÷ 2, where x = base pair size of PCR product.

ESR1 repeat length is bimodal in nature and thus was analyzed based on the presence of a short allele, "s" (less than 18 repeats) or a long allele, "l" (greater than or equal to 18 repeats).

Two *ESR1* SNP polymorphisms are present at position -397 and -351 in the promoter region of the gene, upstream and in linkage disequilibrium with the TA repeat polymorphism. These SNP polymorphisms were analyzed by allelic discrimination with an ABI Prism®7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Allelic discrimination employs two different fluorescently tagged probes, one for each SNP allele. Most often, only the correct probe for each SNP will bind and will give off the corresponding fluorescent signal. By PCR, primers amplify a region that encompasses the SNP in question and the fluorescent signal will be magnified. For a given DNA sample, the resulting signal will be either exclusively from one SNP variant or the other (in the case of individuals homozygous for either SNP variant) or a mid-range signal (in the case of heterozygotes for the SNP polymorphism). This is represented graphically as points (representing each individual) that either amplify only in the X direction (on the X axis), only in the Y direction (on the Y axis) or both in the X and the Y direction (falling on a trend line with a slope of 1). The PCR reaction comprised of 8.25 µl distilled water, 12.5 µl 2x TaqMan® Universal PCR Master Mix, 20µM of each primer 10 µM of each probe and 200 ng of DNA (Applied Biosystems, Foster City, CA). Both SNPs used the same primers, which were 5'-TCCATCAGTTCATCTGAGTTCCAA3' (forward) and 5'-TTCAGAACCATTAGAGACCAATGCT-3' (reverse). The probe sequences for -397C/T were 5'-VIC-CCCAGCCGTTTT-3' (C) and 5'-6FAM-CCCAGCTGTTTT-3' (T). The probe sequences for -351A/G were 5'-6FAM-CCCAACTCTAGACCA-3' (A)

and 5'-VIC-CCCAGCCGTTTT-3' (G) (Koch et al. 2005). The PCR mix was loaded in to a 96 well flat plate sealed with a MicroAmp™ optical adhesive film and run on an ABI Prism® 7000 Sequence Detection System using the allelic discrimination method (Applied Biosystems, Foster City, CA). Analysis of real-time data was performed using ABI Prism® 7000 Sequence Detection software version 1.2.3 (Applied Biosystems, Foster City, CA).

FMR1 repeat polymorphism

The *FMR1* gene maps to Xq27.3 and has a variable CGG repeat sequence in the 5' untranslated region of exon 1. *FMR1* repeat size was calculated in the same manner as *AR* and *ESR1*. The primers used were 5'-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3' for the forward primer and 5'AGCCCCGCACTTCCACCACCAGCTCCTCCA-3' for the reverse primer. The forward primer was labeled with Prism® 310 dye 6-FAM. The 10 µL PCR reaction containing 1µL of digested or undigested DNA was combined with 1x PCR buffer, 1% DMSO, 260 µM dATP , 260 µM dTTP, 260 µM dCTP, 100 µM dGTP, 250 µM 7-deaxa GTP, 1 µM of each primer and 0.1 µM Taq enzyme mix (Expand Long Template PCR System Kit, Roche Diagnostics, Indianapolis, IN). The PCR cycled 25 times with conditions of 97°C for 30 seconds (initial denaturation), 97°C for 30 seconds, 65°C for 45 seconds (additional 20 seconds per cycle) and 68°C for 4 minutes.

3.3 Results

AR CAG repeat length

The distributions of androgen receptor (*AR*) length were not significantly different between the *BRCA1* mutation carriers with cancer, *BRCA2* mutation carriers with cancer,

cancer patients with no mutation and controls (comprised of within-family controls and population controls) (χ^2=8.42, df=9, p=0.51) (Table 3.1, Figure 3.1). There was a slight decrease in short repeat length alleles and a slight increase in long repeat length alleles in cancer patients without a known *BRCA* mutation (<19, 40% deviation from expected value and >22, 35% deviation from expected value). There was also no difference between breast and ovarian carcinomas patients for *AR* repeat length (χ^2=4.5, df=8, p=0.81) (Table 3.2, Figure 3.2).

Table 3.1. *AR* repeat size allele distribution in breast and/or ovarian carcinoma patients and controls.

	Number of AR repeats (Percent)			
Sample Type	**<19**	**19/20**	**21/22**	**> 22**
BRCA1 cancer (n=110 alleles)	26 (24%)	31 (28%)	21 (19%)	32 (29%)
BRCA2 cancer (n=52 alleles)	15 (29%)	15 (29%)	9 (17%)	13 (25%)
Non-BRCA cancer (n=60 alleles)	8 (13%)	17 (28%)	9 (15%)	26 (43%)
Controls (n=246 alleles)	55 (23%)	60 (25%)	50 (20%)	79 (32%)

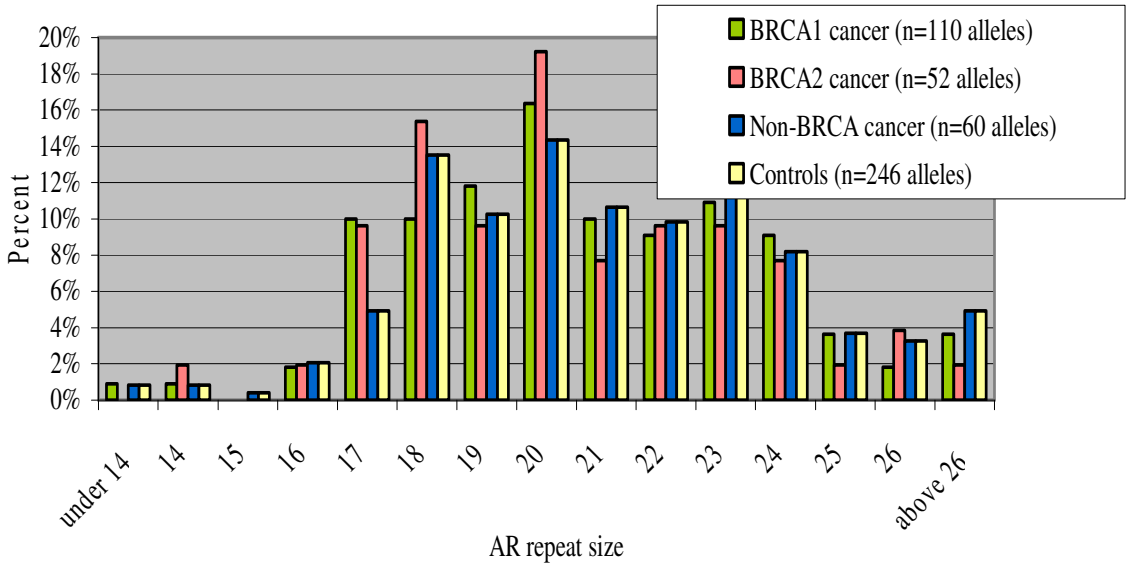

Figure 3.1. Distribution of *AR* alleles in breast and ovarian carcinomas patients divided by mutation status and controls.

Table 3.2. *AR* repeat allele distribution in breast cancer and ovarian carcinoma patients compared to controls.

	Number of *AR* repeats (Percent)			
Sample Type	**<19**	**19/20**	**21/22**	**>22**
Breast Cancer (n=110 alleles)	25 (23%)	24 (22%)	29 (26%)	32 (29%)
Ovarian carcinoma (n=112 alleles)	24 (21%)	20 (18%)	29 (26%)	39 (35%)
Controls (n=246 alleles)	55 (22%)	61 (25%)	50 (20%)	80 (33%)

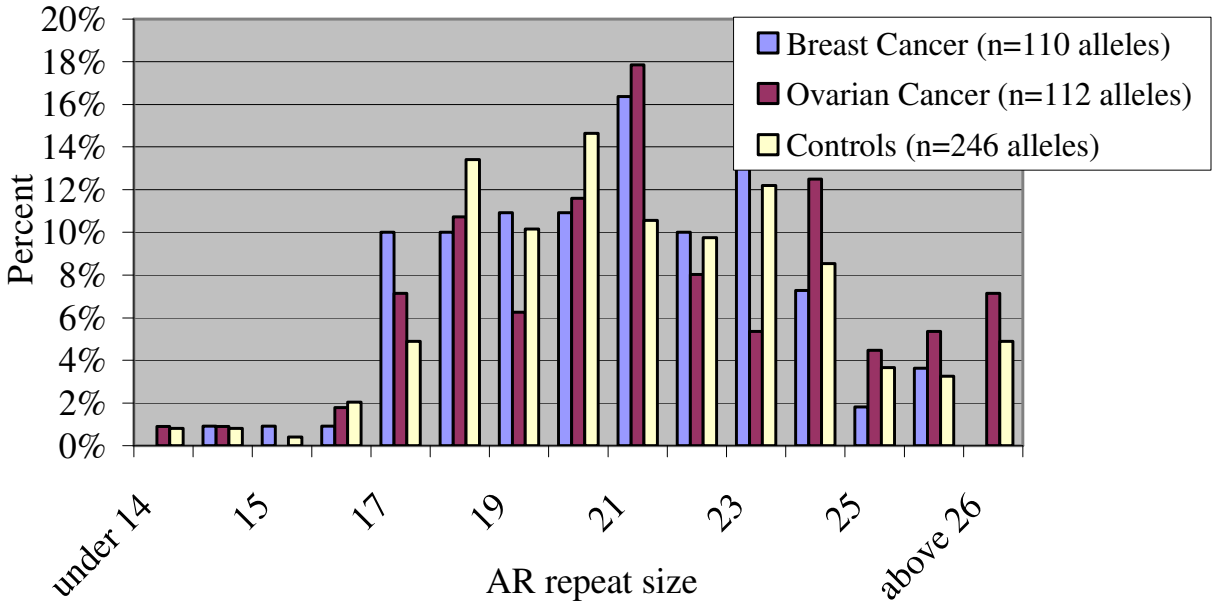

Figure 3.2. Distribution of *AR* alleles in breast and ovarian carcinomas patients and controls.

ESR1 repeat polymorphism

The distribution of *ESR1* alleles was not significantly different between the *BRCA1* mutation carriers with cancer, *BRCA2* mutation carriers with cancer, cancer patients with no mutation and controls (χ^2=17.77, df =12, p=0.13) (Table 3.3, Figure 3.3). The largest peak in most populations occurs at 14 or 15 repeats (Gennari et al. 2005) as was the case for all groups assayed except for the breast and ovarian carcinomas patients with no *BRCA1* or *BRCA2* mutation, where the median was 23 (Figure 3.3, blue). There was a trend towards an increased presence of large alleles in cancer patients with no known

BRCA mutation (>21, 23% deviation from expected value). The distribution of *ESR1* repeat genotypes was not significantly different among groups assayed (χ^2=7.05, df=6, p=0.32) (Table 3.4, Figure 3.4).

Table 3.3. Allele distribution of *ESR1* repeat size in cancer and control groups.

	Number of *ESR1* repeats (Percent)				
Sample Type	**< 15**	**16/17**	**18/19**	**20/21**	**> 21**
BRCA1 cancer (n=110 alleles)	53 (48%)	12 (11%)	3 (3%)	13 (12%)	29 (26%)
BRCA2 cancer (n=52 alleles)	25 (48%)	5 (10%)	4 (8%)	4 (8%)	14 (27%)
Non-BRCA cancer (n=60 alleles)	27 (45%)	5 (8%)	2 (3%)	6 (10%)	20 (33%)
Controls (n=246 alleles)	146 (59%)	7 (3%)	9 (4%)	20 (8%)	64 (26%)

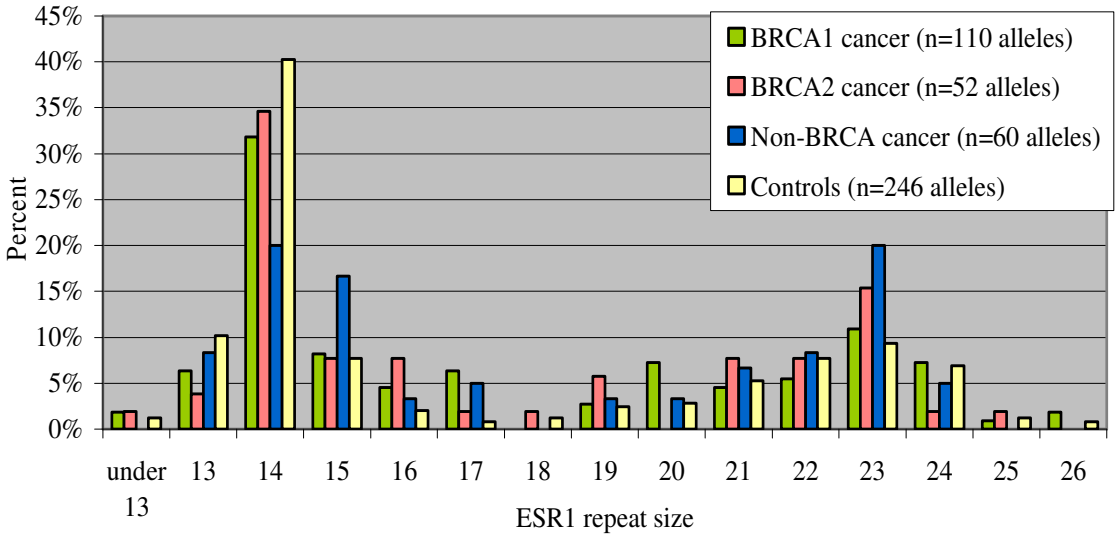

Figure 3.3. Distribution of *ESR1* repeat sizes in all breast and ovarian carcinomas patients and controls.

Table 3.4. Genotype distribution of *ESR1* repeat size alleles in breast cancer and ovarian carcinoma patients (SS: two short alleles, LL: two long alleles, SL: heterozygote).

Sample Type	**SS**	**SL**	**LL**
BRCA1 cancer (n=110 alleles)	19 (35%)	27 (49%)	9 (16%)
BRCA2 cancer (n=52 alleles)	10 (38%)	9 (35%)	7 (27%)
Non-BRCA cancer (n=60 alleles)	6 (20%)	19 (63%)	5 (17%)
Controls (n=246 alleles)	47 (38%)	59 (48%)	17 (14%)

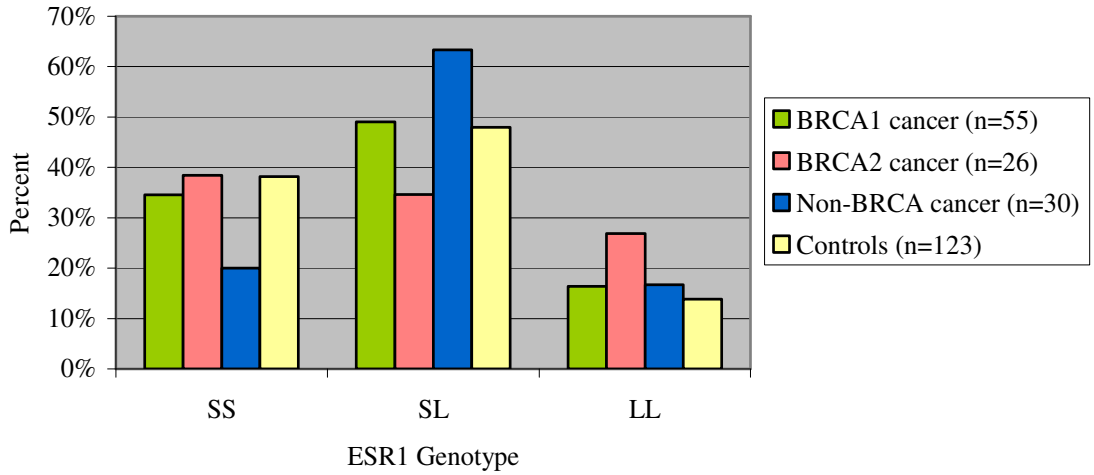

Figure 3.4. Distribution of *ESR1* repeat size genotypes in all breast and ovarian carcinomas patients and controls.

ESR1 SNP polymorphisms

The distribution of the two SNPs, -397T/C and -351A/G, is shown in Figure 3.5. There was a significant difference between the distributions of the combined *ESR1* SNP genotypes within the cancer groups assayed (χ^2=26.63, df=12, p=0.009) (Table 3.5, Figure 3.5). Breast and ovarian carcinomas patients without a *BRCA1* or *BRCA2* mutation have an increased presence of the CCGG genotype compared to the combined controls and to the other breast and ovarian carcinomas groups (135% deviation from expected value).

Table 3.5. Allele distribution of *ESR1* repeat size in cancer and control groups.

Genotype	BRCA1 cancer (n=55)	BRCA2 cancer (n=26)	Non-BRCA cancer (n=30)	Controls (n=123)
CCAA	0 (0%)	1 (4%)	0 (0%)	1 (1%)
CCAG	7 (13%)	4 (15%)	4 (13%)	8 (7%)
CCGG	4 (7%)	2 (8%)	7 (23%)	10 (8%)
CTAA	14 (25%)	0 (0%)	5 (17%)	12 (10%)
CTAG	15 (27%)	9 (35%)	10 (33%)	47 (38%)
TTAA	15 (27%)	10 (38%)	4 (13%)	45 (37%)

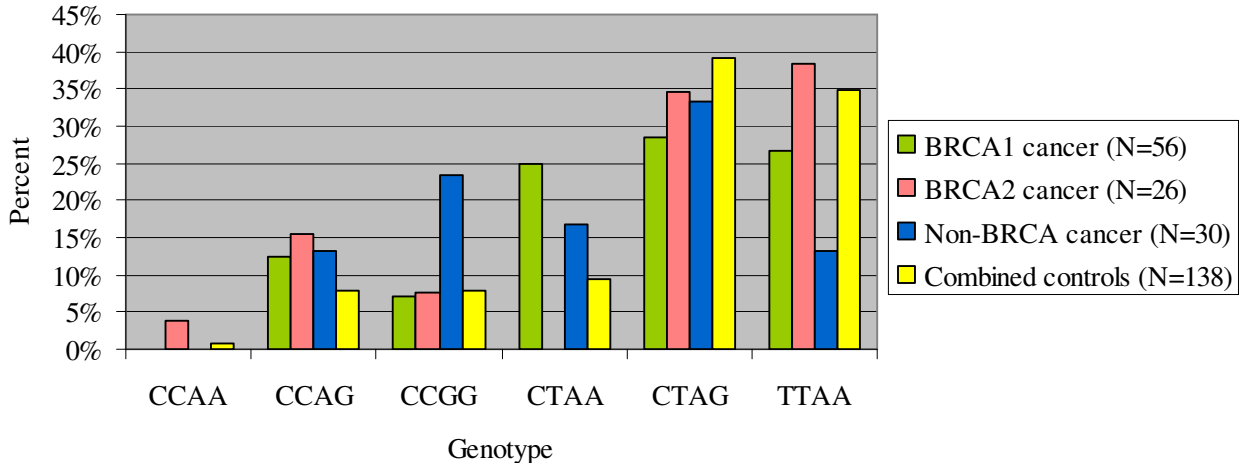

Figure 3.5. The distribution of the combined -397T/C and -351A/G genotypes in breast and ovarian carcinomas patients and controls.

FMR1 repeat polymorphism

The distribution of *FMR1* repeat alleles is shown in Figure 3.6. There is a non-significant increase in alleles bearing large *FMR1* repeats (≥40 repeats) present in *BRCA1* carriers without cancer and in BRCA2 mutation carriers (Table 3.6).

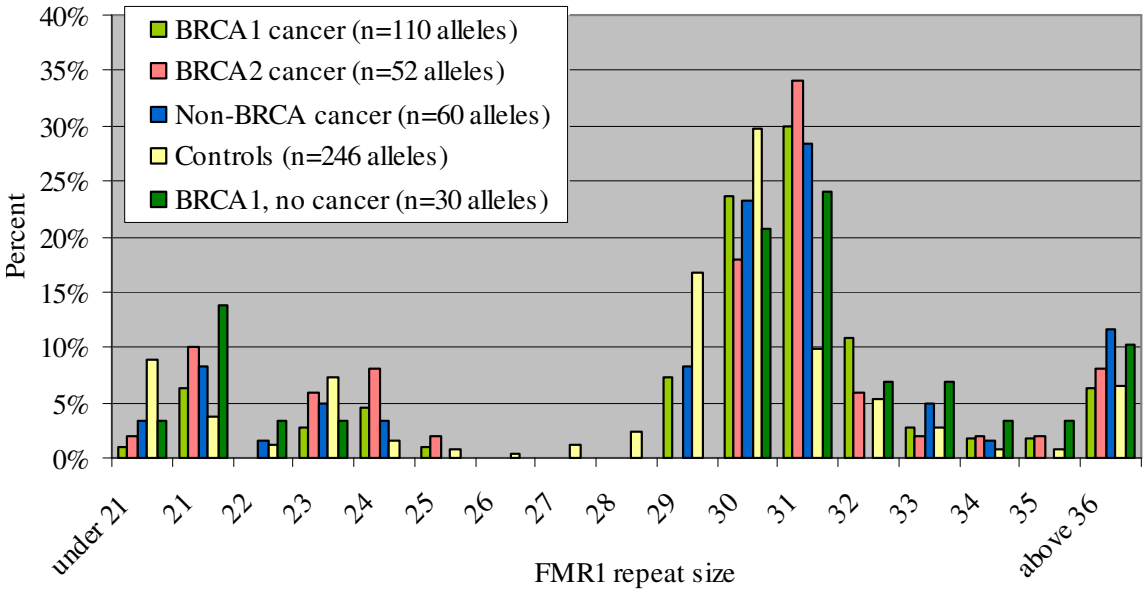

Figure 3.6. Distribution of *FMR1* repeat sizes in all breast and ovarian carcinomas patients and controls.

Table 3.6. The number of large repeat size *FMR1* alleles in breast and ovarian carcinomas patients.

	***FMR1* repeat size**			
Patient Group	**≥35**		**≥40**	
	n	**%**	**n**	**%**
BRCA1 and cancer (N=110 alleles)	9	8%	4	4%
BRCA2 and cancer (N=52 alleles)	5	10%	4	8%
No *BRCA* mutations and cancer (N=60 alleles)	7	12%	2	3%
BRCA1, no cancer (N=30 alleles)	3	10%	3	10%
Controls (N=246 alleles)	18	8%	11	4%

3.4 Discussion

Results from this work suggest that while certain hormone receptor polymorphisms may influence breast and/or ovarian carcinoma risk, the effect is likely modest. Such an effect may be limited to patients without *BRCA1* or *BRCA2* mutations, as it was in this group that the greatest deviations relative to controls were seen.

There was a tendency towards increased long *AR* repeat alleles in ovarian carcinoma patients with no *BRCA1* or *BRCA2* mutation. A marginally significant increased risk of ovarian carcinoma was previously reported when two long *AR* alleles were present (≥22 repeats, OR 1.31, 95% CI 1.01-1.69; Terry et al. 2005). Similarly to the present findings, no association between family history and age of onset with long *AR* repeat lengths was made (Terry et al. 2005, Menin et al. 2001). Increased *AR* repeat length is negatively correlated with both receptor function and amount of mRNA transcribed (Chamberlain et al. 1994, Choong et al. 1996), but the exact role this might play in ovarian carcinogenesis is unclear. *AR* knock out mice exhibit a range of reproductive issues pertaining to ovarian function, though effects to the ovarian surface epithelium are unknown (Hu et al. 2004, Terry et al. 2005).

Recently, a relationship between repeat length and interdomain communication, a necessary interaction for *AR* transactivation, has been made (Buchanan et al. 2004). It

appears that only individuals with repeats in the 16-29 range have satisfactory interdomain communication. This may explain why an effect of *AR* repeat length has been observed within the upper boundary of the normal range for certain breast and ovarian carcinomas patient groups.

Estrogen receptors are most prevalent in female reproductive tissues (Hewitt et al. 2000), so if a polymorphism in *ESR1* influences receptor activity, such a change could result in breast and/or ovarian carcinoma. No significant associations were found between *ESR1* and any breast and/or ovarian carcinoma group studied. A trend toward increased *ESR1* repeat length and a decreased presence of the short/short genotype in cancer patients with no known *BRCA1* or *BRCA2* mutation merits further study with a larger sample size. This trend may indicate a role for *ESR1* polymorphisms in sporadic breast and/or ovarian carcinoma or in familial non-*BRCA* cancer. *BRCA1* mutated breast cancers are most often estrogen receptor negative which may explain why *ESR1* polymorphisms were not associated with the *BRCA1* mutation carrier group.

BRCA1 mutation carrying women with premature ovarian failure and long *FMR1* repeat lengths may be protected against breast and ovarian carcinomas. This is based on the notion that risk of cancer significantly decreases in *BRCA1* mutation carriers after menopause. There was a trend towards an increase in long alleles (≥40 repeats) in *BRCA1* mutation carriers without cancer compared to controls. *BRCA2* mutation carriers with cancer also had a non-significant increase in long *FMR1* repeats, though sample size was small. It would be of value to assess *FMR1* repeat length in a larger population of *BRCA1* mutation carriers who do not develop cancer to assess whether there is a tendency to earlier menopause in this group that is also protective against breast and/or ovarian carcinoma.

No hormone receptor polymorphism was very highly correlated to any breast and/or ovarian carcinoma group. However, a polymorphism that acts as a genetic modifier may not exert a large influence on risk alone. Considering different receptors polymorphisms together in a large cohort of patients and controls may more accurately assess risk.

One inherent problem in studying repeat length polymorphisms between different studies is that the repeat cutoffs used are variable. In this study, cut-offs were chosen based on the distribution of alleles. However, for *AR*, *ESR1* and *FMR1*, the actual repeat size that would create the most functional effect is unknown, making the assignation of cut-offs problematic. In addition, information on ethnic origin of the patients was not available and thus population differences in specific polymorphisms were not accounted for.

Discovering the nature of the female specificity of *BRCA1* cancers could lead to better risk assessment and treatment. Given the epidemiological data on estrogen's effect upon risk of breast and ovarian carcinomas, it is useful to identify variants in genes that effect circulating estrogens that may play a role in cancer development. Overall, polymorphisms in *AR* and *ESR1* may account for a modest increase in breast and ovarian carcinomas risk in those without *BRCA* mutations. Greater sample sizes would allow for better assessment of which polymorphisms may be the most relevant in identifying risk in an individual.

Chapter 4: Discussion

The presence of a mutation in *BRCA1* predisposes a woman to breast and ovarian carcinomas (Antoniou et al. 2003). Since a mutation in a gene integral to basic cellular processes such as DNA repair and cell cycle control leads to female-limited cancers, researchers have sought after some X factor in women to explain this phenomenon. One possibility is the presence of two X chromosomes and the process of XCI, which are usually unique to females. Several lines of evidence have linked X chromosome genetics and epigenetics to breast and ovarian carcinomas, such as a connection between *BRCA1* and *XIST* and X-linked gene overexpression in *BRCA1*-mutated ovarian carcinoma (Ganesan et al. 2002, Jazaeri et al. 2002). Furthermore, it has been postulated that the inactive X chromosome may be partially or fully reactivated leading to tumour progression in breast and/or ovarian carcinoma (Stone et al. 2003).

While X chromosome genetics or epigenetics may be involved in the tissue-specificity of breast and/or ovarian carcinoma, estrogen exposure is known to increase the risk of developing these cancers (MacMahon 2006, Lukanova and Kaaks, 2005; Table 1.1). Hormone receptor variants that affect estrogen levels may therefore influence risk of developing breast and/or ovarian carcinoma. The hormone receptors *AR* and *ESR1* have been associated with *BRCA1* via protein/receptor interactions (Rosen et al. 2005). Therefore, hormonal differences may also affect the penetrance of a *BRCA1* mutation. The data presented in this thesis indicate that neither the X chromosome nor hormone receptor polymorphisms have a simple relationship with the presence of breast and/or ovarian carcinoma.

4.1 X chromosome abnormalities

The loss of inactive X chromosome marks by FISH and X-linked gene overexpression and hypomethylation were observed in both ovarian carcinoma cell lines and in normal OSE from *BRCA1* mutation carriers. Some ovarian carcinoma cell lines assayed lacked an *XIST* signal using FISH, consistent with the lack of inactive X chromosomes in certain breast cancer cell lines (Sirchia et al. 2005). Though most ovarian carcinoma cell lines had excess X chromosomes, *XIST* signal and localization did not conform to that expected by X chromosome number. In addition, DNA methylation was decreased for at least one gene in some of the cell lines and overexpression of at least one X-linked gene was present in all cell lines. While X chromosome abnormalities were present, no clear pattern of consistent alterations emerged using X reactivation markers in ovarian carcinoma cell lines. Thus, while the X chromosome appears to be involved in some way with breast and/or ovarian carcinoma, the nature of the relationship is unclear.

Presumed X reactivation was previously reported in 22% of primary basal-like breast tumours that had lost marks of XCI (as measured by the absence of H3MK27 and *XIST* staining) (Richardson et al. 2006). While the samples assayed were from cases of sporadic breast cancer, they were of the subtype that is most frequently observed in *BRCA1* mutation carriers (Sorlie et al. 2003). In addition, two primary breast carcinomas, a *BRCA1* and a non-*BRCA1* associated case, contained two active X chromosomes and were heterozygous for most X chromosome markers, suggestive of X reactivation (Sirchia et al. 2005).

However, X reactivation only accounts for a minority of the basal-like breast cancer cases lacking an inactive X chromosome (Richardson et al. 2006). The majority of

cases (61%) resulted from loss of the inactive X chromosome and duplication of the active X chromosome (Richardson et al. 2006). Loss of the inactive and duplication of the active X chromosome was also observed in one *BRCA1* protein negative and six *BRCA1* protein positive breast cancer cell lines (Sirchia et al. 2005). In the present study, one cell line lacking *XIST* gene expression and *XIST* signal by FISH was homozygous at four X chromosome markers and was likely derived from a loss and duplication event. Of the basal-like breast tumours lacking an inactive X chromosome, 16% had supernumerary active X chromosome content (Richardson et al. 2005).

Thus, increased active X chromosome copy number relative to normal may be the important factor in the development of breast and/or ovarian carcinoma. This may be the case regardless of whether the active X chromosome was derived by X reactivation, the loss of the inactive X chromosome and duplication of the active X chromosome or the addition of active copies of the X chromosome.

In short-term OSE cultures from *BRCA1* mutation carriers, one sample lacked an *XIST* signal, while both samples overexpressed two X-linked genes. Though heterozygosity of the samples is unknown, it is possible that X reactivation has occurred in the putative normal ovarian tissue and is an early step in ovarian oncogenesis.

The two short-term OSE cultures were obtained from *BRCA1* mutation carriers, while the ovarian carcinoma cell lines used in this study have no known *BRCA1* mutations. Therefore, the changes in X reactivation markers appear to occur whether or not a *BRCA1* mutation is present. This implies that *BRCA1* may not play a role in the X chromosome's involvement in breast and/or ovarian carcinoma. An important question to resolve is whether or not *BRCA1* is directly involved in X chromosome abnormalities observed in breast and ovarian carcinomas. While some suggest a direct link between

XCI and *BRCA1* (Ganesan et al. 2002), others maintain that no relationship exists (Pageau et al. 2007, Xiao et al. 2007). That the observed changes in X reactivation markers were irrespective of *BRCA1* status does not preclude a role for *BRCA1* in breast and/or ovarian carcinoma via some X chromosome effect. A mutation in *BRCA1* might predispose a woman to early X chromosome changes, an explanation that would account for the predisposition of mutation carriers to developing breast and/or ovarian carcinoma at an earlier age.

4.2 Breast and/or ovarian carcinoma and skewed XCI

Previously, increased skewed XCI has been reported in the peripheral blood of breast and ovarian carcinomas patients (Buller et al. 1999, Kristiansen et al. 2002). If X reactivation occurs, it could lead to skewed XCI if the reactivation was allele-specific. However, skewed XCI was not increased in the peripheral blood of *BRCA1* mutation carriers or any other patient group sampled with breast and/or ovarian carcinoma in this study.

The percent of controls with skewed XCI varies greatly among studies (Table 2.4). Skewing above 90% in the general population as assayed here is 7% overall and 11% when age-matched to the patient population. Other studies have reported similar skewing results in the general population (Struewing et al. 2006, Hebling-Leclere et al. 2007). However, the studies reporting a significant association between skewed XCI and breast or ovarian carcinoma observed a lower rate of skewing above the 90% cut-off in controls (Buller et al. 1999, Kristiansen et al. 2002, Kristiansen et al. 2005). Since the tissue studied and the assay used is similar among studies, it is perplexing that the amount of skewing in the controls varies so dramatically. Increased skewed XCI in controls may arise due to population differences, though this has never been reported.

Technical aspects of the assay may also account for low extreme skewing in controls for some studies. For example, some studies do not use the enzyme *RSAI* in correlation with *HpaII*, which may lead to incomplete digestion of DNA (Sharp et al. 2000). Discrepancies concerning the level of skewing in controls among different research groups makes it difficult to compare results between studies.

4.3 How X reactivation could cause breast and/or ovarian carcinoma

There are two ways that X reactivation might cause breast and/or ovarian carcinoma, by overexpression of a cancer-causing gene or by misregulation of global DNA methylation. However, X reactivation might also be a consequence of another mechanism of cancer, rather than the causal element.

Only a subset of X-linked genes are overexpressed in ovarian tumours from *BRCA1* mutation carriers compared to non-*BRCA1* associated tumours (Jazaeri et al. 2004). In addition, *BRCA1* and *XIST* occupy different physical domains on the inactive X chromosome in a subset of cells (Xiao et al. 2007, Pageau et al. 2007). Furthermore, there are X-linked genes that are heterogeneously inactivated among different women (Carrel and Willard, 2005). Thus, one could postulate that *BRCA1* participates in some form of secondary control to silence X chromosome genes beyond XCI and, when mutated, allows partial reactivation of the silent X chromosome and overexpression of an unknown, X-linked tumour promoting gene. A putative candidate for this role is *TIMP1*, an X chromosome gene that is variably inactivated among women (Anderson and Brown, 1999). In the present study, its expression was increased in all ovarian carcinoma cell lines as well as normal OSE cultures from *BRCA1* carriers. These results are consistent with a previous study that observed increased expression of *TIMP1* in *BRCA1*-null

ovarian tumours and subsequent repression with the addition of wild-type *BRCA1* (Jazaeri et al. 2004). It has previously been linked to several cancers including breast cancer (Spatz et al. 2004). Increased *TIMP1* mRNA and protein levels have been associated with negative outcomes in breast cancer (Ree et al. 1997, Schrohl et al. 2003).

Conversely, X reactivation may contribute to breast and/or ovarian carcinoma by misregulating global gene expression. Embryonic stem cells with normal autosomal content and two active X chromosomes require either a critical deletion in the distal region or complete loss of one X chromosome in order to persist in culture (Robertson et al. 1983). In mouse ES cells, the presence of two active X chromosomes leads to global hypomethylation at both repetitive and gene sequences (Zvetkova et al. 2005). Changes in methylation patterns have been observed in several cancers, such that specific genes are hypermethylated while global methylation decreases (Jones and Laird, 1999). Epigenetic changes can silence tumour suppressors, increase the incidences of mutations and cause chromosomal instability (Hoffman and Schulz, 2005). X reactivation causing global hypomethylation, may be an alternative mechanism for breast and/or ovarian carcinoma development.

X reactivation might also be a consequence of some other cancer-causing phenomenon. For example, global misregulation of methylation may cause X reactivation, rather than the reverse as hypothesized above. Assessing the cause from the effect in this situation could be performed by using tumour samples from early stages of ovarian carcinoma compared to normal ovarian tissue.

4.4 Further study of the association between the X chromosome and breast and/or ovarian carcinoma

The markers chosen to study a possible X reactivation event have not lead to conclusive findings. This is partially due to the difficulty of assessing whether genetic and epigenetic changes are causes or consequences of tumour growth in later stage cancers and cell lines. Study of X chromosome changes in normal breast and ovarian tissue from women with a known predisposition to cancer (via *BRCA1* and *BRCA2* mutations) compared to control ovarian tissues could clarify what role early X chromosome abnormalities play in tumour progression. It would also be valuable to study matching blood samples for these cases to observe whether or not the X chromosome changes only occur in breast and/or ovarian tissue.

4.5 The use of cancer cell lines

There are drawbacks of using an ovarian carcinoma cell line as a tumour proxy. While it is useful to study commercially available cell lines in order to compare results among different researchers, karyotype changes can arise via the culture and the passaging of cell lines. For example, in both MCF-7 and Ishikawa cell lines assessed over approximately 100 passages, karyotype heterogeneity was observed by comparative genomic hybridization and by FISH and attributed to extended time in culture (Wenger et al. 2004). In the present work, though gene expression was comparable over 30 passages for one cell line assayed, some variability of karyotype and/or gene expression may have occurred due to culture conditions.

If an X reactivation event occurs early in oncogenesis, it may result in an unstable state. Thus, tumours may bypass this state quickly, perhaps by losing the formerly

inactive X chromosome and duplicating the active X chromosome. Therefore, using tumour cell lines and/or passaging may capture a later cytogenetic time where X reactivation can no longer be observed. In breast cancer cell lines, over 90% of X chromosome markers showed LOH, while primary breast carcinoma samples, though more homozygous than matching blood samples, were only about 50% heterozygous overall (Sirchia et al. 2005). While the cell lines were not matched to primary samples, these results imply that a change has occurred in culture that has increased homozygosity. Thus, cell lines may not be representative of the *in vivo* situation.

Finally, gross karyotypic changes have been observed in ovarian carcinoma cell lines (Appendix 1). While changes in X chromosome content and expression may be important in the development of breast and ovarian carcinomas, assessment of X chromosome abnormalities without the context of the other chromosomal abnormalities within a cell line may have limited value. X chromosome abnormalities observed in this study may be a function of general chromosomal instability rather than a mark of a specific change leading to cancer development. Genomic changes with real effects on tumour progression would be best monitored in samples from early ovarian carcinoma stages rather than cell lines.

4.6 X chromosome abnormalities in other cancers

While it is attractive to suppose that changes in X chromosome content are present only in breast and ovarian carcinomas, aneuploidy, including X chromosome gains, is a common feature of many cancers. Testicular germ cell tumours, hepatoblastoma, chronic neutrophilic leukemia, childhood acute lymphoblastic leukemia and prostate cancer have all been associated with somatic gain of X chromosome content, though no increased X-

linked expression has been observed (Spatz et al. 2004). As mentioned previously, it may be that increased active X chromosome content is important in breast and ovarian carcinomas, rather than X chromosome aneuploidy alone. Colorectal and anal canal cancer patients frequently had gains of active X chromosome content and, depending on their sex, had previously lost either their Y chromosome or their late-replicating, presumed inactive X chromosome (Dutrillaux et al. 1986). Intracranial germ cell tumours also had X chromosome gains in 92% (23 of 25) of samples assayed, while 86% (13 of 16) of the cases assayed for methylation status at three genes were hypomethylated, suggestive of increased active X chromosome content (Okada et al. 2002). Thus, changes in active X chromosome content are not unique to breast and ovarian carcinomas. This may mean that there is a general cancer mechanism that relies on increased active X chromosome copy number. However, with its approximately 1100 known genes, there may be many genes residing on the X chromosome that become cancer-causing when active X chromosome content is increased, leading to different types of neoplasms.

4.7 Are hormone receptor variants associated with breast and/or ovarian carcinoma?

No significant difference in distribution of allele frequencies at hormone receptor polymorphism between any cancer patient group and controls was observed in this study. Trends towards allele frequency differences were most often found in breast and ovarian carcinomas patients with no known *BRCA1* or *BRCA2* mutations, suggesting that hormone receptor gene variants may be more important in sporadic breast and/or ovarian carcinoma.

Since information on the ethnic origin of the patients was not available, population differences in specific hormone receptor polymorphisms could not be accounted for in this study. Overall, the *AR* CAG repeat polymorphism ranges from 9 to 39 repeats, while 91-99% of all individuals regardless of race have repeat lengths from 16-29 repeats (Buchanan et al. 2004). However, there is some variability in repeat length distribution among different populations that could affect the results presented here (Table 4.1).

Table 4.1. *AR* repeat length variability among different populations.

Study	Population	N	Range	Median	Mean(SD)
Haiman et al. 2002	North American (USA-nurses)	969	10-33	21	21.8
Freedman et al. 2005	African Americans	664	11-41	20	20.11(3.39)
	Japanese	476	12-33	23	22.8 (2.98)
	Latinos	574	12-38	23	22.67 (3.21)
	Whites	446	12-35	22	22.02 (2.93)
Dos Santos et al. 2003	White	100	11-30	22	22.1 (3.2)
	Black	100	15-30	21	21.4 (3.1)
	Asian	79	15-30	23	22.9 (3.0)
Hsing et al. 2000	Chinese	300	10-33	23	22.7
Beilin et al. 2001	Australian (caucasian)	456	14-32	22	21.95
Hickey et al. 2002	Australian (caucasian)	831	14-30	-	-
Yaron et al. 2001	Israeli Jewish	44	4-22	18	16.3

There is also population variability in the distributions of the *ESR1* polymorphisms assayed. The *ESR1* TA repeat in the promoter region of the gene has a slightly different median in different populations, with the largest peak at 14 repeats in European populations and 15 repeats in Asian populations (Gennari et al. 2005). In addition, the combined -397T/C and -351A/G genotypes frequencies differ to a great extent between

ethnic groups, particularly between Caucasian, Asian and African American populations (Gennari et al. 2005).

The frequency of breast and ovarian carcinomas also varies among different ethnicities (Table 4.2). Breast and ovarian carcinomas are most common among Caucasian women, though incidence does not necessarily correspond to mortality rates (Jemal et al. 2007).

Table 4.2. The incidences of breast and ovarian carcinomas in different ethnic groups of the U.S. (per 100,000)

Population	Breast Cancer*	Ovarian carcinoma[t]
Caucasian	130.8	13.1
African Americans	111.5	9.0
Asian American/ Pacific Islander	91.2	10.2
American Indian/ Alaska Native	74.4	8.4
Hispanic	92.6	n/a

*Jemal et al. 2007 (Data compiled from 1999-2002)
[t]Goodman et al. 2003 (Data compiled from 1992-1997)
n/a: no data

Although the trends are non-significant, interestingly, patients that did not carry a *BRCA1* or *BRCA2* mutation were less similar to controls than patients with a mutation. Firstly, a non-significant increase in skewed XCI was observed in non-mutation carriers. This agrees with the previous finding of increased skewed XCI in young patients with familial non-*BRCA1*/*BRCA2* breast cancer (Kristiansen et al. 2005). Secondly, the prevalence of long *AR* repeat alleles was increased in ovarian carcinoma patients with no *BRCA1* or *BRCA2* mutation. Thirdly, increased *ESR1* repeat length and a decreased presence of the short/short genotype were observed in breast and ovarian carcinomas patients with no *BRCA1* or *BRCA2* mutation. These trends may indicate that XCI skewing and *AR* and *ESR1* repeat length polymorphisms are genetic modifiers. Also, such high risk in *BRCA1* carriers may obscure the effect of such modifiers. This may

explain why the strongest differences would be observed in the cancer group containing patients with no other known genetic predisposition to breast and/or ovarian carcinoma. However, the mean age of this group is higher than other groups assayed since they tended to have cancer later in life and skewing is known to increase with age (Hatakeyama et al. 2004), so the trend in the case of XCI skewing may be incidental.

Overall, this study suggests that there is merit in looking at genetic modifiers of breast and ovarian carcinomas within genes that affect hormonal exposure. Here, three genetic polymorphisms within two different hormone receptor genes, *AR* and *ESR1*, were assayed. However, even among these two genes, 13 different genetic polymorphisms have been reported, 2 repeat polymorphisms in *AR* and 11 SNP polymorphisms and 2 repeat polymorphisms in *ESR1* respectively (Gennari et al. 2005). Moreover, there are many genes that have a reputed involvement with estrogen exposure. Thus, a more comprehensive approach to identify a larger number of candidate genes would be valuable. SNP data could also be ascertained in order to correct for ethnic differences.

4.8 *FMR1* length, reproductive health and breast and/or ovarian carcinoma risk

The risk of breast and/or ovarian carcinoma decreases in *BRCA1* mutation carriers after menopause (Nkondjock and Ghadirian 2004). Thus, *BRCA1* mutation carrying women with premature ovarian failure and long *FMR1* repeat lengths may be protected against breast and ovarian carcinomas. Here, there was a non-significant trend towards an increase in long alleles (≥40 repeats) in *BRCA1* mutation carriers without cancer compared to controls, though the sample size was low (N=15). It would be valuable to assess *FMR1* repeat length and early menopause in a larger population of *BRCA1*

mutation carriers who do not develop cancer to assess whether the tendency for earlier menopause in this group is also protective against breast and/or ovarian carcinoma.

4.9 Conclusions

An X reactivation event that promotes the overexpression of some tumour-enhancing gene or suite of genes is an attractive hypothesis to explain the etiology of breast and ovarian carcinomas. A relationship between *BRCA1* and XCI could explain the cancer predisposition in women with *BRCA1* mutations. While there are indications that X chromosome abnormalities are present in breast and ovarian carcinomas cases, a partial or full X reactivation event has not been conclusively shown. Furthermore, estrogen exposure changes via hormone receptor polymorphisms could affect breast and ovarian carcinomas risk. Though hormone receptors polymorphisms do not appear to be largely different in patient and control groups, they may contribute to risk in patients with no known *BRCA1* or *BRCA2* mutations. Early detection of breast and/or ovarian carcinoma risk is important for the prevention and the treatment of these diseases. The identification of other genetic markers besides *BRCA1* and *BRCA2* mutations is a worthwhile pursuit as it may lead to more effective screening of at-risk women.

Chapter 5: References

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Appendix 1.Information on cell lines used in this study.

Cell Line	Known/Relevant Information
A2780	**Patient Info:** Ovarian epithelial adenocarcinoma from an untreated patient **Molecular Biology:** *PTEN* mutation (unknown origin), no *BRCA1* mutation, microsatellite instability at one repeat assayed (Sanger Institute – Cancer Cell Line Project) **Company and Product Number**: ATTC, ECACC # 93112519 **Paper :** Behrens et al. 1987
CaOV3	**Patient Info:** Ovarian Tumour from a 54 year old Caucasian
SKOV3	**Patient Info:** Epithelial ovarian adenocarcinoma, cisplatin sensitive from a 64 year old caucasian woman **Cytogenetics:** Hypodiploid (mode N=43 in 63% of cells). X either single or paired. **Or**: Hyperdiploid, triploid or tetraploid, trisomy X (SKY-NC160) **Or**: Hypodiploid to hypotetraploid (ECACC) **Company and Product Number:** ATTC, ECACC # 91091004
OVCAR2	**Patient Info:** Ovarian tumour from a cisplatin-refractory patient **Cytogenetics:** Aneuploid , chromosome counts sub to near-triploid 11,13,14,15,16,17,22 under-represented/ present as marker chromosomes
OVCAR3	**Patient Info:** Ovarian adenocarcinoma from a cisplatin-refractory treated patient (60 year old Caucasian) **Cytogenetics:** Aneuploid, near-triploid, rearrangements present, 3 Xs (SKY – NC160) **Molecular Biology**: Somatic *TP53* mutation, no *BRCA1* mutation found, no known microsatellite instability (Sanger Institute – Cancer Cell Line Project) **Pathology:** AR+, ER+, PR+ **Company:** ATTC **Paper:** Hamilton et al. 1983

Cell Line	Known/Relevant Information (continued)
OVCAR5	**Patient Info**: Untreated ovarian tumour **Cytogenetics:** Aneuploid, trisomy for chromosomes 2, 7, 8, 11, 15, 17, 19, 20, 22, monosomy for chr. 13, 14, 18, X (SKY-NC160) **Molecular Biology:** Somatic *KRAS* mutation, *CDKN2A* mutation of unknown origin, no *BRCA1* mutation, no known microsatellite instability (Sanger Institute – Cancer Cell Line Project) **Company**: MIISB
OVCAR8	**Patient Info:** Ovarian carcinoma from a platinum-refractory patient **Cytogenetics**: Complex rearrangements, aneuploidy, monosomy X (SKY – NC160) **Molecular Biology:** *ERBB2*, *TP53* mutations of unknown origin, no *BRCA1* mutation, no known microsatellite instability (Sanger Institute – Cancer Cell Line Project)
OVCAR10	**Patient Info:** Ovarian tumour from a platinum-refractory patient
Short term ovarian surface epithelium (OSE) cultures from *BRCA1* mutation carriers	
FAM1 (OSE 547F)	**Patient Info:** Scraping from OSE of both ovaries of a 48 year old *BRCA1* mutation carrier with previous history of breast cancer
FAM2 (OSE 563F)	**Patient Info:** Scraping from OSE of one ovary of a 48 year old *BRCA1* mutation carrier with previous history of bilateral breast cancer
Controls	
IOSE 397	Immortalized OSE sample
GMO4626	Three X cell line