BRCA1 promoter methylation: the influence on gene expression and the effect of long term drug treatment

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Abbreviations

BARD1	BRCA1-associated RING domain protein 1
BC	Breast Cancer
BRCA1	Breast cancer susceptibility gene 1
BRCT	BRCA1 C Terminus domain
BRIP1	BRCA1 interacting protein 1
CGI	CpG Island
CpG	Cytosine-phosphate-guanine
ddH_2O	Double distilled water
DMSO	Dimethyl sulfoxide
DSB	Double stranded break
EMEM	Eagle's Minimum Essential Medium
ER	Estrogen receptor
Her2	Human epidermal growth factor receptor 2
HR	Homologous recombination
HRP	Horseradish peroxidase
HRR	Homologous recombination repair
MSP/USP	Methylation specific PCR/Unmethylated specific PCR
MSRE	Methylation specific restriction enzyme
NGS	Next generation sequencing
NLS	Nuclear localization signal
OC	Ovarian Cancer
PALB2	Partner and localizer of BRCA2
PARP	Poly-ADP-ribose polymerase
PARPi	PARP inhibitor
PBS	Phosphate buffered saline
PR	Progesterone receptor
qPCR	Quantitative PCR
RE	Restriction enzyme
RPMI	Rosewell Park Memorial Institute
SAM	S-Adenosyl methionine
TAE	Tris base, acetic acid and saline
TBST	Tris buffered saline with tween
TF	Transcription factor
TNBC	Triple negative breast cancer
TOPBP1	DNA topoisomerase 2 binding protein 1

Abstract

Breast cancer is the most common type of cancer among woman all over the world, with over 1.67 million new cases in 2012. Heritable breast cancer is closely linked to mutations in the tumor suppressor gene *BRCA1*, with up to 80% lifetime risk for developing breast cancer among women harboring a mutation in this gene. However, most breast cancer cases are sporadic and somatic mutations of the *BRCA1* gene are rare. Furthermore, some tumors show BRCAness, despite being *BRCA1* wild-type. Thus, it is of great interest to assess alternative mechanisms for inactivation of the *BRCA1* gene, and addressing the missing causality of many breast cancers. Furthermore, it is of great interest to assess the mechanisms of drug resistance, a major challenge in cancer treatment today, where *BRCA1* may play an important role.

The overall aim of this thesis is to increase the understanding of the biological role of *BRCA1* promoter methylation in breast cancer. Three sub aims for the present project were outlined; 1) Quantify the *BRCA1* α and β transcripts and the total BRCA1 protein levels and relate the expression data to the methylation pattern in the *BRCA1* promoter region in a panel of breast cancer cell lines. 2) Investigate how the total expression levels, as well as the ratio between the α and β transcripts are affected by alterations in the α and β promoter region of *BRCA1*, including methylation of specific CpGs as well as the polymorphisms rs71361504 and rs799905. 3) Investigate the effect of long term treatment with the drugs olaparib and doxorubicin on the *BRCA1* promoter methylation in SKBR3 breast cancer cells as a potential cause of drug resistance.

The study showed a weak correlation between *BRCA1* methylation pattern and *BRCA1* mRNA expression. No correlation was observed between the methylation pattern and protein expressed or between mRNA levels and protein expression. Analysis of polymorphisms rs71361504 and rs799905 found in the *BRCA1* promoter showed that the two variants seemed to counter-balance each other, giving equal luciferase expression levels when differing in two positions and lower expression levels when intermediate variants were studied. Finally, long term drug treatment of the cell line SKBR3 did not alter the methylation levels in the *BRCA1* promoter, consequently demethylation seems not to be a mechanism for drug resistance in the experimental setup tested in this study.

1 Introduction

1.1 Cancer

Cancer is a major global health problem. In 2012, 8.2 million people died of cancer (mortality), 14.1 million new cancer cases were reported (incidence), and 32.6 million people were living with cancer (prevalence) worldwide (IARC, 2012). In Norway, a total of 32 592 new cancer cases were diagnosed in 2015 (Kreftregisteret.no, 2017).

Cancer is a group of diseases characterized by uncontrolled cell growth. A cancer cell evolves in a progressive manner, gradually acquiring properties necessary for neoplastic development. The cancer specific properties have, for simplicity, been classified into ten different "hallmarks" of cancer (Figure 1.1) (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011) These hallmarks of cancer include genomic instability and mutation, tumor promoting inflammation, production of signals that sustain proliferation and evade growth repression. Further, a developing cancer cell needs to escape programmed cell death and enable replicative immortality, as well as induce angiogenesis, activate invasion and metastasis, deregulate cellular energetics and avoid immune destruction.

Cancer is regarded as a genetic disease at the cellular level caused by accumulation of genetic and epigenetic alterations providing growth advantages over neighboring cells. The development of cancer is largely governed by the functions of tumor suppressor genes and proto-oncogenes. Tumor suppressor genes protect cells from transforming into malignant cancer cells while proto-oncogenes can potentially induce cancer (become oncogenes) if hyper-activated. Alterations that inactivate a tumor suppressor gene, or activate or amplify a proto-oncogene, can contribute to malignant transformation of cells. Such alterations, directly contributing to cancer development and growth, are called "drivers", while the majority of alterations found in cancer cells are "passengers", not contributing to cancer development (Stratton et al., 2009, Greenman et al., 2007, Sjoblom et al., 2006).

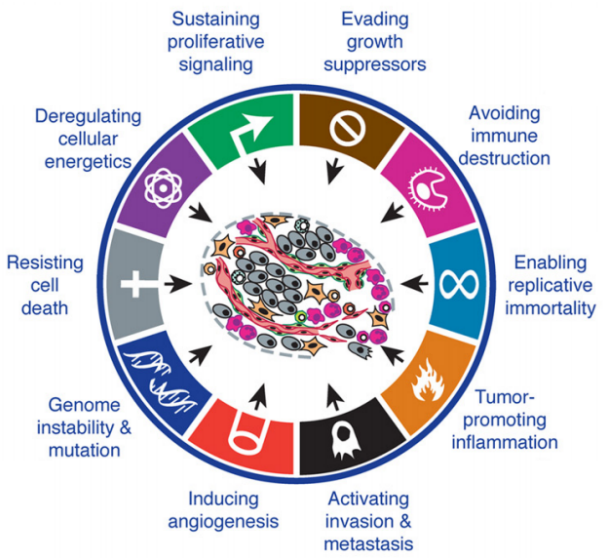

Figure 1.1: The hallmarks of cancer are ten properties characterizing cancer cells as described by Hanahan and Weinberg in 2000 and 2011. The properties are acquired in a progressive matter, by genetic and/or epigenetic changes in the DNA. Modified from (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011).

1.2 Breast cancer

Among all cancer forms, breast cancer is the second most common and the most common for women. In 2012, 1.67 million new cases of breast cancer were diagnosed worldwide (IARC, 2012). In Norway, 3439 new cases of breast cancer were registered in 2015, making up >10% of all new cancer diagnosis that year (Kreftregisteret.no, 2017). The breast cancer prevalence is increasing in developed countries, owing to both increased incidence (linked to western lifestyle) as well as improved screening programs/early detection (Ma and Jemal, 2013, Desantis et al., 2016, Senkus et al., 2013). However, breast cancer is still the leading cause of cancer-related deaths among European women (Senkus et al., 2013).

1.2.1 Breast cancer classification

Over the last decades, it has become evident that breast cancer is not a single disease, but rather a diagnosis that can be divided into many subclasses. The most common stratifications are to subgroup breast cancers according to their hormonal receptor status, that is, whether they express receptors for estrogen (ER) and/or progesterone PR). Further, an important stratification parameter (for treatment choice) is whether the tumor cells overexpress the Her2 receptor (see section 1.2.1). Breast cancers may also be divided according to histological type, with the two main forms being ductal and lobular carcinomas. Based on gene expression profiles, breast cancers have also been

divided into five subtypes with specific phenotypes and clinical outcomes; Luminal A, Luminal B, Her2 over-expression, Basal-like, and Normal-like. Luminal A is predicted to have the best survival outcomes, while Basal-like, which often include a triple negative receptor status (ER, PR, Her2), is associated with the poorest disease prognosis (Sorlie et al., 2003, Sorlie et al., 2001, van 't Veer et al., 2002, van de Vijver et al., 2002, Perou et al., 2000).

1.2.2 Factors contributing to breast cancer development

Cancer is induced by multiple factors, but the exact cause is not fully understood. However, it is well established that the risk of breast cancer is influenced both by environmental and genetic factors. Some of the most important environmental factors linked to breast cancer risk are age, reproductive and hormonal aspects, diet/obesity, lack of physical exercise, alcohol consumption and exposure to X- and γ-radiation (Stewart, 2014). Many of these factors are associated with the western life style (Buell, 1973), and the western part of the world is also where the breast cancer incidence is the highest (IARC, 2012).

While most breast cancer cases are considered to be sporadic (i.e not caused by high risk genetic factors), a small fraction (1-5%) of all breast cancers (Davies et al., 2017) (2% in Norway (kreftforeningen.no)) are linked to inherited genetic variants predisposing to high risk of breast cancer. The best example is germ line mutations in the *BRCA1* gene that predispose women to breast as well as ovarian cancer (Rohini et al., 2011). Women harboring mutations in *BRCA1* have a lifetime risk of 50-80% for developing breast cancer and 30-50% for ovarian cancer (Rahman and Stratton, 1998, Antoniou et al., 2003). Other genes in which germline mutations are associated with risk of breast cancer, are *TP53*, *CHEK2*, *PTEN* and more.

1.2.3 Breast cancer treatment

Breast cancer treatment options include surgery, chemotherapy, radiation, endocrine therapy, and targeted therapy. Recently, immunotherapies have also been applied in clinical trials (Ling et al., 2017, Curigliano et al., 2016). Several factors affect the choice of treatment such as tumor stage/grade, lymph node and hormonal receptor status, Her2-status, age (menopausal status) and patient's health and own preference

(Senkus et al., 2013). In recent clinical trials regarding sporadic cancers, mutation status of specific genes (such as *TP53*) is also included as a parameter for treatment choice. Most breast cancers are treated with surgery followed by radiation. Chemotherapy is generally used as adjuvant therapy and in relation to metastasis and relapse, as well as for patients that for various reasons cannot go through surgery. For large tumors, chemotherapy is also used prior to surgery (neo-adjuvant treatment) (kreftforeningen.no).

Doxorubicin is a classical chemotherapeutic drug used in breast cancer as well as many other cancer forms. Doxorubicin is an anthracycline with cytotoxic mechanisms involving DNA intercalation, topoisomerase inhibition and generation of free oxygen species, resulting double stranded breaks (DSB). This, in turn, leads to cell cycle arrest, senescence or apoptosis (Niethammer and Bruchelt, 1998).

Poly ADP ribose polymerase (PARP) inhibitor, is a newer and more targeted therapy which is given to patients with a *BRCA1* mutation (Rafii et al., 2017) and has also been suggested to patients displaying other types of *BRCA1* deficiency, including hypermethylation in the *BRCA1* promoter (Veeck et al., 2010). PARP is a group of proteins involved in single stranded DNA repair trough base-excision repair. If the PARP-protein is inhibited, single stranded breaks will eventually turn into double stranded (ds) breaks. In normal cells, ds DNA breaks (DSB) are repaired by mechanisms including *BRCA1* dependent homologous recombination (HR) repair. Hoewever, when treating *BRCA1/2*-mutated and potentially methylated cells displaying deficient DSB repair system with PARP-inhibitor (PARPi), this will not happen and the targeted cell will die (figure 1.2) (Fong et al., 2010). This treatment model utilizes a defect already present in the cell as an advantage for selectively killing the cancer cells, a concept termed synthetic lethality. Olaparib is an example of a promising PARP-inhibitor, and several clinical trials are testing the effect of this drug (Murata et al., 2016, Miller and Ledermann, 2016).

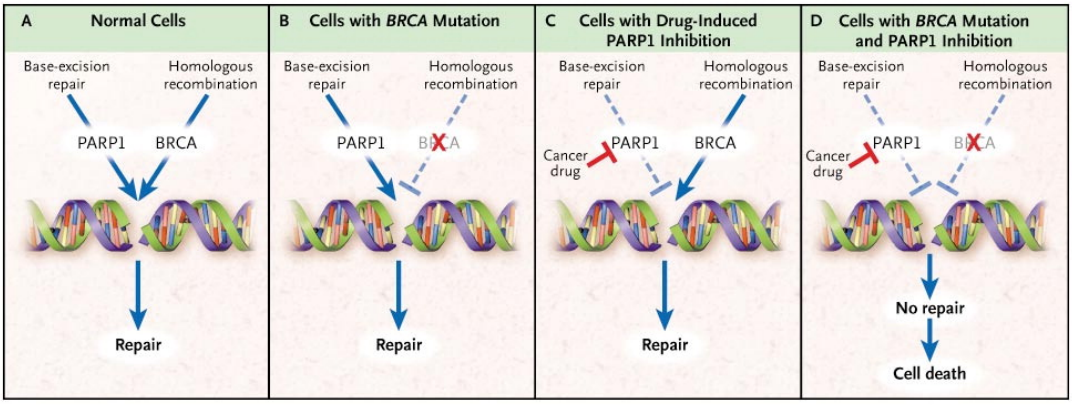

Figure 1.2: Overview of the role of PARP1 and BRCA1/2 in synthetic lethality. DNA damage can be repaired by several mechanisms involving different molecules, normal cells should have both base-excision repair and homologous recombination (**A**). DNA can still be repaired by base-excision repair if *BRCA* is mutated (**B**), and by homologous recombination if PARP1 is inhibited (**C**). A cancer cell can be targeted by synthetic lethality when *BRCA* mutated cells are treated with PARP1 inhibitor, and when neither base-excision repair, nor homologous recombination can be performed, the DNA will not be repaired and the cell will die (**D**). Reproduced with permission from (Iglehart and Silver, 2009), Copyright Massachusetts Medical Society.

Despite many efficient treatment options, there are still major challenges in current breast cancer management. One such challenge is the development of drug resistance, which is the main cause of unsuccessful treatment and subsequent death (Foo and Michor, 2009, Lonning and Knappskog, 2013). In order to improve treatment results, a deeper understanding of the underlying mechanisms and pathways for development of treatment resistance within the cancer cell is needed. Both genetic and epigenetic processes should to be considered, however, epigenetics is of particular interest due to its highly dynamic nature.

1.3 Breast cancer gene 1

Breast cancer susceptibility gene 1 (*BRCA1*) was identified in 1990 and isolated and cloned in 1994 (Miki et al., 1994, Hall et al., 1990). The *BRCA1* gene is located on chromosome 17q21.3, consists of 23 coding exons and encodes a large protein of 220 kDa, consisting of 1863 amino acids (figure 1.3). The *BRCA1* gene is partially duplicated, resulting in a *pseudo BRCA1* gene containing only exon 1A, 1B and 2. *Pseudo BRCA1* is located of the same chromosomal band, separated from the wild-type *BRCA1* by the *NBR2* gene (figure 1.3 A). Notably, the pseudo *BRCA1* gene has also been found to be expressed in malignant cells (Pettigrew et al., 2010).

1.3.1 BRCA1 protein domains and function

The BRCA1 protein contains several functional domains including a N-terminal RING domain involved in heterodimerization of BRCA1/BARD1, providing E3 ubiquitin ligase activity. Two nuclear localization sequences (NLS) are located towards the N terminus, allocating BRCA1 to the nucleus. A coiled-coil domain found towards the C-terminus of the protein is involved in binding to Partner and localizer of BRCA2 (PALB2). Two BRCA1 C-terminal (BRCT) domains that bind proteins involved in transcription and DNA damage response (Rohini et al., 2011, De Siervi et al., 2010) are also located in the C-terminal end (figure 1.3 B). The central region of BRCA1 does not contain any known functional domains, which allows for a wide array of structural changes and the possibility to bind a range of other molecules (Savage and Harkin, 2015). BRCA1 phosphorylation is involved in subcellular localization of the protein (Scully and Livingston, 2000, Scully et al., 1997, Brodie and Henderson, 2010). Through its protein domains, BRCA1 interact with a myriad of other proteins resulting in numerous large protein complexes which participates in several important processes including cell cycle regulation, regulating of transcription, ubiquitination, chromatin remodeling, mRNA splicing, apoptosis, maintenance of genome integrity and repair of dsDNA breaks through homologous recombination (HR) (Savage and Harkin, 2015), the latter being among the most relevant function with respect to BRCA1 tumor suppressor activities.

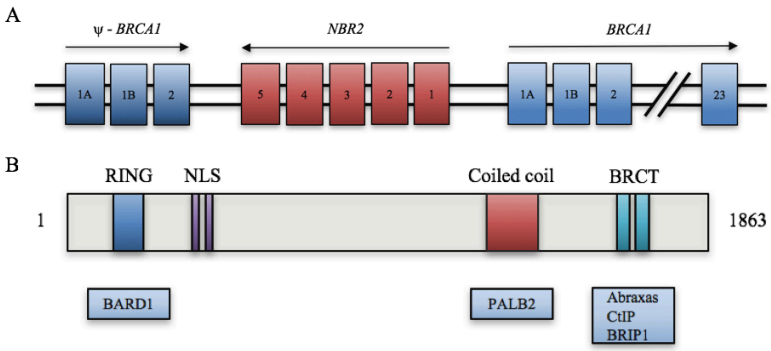

Figure 1.3: Schematic presentation of *BRCA1* gene localization and functional domains of BRCA1 protein. A) The ψ*-BRCA1* is located upstream to *BRCA1* on the negative strand in the human genome. *NBR2* is located between the two genes and is transcribed in the positive direction. **B)** Protein domains and binding partners for the BRCA1 protein.

DSB is considered to be one of the most hazardous types of DNA damage, and can be repaired trough two major pathways called homologous recombination (HR) and non-homologous end joining (NHEJ). It is well established that BRCA1 plays a central role

in HR as illustrated in figure 1.4 (Rohini et al., 2011). DSB are detected by sensor molecules, which signal mediators that in turn can activate effectors and allowing repair of the damage. BRCA1 is considered a mediator; it binds to several other proteins and allow recruitment of molecules involved in HR. One of the first responses to DSB is phosphorylation of histone H2A.X, which starts a cascade of reactions leading to binding of complex abraxas-RAP80 and promotion of BRCA1. BRCA1 is important for recruiting a myriad of molecules and complexes involved in DSB-repair, as well as in strand resection by interactions with CtIP and MRN (MRE11-RAD50-NBS1). Furthermore, BRCA1 interacts with PALB2 and BRCA2 which lead to RAD51 mediated HR by invasion of sister chromatid. DNA is synthesized by a DNA polymerase and the strands can be relegated. The BRCA1-BRIP1-TOPBP1 complex is associated with DNA repair, but the exact mechanisms are unknown (Savage and Harkin, 2015, Ronit et al., 2002, Rohini et al., 2011) (figure 1.4).

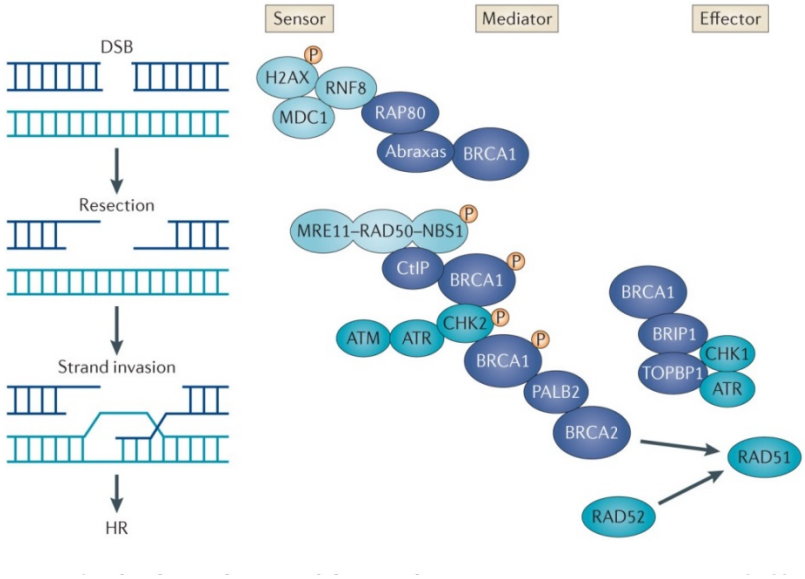

Figure 1.4: The role of BRCA1 in homologous recombination (HR). BRCA1 is involved in double-stranded break (DSB) repair. DSB are detected by sensors (light blue) which lead to a cascade of reactions and the recruitment of BRCA1, which is considered a mediator (dark blue). BRCA1 is involved in recruiting molecules, involved in HR repair. BRCA1 bound to relevant complexes, is involved in both resection and strand invasion by binding to various effectors (turquoise). Reproduced with permission from (Rohini et al., 2011), Copyright Nature Publishing Group.

1.3.2 The *BRCA1* promoter region

BRCA1 transcription is under regulation of two promoters; α and β, which generate two different transcripts containing either exon 1a or exon 1b, respectively (Xu et al., 1995, Xu et al., 1997). The two *BRCA1* transcripts (called *BRCA1* α and β transcript) differ in length and nucleotide sequence, but the protein products arising from the transcripts

are identical independent on whether the transcript contains exon 1a or exon 1b as the start codon for translation is located in exon 2. Promoter α, consisting of just over 200 bp, spans a slightly larger region than promoter β, which consist of approximately 150 bp.

The *BRCA1* promoter does not contain a classical TATA box, however, several transcription factor binding sites associated with TATA-less promoters are found in the *BRCA1* promoter region. Transcription factor binding sites for specific protein 1 (Sp1), the cyclic AMP responsive element binding (CREB) protein (Hockings et al., 2008, Mancini et al., 1998), RIBS element, E2F transcriptions factor family and more (Mueller and Roskelley, 2003, Xu et al., 1995). *BRCA1* is also found to be regulated by estrogen (E2) through a non-classic activation pathway. *BRCA1* lacks the classic E2-response elements, but E2-liganded-ERα can bind to the p300 co-activator, which can interact with Jun/Fos transcription factors which bind to a AP-1 site located in the *BRCA1* promoter (Shukrun et al., 2014, Jeffy et al., 2005, Xu et al., 1997).

Interestingly, the *BRCA1* α transcript is found to be expressed in both normal and cancerous breast tissue, while the *BRCA1* β transcript is only found to be expressed in breast cancer tissue (Xu et al., 1995, Sobczak and Krzyzosiak, 2002). These findings indicate that at some point during neoplastic development, *BRCA1* transcription is deregulated, resulting in the activation of promotor β. Altered dynamics of methylation of CpG dinucleotides located in the promotor region of *BRCA1* could potentially be among the mechanisms involved in the switch from α to β promoter. Altered dynamics of methylation of CpG dinucleotides located in the promotor region of *BRCA1* could potentially be among the mechanisms involved in the switch from α to β promoter. Furthermore, the expression of *BRCA1* α transcript has been found to be 6-150 times higher than the *BRCA1* β transcript (Fernandes et al., 2014, Xu et al., 1997), where possible explanations could be regulation by different transcription factors. In addition, translation of the BRCA1 WT β transcript has also been shown to be less efficient, suggested explanations involve alternative and suboptimal start codons that allow premature initiation and termination, stable secondary structures within the β transcript which interfere with assembly of pre-initiation complex and 40S ribosomal subunit scanning of mRNA (Sobczak and Krzyzosiak, 2002).

1.3.3 *BRCA1* alterations in breast cancer

The *BRCA1* gene is closely linked to cancer, due to the very high risk of female cancers including breast and ovarian cancer, among carriers of germline mutations. Of a total of 1277 entries in the *BRCA1* Database provided by The University of Utah, most of the germline mutation variants that are observed for *BRCA1* are deletions (43%), followed by nonsense mutations (20%) and insertions (15%) among other (www.arup.utah.edu, 2017).

Regarding somatic mutations, a total of 548 unique cancer samples are registered with *BRCA1* mutations, in the catalogue of somatic mutation in cancer database (COSMIC v.81) (Forbes et al., 2017). The most common somatic alteration of *BRCA1* gene in cancer is missense substitutions (60%), synonymous mutations (17%) and nonsense mutations (10%). Copy number variation (CNV), large genomic rearrangements (LGR) and loss of heterozygosity (LOH) are genetic alterations that also are observed in *BRCA1* cancers (Ewald et al., 2009, Fridlyand et al., 2006, Hampton et al., 1994). Other mutations detected include frameshift insertion and deletion, in-frame deletion and more. However, somatic mutations of *BRCA1* in sporadic breast cancer are rare (Catteau and Morris, 2002), yet decreased expression of *BRCA1* mRNA and protein is still observed in breast tumors despite the absence of mutations of the *BRCA1* gene (Thompson et al., 1995, Hasan et al., 2013). In addition, cancers might have global mutational profiles reflecting deficient HR repair even if they do not harbor mutations in *BRCA1*. This phenomenon is termed "BRCAness" and is defined by "*traits that some sporadic cancers share with those occurring in either BRCA1 or BRCA2-mutation carriers*" (Davies et al., 2017, Turner et al., 2004). This indicates that mechanisms other than mutations must be involved to cause inactivation or down-regulation of *BRCA1*. DNA methylation in the promoter region is proposed as such an alternative mechanism for silencing of tumor suppressor genes (Rice et al., 1998), potentially explaining some of the missing causality of breast cancer.

1.4 Epigenetics and DNA methylation

"The term epigenetic includes regulatory mechanisms that influence gene expression without any changes in the sequence of the DNA" (Gaal and Olah, 2014). Epigenetic

changes have the potential to alter gene expression through several mechanisms; the main mechanisms include DNA methylation and covalent tail modifications of histones (mostly methylation and acetylation). DNA methylation is the most studied epigenetic modifications in the human DNA. It involves covalent binding of a methyl group (-CH_3) to the 5-position of the pyrimidine ring of a cysteine (C) nucleotide in primarily CpG dinucleotides (where p stands for the phosphate bond between the nucleotides), resulting in 5-methylcytosine (m5C). The reaction adding methyl to DNA is catalyzed by the DNA methyl transferase (DNMT) enzyme family (figure 1.5).

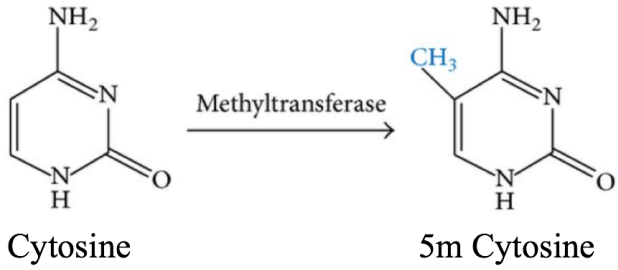

Figure: 1.5: Methylation of cysteine by metyltransferase. Methylation of the cytosine pyrimidine ring catalyzed by the enzyme methyltransferase.

Methylation of CpGs has been found to be the most common form of methylation in humans. Approximately 50% of the human genes contain short stretched with high content of CpG dinucleotides termed CpG islands (CGI), these are usually located in promoters, while the rest of the genome is generally depleted of CpGs (Jones, 2012). Hyper- or hypomethylation of a promoter region can be involved in transcriptional silencing or activation of a gene, respectively. Genes that are transcribed in a normal manner generally have unmethylated promoter regions. (Ali et al., 2011, Kloten et al., 2013). Methylation in the promoter region can lead to silencing of genes by several mechanisms. One of the mechanisms involves direct blocking of transcription factor binding sites by methylation within or close to these sites. A second model proposes that methylation attracts proteins that specifically bind and consequently block the access of other factors required for gene expression. Additionally, DNA methylation can be involved in compact packing of chromatin resulting in inactive regions (heterochromatin).

While methylation in promoter regions are associated with silencing of genes, methylation of the gene body is associated with actively transcribed genes (Wolf et al.,

1984). Most gene bodies have only a few CpGs, but these are generally methylated. Methylation is known to be involved in several processes like embryonic development (Li et al., 1992), imprinting (Wolf et al., 1987) and X chromosome inactivation (Riggs, 1975) as well as and regulation of diseases, including cancer (Vardhman et al., 2011, Haoyang, 2013, Nguyen et al., 2010, Kulis and Esteller, 2010)

1.4.1 The role of DNA methylation in cancer

Abnormalities like loss and gain of methylation, hypo- and hypermethylation, respectively, are widely associated with cancer development. A global reduction of methylation, along with hypermethylation of the promoter of tumor suppressor genes are phenomena commonly observed in cancer (Gama-Sosa et al., 1983, Eden et al., 2003). Genome wide demethylation can lead to chromosome instability due to activation of transposable elements/reteroviral elements (Hansmann et al., 2012) and recent sequencing efforts have revealed a large number of structural rearrangements, (involving genes, exons and regulatory elements) in cancer genomes to be associated with, and possibly caused by, re-activated retroviral elements (Tubio et al., 2014). Aberrant hypermethylation in the promoter region is among the most common way of silencing of tumor suppressor genes and represent an alternative inactivating mechanism to mutations. Aberrant hypermethylation in the promoter region has been described for several tumor suppressor genes in breast cancer including *CDH1, RASSF1A* and *BRCA1* (Cho et al., 2010). Cytosine methylation status can also affect cancer by oncogenic point mutations due to spontaneous deamination of methylated cysteines. The focus in this thesis is on methylation in the promoter of *BRCA1*.

1.4.2 Aberrant methylation of *BRCA1* promoter in breast cancer

The tumor suppressor gene *BRCA1* can be epigenetically silenced by hypermethylation within the promoter (Esteller et al., 2000, Esteller et al., 2001, Hasan et al., 2013) and experimental demethylation of *BRCA1* leads to reactivation of the gene (Choudhury et al., 2016). Furthermore, significantly higher levels of methylation among CpGs in the *BRCA1*promoter have been found in breast cancer compared to normal tissue (Zhang and Long, 2015, Ali et al., 2011). Hypermethylation of the *BRCA1* promoter is found in approximately 5-65% of sporadic breast cancers (Ignatov et al., 2013, Buyru et al., 2009, Birgisdottir et al., 2006). The range in frequency might be explained by factors

including methylation detection method, CpGs/area analyzed and potential contamination by unmethylated normal tissue. Some studies have analyzed the methylation pattern of individual CpGs in the *BRCA1* promoter region of breast cancer and ovarian cancer, revealing the methylation pattern to be highly heterogeneous (Rice et al., 2000, Wilcox et al., 2005, Scott et al., 2016, Hansmann et al., 2012).

In patients carrying *BRCA1* germline mutations, it is believed that a "second hit" is needed for inactivation of the wild-type *BRCA1* allele and for development of cancer (Birgisdottir et al., 2006, Chenevix-Trench et al., 2006). Some studies have proposed that methylation of *BRCA1* promoter may serve as this second hit in tumors of such patients (Tapia et al., 2008, Esteller et al., 2001). Phenotypically, *BRCA1*-methylated tumors are similar to those occurring in carriers of germline *BRCA1* mutations, thus displaying "BRCAness". *BRCA1* promoter methylation in breast cancer patients has been found to correlate with reduced expression levels of both mRNA (Galizia et al., 2010, Rice et al., 2000, Hasan et al., 2013) and protein (Scott et al., 2016, Wu et al., 2016), confirming aberrant promoter methylation to be an important inactivating mechanism. Furthermore, *BRCA1* promoter methylation is suggested to be involved in initiation of tumor development, and that it could be used as a biomarker for early detection of sporadic breast cancer (Hosny et al., 2016, Ali et al., 2011, Cho et al., 2010, Hansmann et al., 2012).

Recent meta-analyses reported *BRCA1* promoter methylation to be associated with various clinico-pathological features such as lymph node metastasis, histological grade 3, triple-negative phenotype as well poor survival of patients with breast cancer (Zhang and Long, 2015, Wu et al., 2013, Birgisdottir et al., 2006). These findings indicate that hypermethylation of the *BRCA1* gene promoter could be an important marker for prognosis.

Triple negative breast cancer (negative for ER, PR and Her2) is an aggressive subtype which is difficult to treat and often associated with loss of function of the *BRCA1* gene, either through mutation, loss of heterozygosity, or methylation (Lips et al., 2013). Identification of predictive markers involved in this type of cancer is crucial to improve breast cancer survival. Several studies have suggested that tumors with *BRCA1* promoter methylation have features similar to those harboring *BRCA1* mutations, and

therefore may be suitable for the same targeted therapies, such as platinum based chemotherapy agents and PARP-inhibitors. Consequently, methylation status could be used as a biomarker for treatment strategy decisions (Sharma et al., 2014, Veeck et al., 2010).

1.4.3 Methods for detection of methylation

Numerous methodological approaches exist for detecting DNA methylation including methylation-sensitive restriction enzymes (MSREs), methylation-specific PCR (MSP/USP PCR), pyrosequencing and methylation specific next generation sequencing (NGS) (Delpu et al., 2013). MSREs was one of the first methods used, and this method allowed identification of exact CpG position within the DNA, however, detected CpGs are limited to restriction sites in the given region.

A number of the methods for analyzing DNA methylation utilize bisulfite converted DNA as starting material. Methylation involves binding of a chemical group to DNA, and does not induce a change within the DNA backbone, consequently ordinary sequencing cannot detect methylation. Bisulfite conversion offers a solution to the problem of detection. When treating DNA with sodium bisulfite, methylated cytosine nucleotides are left unchanged, while unmethylated cytosine are chemically deaminated, thus turning unmethylated Cs into uracil, which then can be detected as thymine after PCR (figure 1.6) (Frommer et al., 1992) (Hayatsu, 2008, Wang et al., 1980). In this way, a previously undetectable epigenetic marker/chemical tag is turned into change in the nucleotide sequence detectable by sequencing.

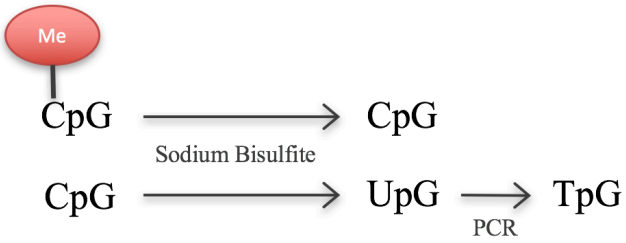

Figure 1.6: Bisulfite conversion of methylated and unmethylated CpG's. When treated with sodium bisulfite, methylated Cs remain unchanged while unmethylated Cs are deaminated to Us, which can be detected as Ts after PCR and sequencing.

MSP/USP PCR, utilize bisulfite converted DNA and is an easy and low cost PCR based method dependent on 5-10 CpGs covered by the primers. A drawback with the method

is that methylation status can only be collected for the area covered by the primers. In addition, due to the need for separate assays for methylated versus unmethylated specific amplifications, assessment of the methylation ratio is challenging. Pyrosequencing is a technique that also utilize bisulfite converted DNA, it is rapid and can be standardized. Even if qPCR is more sensitive, this method has most of the same drawbacks as MSP/USP PCR. However, many labs currently use pyrosequencing as the standard for quantification of DNA methylation, but improved NGS methylation protocols are being developed.

NGS methods give the best coverage of CpGs and read depth, but the methods are still rather expensive and demand advanced bioinformatical processing of data. The Roche NimbleGen protocol SeqCap Epi Target Enrichment of bisulfite treated DNA allows methylation assessment at single-base resolution for all possible methylation combinations on both strands. However, in the context of NGS and methylation, mapping is extra challenging because one region could be fully methylated, partly methylated or unmethylated. In addition, due to bisulphite conversion the upper and lower strand are no longer complementary, and the genome is doubled. Another challenge is polymorphic variants in CpG positions, which is important to identify because true C that have been changed to T in the evolution cannot be separated from C to T substitutions caused by bisulfite conversion. These positions might thus be mistakenly interpreted as unmethylated C's. NGS data can be analyzed in context of SNPs by algorithms that uses known SNPs in the genome and comparison of the obtained reads. The output NGS data also allow calculation of percentage methylation at individual CpG and in specific regions of interest.

It is likely that methods for analysis of DNA methylation will be improved in the near future. Although still hampered by a high error-rate, one of the most promising approaches is to apply nanopore sequencing capable of distinguishing five different bases in a DNA strand (A, G, T, C and mC), thereby merging genetic and epigenetic analyses into a single experiment, without prior bisulfite conversion of DNA (Simpson et al., 2017).

2 Aim

Based on the given background knowledge, and the overall aim to increase the knowledge of the biological role of *BRCA1* promoter methylation, the three sub aims of this master thesis are:

1. Quantify the *BRCA1* α and β transcripts and the total BRCA1 protein levels and relate the expression data to the methylation pattern in the *BRCA1* promoter region in a panel of breast cancer cell lines.

2. Investigate how the total expression levels, as well as the ratio between the α and β transcripts is affected by alterations in the α and β promoter region of *BRCA1*, including methylation of specific CpGs as well as polymorphisms rs71361504 and rs799905.

3. Investigate the effect of long term drug treatment with drugs olaparib and doxorubicin on *BRCA1* promoter methylation in SKBR3 breast cancer cells.

3 Material

3.1 Cell culturing

Table 3.1.1: Cell lines with description of tissue, sub-class and receptor status*

Cell line	ATCC® ID	Tissue	Sub-class	Receptor status
SKBR3	ATCC HTB-30	breast	Her2 over express	ER-, PR-, Her+
ZR-75-1	ATCC CRL-1500	breast (metastatic)	Luminal B	ER+, PR+, Her+
UACC-3199	ATCC CRL-2983	breast (metastatic)	Unknown	ER-, PR-, Her+
BT-549	ATCC HTB-122	breast	Basal like	ER-, PR-, Her-
T-47D	ATCC HTB-133	breast (metastatic)	Luminal A	ER+, PR+, Her-
HCC38	ATCC CRL-2314	breast	Unknown	ER-, PR-, Her-
MCF7	ATCC HTB-22	breast (metastatic)	Luminal A	ER+, PR+, Her-
MDA-MB 231	ATCC HTB-26	breast (metastatic)	Basal-like	ER-, PR-, Her-
MDA-MB 468	ATCC HTB-132	breast (metastatic)	Basal like	ER-, PR-, Her-

*Sub-class and receptor status retrieved from ATCC and article "Choosing the right cell line for breast cancer research" (Holliday and Speirs, 2011).

Table 3.1.2: Cell lines and corresponding medium reagents

Cell line	Medium* (Supplier: Lonza)	FBS (Sigma-Aldrich)	L-Glutamine (Lonza)	Extra components added/notes (Sigma Aldrich)
SKBR3	McCoy's 5a	10%	-	-
ZR-75-1	RPMI-1640	10%	4 mM	-
UACC-3199	Leibovitz's L-15	5%	2 mM	0.01 mg/mL transferrin 0.01mg/mL insulin 5 µg/mL catalase 3.6 µg/mL hydrocortisone
BT-549	RPMI-1640	10%	4 mM	0.023 U/mL bovine insulin
T-47D	RPMI-1640	10%	4 mM	0.2 U/mL bovine insulin
HCC38	RPMI-1640	10%	4 mM	-
MCF7	EMEM	10%	4 mM	-
MDA-MB 231	RPMI-1640	10%	4 mM	-
MDA-MB 468	Leibovitz's L-15	10%	4 mM	Cultured without CO2

* All media were prepared with 5% PenStrep (Life technologies, 15070-063). When required, cells were split with trypsin-EDTA (Lonza17-516F).

Table 3.1.3: Drugs for cell treatment

Name	Supplier (Cat #)
DMSO	Sigma Aldrich (D2650)
Olaparib	Selleckchem (S1060)
Doxorubicin	Nycomed pharma (417154)

3.2 Transfection

Table 3.2.1: Plasmids and transfection reagents

Name	Supplier (Cat #)
OPTI-MEM®	Life Technologies (31985062)
Lipofectamine LTX Reagent and PLUS reagent	Invitrogen (15338100)
pGL4.10[*luc2*] Vector	Promega (E665A)
pGL4.13[*luc2*/SV40]	Promega (E6681)
pCMVcytoEGFP	In house (generated by Marc Niere & Stian Knappskog)

3.3 Cloning

Table 3.3.1: Reagents and kits used for bacterial transformation and cloning

Name	Supplier (Cat #)
TOPO TA Cloning kit including: - One Shot Top 10 cells (*E. coli**) - pCR2.1-TOPO vector	Invitrogen, Life technologies (K4500-40)
S.O.C Medium	Invitrogen (15544-034)
Ampicillin	Bristol-Myer Squibb (056432)
X-Gal	Sigma Aldrich (B4252)
1x CutSmart NEB Buffer	New England BioLabs (B72045)
KpnI-HF	New England BioLabs (R3142)
HindIII-HF	New England BioLabs (R3104)
T4 DNA Ligase	TaKaRa (2011A)
10x T4 DNA ligase buffer	TaKaRa (2011A)
Shrimp Alkaline Phorphatase (rSAP)	New England BioLabs (M0371S)

*E.coli species: F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G

3.4 DNA, RNA and protein extraction and purification

Table 3.4.1 Reagents and kits used for DNA, RNA and protein extraction

Name	Sample	Supplier (Cat #)
QIAmp DNA Mini Kit	DNA	Qiagen (51306)
TRIzol Reagent	RNA	Life Technologies (15596-018)
Chloroform	RNA	VWR (97064-678)
Isopropanol	RNA	Sigma Aldrich (I9516)
Protein lysis buffer - 50 mM Tris-HCl (pH 7.5) - 150 mM NaCl - 0.1% SDS - 1% Deoxycholate - 1% Triton X-100	Protein	Sigma Aldrich
To 10 ml of Protein lysis buffer added: - ULSTRA, Mini, Protease Inhibitor Cocktail Tablet - PhosSTOP Phosphate Inhibitor Cocktail tablet		Roche (05892970001) Roche (04906837001)
QIAprep Spin, mini-prep kit	Plasmid	Qiagen (27016)
Illustra GFX PCR DNA and Gel Band Purification kit	DNA	GE Healthcare (28-9034-70)
HiSpeed Plasmid Purification MaxiPrep	DNA	Qiagen (21663)

3.5 DNA bisulfite conversion

Table 3.5.1 Kit and controls used in bisulfite conversion of DNA

Name	Supplier (Cat #)
EZ DNA Methylation-Gold Kit	Zymo Research (D5005)
Cp Genome Universal Methylated DNA (pos. control)	Millipore (S7821)
CRL-5803 DNA (negative control)	Gift from Mohn lab

3.6 PCR systems

Table 3.6.1: Polymerases and buffer components

Name	Supplier (Cat #)
AmpliTaq Gold polymerase Kit	Applied Biosystems (4311820)
2 mM dNTP	TaKaRa (4026, 4027, 4028, 4029)
VWR Taq DNA polymerase Kit	VWR (5101600-0100)
10 mM dNTP	TaKaRa (4026, 4027, 4028, 4029)
DNA template (purified from pooled blood samples from five health individuals)	Gift from by Elisabet Ognedal Berge, Mohn lab
DyNAzymes EXT	BioRad (172-5300)
LightCycler 480 Probes Master	Roche (04887301001)

3.7 Primers

Table 3.7.1 Primers* used for PCR amplification and Sanger sequencing

Name	Direction	Sequence (5'→3')	Annealing temp (°C)
Primers used for PCR amplification of *BRCA1* promoter from bisulfite converted DNA (cell lines) for TA cloning			
CpG Island F1	F	GAATTTTTTTAAATTTTTTTAGTG TG	51.5
CpG Island F2	F	ATTTTTAGTAATTTAGGTTG	48.5
CpG Island R1	R	TCCAATAAATAAATTAAAAACC	48.5/51.5
Region A (RICE ytre)	F	GGGGTTGGATGGGAATTGTGA	55
Region A (RICE ytre)	R	CTCTACTACCTTTACCCAAAAACA	55
Region A (RICE indre)	F	GTTTATAATTGTTGATAAGTATAAG	57
Region A (RICE indre)	R	AAAACCCCACAACCTATCCC	57
Region B (Fr. E)	F	TTGGGTGGTTAATTTAGAG	55
Region B (Fr.E)	R	CTCAATACCCCCTTCCTAATCCTC	55
Primers used for PCR screening and sequencing of TA cloned bacterial colonies			
M13 Forward	F	GTAAAACGACCCCCAG	50
M13 Reverse	R	CAGGAAACACCTATGAC	50
Primers used for PCR amplification of *BRCA1* promoter from blood DNA for classical cloning into pGL4.10[*luc2*]			
BRCA1_KpnI	F	TGGCGGTACCGTACGTATCTTTTTAAG	56
BRCA1_HindIII-R long	R	GGGCGCAAGCTTTTCTTTCTGTTCCAATG	56
Primers used for sequencing of *BRCA1* promoter cloned into pGL4.10[*luc2*]			
RV primer 3	F	TAGCAAAATAGGCTGTCCC	50
pGL4.10 MCS R	R	TGG CTTACCAACAGTACC	50
BRCA1 F2	F	GGCAAACTCAGGTAGAATTC	50
BRCA1 promoter sekv S1	F	CGTGAGCTCGCTGAGACTTCC	50
BRCA1 lin.v.sekv S3	F	GGGTTGGCAGCAATATGTGA	50
Primers for site-directed mutagenesis			
SNP intro V1.1	F	TATTCTTTGAGGGGGGGTAGG	60
SNP intro V2.1	F	TATTCTTTGACGGGGGGTAGGGG	60
SNP intro common	R	CCCATCTGTCAGCTTCGG	60
Primers used for introduction of site specific methylation✦			
CpG 33 Met	F	5'Phos CCTCCATTAGGGC^{m}GGAAAGAGTGGGGG	53
CpG 33	F	5'Phos CCTCCATTAGGGCGGAAAGAGTGGGGG	53
CpG 33	R	5'Phos TCTCCAGTTTCGGTAAATATAAGTAATAAGG	53
CpG 43-44 Met	F	5'Phos CCAGAGCCCC^{m}GAGAGAC^{m}GCTTGGCTC	63
CpG 43-44	F	5'Phos CCAGAGCCCCGAGAGACGCTTGGCTC	63
CpG 43-44	R	5'Phos ATTGGCCACCCAGTCTGCCCCCGG	63
CpG 48-52 Met	F	5'Phos TTCC^{m}GTGGCAAC^{m}GGAAAAGC^{m}GC^{m}GGGAATTAC	60
CpG 48-52	F	5'Phos TTCCGTGGCAACGGAAAAGCGCGGGAATTAC	60
CpG 48-52	R	5'Phos ACCAAGGGGCTACCGCTAAGCAGCAGCC	60

* Supplier: Sigma Aldrich

✦ A methylated cytosine is marked as **C^{m}**

Table 3.7.2 Primers* and probes* used in quantitative PCR (qPCR)

qPCR assay	Sample type and direction	Sequence (5'→3')	Annealing temp (°C)
RPLP2	Primer F	GACCGGCTCAACAAGGTTAT	55
	Primer R	CCCCACCAGCAGGTACAC	55
	Probe	Cy5-AGCTGAATGGAAAAAACATTGAAGACGTC-BBQ	55
BRCA1 WT total	Primer F	TGAAGCAGCATCTGGGTG	55
	Primer R	GCTTCTAGTTCAGCCATTTCCTG	55
	Probe	6FAM-AGACTGCTCAGGGCTATCCTCTCAG-BBQ	55
BRCA1 WT α	Primer F	GCGTGAGCTCGCTGAGACTTC	61
	Primer R	TGTGGAGACAGGTTCCTTGA	61
	Probe	6FAM-AGAGGGTGAAGGCCTCCTGAGCG--BBQ	61
BRCA1 WT β	Primer F	GACAGAGCGAGACTGTCTCAAAA	55
	Primer R	ACAGGTTCCTTGATCAACTCCAG	55
	Probe	6FAM-AGCCGGTGTTTTTTGTTTTGTTTTGTTTTGTTTTGT TTTGAG-BBQ	55
BRCA1 Pseudo α	Primer F	ACGTGACTGCGCGTCGTG	61
	Primer R	CGCAAACAGCAGATAAATCTATCTCTTTCTG	61
	Probe	6FAM-CCAGAACGTCTCAGCGAGCTCACGACG-BBQ	61
BRCA1 Pseudo β	Primer F	GACACTCCGTCTCAAAAAC	55
	Primer R	GCAAACAGCAGATAAATCTATCTC	55
	Probe	6FAM-AGCCGGTGTTTATTTCTTTGTTTGTTT-BBQ	55

*Supplier: TIB MOLBIOL

3.8 Sequencing

Table 3.8.1 Kit used in Sanger Sequencing

Name	Supplier (cat #)
BigDye Terminator v1.1 cycle sequencing kit	Thermo F. Scientific (4336774)

3.9 Gel electrophoresis

Table 3.9.1: Agarose gel electrophoresis reagents

Name	Supplier (cat #)
Agarose	Fisher Scientific (10366603)
GelRed nucleic acid stain	Biotium (41003-1)
GeneRuler DNA ladder	Fermentas (SM0331)
φX174 Hae III digest	TaKaRa (3405A)
TAE-buffer	(see table 3.13.1)

Table 3.9.2: (SDS)-Polyacrylamide gel electrophoresis (SDS-PAGE) reagents

Name	Supplier (cat #)
10% Mini-PROTEAN TGX Precast Gel	Bio-Rad (4561036S)
Trans-blot Turbo Mini Nitrocellulose transfer pack	Bio-Rad (170-4158)
Precision plus Protein WesternC Blotting Standard	Bio-Rad (161-0376)
PS11 protein marker	GeneONe (310005)
Mercaptoetanol	Sigma Aldrich (M6250)
PAGE Buffer	(see table 3.13.1)

3.10 Immunoblot and immunofluorescence

Table 3.10.1: Antibodies

Primary Antibody	Dilution*	Host	Supplier (cat #)	Application
Anti-BRCA1	1:1000	Rabbit	Millipore (07-434)	Western blot
Anti-Actin	1:100	Rabbit	Sigma Aldrich (A2066)	Western blot
Phospho-Histone H2a.X (Ser140)	1:1500	Mouse	Thermo Fisher Scientific (MA1-2022)	Immunofluorescence
Secondary Antibody	**Dilution**	**Host**	**Supplier (cat #)**	**Application**
Anti-Rabbit ECL HRP	1:1000	Donkey	GE Healthcare (NA934V)	Western immunoblot
Alexa Fluor 488 goat anti-mouse IgG	1:200	Goat	Life Technologies (a-11001)	Immunofluorescence assay

*Diluted in TBS-tween for western blotting and PBS for immunofluorescence (table 3.13.1)

Table 3.10.2: Reagents and buffers used in immunoblot assay

Name	Supplier (cat #)
Trans-Blot Turbo Midi Nitrocellulose Transfer Pack	Bio-Rad
Tween20	Sigma Aldrich (P1379)
BSA	Sigma Aldrich (A2153)
SuperSignal West Pico Chemilumeniscence Substrate	Thermo Scientific (34080)
SuperSignal West Femto Chemilumeniscence Substrate	Thermo Scientific (34095)

Table 3.10.3: Reagents used in immunofluorescence assay

Name	Supplier (cat #)
Poly-L-Lycine	Sigma Aldrich (P4832)
37% Formaldehyde	Sigma Aldrich (252549)
Triton-X100	Sigma Aldrich (T8787)
VectaShield HardSet Antifade Mounting medium with DAPI	Vector Laboratories (H-1400)

3.11 Site-specific methylation and Luciferase assay

Table 3.11.1: Reagents used in site-specific methylation

Name	Supplier (cat #)
Herculase II fusion polymerase Kit	Agilent (600675)
Taq DNA ligase	New England BioLabs (M0208S)
Taq DNA ligase reaction buffer	New England BioLabs (M0208S)
NAD^+	New England BioLabs (B90007S)
dNTP	TaKaRa (4026, 4027, 4028, 4029)
T4 DNA ligase	TaKaRa (2011A)
T4 Poly Nucleotid Kinase	New England Biolabs (M0201S)
T4 polynucleotide buffer	New England Biolabs (B0201S)
DpnI	New England Biolabs (R0176S)
10x NEB Buffer 2	New England Biolabs (B7002S)
T7 exonuclease	New England Biolabs (M0263S)
10x NEB Buffer 4	New England Biolabs (B7004S)
Dnmt1	New England Biolabs (M0230S)
Shrimp Alkaline Phosphatase (rSAP)	New England Biolabs (M0371S)

Table 3.11.2: Kit used for luciferase assay

Name	Supplier (cat #)
Dual-Glo® Luciferase Assay System	Promega (E2920)

3.12 Long term cell culture experiment

Table 3.12.1: Kits for cell proliferation assay, mycoplasma testing and STR profiling

Name	Supplier (cat #)
GlobalFiler ID-X PCR Amplification Kit	Applied Biosystems (4476137)
GeneScan 600 LIZ Size Standard v2.0	Applied Biosystems (4408399)
Venor GeM Mycoplasma Detection Kit	Sigma Aldrich (MP0025)
WST-1 Cell proliferation Assay Kit	Roche Applied Science (05015944001)

Table 3.12.2: Kits and reagents used in next generation sequencing

Name	Supplier (cat #)
SeqCap EPI Accessory kit v.2	NimbleGen (07145519001)
KAPA Library Preparation Kit	KAPA Biosystems (07137974001)
SeqCap Adapter Kit A	NimbleGen (07141530001)
EZ DNA Methylation Lightning Kit	Zymo Research (D5030)
SeqCap HE-Oligo Kit A	NimbleGen (06777287001)
SeqCap EZ Hybridization and Wash Kit	NimbleGen (05634261001)
SeqCap EZ Pure Capture Beads	NimbleGen (06977952001)
SeqCap Epi Choice Enrichment Kit	Nimblegen (07138989001)
MiSeq Reagent kit v2, 300 cycles	Illumina (MS-102-2002)
Qubit dsDNA Broad Range Assay Kit	Thermo Fisher Scientific (Q32850)
Qubit dsDNA High Sensitivity Assay Kit	Thermo Fisher Scientific (Q32854)
Bioanalyazer DNA 1000 Kit (DNA Chip)	Agilent Technologies (5067-1504)
Bioanalyzer High Sensitivity Kit (+HS DNA Chip)	Agilent Technologies (5067-4626)
Phi X	Illumina (15017666)

3.13 Buffers and Chemicals

Table 3.13.1: General solutions and buffers

Name	Components	Supplier	Application
10x TAE Buffer	48.4 g Tris base, 20ml 0.5 M EDTA (pH 8.0), 11.44 ml Glacial acetic acid. ddH_2O to 1L	Sigma Aldrich (EDTA – VWR)	Agarose Gel electrophoresis
10x Loading dye	5 mL ddH_2O, 5 mL glycerol, Bromo phenol blue	Sigma Aldrich	Agarose Gel electrophoresis
10x PAGE Buffer	150g Tris, 710 g Glycine, 500 mL 10% SDS, ddH_2O to 5 L	Sigma Aldrich	SDS-PAGE
3x SDS – Gel Loading Dye	7.5 mL 1M Tris-HCl (pH 6.8), 15 ml 20% SDS, 317 mL 87% glycerol, 0.15 g Bromo phenol blue, 5 mL MeOH, ddH_2O to 50 mL		SDS-PAGE
10x PBS	80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4, ddH_2O to 1 L.	Sigma Aldrich (KCl –VWR)	Cell culturing
10x TBS	24.2 g Tris base, 80 g NaCl, adjust pH to 7.5 with HCl, ddH_2O to 1L		immunoblotting
LB-medium	10 g peptone, 5 g bacto yeast extract, 10 g NaCl, 950 mL ddH_2O Shake until solution is clear, adjust pH to 7.0 with NaOH. ddH2O to 1 L	Sigma Aldrich (NaOH – VWR)	*E.coli* culturing
LB-Agar plates	10 g Tryptone, 5 g yeast extract, 4 mL 1N NaOH, 15 g Agar, 5 g NaCl. Autoclaved, add 100 µg/mL ampicillin. ddH_2O to 1L.	(Trypton, NaOH – VWR)	*E. coli* culturing
Low-TE Buffer	Commercial	Invitrogen	STR-profiling, NGS libr. prep
X-gal (20 mg/mL)	100 mg X-gal, 5 mL Dimethylformamide	Sigma Aldrich	Blue-white screening
Rectified ethanol		Antibac (600051)	Multipurpose
Absolutte ethanol		Antibac (600068)	Multipurpose

3.14 Instrument and Software

Table 3.14.1: Instruments

Name	Supplier	Application
Microsentrifuge (16.000 g capability)	Multiple Vendors	Multipurpose
NanoDrop ND-100 Spectrophotometer	Qiagen	Conc. measurements
Qubit®3.0 Flurometer	Thermo F. Scientific	Conc. measurements
Scepter™ 2.0 Handheld Automated Cell Counter	Millipore	Cell culture work
Mastercycler gradient (nexus)	Eppendorf	PCR
Light Cycler® 480 II	Roche	qPCR
FLUOstar Omega plate reader	BMG LABTECH	Bioluminescence
Leica DM RTX microscope	Leica	Immunofluorescence
Leica Fw4000 microscope	Leica	Immunofluorescence
Molecular Imager Gel Doc EZ Imager	Bio-Rad	Agarose gel electrophoresis
3500XL Genetic ANAlyser	Thermo F. Scientific	STR Profiling
Trans-Blot® Turbo™ Transfer System	Bio-Rad	Western blot
Applied Biosystems 3730 capillary sequencer	Applied Biosystems	Sanger sequencing
Bioanalyzer 2100	Agilent Technologies	NGS
MiSeq deep sequencing system	Illumina	NGS
DNA Vacuum Concentrator	Multiple Vendors	NGS
Covaris Ultra Sonicator	Covaris	NGS
Water Bath	Multiple Vendors	NGS

Table 3.14.1: Software

Name	Supplier	Application
Light Cycler 480 software	Roche	RT-qPCR
Sequence Scanner	Applied Biosystems	Sequence alignment
4Peaks	Nucleobytes	Sequence alignment
ClustalX v1.83	Conway Institute UCD Dublin	Sequence alignment
GneSnap	SynGene	Agarose gel electrophoresis
Omega	BMG LABTECH	Bioluminescence
SPSS	IBM	Statistics

4 Methods

4.1 Cell culturing

4.1.1 Propagation

The breast cancer cell lines SKBR3, ZR-75-1, UACC-3199, BT549, T-47D, HCC38, MCF-7, MDA-MB 231, and MDA-MB 468 were cultured in recommended medium supplemented with 5-10 % FBS and 5% Pen/Strep, as listed in table 3.1.2. The cells were incubated in humidified air at 37˚C with 5% CO_2 or without CO_2. At 90% confluency, cells were split using trypsin-EDTA which causes the cells to detach from growth surface. Trypsin activity was neutralized by addition of fresh growth medium before re-seeding of cells.

4.1.2 Harvesting of cells for DNA, RNA and protein analysis

A homogenous cell suspension was transferred to 1.5 mL tubes and centrifuged (600 G, at 4°C for 5 min) before medium was removed. All samples were immediately cooled on ice. Cell pellets for DNA isolation were frozen at -20˚C. Cells collected for RNA analysis were lysed in 0.5 mL TRIzol and frozen at -80˚C. Samples for protein analysis (0.5 million cells) were dissolved in 0.2 mL protein lysis buffer (table 3.4.1) and frozen at -20˚C.

4.1.3 Long term drug treatment of cell cultures

SKBR3 cells were cultured (section 4.1.1) under the exposure of various doses of olaparib and doxorubicin for a period of 11-13 weeks (figure 4.1.). Each drug was dissolved in Dimethyl sulfoxide (DMSO), consequently a control sample treated with DMSO only was included in the long-term drug treatment. The final concentration of DMSO for all drug settings and DMSO treatment alone was 0.025%. Medium including drug was changed twice a week, and the cells were split when the confluence reached ~90%. Medium and cells were harvested for subsequent analyses.

When the long term experiment was initiated, concentrations much lower than half maximal inhibitory concentration (IC_{50}) (described in section 4.2) were chosen due to the long time-period the cells would be exposed to the drugs (figure 1.4). Olaparib treatment was started at 1 μM and gradually increased to 8 μM, while doxorubicin treatment was started at 0.002 μM and increased to a maximum of 0.012 μM. The first

increase in drug concentration was done after four weeks, at which time a backup line was separated from the experiment line, in case the cells could not handle increase. The drug concentrations of doxorubicin and olaparib in the experiment lines were increased once a week until cell proliferation drastically decreased (max concentration the cells could handle), the drug concentrations were then decreased to allow more rapid proliferation again. Cells were harvested for DNA analysis for both drugs and both lines, as seen in figure 4.1 (filled squares). Olaparib treated cell were harvested at 11 time points for the experiment line and 12 time points for the backup line. Cells were harvested at 7 time points for the experiment line and 12 time points for the backup line for the Doxorubicin treated cells. Time point zero was harvested before addition of drug, and is the same for all treatment settings.

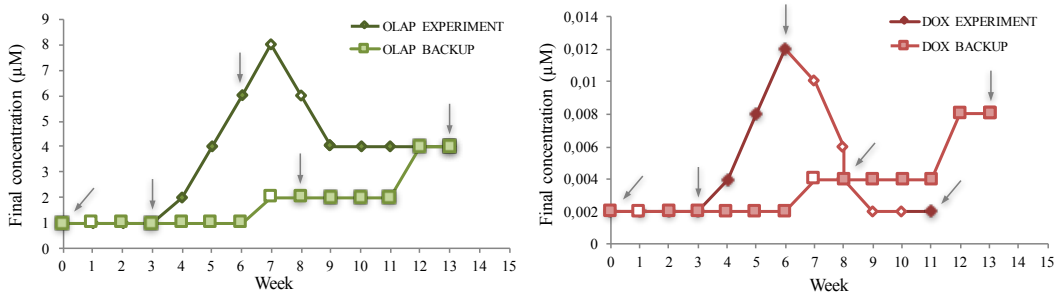

Figure 4.1: Overview of long term treatment of SKBR3 cells. SKBR3 cells were treated over a time-period of 11-13 weeks with the PARP-inhibitor olaparib (1-8 µM) and the chemotherapy doxorubicin (0.002-0.012 µM). The treated cells were split in two settings for each of the drugs; one experiment line and one backup line, indicated with dark and bright colors, respectively. Concentration (µM) was plotted against weeks of treatment. Time points of harvesting are marked with filled squares, and the samples analyzed by next-generation sequencing (explained in section 4.23) are marked with arrows. Harvesting at week zero was performed before addition of drugs.

4.1.4 Mycoplasma test

Mycoplasma test was performed on cell culture medium using the Venor GeM Mycoplasma Detection Kit by following the manufacturer's instructions. The kit tests for all the known Mycoplasma, Acholeplasma and Ureaplasma species. Positive control DNA and Internal control DNA was included in all assays. The internal control containing a 191 bp DNA fragment, was co-amplified along with the samples.

4.1.5 STR Profiling

The GlobalFiler ID-X PCR Amplification Kit was used to confirm the identity and absence of cross contamination of cell lines of interest. The protocol was performed according to the manufacturer's instructions. DNA samples (1 ng) were prepared in

Low-TE buffer. In each run, negative control (Low-TE buffer), positive control (DNA control 007) and an allelic ladder were run in parallel. GeneScan 600 LIZ Size Standard v2.0 was added to all samples analyzed (internal size standard). The samples were run on a 3500 XL Genetic ANAlyser at Gade Laboratory for Pathology, Haukeland University Hospital, and analysed using the GeneMapper ID-X software.

4.2 WST-1 assay

The cell proliferation reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) was used to evaluate the cytotoxic effect, measured as $IC_{50,}$ of the drugs doxorubicin and olaparib on the SKBR3 cell line. SKBR3 cells were seeded on a 96-well plate (6000 cells/well) and incubated with medium containing 0.02 µM - 0.6 µM doxorubicin and 100 µM - 1000 µM olaparib for 96 h. DMSO treated cells were included in the assay to calculate background noise. Each treatment was performed with four parallels, and the assays were repeated three times for each drug. The cells were incubated at 37°C with WST-1 reagent (100 µL) for 1.5 h and the samples were measured by use of FLUOstar Omega plate reader (BIO LABTECH). DMSO treated cells were set as blank.

4.3 Immunofluorescence assay

SKBR3 cells (120 000) were seeded on poly-L-lysine pre-treated coverslips. SKBR3 cells were treated with the drugs olaparib (1-2 µM), doxorubicin (0.002-0.004 µM) or DMSO (control) for 48 h. Additional controls to check the specificity of the antibody were included in the assay; cells treated with the highest dose (2 µM for olaparib and 0.004 µM for doxorubicin) were incubated with primary antibody only and secondary antibody only to look for any detectable signal. The cells were washed with 1x PBS (500 µL) twice, before fixation with 4% formaldehyde in 1% PBS (1mL) for 15 min at RT. The cells were again washed 3x 5 min with PBS (500 µL) at RT, followed by permeabilisation using 0.1% Triton-X-100 in ddH_2O (1 mL) for 10 min at RT. After washing with 1x PBS (500µL), blocking was performed by incubating the cells with 1% BSA in 1x PBS (30 µL) for 30 min. The cells were then incubated with the primary antibody Phospho-Histone H2A.X (Ser140) anti-mouse, 1:1500 dilution in 1% BSA in 1x PBS for 1 hour at RT. The cells were washed 3x 10 min in 1x PBS (30µL) at RT before incubation with 1:200 diluted secondary antibody Alexa Fluor 488 goat anti-

mouse IgG (2 mg/mL), tagged with fluorescein isothiocyanate (FITC) in 1% BSA in 1x PBS for 1 hour in the dark at RT. Cells were washed in same manner as after primary antibody incubation before mounting using Vectashield HardSet antifade mounting medium with DAPI on a microscope slide. Samples were stored in the dark at 4°C overnight before pictures were taken using a Leica DM RTX microscope and the Leica Fw4000 software. To count the total number of cells DAPI stain was used, the positive cells were characterized by green fluorescent signal (FITC) and granulation. A minimum of 100 cells were counted for each sample and the experiment was repeated three times. Average and standard deviation of three parallel experiments were estimated (statistical analyses are described in section 4.22).

4.4 Transfection

Lipofectamine LTX Reagent with PLUS reagent was used for transfection of cultured cells. For each sample, two tubes containing optimum medium (400 µL, room temperature) were prepared. To one of the tubes, Plus reagent (5 µL) and a total of 1000 ng plasmid DNA (700 ng *BRCA1* promoter-pGL4.10[*luc2*] + 300 ng pCMV-cytoEGFP) was added. In the second tube, Lipofectamine LTX (10 µL) was added. After 10 min incubation, the contents of the two tubes were mixed followed by incubation for 20 minutes. The transfection mixture (100 µL) was added to the cells (10 000 cells in 100 µL medium mix) in 96-well plate (Greiner BioOne, 96-wells, white (transparent bottom)). Transfection reagents were removed and replaced with fresh medium after 24 h. As positive and negative controls for luciferase expression, the vector pGL4.13[*luc2*/SV40] containing the strong SV40 promoter and reporter gene luciferase and the empty vector pGL4.10 were used, respectively. Each sample was co transfected with the eGFP expressing pCMV-cytoEGFP vector in order to enable correction for transfection efficacy. The experiment was repeated seven times.

4.5 DNA extraction from cell pellets

DNA was extracted from harvested cell pellets by using the QIAGEN QIAamp DNA Mini Kit for DNA purification according to the manufacturer's instructions, samples were eluted in elution buffer.

4.6 Determination of RNA and DNA concentration

Determination of both DNA and RNA concentrations were performed for the samples of interest by use of NanoDrop spectrophotometer. Average of three measurements was used for concentration determination for each sample.

Qubit dsDNA BR Assay Kit were used to determine concentration of samples for Methylation specific massive parallel sequencing (section 4.23). Measurements were performed according to manufacturer's protocol.

4.7 Bisulfite conversion of DNA

Bisulfite conversion of purified DNA for investigation of methylation was performed by EZ DNA Methylation-Gold Kit using 200 - 500 ng input DNA. Cp Genome Universal Methylated DNA (methylated positive control), CRL-5803 lung carcinoma cell line DNA (negative control) and ddH_2O (negative control) were included in each set up.

4.8 PCR amplification

AmpliTaq Gold polymerase (Table 3.6.1) was used for PCR amplification of the *BRCA1* promoter region from bisulfite converted DNA obtained from breast cancer cell lines (subsequent to TA cloning) for determination of the methylation pattern. As the bisulfite treatment causes DNA to fragment, the region of interest was amplified as three shorter fragments in separate PCR reactions, termed CpG Island, Region A and Region B. The fragments were PCR amplified using 200-500 ng (2-3 µL) bisulfite converted DNA (section 4.7) as template in 1st round of PCR. Fragment CpG Island and Promoter A were amplified by nested PCR, while only one round of amplification was performed for Region B. Primers are described in table 3.7.1 and an overview of reaction components and PCR programs is shown in tables 4.8.1-4.8.4.

Table 4.8.1 Reagents used for PCR amplification with AmpliTaq Gold polymerase

Reagents	**Final conc.**
AmpliTaq Gold polymerase (5 U/µL)	0.5 µL
10x Gold PCR Buffer u/$MgCl_2$	5.0 µL
$MgCl_2$ (25) mM	3.0 µL
dNTP (2 mM)	2.5 µL
Forward primer 10 mM	1.0 µL
Reverse primer 10 mM	1.0 µL
Bisulfite converted DNA template or 1. Round PCR product	1-3 µL
ddH_2O	total volume of 50 µL

Table 4.8.2: PCR programs used for amplification of fragment CpG Island

PCR program: CpG Island 1.r		
94 °C	5 min	
94 °C	30 sec	
51 °C	30 sec	35 cycles
72 °C	50 sec	
72 °C	5 min	
10 °C	Hold	

PCR program: CpG Island 2.r		
94 °C	5 min	
94 °C	30 sec	
48.5 °C	30 sec	40 cycles
72 °C	50 sec	
72 °C	5 min	
10 °C	Hold	

Table 4.8.3: PCR programs used for amplification of fragment Region A

PCR program: Region A 1.r		
95 °C	5 min	
95 °C	1 min	
55 °C	3 min	35 cycles
72 °C	1 min	
72 °C	5 min	
10 °C	Hold	

PCR program: Region A 2.r		
95 °C	5 min	
95 °C	1 min	
57 °C	3 min	35 cycles
72 °C	1 min	
72 °C	5 min	
10 °C	Hold	

Table 4.8.4: PCR programs used for amplification of fragment Region B

PCR program: Region B		
95 °C	5 min	
95 °C	1 min	
55 °C	3 min	40 cycles
72 °C	1 min	
72 °C	5 min	
10 °C	Hold	

The VWR Taq DNA polymerase (table 3.6.1) was used for PCR amplification in two settings; 1) For screening of bacteria colonies resulting from TA cloning of bisulfite convert DNA corresponding to *BRCA1* promoter in breast cancer cell lines, and 2) PCR amplification of the *BRCA1* promoter from non-converted DNA purified from blood for subsequent cloning into the vector pGL4.10[*luc2*] (table 3.2.1) using restriction

enzymes (RE). Primers are listed in table 3.7.1 and reaction components and PCR programs are described in table 4.8.5 and 4.8.6-4.8.7.

Table 4.8.5 Reagents used for PCR amplification with VWR Taq DNA polymerase

Reagent	Volume
VWR Taq DNA polymerase (5U/µL)	0.25 µL
10 x Key Buffer	2.5 µL
dNTP (0.8 µM)	0.5 µL
M13 F (10 µM)	0.5 µL
M13 R (10 µM)	0.5 µL
DNA template	X µL
ddH2O	to a total of 25 µL

Table 4.8.6: PCR programs for screening of bacteria colonies following TA cloning of bisulfite converted DNA

PCR program: Screening PCR for bacterial colonies		
95 °C	10 min	
95 °C	30 sec	
52 °C	30 sec	35 cycles
72 °C	1 min	
72 °C	5 min	
10 °C	Hold	

Table 4.8.7: PCR program for amplification of *BRCA1* promoter from blood DNA for subsequent cloning into pGL4.10[*luc2*] using restriction enzymes

PCR program: RE Cloning		
95 °C	10 min	
95 °C	30 sec	
56 °C	30 sec	40 cycles
72 °C	2 min	
72 °C	5 min	
10 °C	Hold	

4.9 Agarose gel electrophoresis

Agarose gel electrophoresis was performed for analysis of DNA products. GelRed (0.01%) was added to 1-2% agarose dissolved in 1x TAE-buffer. The polymerized gel was transferred to a running chamber with TAE-buffer. Samples were loaded with 6x DNA loading buffer (final concentration 1x) and the gels were run at 100 V for approximately 1 h. The Molecular markers HaeIII digest and Generuler DNA ladder mix with pre-determined fragment sizes were run in parallel and used to estimate the size of the DNA in the samples. Molecular Imager Gel Doc EZ Imager from Bio-Rad

was used along with the software GeneSnap from SynGene to visualize and document the DNA fragments by UV light.

4.10 Gel purification

Samples were loaded and run on an 1% agarose gel (section 4.9). Bands of interest were cut from the gel using a scalpel and purified using Illustra GFX PCR DNA and Gel Band purification kit.

4.11 TOPO TA Cloning

PCR products (1-5 µL; 100-500 ng) were TOPO TA cloned into pCR2.1-TOPO vector followed by transformation into One Shot TOP 10 *E. Coli* chemically component cells according to instruction from the manufacturer (TOPO TA cloning kit). Samples older than one week were re-adenylated before cloning by addition of dNTP (10mM) and Dynazyme Ext (1 U/µL) to the PCR product and incubated at 72°C for 30 min. *E. Coli* cells were plated on LB-plates containing X-gal (for blue/white screening) and ampicillin. Screening PCR followed by agarose gel analysis (1%) was used for selection of colonies that contained insert of correct size as described in in section 4.8 and 4.9.

4.12 Restriction Enzyme cloning

The vector pGL4.10[*luc2*] and PCR product containing *BRCA1* promoter amplified from non-converted blood DNA were cut with the restriction enzymes KpnI-HF and HindIII for 1 h at 37°C. Reaction components are listed in table 4.12.1-4.12.2.

Table 4.12.1: Reagents used for RE cutting of vector pGL4.10[*luc2*]

Reagent	Volume
10x CutSmart NEB Buffer	2 µL
KpnI-HF 20 U/µL	1 µL
HindIII-HF 20 U/µL	1 µL
Vector pGL4.10[*Luc2]* (1µg/µL)	1.3 µL
ddH_2O	To a total of 20 µL

Table 4.12.2: Reagents used for RE cutting of PCR amplified BRCA1 promoter

Reagent	Volume
10x Cutsmart NEB Buffer	5 µL
KpnI-HF 20 U/µL	1 µL
HindIII-HF 20 U/µL	1 µL
PCR product	12.5 µL
ddH_2O	To a total of 50 µL

To prevent re-ligation of the vector pGL4.10[*luc2*] after restriction enzyme cutting, the vector was dephosphorylated by rSAP (1.0 µL, 1.0 U) at 37°C for 45 min, followed by heat inactivation at 80°C for 20 min. The cut and dephosphorylated vector, as well as the *BRCA1* promoter PCR product was purified by agarose gel purification (section 4.10). The *BRCA1* promoter PCR product was ligated into the vector pGL4.10 by T4 DNA ligase at 16°C overnight using a vector:insert molar ratio of ~1:10. Reaction components are listed in table 4.12.3.

Table 4.12.3 Reagents used for ligation of *BRCA1* promoter PCR product into pGL4.10[*luc2*]

Reagent	Volume
T4 DNA Ligase (350 U/µL)	1 µL
10x T4 DNA ligase buffer	2 µL
pGL4.10[*luc2*] cut with KpnI-HF/HindIII-HF (60.2 ng/µL)	0.9 µL
BRCA1 promoter PCR cut with KpnI-HF/HindIII-HF (23.7 ng/µL)	9.7 µL
ddH_2O	to a total of 20 µL

The ligation mixture (0.5 µL) was transformed into competent TOP 10 *E. coli* cells as described in section 4.11.

4.13 Bacteria culturing

Bacteria colonies harboring plasmid containing insert of correct size were selected and cultured in LB-medium with ampicillin (0.05%) at 37°C and 250 RPM overnight. Cultures of 1 mL and 150 ml LB-medium were prepared for miniprep and maxiprep, respectively.

4.14 Plasmid purification

Plasmids were purified from bacteria cultures using QIAprep Spin, miniprep kit or HiSpeed Plasmid purification, Maxi Prep kit. Both protocols were performed according to manufacturer's descriptions. The resulting DNA concentrations were determined by NanoDrop.

4.15 Sanger Sequencing

Sanger sequencing was performed using the BigDye Terminator v1.1 cycle sequencing kit and the relevant primers (table 3.7.1). Capillary electrophoresis and data collection were performed on an Applied Biosystems 3730 capillary sequencer at the Center of Medical Genetics and Molecular Medicine at Haukeland University Hospital. All sequences were analyzed in 4Peaks/Sequence Scanner and compared to reference sequence GenBank U37574.1 using Clustal X (v2.1). Reaction components and PCR program are listed in table 4.14.1-4.14.2.

Table 4.14.1 Reagents used for Sanger Sequencing

Reagents	Volume
5x BigDye Terminator v1.1 Sequencing Buffer	5 µL
BigDye Terminator v1.1 Ready reaction Mix	0.5 µL
Primer (10 µM)	1 µL
DNA template	X µL
ddH_2O	To a total of 10 µL

Table 4.14.2 PCR program used for Sanger Sequencing

PCR program: Sanger sequencing		
94 °C	5 min	
94 °C	15 sec	
50 °C	5 sec	30 cycles
60 °C	4 min	
10 °C	Hold	

4.16 RNA purification

RNA was purified from cell pellets using the TRIzol Reagent according to the manufacturer's instructions. Chloroform ($CHCl_3$) was added to the TRIzol lysate, resulting in a separation of the fluid into three distinct layers; an upper aqueous layer containing RNA, an intermediate layer, and a lower organic layer containing DNA. Isopropanol was used to precipitate the RNA from the aqueous layer. The only exception from the recommended protocol was that samples were incubated in isopropanol for 40 min at -20°C, instead of 10 min at RT. The RNA concentration was determined by Nanodrop and the samples were stored at -80˚C.

4.17 cDNA synthesis

Purified RNA (500 ng) was converted to complementary DNA (cDNA) by using the Quanta Biosciences qScript dDNA Supermix Kit. Protocol was performed according

to the instructions of the manufacture. Samples were diluted 1:10 in nuclease free ddH_20 and stored at -20°C.

4.18 qPCR

Quantitative PCR (qPCR) was performed to determine the expression levels of transcripts of interest by use of LightCycler 480 Probes Master and a hydrolysis probe assay containing a fluorescence reporter dye and quencher. Position of primers and probes, as well as the specific nucleotide sequence can be found in appendix 1 (figure A.1) Relative mRNA levels of the samples were calculated by using in-run standard curves (kindly provided by Elisabet Ognedal Berge) to convert crossing point (CP) values for the gene of interest to concentrations. All CP values above 40 were considered below the sensitivity limit for mRNA detection, and concentrations for such samples were set to zero. A minimum of five runs were performed for each assay and the extreme upper and lower values were excluded when calculating average relative mRNA levels. For each assay, an internal calibrator (pooled cDNA from five heathy donors, kindly provided by Elisabet Ognedal Berge) was included in every parallel run to correct for potential run-to-run variations. The housekeeping gene *RPLP2* (encoding ribosomal protein P2) was used as a reference gene to correct for any differences between the samples in each assay caused by variation in amount of input cDNA (loading control). The samples were run on a LightCycler 480 machine and analyzed in the corresponding Light Cycler 480 software (version 1.5.1).

Exact comparisons of data across assays (e.g. concentrations of *BRCA1* WT α versus *BRCA1* β or versus *BRCA1* WT total) were not possible due to different reaction efficacies between the different assays. Thus, for this purpose, a ΔΔCP-approach was applied, assuming a perfect (2-fold per cycle) efficacy of all assays. In this method, the difference in CP-values between the respective target genes (*BRCA1* WT α or WT β) and the reference gene (*BRCA1* WT) total were first calculated (equation 4.1). Then, these ΔCT-values were converted to relative concentrations by applying 2 as the efficiency number, instead of using standard curves (Δ concentration between two transcripts = $2^{\Delta CP}$). Primers and probes are listed in table 3.7.2, reaction components are described in tables 4.18.1 and 4.18.2, and the thermocycler program are described in table 4.18.3.

Equation 4.1

$$\Delta CT = CT\ (target\ gene) - CT\ (reference\ gene)$$

Table 4.18.1: Reagents used for preparations of primer/probe mix

Reagent	Volume
Forward Primer (50 µM)	10 µL
Reverse primer (50 µM)	10 µL
Probe (50 µM)	2.5 µL
ddH_2O	to a total of 100 µL

Table 4.18.2: Reagents used for preparations of reaction master mix

Reagent	Volume
2x LightCycler 480 probes master	10 µL
Primer/probe mix (specific for each assay, table 3.7.2)	2 µL
Template	5 µL
ddH_2O	to a total of 20 µL

Table 4.18.3: qPCR program

qPCR program: *BRCA1* mRNA levels		
95 °C	5 min	
95 °C	10 sec	50 cycles
X °C	25 sec	
40 °C	10 sec	

*Annealing temperature (X) for each specific reaction is listed in table 3.7.2

4.19 Western blot analysis

The relative protein levels were analyzed by Western blot (WB) analysis. Cell pellets lysed in protein lysis buffer (20 µL) was boiled with sample loading buffer (10µL, see table 3.13.1) at 95°C for 10 min. Protein samples were loaded on a 10% polyacrylamide gel along with BioRad Precision plus standard or molecular size ladder PS11. Samples were run at 100 V for 10 min followed by 150 V for 45 min. Commercial blot membrane was prepared, and the proteins were transferred from the gel to the nitrocellulose membrane by use of the Trans-Blot Turbo Transfer System. The membrane was blocked in 5% BSA diluted in 0.05% TBST for 1h at RT. Excess BSA was removed by a quick wash in 0.05% TBST before the membrane was incubated with primary antibody.

For BRCA1 protein analysis, a 1:1000 dilution (in 0.05% TBST) of the primary antibody Anti-BRCA1 (Millipore, Rabbit antiserum) was incubated overnight at 4°C on shaker. The membrane was washed in 0.05% TBST (3x10 min on shaker at RT)

before incubation with 1:1000 dilution (in 0.05% TBST) of the secondary antibody Anti-Rabbit ECC HRP (GE Health Care) 1h at RT. The membrane was again washed with 0.05% TBST (3x 10 min on shaker at RT) and 1x TBS (1x 5 min on shaker at RT) before development using chemiluminescent substrate horseradish peroxidase (SuperSignal West Pico (200 µL) and Femto (600 µL)) on FUJIFILM-LAS 4000 (~60 s exposure).

To ensure equal loading of the samples, the membrane was again washed before incubation (1h, on shaker at RT) with 1:100 (in 0.05% TBST) of the primary antibody anti-actin (Sigma A2060 Rabbit). The membrane was washed with 0.05% TBST (3x 10 min on shaker at RT) before incubation (1h on shaker at RT) with 1:1000 dilution (in 0.05% TBST) of secondary anti-body Anti-Rabbit ECC HRP (GE Health Care). The membrane was then washed in 0.05% TBST (3x 10 min on shaker at RT) as well as with 1x TBS (1x 5 min on shaker at RT) before developed as described above with exposure time ~2 sec.

4.20 Site specific methylation

Introduction of DNA methylation at specific sites of interest in the *BRCA1* promoter-pGL4.10[*luc2*] construct was performed as described by Han *et. al* (Han et al., 2013). The protocol consists of four main steps: 1) combined PCR and ligation reaction, 2) digestion by DpnI and T7 exonuclease treatment, 3) methylation by Dnmt1, and 4) verification by bisulfite sequencing (figure 4.2).

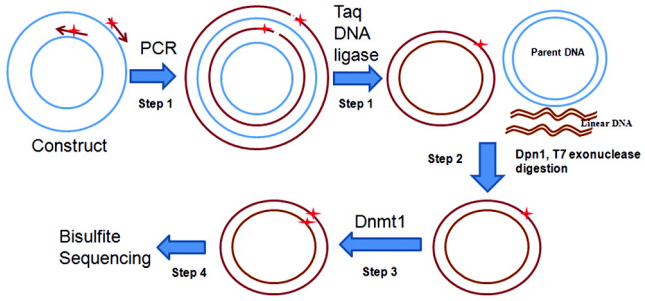

Figure 4.2 Overview of site-specific methylation protocol as described by Han *et. al.* The protocol consists of four main steps. Step 1: In the combined PCR and ligation reaction, primers designed to harbor methylation in positions of interest are used to amplify vector construct by PCR. The DNA polymerase creates a nicked template which is ligated directly by Taq DNA ligase. Step 2: Digestion of parental DNA and un-ligated DNA by DpnI and T7 exonuclease, respectively. Step 3: Methylation of hemi-methylated template by Dnmt1. Step 4: Verification of methylation pattern by bisulfite sequencing. Methylated CpG's are marked with a red cross in the figure. Reproduced with permission from (Han et al., 2013), copyright Taylor & Francis group.

In the first step, primers with 5'-end phosphorylation, designed to harbor methylation at CpG site of interest (see table 3.7.1) were used in combination with a DNA polymerase without stand displacement activity, to generate a complementary DNA strand with a nick which could subsequently be ligated by Taq DNA ligase. Gradient PCR was performed prior to experiment start to determine best annealing temperatures for each primer set. For each PCR using methylated primers, an additional PCR reaction with corresponding unmethylated primers was performed as a negative control Reaction components are described in table 4.20.1 and PCR program in table 4.20.2.

Table 4.20.1: Reagents used for combined PCR and ligation

Reagent	Volume
Herculase II fusion polymerase	0.5 µL
5x Herculase II reaction buffer	10 µL
Taq DNA ligase (40 U/µL)	4 µL
NAD+ (50mM)	1 µL
dNTP (0.8 M)	5 µL
Forward primer, with/without methylation (10 µM)	1.25 µL
Reverse primer, without modification (10 µM)	1.25 µL
BRCA1 promoter -pGL4.10[*luc2*] (10 ng/µL)	1 µL
ddH_20	Total volume of 50 µL

Table 4.20.2: PCR program for combined PCR and ligation

PCR program: Combined PCR and ligation		
98°C	2 min	
98 °C	20 sec	35-40 cycles
*	30 sec	
72°C	3.5 min	
65 °C	5 min	
4 °C	Hold	

*Annealing temperatures according to primer are listed in table 4.7.1

The sample was purified using GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) by following the manufacturer's instructions (section 4.10). Samples were eluted with 2x 25µL sterile nuclease-free water and treated with DpnI at 37°C for 1 hour to digest the parental plasmid. Reaction components are listed in table 4.20.3.

Table 4.20.3: Reagents used for DpnI treatment of PCR-ligation product

Reagent	Volume
DpnI (20 µL)	1 µL
10x NEB Buffer 2	5 µL
PCR-ligation product	45 µL (50 µL total reaction volume)

The DpnI treatment was followed by a second round of clean up in the same manner as previously described (section 4.9). In order to remove un-ligated linear PCR product, T7 exonuclease was added, and the reaction mixture was incubated at 25°C overnight. Reaction components are listed in table 4.20.4.

Table 4.20.4: Reagents used for T7-exonuclease treatment of DpnI-treated PCR-ligation product

Reagent	Volume
T7 exonuclease (10 U/µL)	2 µL
10x NEB Buffer 4	5 µL
DpnI treated PCR-ligation product	43 µL(50 µL total reaction volume)

Samples were purified by GFX PCR DNA and Gel Band purification kit (GE Healthcare) and run on agarose gel (1%) with DNA ladder GeneRuler (Fermentas) for visual inspection.

Finally, Dnmt1, which is specific for hemi-methylated DNA, was added to methylate the complementary strand at the positions corresponding to the methylated sites in the primers. The reaction was performed at 37°C for 4 hours. After two hours, additional S-Adenosyl methionine (SAM) was added. Reaction components are listed in table 4.20.5.

Table 4.20.5: Reagents used for Dnmt1 treatment of T7 and DpnI treated PCR –ligation product

Reagent	Volume/amount
10x Dnmt1 reaction buffer	5 µL
BSA (5mg/mL)	1 µL
SAM (8 mM	1 µL x2 (added at two time points)
Dnmt1 (2 U/µL)	2 µL
T7 and -DpnI treated PCR-ligated product	2 µg
ddH_2O	Total volume of 50 µL

After purification, the samples were bisulfite converted (section 4.7) and sequenced (section 4.15).

4.21 Site-directed mutagenesis

Site-specific methylation was performed using Q5 Site-Directed Mutagenesis Kit Protocol and relevant primers (table 3.7.1). Reaction components are listed in table 4.21.1 and the PCR program is listed in table 4.21.2.

Table 4.21.1 Reagents for site-directed mutagenesis

Reagent	Volume
Q5 Hot Start High-fidelity 2x Master Mix	12.5 µL
Forward primer (10 µM)	1.25 µL
Reverse primer (10 µM)	1.25 µL
Template DNA (1-25 ng)	1 µL
Nuclease-free water	Total volume of 50 µL

Table 4.21.2 PCR program for site-directed mutagenesis

PCR program: site-directed mutagenesis		
98 °C	30 s	
98 °C	10 s	25 cycles
X °C	30 s	
72 °C	3.5 min	
72 °C	2 min	

X: annealing temperature for each specific primer used is listed in table 3.7.1.

4.22 Luciferase assay

The Dual-Glo Luciferase assay system was performed to assess variations in firefly luciferase expression levels due to variation within the *BRCA1* promoter. The vector pCMV-cytoEGFP was included for normalization of transfection efficiency, cell count differences and pipetting variations. Approximately 48 h after transfection, GFP was measured before addition of luciferase reagents. Each sample was analyzed in six parallels in each of the seven assays on a FLUORstar Omega plate reader.

4.23 Methylation specific massive parallel sequencing

4.23.1 Library preparations

Library preparations were performed using the Roche NimbleGen SecCap Epi Enrichment System, following the protocol recommended by Roche (Version 1.2, appendix 2) in combination with custom made probe design (SeqCap Epi Choice). Reagents and instruments are listed in table 3.12.2 and 3.14.1, respectively. In short, purified sample DNA (1 µg), concentration determined by use of Qubit dsDNA BR Assay kit (section 4.6), were mixed with bisulfite conversion control (Lambda DNA) and fragmented followed by end repair and A-tailing to permit subsequent adapter ligation (universal and index adapters). The DNA was then bisulfite converted followed

by pre-hybridization PCR. During hybridization, the samples were mixed with a custom-made probes design, allowing analysis only of regions of interest. The probes, designed by Elisabet Ognedal Berge in collaboration with Roche, are intended to target 358 regions from the promoter region of 283 tumor suppressor genes. The selection of genes was based on cancer gene lists from the Welcome Trust Sanger Institute and Roche (Comprehensive Cancer Design), as well as manual literature search to cover as many tumor suppressor genes as possible, independent of cancer type (PanCan). For each gene, a region spanning from approximately 1500 bp upstream to 500 bp downstream from the transcription start site (TSS) was covered. The probes were designed to bind target DNA in all possible methylation configurations (fully methylated, partially methylated and completely unmethylated) on both DNA strands, enabling correction for potential overlap between CpGs and SNPs. The probes, bound to the target region, were marked with biotin and captured using streptavidin coated beads. After post-hybridization PCR, samples were pooled and mixed with PhiX library (10% spike-in). The multiplex DNA library samples were run on the Illumina MiSeq instrument (Figure 4.3).

4.23.2 Clustering and sequencing

Cluster generation and sequencing were performed in fully automated processes on a MiSeq massive parallel sequencer (Illumina). Sequencing was set to generate paired end reads, with a 100 bp length per read.

4.23.3 Bioinformatic data analysis

Analysis of data generated by the NimbleGen SeqCap Epi Enrichment System was performed by Zuzana Sichmanova, using publically available open source analysis tools as described in (appendix 3) and figure 4.3.

In short, the output from the Illumina MiSeq runs are provided as FASTQ files, a text-based format for storing both a biological sequence and its corresponding quality scores (Phred quality score). Before mapping, reads were trimmed for adapters and sequences of poor quality (QC< 20) as well as reads shorter than 75 bp by Trimmomatic. The reads were then aligned using the bisulfite mapping software BSMAP. After sorting the reads into top and bottom strands, the program Piccard was used to remove duplicates

based on identical 5' starting point, before top and bottom strands again were merged. Bamtools was used to filter for properly paired reads, restricting the analysis only to reads pairs where the two mates are mapped at a correct orientation and at a distance corresponding to the insert size of the library. To avoid bias, overlap between read 1 and read 2 in a pair caused by difference in library insert size, was clipped using BamUtil. Various metrics were calculated including count on target reads and depth of coverage by the program Picard. The methylation percentage of each individual CpG was determined by use of BSMAP, which also determines the bisulfite conversion efficiency. Correction for potential SNPs located in CpGs was performed using the program BisSNP.

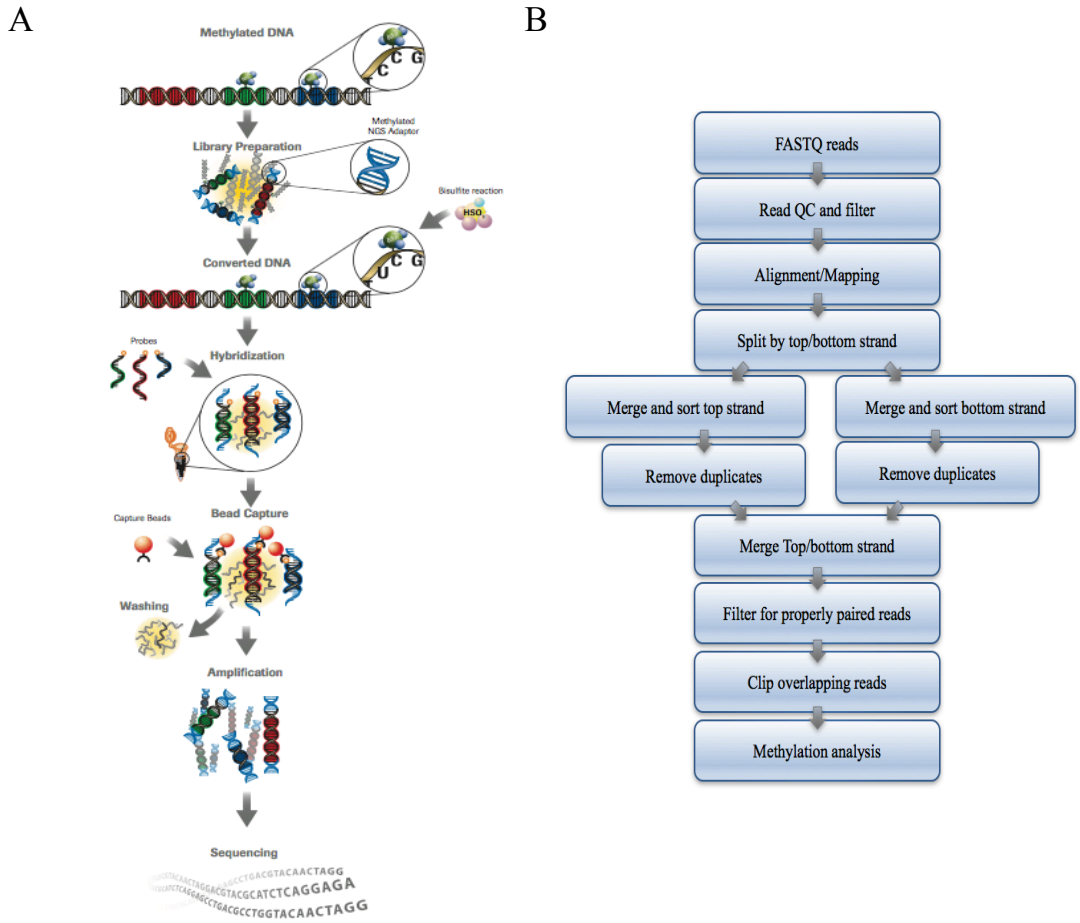

Figure 4.3: Overview of wet-lab and dry-lab workflow for Roche NimbleGen SecCap Epi Enrichment Systems. A) Library preparations was performed on methylated DNA to enable adaper ligation. Bisulfite conversion was performed on the DNA sample before hybridization with custom made probes, designed to bind all possible methylation configurations. Probes bound to target DNA was captured using streptavidin coated beads before amplification by PCR and subsequent sequencing on Illumina MiSeq. **B)** After sequencing, the resulting FastQ files were extracted and run through a bioinformatic pipeline to extract information about the methylation pattern of the promoter in the samples of interest. All tools used are publically available open source tools. The pipeline was designed in collaboration with Roche and analysis was performed by an bioinformatician.

4.24 Statistical analyses

Categorical variables were compared between groups using Pearson's Chi-squared test. Continuous variables were compared between groups using unpaired student's t-test. Correlation between two continuous variables was estimated by Spearman test (Spearman's Rho). All p-values were reported as two-sided and p-values <0.05 were considered significant. All statistics were performed using SPSS (v.23; IBM) and Excel.

5 Results

Experiments were performed to increase the understanding of the biological roles of *BRCA1* promoter methylation, both in terms of global methylation of the promoter region and in terms of methylation of individual CpGs. With this overall aim in mind, three sub aims for the present project were outlined; 1) Assessment of *BRCA1* methylation patterns in a panel of breast cancer cell lines and analysis of correlation between methylation in *BRCA1* promoter region and mRNA and protein expression. 2) Investigate how the ratio between the α and β transcripts is affected by alterations in the α and β promoter region of *BRCA1*, including methylation of specific CpGs as well as polymorphic variants existing in the general population. 3) Analyze the effect of long term drug treatment on methylation levels in the *BRCA1* promoter.

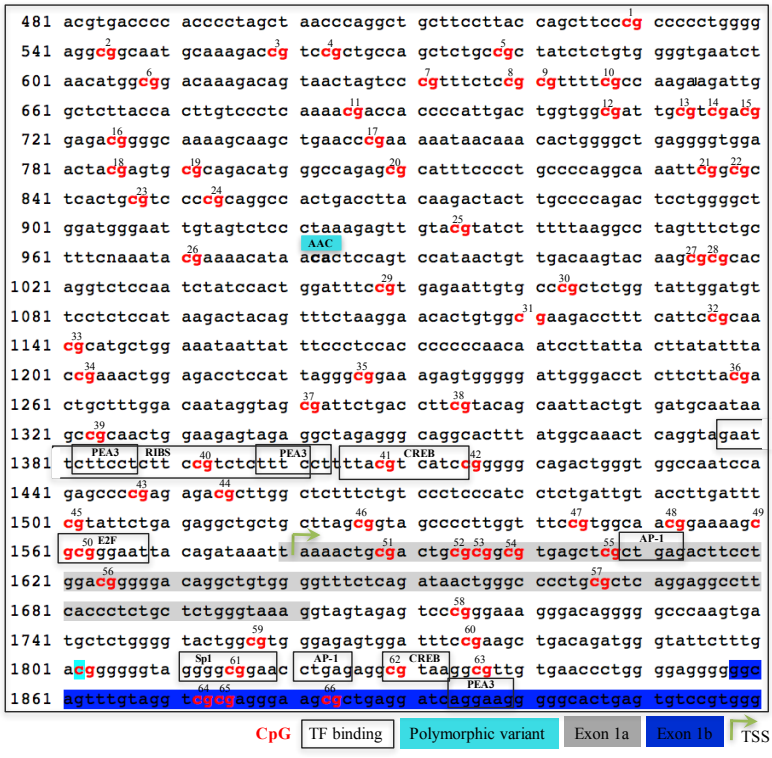

Figure 5.1: CpGs and potential transcription binding sites in *BRCA1* promoter region. CpGs assessed in this study are marked in read and number from 1-66, with one CpG not included in the numeration due to a SNP (marked in turquoise, position 1802) where the C nucleotide can be substituted with G. Potential transcription factor binding sites, found described in the literature, are marked with boxes and corresponding name inside the box, exon 1a is marked with grey color and exon 1b is marked with blue color, transcription start site is marked with a green arrow. If present, polymorphism AAC is inserted between the A and C, nucleotides marked in bold, positions 982 and 983 respectively.

5.1 Assessment of *BRCA1* methylation patterns in a panel of breast cancer cell lines and analysis of correlation between methylation in *BRCA1* promoter region and mRNA and protein expression

In this section, experiments for analysis of the methylation pattern in the *BRCA1* promoter region as well as mRNA and protein levels for a panel of nine breast cancer cell lines are described. All cell lines were choses on the basis that they were breast cancer cell lines and had wild type (WT) *BRCA1* (Hollestelle et al., 2010, Elstrodt et al., 2006), further characteristics of each cell line is given in table 3.1.1. These cell lines were cultured by Elisabet Ognedal Berge and Reham Helwa in the period from December 2015 to April 2016.

5.1.1 Methylation in *BRCA1* promoter in a panel of breast cancer cell lines

For assessment of *BRCA1* promoter methylation at the individual CpG-level, three fragments were amplified from bisulfite converted DNA from a panel of nine breast cancer cell lines. These fragments, covering different but overlapping regions of the *BRCA1* promoter, were termed CpG Island, Region A and Region B. The amplification of all three regions yielded products of the expected molecular sizes: 552, 660, and 490 bp, respectively (figure 5.2).

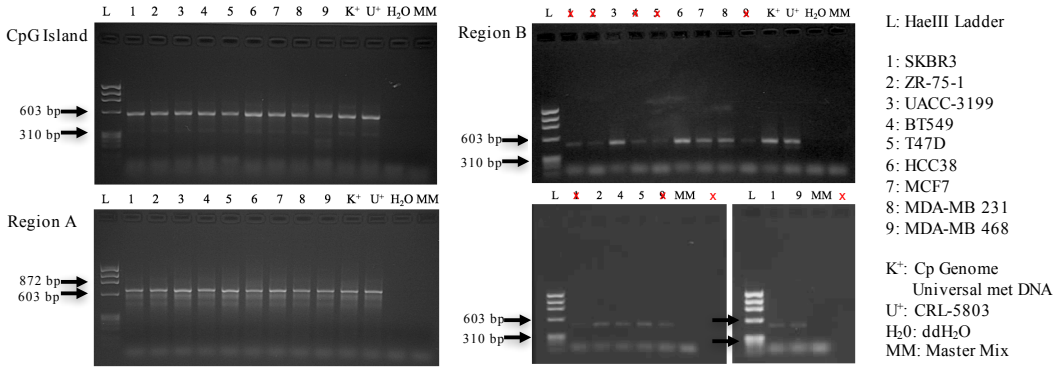

Figure 5.2: *BRCA1* promoter region amplified from bisulfite converted DNA for nine breast cancer cell lines. The region was amplified in three separate PCR reactions named Island, Region A and Region B. CpG Island and Region A were amplified by nested PCR and the PCR product from the second round is shown in the figure. The positive controls CpG Genome Universal methylated DNA (K^+) and negative controls (U^+ (cell line CRL-5803)), ddH_2O and master mix (MM) were included. All nine cell lines were amplified in one set up for fragment CpG Island and Region A, while PCR products were retrieved from three sett ups for fragment Region B. Samples that were not used or well that are empty are marked with and red x. Samples were run in a 1% agarose gel with DNA marker φX174-Hae III.

Each PCR product was subsequently inserted into the pCR2.1-TOPO vector by TA-cloning (section 4.11) used for transformation of *E.coli* cultures. The resulting colonies

were analyzed by PCR screening (section 4.8). Colonies harboring plasmid with insert of correct size for each of the fragments were selected and cultured before purification (mini-prep, section 4.14) and Sanger sequencing (section 4.15). A minimum of 20 colonies revealing sequences of high quality for each fragment and cell line were obtained and aligned with reference sequence U37574.1 (GeneBank), all together covering a region spanning from -1089 to +330 relative to transcription start site, including a total of 66 CpG dinucleotides (figure 5.3). The CpGs are located in positions between -1052 to +302, relative to transcription start site of *BRCA1* in GeneBank sequence U37574.1. For further assessment the CpGs were numbered 1-66 with CpG 1 being in position -1052 and CpG 66 being in position +302. Nucleotide sequence and position of all 66 CpGs can be found in figure 5.1.

The bisulfite conversion rate was calculated to be 99.63%, found by counting the number of Cs and Ts in the cloned regions (in the 20 colonies) for all positions in the reference sequence harboring a C nucleotide outside of CpGs, as well as the number of unconverted Cs (corresponds to methylated Cs) and converted Ts (corresponds to unmet Cs) in each CpG position. The number of Ts divided by the total number of C + Ts defines the bisulfite conversion efficiency.

The data obtained for CpG 26 for cell line SKBR3 and MDA-MB 468 were excluded because the CpGs were in the end of the obtained sequences, consequently the read was of low quality and the data in the specific positions may be unreliable.

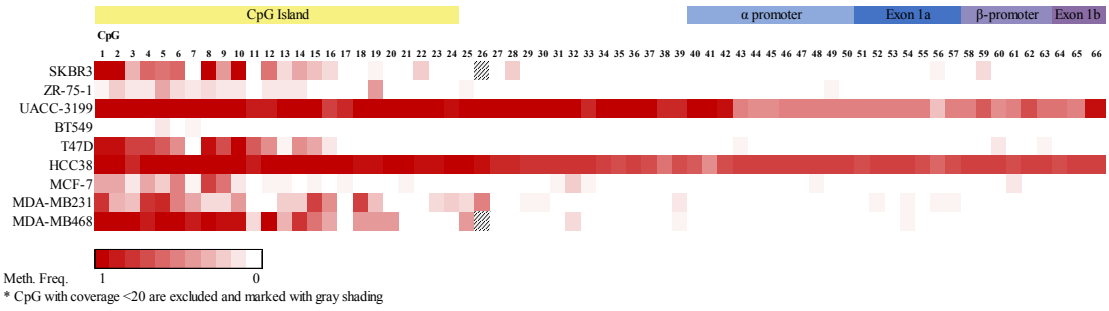

Figure 5.3: Methylation pattern in the *BRCA1* promoter region in breast cancer cell lines. Methylation pattern in *BRCA1* promoter determined by PCR amplification from bisulfite treated DNA followed by TA cloning and Sanger sequencing. Elements of the *BRCA1* promoter region is marked in the top of the figure and cell lines are marked to the left. Each CpGs is covered with 20 individual clones and the methylation ratio is indicated as a colored scale with completely methylated as red and no methylation as white. The cloned *BRCA1* promoter area covers 66 CpG.

The selected cell lines were found to have a varying degree of methylation in the analyzed region of the *BRCA1* promoter (figure 5.3). Highest frequency of methylated CpGs was found in the CpG island region, while the methylation levels in the remaining regions were modest. The exception from this pattern was the cell lines UACC-3199 and HCC38; both these cell lines were extensively methylated throughout most of the studied region, but the highest methylation frequency was still observed within the CpG island. The cell line BT549 was close to unmethylated in the same region, with only 2 of the 66 CpGs having some methylation. A low methylation frequency was also observed for cell line ZR-75-1. The remaining cell lines had intermediate methylation levels, where a clear pattern emerged with more methylation in CpG island relative to region A and B. BT549 and HCC38, the cell lines with the lowest and highest methylation frequency, respectively, are both triple negative with respect to ER, PR and Her2 status. UACC-3199, also found to be extensively methylated are ER and PR negative, but Her2 positive. Other cell lines that are ER and PR negative include SKBR3, MDA-MB 231 and MDA-MB 468, these were found to have an intermediate methylation frequency. As such, the observed methylation patterns did not seem related to breast cancer subtype in terms of hormonal receptor status and/or triple negativity.

5.1.2 *BRCA1* mRNA expression in breast cancer cell lines

Experiments for detection of *BRCA1* mRNA expression levels were performed to assess the effect of methylation status in the *BRCA1* promoter region of nine cell lines. qPCR was performed to quantify the relative total mRNA levels of *BRCA1* WT as well as specific assays to quantify the levels of the two transcripts from each of the promoters (*BRCA1* WT α and *BRCA1* WT β). In addition, mRNA potentially expressed from the *BRCA1* pseudogene were analyzed (*BRCA1* pseudo α and *BRCA1* pseudo β), as described in section 4.18.

To eliminate potential cross reactions between the assays due to sequence similarity, the *BRCA1* WT α assay was performed using *BRCA1* pseudo template cDNA (14 ng) and the *BRCA1* pseudo α assay was performed with *BRCA1* WT α cDNA template (14 ng). The same cross over experiments were performed for *BRCA1* WT β and *BRCA1* pseudo β assays (table 5.1.1). A cut off for mRNA detection levels was set to CP-value equal or above 40. All cross-reaction measurements were found to be negative. Taken

together, these results indicated that despite the sequence similarities between *BRCA1* WT and pseudo gene, all assays had adequate specificity.

Table 5.1.1: Overview of qPCR *BRCA1* WT and pseudo gene cross reactions

Primer/probe	Template (14 ng)	CP-value	Δ CP-values
WT α	WT α Pseudo α	26.87 Negative	-
Pseudo α	Pseudo α WT α	25.68 45.00 (Negative)	19.32
WT β	WT β Pseudo β	25.71 Negative	-
Pseudo β	Pseudo β WT β	30.65 45.00 (Negative)	14.35

All data (recorded as CP-values; i.e maximum 2nd derivative point on the reaction curves) were converted to relative concentrations by use of an in-run standard curve, specific for each assay. Relative concentrations were then adjusted for run-to-run variations by a fixed calibrator (pooled cell line cDNA) included on each reaction-plate. Finally, the data for each sample were corrected for the corresponding levels of mRNA for the ribosomal protein RPLP2. The relative mRNA levels of *BRCA1* WT total, *BRCA1* WT α and *BRCA1* WT β for the nine breast cancer cell lines are presented in figure 5.4. In general, the vast majority of the *BRCA1* mRNA was found to be *BRCA1* WT α compared to a smaller fraction of *BRCA1* WT β, extending from 185 fold to 28 fold (median of 76 fold) higher for *BRCA1* WT α than WT β within each cell line (figure 5.4 A). Due to major differences, none of the *BRCA1* WT β levels are visible in the figure 5.4 A. The overall expression levels for *BRCA1* WT α of the different cell lines were similar to the expression levels of *BRCA1* WT total.

Regarding the differences in *BRCA1* mRNA expression between the nine breast cancer cell lines, a 16-fold difference in *BRCA1* WT total mRNA concentration was observed between cell line SKBR3 (lowest expression levels) to cell line T-47D (highest expression levels). The cell lines with the lowest *BRCA1* WT total expression was SKBR3, MDA-MB 231 and HCC38, respectively, while the cell lines with the highest expression levels were T47D, ZR-75-D and BT549, respectively. When looking at the *BRCA1* WT experiment, the greatest difference in mRNA levels for *BRCA1* WT α was observed for cell line MDA-MB 231 compared to cell line T-47D with a 25-fold increase for cell line T-47D.

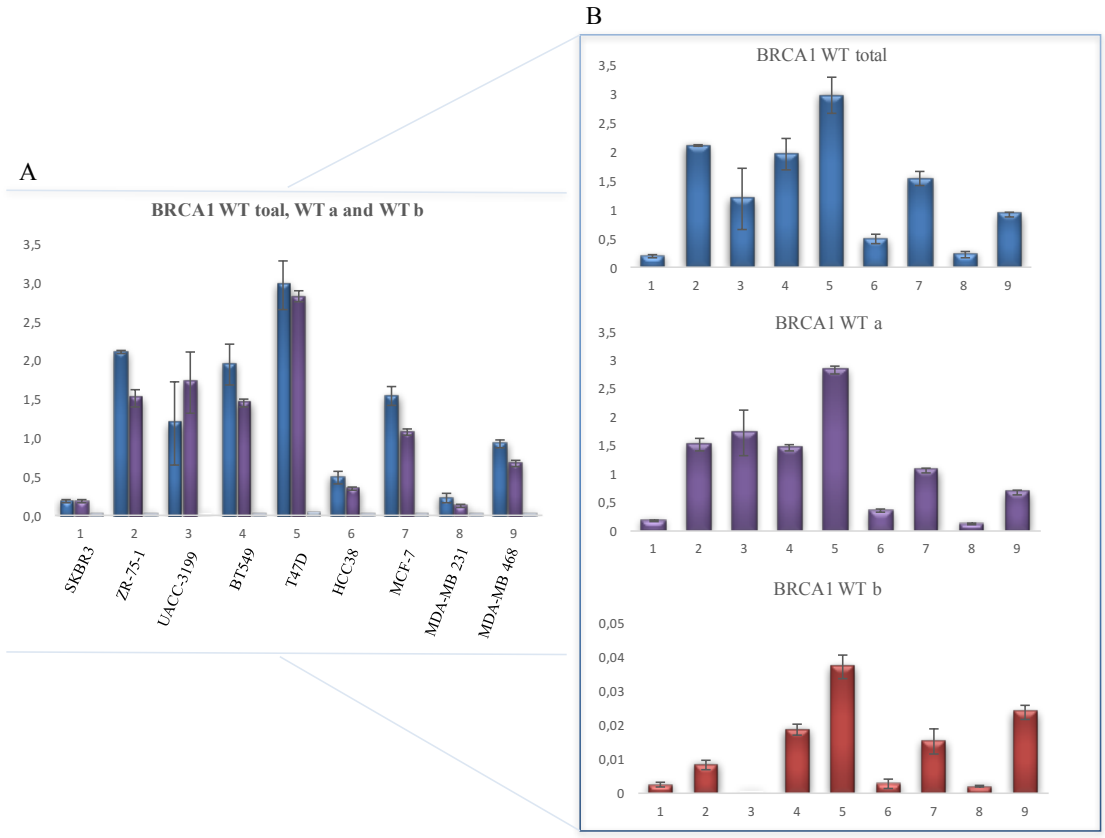

Figure 5.4: Relative concentrations of *BRCA1* mRNA for nine breast cancer cell lines. mRNA levels were measured for *BRCA1* WT total, *BRCA1* WT α and *BRCA1* WT β using LightCycler 480 Probes Master with hydrolysis probe assay. Numbers 1-9 correspond to cell lines SKBR3, ZR-75-1, UACC-3199, BT549, T47D, HCC38, MCF-7, MDA-MB 231 and MDA-MB 468, respectively. The mRNA expression levels of *BRCA1* WT total (dark blue), *BRCA1* WT α (purple) and *BRCA1* WT β (red) relative to each other **(A)**. *BRCA1* WT total, *BRCA1* WT α and *BRCA1* WT β alone (**B).** Relative mRNA concentration is plotted against the nine cell lines. All data are corrected by both internal calibrator as well as for housekeeping gene *RPLP2*. Relative mRNA levels were calculated by the use of a specific standard curve for each assay. For comparison of data across assays a ΔΔCT-approach was applied, where ΔCP-values of respective target genes were first calculated, then these were converted to relative concentrations by applying 2 as the efficiency number (Δ concentration between two samples = $2^{\Delta CP}$), as explained in section 4.17.

When comparing relative mRNA levels between two different transcripts (α and β) for the nine cell lines, it is evident that the mRNA expression pattern for *BRCA1* WT β is much lower than *BRCA1* WTα (figure 5.4 B). In general, however, the distribution of *BRCA1* WT β expression among the cell lines were similar to relative levels of *BRCA1* WT α, except for the cell line UACC-3199 which had no detectable mRNA for *BRCA1* WT β transcript and cell line MDA-MB 468, which had more expressed β transcript than expected according to α expression. Assessing the association between the levels of the two transcripts, a clear trend was observed, however, the data did not reach statistical significance (p>0.3), probably due to the low number data points / cell lines (n=9; figure 5.5).

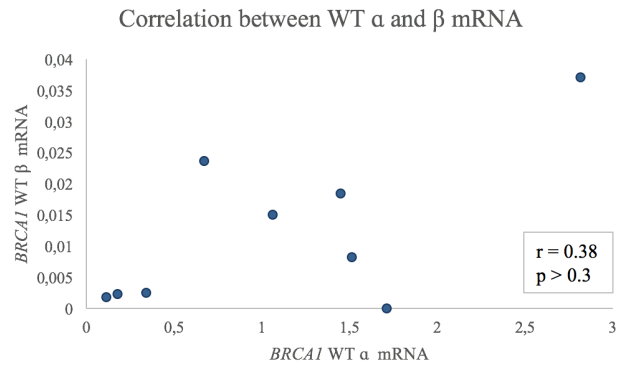

Figure 5.5: Correlation between *BRCA1* WT α mRNA and BRCA1 WT β mRNA expression levels. Relative concentrations were plotted against each other with WT α on x-axis and WT β on y-axis. Spearman's Rho (r) were calculated to assess the rank correlation (r=0.38) (p>0.3).

Regarding *BRCA1* pseudo mRNA expression, the qPCR analysis showed some expression for *BRCA1* pseudo α, however, the detected CP-value for all of these cell lines were above 40, and when the assay was repeated, the expressed cell lines did not show consistency. According to the sensitivity threshold set for this experiment, the qPCR analysis showed no detectable expression of pseudo *BRCA1* α nor, pseudo *BRCA1* β mRNA in this experiment (figure 5.6).

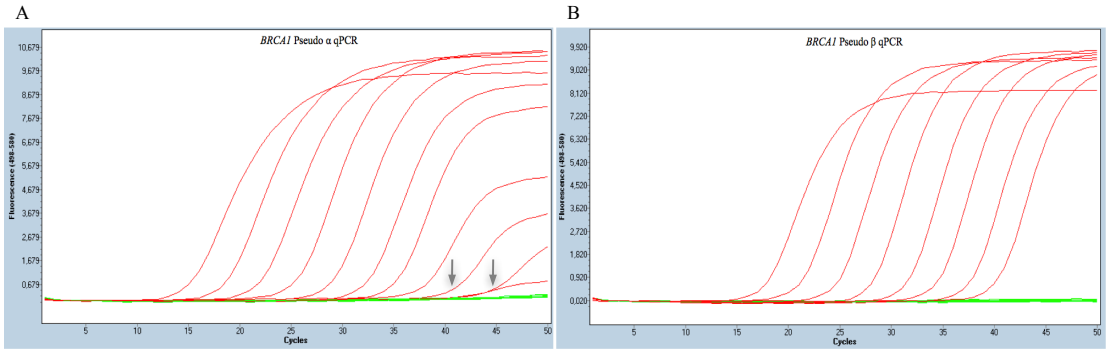

Figure 5.6: *BRCA1* pseudo α (A) and β qPCR (B) measurements. Detected fluorescent signal was plotted against cycles. Each assay was run with a specific standard curve composed of eight concentrations. Samples with positive signal had a red color, while negative samples were green. Three *BRCA1* Pseudo α samples gave positive signal, however all samples had CP-values above 40 (samples are indicated by arrows). Master mix and water was included as negative controls.

The *BRCA1* WT total qPCR data (figure 5.4) were compared to the methylation pattern found for the corresponding breast cancer cell lines (5.3), however, no clear correlation could be observed for the overall methylation pattern of the cell lines and the corresponding mRNA expressed levels. The cell lines with the most extensive methylation, namely UACC-3199 and HCC38, had intermediate to low levels of detected mRNA, where HCC38 showed less *BRCA1* expression than UACC-3199.

However, both cell lines SKBR3 and MDA-MB 231, with intermediate methylation frequency, had lower *BRCA1* WT mRNA expression than HCC38. The cell line with highest mRNA expression levels, T-47D, had similar methylation pattern as the two cell lines with the lowest mRNA expression, SKBR3 and MDA-MB 231.

When assessing smaller regions and their association to *BRCA1* WT mRNA expression, a weak correlation could be found between the methylation of CpGs 22-24 and mRNA levels. SKBR3, MDA-MB 231 and HCC38, methylated in CpGs 22, 23-24 and 22-24 respectively, generally had lower mRNA expression than the unmethylated. Cell lines UACC-3199 was also methylated in CpG 22-24 but with a slightly higher mRNA expression level. However, the expression level was still lower than the samples that have no detected methylation in the same positions. A weak association could also be seen for methylation of CpG 56 and *BRCA1* WT total mRNA expression, as the cell lines harboring some methylation in this position had lower expression levels of *BRCA1* WT total mRNA than cell lines that were unmethylated in the same CpG. However, among the cell lines having the lowest WT total mRNA expression (SKBR3 and MDA-MB 231) only a small percentage of the 20 molecules covered were found to be methylated in CpG 56.

Taken together, no correlation is prominent when looking at the overall methylation pattern and *BRCA1* WT total mRNA expression, however a weak link might be seen for smaller regions or individual CpGs and the *BRCA1* WT total mRNA expression.

5.1.3 BRCA1 protein expression in breast cancer cell lines

Western blot analysis was performed to investigate the BRCA1 protein levels in the different breast cancer cell lines. For all nine cell lines, a band corresponding to the expected size of BRCA1 protein (220 kDa) was detected (figure 5.7). Most of the cell lines had a similar expression of BRCA1 protein, except from HCC38 and MDA-MB 231, which showed approximately 2-fold increased expression, and MCF-7, which had close to no detected protein. Several additional bands were observed around 70 kDa, possibly due to splice variants, partial degradation or unspecific binding of the antibody. The loading control actin (42 kDa) showed similar loading of the nine samples. When comparing the observed expression levels to the detected methylation

levels, no clear association could be observed between the *BRCA1* promoter methylation pattern and BRCA1 protein expression among the cell lines.

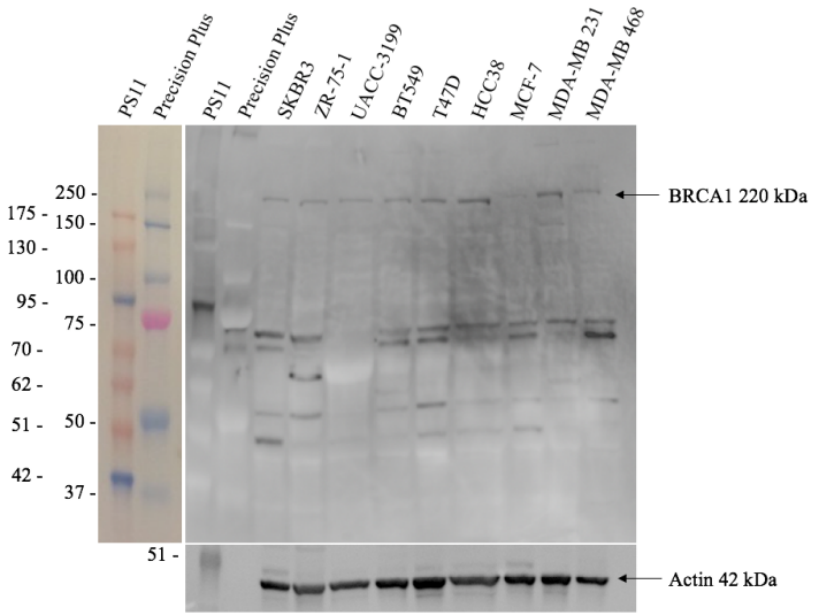

Figure 5.7: Western blot analysis of BRCA1 protein expression in nine breast cancer cell lines. Protein lysate of the nine cell lines were run on a on a 10% polyacrylamide gel along with molecular size ladders PS11 and Precision Plus followed by western blot analysis. The BRCA1 protein (220 kDa) and marked with an arrow in the top of the gel picture. Acting (42 kDa) was used as a loading control. The ladders are detection by both white light and chemilumeniscence.

5.2 Effect of alterations in the α and β promoter, including site-specific methylation and polymorphic variants, on *BRCA1* expression

The aim of the second part of this thesis was to investigate how the *BRCA1* expression levels, as well as ratio between the α and β transcripts was affected by alterations in the α and β promoter region of *BRCA1*. More specifically, CpG site-specific methylation as well as two common polymorphisms in *BRCA1* promoter region were to be assessed by generating and transfecting luciferase reporter constructs with different methylation patterns for each of the *BRCA1* promoter-pGL4.10[*luc2*] polymorphism versions and analyze the effect on protein expression by bioluminescence assays.

5.2.1 Introduction of site-specific methylation in the *BRCA1* promoter to assess effect on promoter strength and ratio of α versus β transcript

For this purpose, the *BRCA1* promoter region (1946 bp) was amplified (section 4.8), yielding a PCR product of expected size (figure 5.8 A). The *BRCA1* promoter region

was subsequently cloned into the luciferase expression vector pGL4.10[*luc2*] (4242 bp) using restriction enzymes as described in section 4.12.

Sequencing of plasmids containing the *BRCA1* promoter region from DNA originating from pooled blood samples of five healthy donors, showed the presence of two construct differing in the polymorphic positions rs71361504 (AAC/-) and rs799905 (G/C) (figure 5.1 and 5.9). For simplicity, the construct harboring the deletion variant of AAC in position rs71361504 and C in position rs799905 were named *BRCA1* promoter-v1.1-pGL4.10[*luc2*] (or simply just version 1.1). The construct harboring the combination of AAC and G in the respective positions were called *BRCA1* promoter-v2.1-pGL4.10[*luc2*] (or simply just variant 2.1) in the text. Both were to be used as template for introduction of site-specific methylation, however, the site-specific methylation protocol was first tested on the major allele (most frequent in the population) *BRCA1* promoter-2.1-pGL4.10[*luc2*] constructs.

To introduce site-specific methylation in the *BRCA1* promoter-2.1-pGL4.10[*luc2*] constructs, the protocol described in methods section 4.20 was followed. The primers used harbored methylated cytosines at positions of interest located within the α and β promoters (CpG 33, 43-44 and 48-52 - corresponding to the same CpG numbers as in the cloning experiment). A critical quality control step during this protocol was detection of correct DNA band post T7 exonuclease treatment, for removal of unligated DNA. Our data revealed the presence of a weak band of approximately 8000 bp in the aliquot from pre-T7 exonuclease treatment (figure 5.8 B). However, no band was observed post-exonuclease treatment, most likely due to unsuccessful ligation during the combined PCR and ligation reaction.

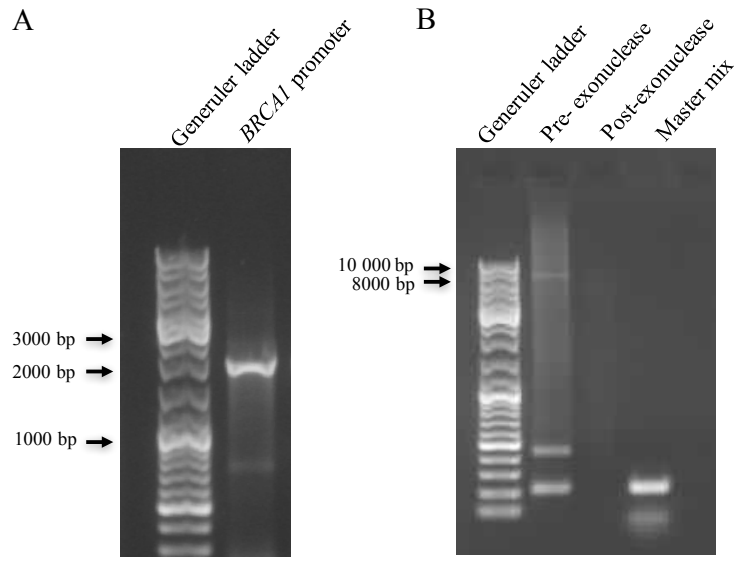

Figure 5.8: PCR amplification of *BRCA1* promoter region and introduction of site specific methylation into the construct *BRCA1* promoter-pGL4.10[*luc2*]. PCR amplification of *BRCA1* promoter for subsequent cloning into vector pGL4.10[*luc2*] for generation of the construct *BRCA1* promoter-2.1-pGL4.10[*luc2*] **(A).** After the combined PCR-ligation reaction in the first step of the site sp methylation protocol, the products were treated with DpnI and T7 exonuclease. Aliquots from pre-exonuclease and post-exonuclease treatment were run on a 2% agarose gel along with the DNA ladder Generuler. Master mix was included as negative control **(B)**.

Several attempts were made to improve the PCR-ligation reaction: modifications that were tested include addition of polynucleotide kinase before adding extra ligase after the combined PCR-ligation reaction, testing of purification both with and without prior separation on gel, reduced purifications steps and reduced exonuclease treatment (down to 30 minutes). However, none of these modifications led to successful PCR-ligation reaction. The original plan to continue with DnmtI treatment was therefore abandoned, and the subsequent steps were never performed. The full focus of this sub-aim was shifted to explore the potential differences in *BRCA1* promoter strength caused by the two polymorphisms detected in the general population.

5.2.2 Effect of SNP variants in the *BRCA1* promoter on protein expression

The original aim was to produce and transfect a repertoire of luciferase reporter constructs with different methylation patterns for each of the *BRCA1* promoter-pGL4.10[*luc2*] polymorphism variants and analyze the effect on protein expression by bioluminescence assays. However, as the protocol for introduction of site-specific methylation did not succeed, the focused was moved to analyzing the effect of polymorphisms in the *BRCA1* promoter on the expression level. The polymorphisms

were different in two positions; thus, intermediates variants were made by site directed mutagenesis (section 4.21) and named variant 1.2 and 2.2 (figure 5.9).

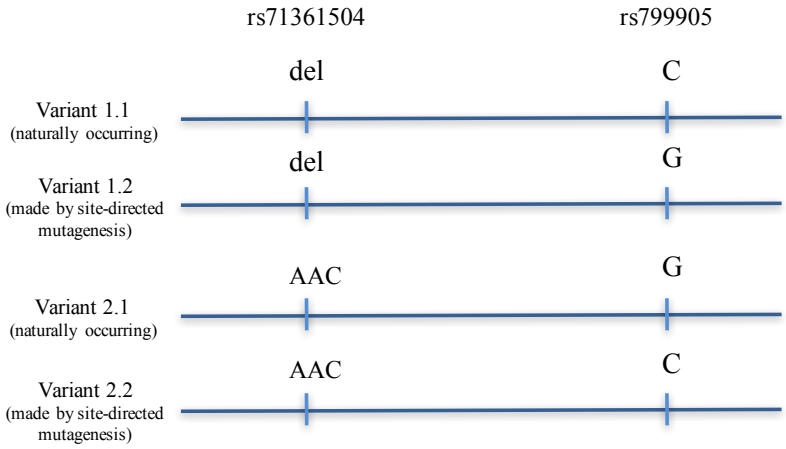

Figure 5.9: Schematic presentation of *BRCA1* promoter pGL4.10[*luc2*] SNP version 1.1, 1.2, 2.1 and 2.2. *BRCA1* promoter-pGL4.10[*luc2*] variant 1.1 and 2.1 were detected by sanger sequencing of pooled blood DNA, while variant 1.2 and 2.2 were made by site-directed mutagenesis. The polymorphisms rs71361504 and rs799905 were in positions Chr17:43125989-90 and Chr17:43125170, respectively, in human genome version 38, corresponding to -598 and +221 relative to transcription start site in reference sequence U37574.1 (GeneBank).

The *BRCA1* promoter-pGL4.10[*luc2*] vector constructs containing the different polymorphic combinations in the *BRCA1* promoter, were transfected into MCF7 cells for subsequent bioluminescence measurements. However, before comparing the expression levels from the different constructs, the assay was optimized in terms of substrate incubation time. Thus, the luciferase signal generated in cells transfected with construct *BRCA1* promoter-V.2.1-pGL4.10[*luc2*] (major allele) was measured at 1, 2, 5, 7 and 10 minutes after addition of substrate. The measurement showed a peak in the signal strength after 2 minutes of incubation, and which the following measurements showed a steady decrease in the luciferase signal (figure 5.10 A). Based on these results, subsequent luciferase assay measurements for the all *BRCA1* promoter-pGL4.10[*luc2*] polymorphic-versions were performed after 2 minutes incubation with substrate.

A total of seven luciferase assays were performed for the four *BRCA1* promoter-pGL4.10[*luc2*] variants 1.1, 1.2, 2.1 and 2.2 as described in methods section 4.20. Each sample was measured in six parallels for each experiment, and the measurements were normalized according to corresponding GFP measurements. Mean, standard deviations and relative standard deviation (Coefficient of variation) were calculated for all

constructs within the six parallels both before and after normalization. Median relative standard deviation was found to be 11.35% with a range from 4.48% to 24.50% after normalization. Luciferase expression strength were found by calculating a new average composed of the mean signal value for each variant in each of the seven experiments performed, followed by calculation of standard deviations (figure 5.10 B). Version 2.1, which is the major allele in the Caucasian population were set as a reference (100%) and the signal strength of the other three variants were calculated accordingly.

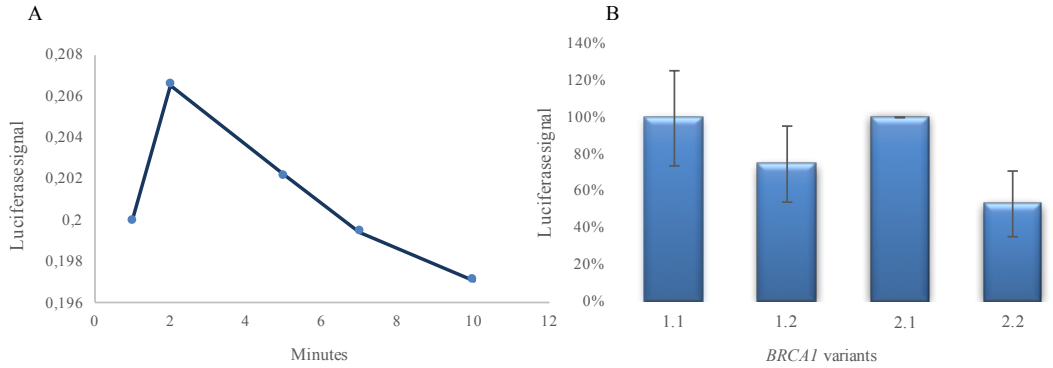

Figure 5.10: Bioluminescence measurements for *BRCA1* promoter SNP variants pGL4.10[*luc2*] 1.1, 1.2, 2.1 and 2.2. In order to find the optimal time for measurements of bioluminescence after addition of substrate, a bioluminescence assay was performed for the major allele *BRCA1* promoter variant 2.1 measuring the bioluminescence signal 0.5, 2, 5, 7 and 10 min after addition of substrate. The assay show a steady decrease in signal over time. For the following assays, the reactions were measured 2 minutes after addition of the substrate **(A)**. The luciferase measurements of *BRCA1* variant 1.1, 1.2, 2.1 and 2.2. All samples were normalized according to co-transfected eGFP, and the calculation are based on six parallels in seven assays Version 2.1, the major allele in the Caucasian population, was set as reference and the other SNP-versions were calculated accordingly **(B)**.

When assessing the influence of rs799905 and rs71561504 on gene expression, the bioluminescence assay for the versions 1.1, 1.2, 2.1, and 2.2 were compared. When comparing the two variants of rs799905 (G compared to C), the nucleotide identity in the other polymorphic position, rs71561504, was kept identical. As rs71561504 exists in two forms (AAC/-), the comparison of C and G in rs799905 was performed both with the -/- version and AAC/AAC version of rs71561504. The luciferase expression was found to be significantly higher for the construct harboring the C-allele (1.1) versus the G-allele in construct variant (1.2) (p=0.0454) (both construct harboring the deleted version of rs71561504). The opposite was shown for construct variant 2.1 (G) versus variant 2.2 (C), where the G-allele showed significant higher luciferase expression compared to the C-allele (p=8.17E-06) (both construct harboring the AAC/AAC version of rs71561504). A similar strategy was chosen when comparing the two

variants of the rs71561504 (AAC+ compared to AAC-). The variant without the deletion, variant 2.1 (AAC+), had a significantly higher expression than variant 1.2 (AAC-) (p=0.0092) (both harboring version G of the rs799905). In the next comparison, version 1.1, with deletion (AAC-) had significantly higher expression than version 2.2 (AAC+) (p=0.0012) (both harboring version C of the rs799905)), again showing opposing results (figure 5.9 and table 5.2.1).

Notably, version 1.1 (AAC) and 2.1 were the two versions found natural occurring in healthy individuals and these two versions yield a similar luciferase expression (p=0.6126). Version 1.2 and 2.2 were generated by site-directed mutagenesis, and no literature could be found about their existence in the population. Both yielded lower luciferase expression levels than the naturally occurring versions, with variant 2.2 having significantly lower expression signal than variant 1.2 (p = 0.0416).

Table 5.2.1: Effect of polymorphic variants on luciferase activity

SNP Version	rs71361504	rs799905	p-value
1.1	AAC -	C	0.0454
1.2	AAC -	G	
2.1	AAC +	G	8.17E-06
2.2	AAC +	C	
1.2	AAC -	G	0.0092
2.1	AAC +	G	
1.1	AAC -	C	0.0012
2.2	AAC +	C	
1.1	AAC -	C	0.6125
2.1	AAC +	G	
1.2	AAC -	G	0.0416
2.2	AAC +	C	

5.3 Analyzing the effect of long term drug treatment on methylation levels in the *BRCA1* promoter

The aim of the third part of the present project was to assess the potential effects of long term treatment with olaparib and doxorubicin on the methylation pattern of the *BRCA1* promoter in breast cancer cell line SKBR3. As such, differences in *BRCA1* methylation were to be assessed as a potential mechanism of resistance to the drugs. The cell line SKBR3 was chosen for this experiment, as it has previously been reported to be methylated in *BRCA1* promoter (Cai et al., 2014). SKBR3 cells were exposed to the drugs for an extended period of time at increasing concentrations (figure 4.1), with

the lowest doses confirmed to exert an effect by immunofluorescence assay. The effect over time on the methylation pattern was analyzed by NGS.

5.3.1 Cell proliferation assay for determination of IC_{50} for olaparib and doxorubicin.

Before initiating the long term exposure of SKBR3 cells to doxorubicin and olaparib, the IC_{50} was determined for each of the drugs. To estimate IC_{50} for olaparib (100-1000) and doxorubicin (0.02-0.06 µM) in SKBR3 cell line, cell proliferation assay WST-1 was performed as described in methods. Treatment with both drugs yielded expected dose-response curves, enabling estimates of the IC_{50} points to be made. The IC_{50} of olaparib and doxorubicin was determined to be approximately 260 µM and 0.06 µM respectively (figure 5.11).

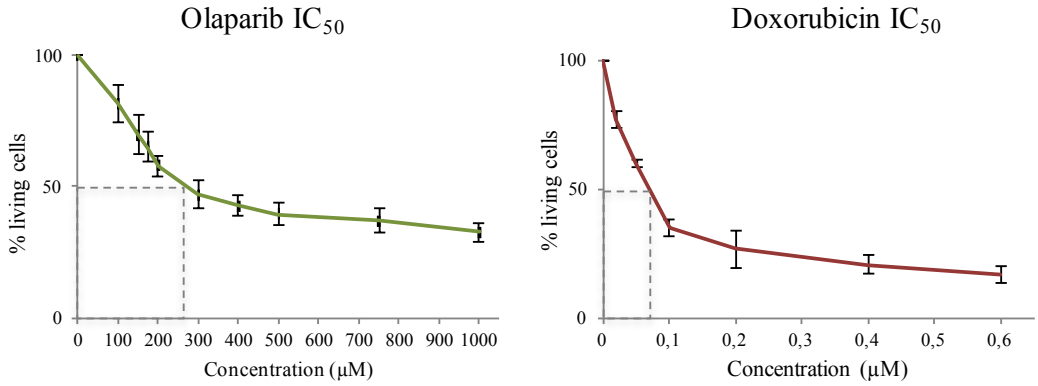

Figure 5.11: Determination of IC_{50} for the olaparib and doxorubicin in SKBR3 cells. Cells were treated with DMSO, olaparib and doxorubicin over a period of 72h before cell proliferation assay WST-1 was performed**.** Percentage of living cells were plotted against the drug concentration. IC_{50} was determined to be 260 µM and 0.06 for olaparib and doxorubicin, respectively.

5.3.2 Immunofluorescence assay for detecting dsDNA breaks

As the aim of this part of the study was to analyze the effect of long time drug exposure, the initial drug doses needed to be far below IC_{50}. To verify that the lowest doses chosen (1 µM for olaparib and 0.002 µM for doxorubicin) exerted an effect on the cells, immunofluorescence assay detecting dsDNA breaks was performed as described in methods (section 4.3). SKBR3 cells were treated with the drugs olaparib (1-2 µM) or doxorubicin (0.002-0.004 µM) for 48 h. A representative selection of images from the experiment is shown in figure 5.12 A. All treatment settings gave positive signals, and both the 0.002 µM and 0.004 µM doxorubicin and 1 µM and 2 µM olaparib resulted in

a positive signal for staining. Neither of the negative controls showed any FITC signal (figure 5.12 B).

The percentages of positive cells were calculated. In the reference sample treated with DMSO, 18% cells were found to be positive, compared to 32% and 43%, for 1µM and 2µM olaparib and 37% and 38% for 0.002 µM and 0.004 µM doxorubicin, respectively (figure 5.12 C). Significantly higher number of dsDNA breaks in were found in SKBR3 cells treated with 1 and 2 µM olaparib as well as a 0.002 and 0.004 µM doxorubicin compared to cells treated with reference (DMSO) ($p \leq 0.001$). These findings showed that the lower doses of both drugs, 0.002 µM doxorubicin, and 1 µM olaparib could be used as initial drug concentrations for long-term treatment of SKBR3 as they both exert an effect on the DNA.

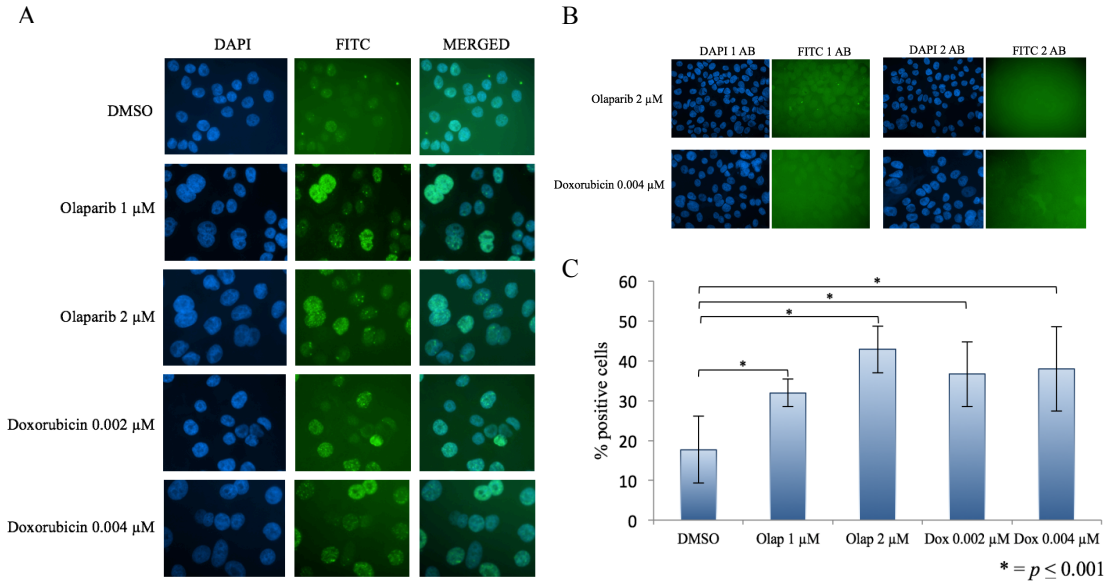

Figure 5.12: Immunofluorescence assay for detecting dsDNA breaks in SKBR3. Immunofluorescence assay was performed on SKBR3 cells treated with olaparib (1-2 µM) and doxorubicin (0.002-0.004 µM) to detect an effect of the drugs at the specific concentrations. DMSO was included as a reference. First column show DAPI staining of the nucleus, FITC staining in the second column and the merged pictures in column three **(A)**. Treated cells incubated with primary antibody (1AB) only or secondary antibody (2AB) only were included in the assay to show the specificity of the antibodies **(B)**. Percentage positive cells were calculated, followed by estimation of the average and standard deviation of three parallel experiments. All treatments were calculated to be significantly higher than the DMSO control ($p \leq 0.001$) **(C)**.

5.3.3 Mycoplasma testing of doxorubicin and olaparib treated SKBR3 cells

Due to the long duration of the drug treatment experiment, mycoplasma test was performed on medium from the initial and final harvesting for both drugs (section 4.1.4). When the initial drug dose was increased a backup line was separated from the

experiment line, which led to four treatment settings (olaparib experiment, olaparib backup, doxorubicin experiment and doxorubicin backup), as well as the control treatment (DMSO). All treatment settings were tested. As expected, the positive controls show a band between 267-277 bp, and the negative control show only the expected band for the internal control of 191 bp (figure 5.13). None of the samples from the SKBR3 harvesting tested positive for mycoplasma contamination.

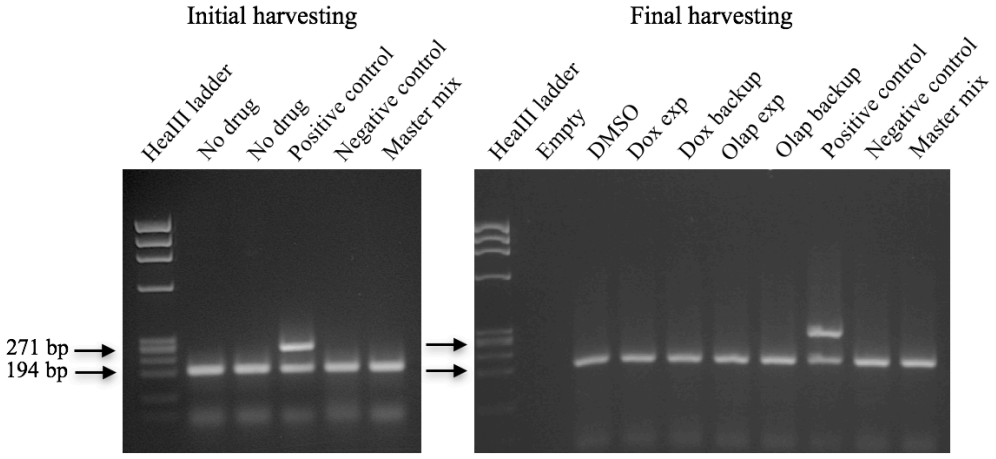

Figure 5.13: Mycoplasma test of SKBR3 cells. Medium harvested from SKBR3 cell cultures were tested for mycoplasma by using Venor GeM Mycoplasma Detection Kit. Medium were collected at the initial harvesting (no drug) as well as for the final harvesting of each drug; DMSO, olaparib (Olap) and doxorubicin (Dox) from both the experiment line and the backup line were tested. Internal control (191 bp) was added to each sample. Postive (DNA fragment of *Mycoplasma orale* genome) and negative control (mastermix) were run in parallel. The samples were run on a 2% agarose gel with φX174-HaeIII digest as molecular marker.

5.3.4 STR profiling of SKBR3 cells from long term experiment

In addition to the mycoplasma test, the long term nature of this experiment required validation of the cell lines identity both before initiation of drug treatment and after completion of the entire treatment period; the latter in order to detect any potential cross-contamination with other cells during the experiment. The cell line SKBR3 was therefore tested for correct identity by STR Profiling (DNA "fingerprinting"; section 4.1.5). Samples corresponding to the first harvest, containing no drug, as well as the final harvest for all treatment settings were STR profiled. All samples from first and last harvesting for both drugs (experiment and backup line), as well as the DMSO control were found to have a STR profile matching the STR profile of SKBR3 provided by ATCC (Table 5.3.1).

Table 5.3.1: Overview of STR profiling for SKBR3 test samples.

Locus designition	STR: position	No drug	DMSO	Olaparib experiment	Olaparib backup	Doxorubicin experiment	Doxorubicin backup
Amelogenin	X *	X	X	X	X	X	X
CSF1PO	12	12	12	12	12	12	12
D13S317	11, 12	11, 12	11, 12	11, 12	11, 12	11, 12	11, 12
D16S539	9	9	9	9	9	9	9
D5S818	9, 12	9, 12	9, 12	9, 12	9, 12	9, 12	9, 12
D7S820	9, 12	9, 12	9, 12	9, 12	9, 12	9, 12	9, 12
THO1	8, 9	8, 9	8, 9	8, 9	8, 9	8, 9	8, 9
TPOX	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11
vWA	17	17	17	17	17	17	17

* X refers to the female X chromosome

5.3.5 BRCA1 promoter methylation by methylation specific NGS

Long term drug treatment of the cell line SKBR3 was performed to assess potential alterations in the methylation pattern in *BRCA1* promoter region to further link this to drug resistance. SKBR3 cells were treated with increasing concentrations of the drugs olaparib (1-8 μM) and doxorubicin (0.002-0.012 μM) for a period of 11-13 weeks as described in methods, section 4.1.3. Multiple samples from this experiment were subjected to detailed methylation analyses by massive parallel sequencing (NGS) of the promoter regions of 283 tumor suppressor genes on bisulfite converted DNA. The harvesting time-points for the samples selected for NGS analysis are given in table 5.3.2 and figure 4.1.

Table 5.3.2: Overview of harvesting time points for treated SKBR3 cells.

Week	DMSO treatment	Olaparib experiment	Doxorubicin experiment	Olaparib backup	Doxorubicin backup
0	x	x	x	x	x
3	x	x	x	x	x
6	x	x	x		
8	x			x	x
11	x		x		
13	x	x		x	x

Control samples treated with DMSO were selected for corresponding time points for every selected olaparib or doxorubicin treated sample. A summary of the results of methylation specific massive parallel sequencing (described in section 4.23) is provided in Appendix 1 (table A.2). A series of parameters, used to ensure high quality data, were extracted from the sequencing run. The combined Watson and Crick duplication percentage (data from both original strands) varied from 2.53% to 7.06%, except for the doxorubicin experiment sample harvested in week three (referred to as Dox-week

3), which had a duplication percentage of 26.68. The mean capture target coverage ranged from 122.69x to 279.35x, except for sample Dox-week 3, which had a mean capture target coverage of 53.04x. *BRCA1* was the focus for this thesis, and only CpGs in the *BRCA1* promoter were studied. Mean coverage for CpGs of interest within the *BRCA1* promoter ranged from 149.21x to 27.42x, with an average of 84.92x. All samples except Dox-week 3 had a mean coverage above 60x. The bisulfite conversion efficiency (C to T) of the internal Lambda DNA control were found to be on average 99.45% (range 99.17% - 99.57%) in the analyzed samples. All in all, these measures indicated sequencing data of high quality and allowed further biological interpretations to be made.

For each sample, the methylation frequency for 66 CpGs located in the *BRCA1* promoter region were extracted from the NGS data, corresponding to the same CpG's analyzed in the plasmid bisulfite sequencing experiment described in section 5.1.1. A heat map was constructed, showing the methylation frequency for the 66 CpGs in the samples corresponding to SKBR3 treated with DMSO, doxorubicin experimental and backup line as well as olaparib experimental and backup line (table 5.3.2, figure 5.14 A). In general, CpG 1-15 located in the CpG Island were mainly methylated, while the rest of the CpGs were largely unmethylated. The findings show only small differences between first and final harvesting observed for any of the experimental or backup lines when comparing to the DMSO treated control samples. Methylation levels seem to be decreasing slightly over time for all samples in the CpG Island region, but this also includes the DMSO control samples. Thus, the overall impression is that no major differences can be detected, while the minor changes observed are not caused by doxorubicin or olaparib.

Further, calculating ratios of methylation levels in individual CpGs (1-15) in the last time point versus time point zero, showed that most of the individual CpGs were in general demethylated over time, for all treatments including the DMSO control sample (figure 5.14 B). Thus, the potential differences do not seem to be related to the drug treatments. Apart from the CpGs within the CpG island, the rest of the promoter was largely unmethylated. In total, a small decrease in methylation of some CpG with the CpG Island was observed over time when treating with doxorubicin and olaparib, however, a clear effect compared to the DMSO treated sample was lacking.

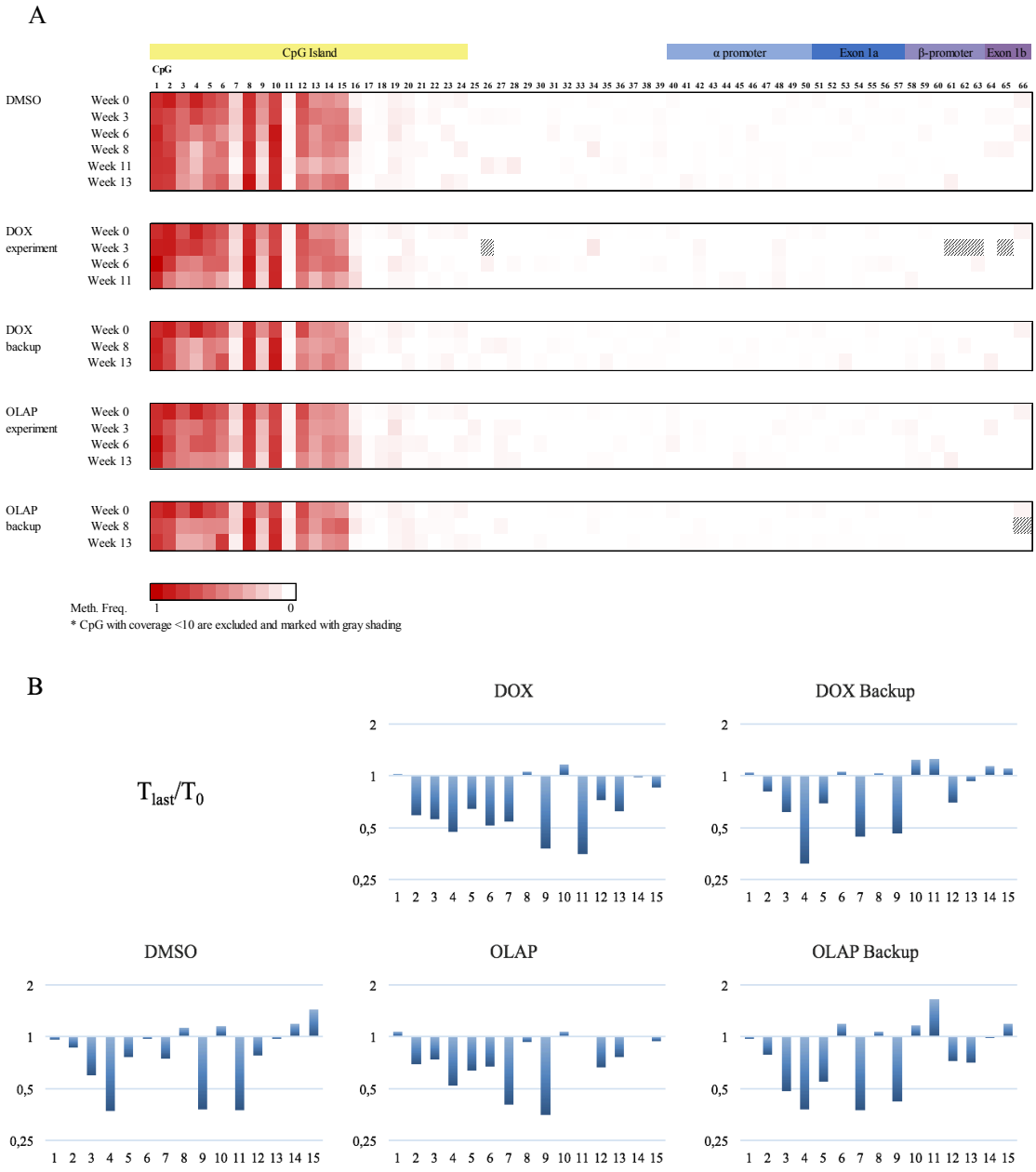

Figure 5.14: Overview of methylation pattern in SKBR3 cells treated with olaparib and doxorubicin detected by NGS. The breast cancer cell line SKBR3 was treated with olaparib and doxorubicin over a time period of 11-13 week. DMSO was included as a reference and harvested at time points equal to the harvesting of drug treated samples. The drug treated samples were split in one experiment line and one backup line for each of the two drug; doxorubicin experiment line (0.002-0.012 µM), doxorubicin backup line (0.002-0.008 µM), olaparib experiment line (1-8 µM) and olaparib backup line (1-4 µM). Samples were harvested and deep sequenced. **A)** Harvesting time points for the specific samples are indicated to the left in the figure. Methylation frequency of *BRCA1* promoter were detected by use of Roche NimbleGen SecCap Epi Enrichment systems in combination with custom made probe panel. CpG 1-66 and the corresponding position within the *BRCA1* promoter region is listed in the top of the figure. Methylation frequency is presented in a range from one to zero, where more methylated samples have a darker red color. Samples with coverage below 10x were excluded from the data and are marked as gray shaded squares in the figure. **B)** The change in methylation over time for each of the 5 treatment lines was calculated by dividing the methylation frequency for the last harvesting (T_{last}) by the methylation frequency at the first harvesting time point (T_0). The ratio was plotted for CpG 1-15.

6 Discussion

The interplay between cancer and epigenetics raises a multitude of complex questions that researchers all over the world are trying to address. The connection between breast cancer, *BRCA1,* and methylation is only one topic on an endless list of matters needed to be answered to be able to get a deeper understanding of the cancer disease. Both *BRCA1* and methylation are topics that have been extensively studied, separately and in combination, however, a lot of uncertainties are associated with both themes, and a great deal of research remains. The background for studying methylation in the *BRCA1* promoter is based on the knowledge of the severe breast cancer risk associated with germline *BRCA1* mutations as well as the low *BRCA1*-mutational rate found in sporadic breast cancer even though the mutational profiles can display BRCAness. Furthermore, with drug resistance as a major challenge in cancer treatment, as well as an increasing interest in the PARP-inhibitor and its potential to be used for all cancers showing deficient DSB-repair, it is of great interest to assess methylation as a possible mechanism for drug resistance with treatment of both the PARP-inhibitor olaparib and the chemotherapy doxorubicin. In this study, we aimed to increase the understanding of the biological role of *BRCA1* promoter methylation in breast cancer, by addressing the subject from several angles; 1) Analysis of correlation between methylation in *BRCA1* promoter and *BRCA1* mRNA and protein expression in breast cancer cell lines. 2) Investigate the potential effects of alterations in the *BRCA1* promoter region, including methylation of specific CpGs as well as polymorphism variants, on *BRCA1* promoter strength and ratio between α and β transcripts. 3) Analyze the effect of long term drug treatment on methylation levels in the *BRCA1* promoter.

6.1 *BRCA1* promoter methylation pattern linkage to mRNA and protein expression

In the first part of this study, we wanted to assess the potential links between DNA methylation in the *BRCA1* promoter, RNA and protein expression. The results revealed a large variability in methylation and expression of both RNA and protein levels in a panel of breast cancer cell lines. However, only weak associations could be found between the methylation pattern in the *BRCA1* and the corresponding mRNA levels,

while no associations between methylation pattern and BRCA1 protein expression were observed.

The 66 CpGs analyzed in this study were distributed between nucleotides 1052 upstream to 302 downstream of *BRCA1* transcription start site, covered both the α and the β promoters, as well as a CpG Island located further upstream within the *BRCA1* promoter region. The highest frequency of methylation was observed in the CpG Island for all nine cell lines, which may be expected, considering what is known about hypermethylation of CpG Islands and silencing of tumor suppressor genes such as *BRCA1* in breast cancer (Hasan et al., 2013, Hosny et al., 2016). A considerable variation in the methylation frequency between the cell lines were found; the cell lines UACC-3199 and HCC38 were highly methylated in the whole region studied, while the cell lines BT549 and ZR-75-1 were found to be close to unmethylated in the same region. None of the CpG-positions stood out as completely methylated or unmethylated when comparing all cell lines, thus revealing a highly heterogeneous methylation pattern within the *BRCA1* region, which is in line with previous descriptions (Wilcox et al., 2005, Zhang and Long, 2015, Hansmann et al., 2012).

The qPCR measurements of *BRCA1* mRNA in the breast cancer cell line panel revealed large differences in the expression levels of *BRCA1* WT α and *BRCA1* WT β, with the expression levels of the α-transcript being higher than the β-transcript in all cell lines studied. These findings are consistent with previous studies that report the relative levels of *BRCA1* WT α to be 6-150 times higher than *BRCA1* WT β in tumor tissue and cell lines (Xu et al., 1997, Fernandes et al., 2014). All cell lines, except UACC-3199, expressed the β transcript. In general, this finding is in line with previous findings indicating that the β transcript is only expressed in malignant cells, since all cell lines are derived from breast carcinomas (Sobczak and Krzyzosiak, 2002, Xu et al., 1995). Furthermore, when comparing relative mRNA expression of *BRCA1* WT α to *BRCA1* WT β for each cell line, the expression pattern between the two transcripts seem to be following each other, except for cell line UACC-3199 for which no *BRCA1* WT β mRNA transcript was detected. The correlation for most of the cell lines looks to be strong when the values are plotted in a graph, however, the values did not reach statistical significance, possibly due to low number of observations (figure 5.5). This

suggest that the regulation of α and β not are completely independent, but that the two transcripts might be co-regulated. According to our knowledge, this is a novel finding, not described in existing literature. Moreover, transcription of the pseudo gene, detected by both qPCR and luciferase assay, has been described by one group (Pettigrew et al., 2010). However, none of the cell lines analyzed in this study showed *BRCA1* pseudo α nor β mRNA levels within the detectable range when measured by qPCR analysis.

The findings of a minor association between methylation pattern and *BRCA1* WT total mRNA expression in this study were only partially in line with the data in existing literature. As mentioned above, cell lines UACC-3199 and HCC38 were found to be extensively methylated over the whole studied region, which according to the literature is associated with lower gene expression (Hasan et al., 2013), and indeed this was the findings for the cell line HCC38. However, cell line UACC-3199 had intermediate expressed mRNA levels, not consistent with what would be expected. When looking at the opposite end of the scale, the cell lines BT549 and ZR-75-1 displayed low degree of methylation, expression level would be expected to be higher than for the methylated cell lines, and in fact, both cell lines had higher *BRCA1* WT total mRNA expression levels compared to all but one cell line. Cell line T-47D, which was found to be intermediate methylated, had the highest mRNA expression for both *BRCA1* WT α and β.

Smaller regions and individual CpGs were further studied to connect methylation in transcription factor binding sites to expression levels (relative positions can be found in figure 5.1). However, no obvious correlation was found even though several transcription factor binding sites have previously been reported to be affected by methylation in the promoter region of *BRCA1* (Choudhury et al., 2016, Shin et al., 2013). If the methylation frequency is not taken into consideration, but simply determining if methylation is present or not, then methylation of CpGs 22-24 might be linked to *BRCA1* WT total mRNA levels. No transcription factor binding sites are described in the literature to be in immediately proximity to the position of these CpG sites, however, as the DNA is flexible, these sites have the potential to affect transcription factors binding to sites more distantly away from their own positions. Moreover, even though only a low frequency of methylation was found, methylation at

CpG 56 was consistent with lower *BRCA1* WT total mRNA expression. This CpG is positioned close to an AP-1 binding site involved in *BRCA1* regulation by estrogen trough a non-classical activation pathway (figure 5.1). Other transcription factors previously identified to be affected by methylation in *BRCA1* include Sp1, Egr-1 CTCF, E2F1 and E2F6 and CREB (Choudhury et al., 2016, Xu et al., 2010, Debora et al., 1998), but a direct link to any of these was not found in this study. These findings could be explained by variations between the cell lines, other than methylation status, such as varying expression levels of different transcription factors in the specific cell lines. No correlation was found between the receptor status/breast cancer sub-class and methylation pattern. Usually it is triple negative breast cancer that is associated with BRCAness, however, no correlation was found here.

While the *BRCA1* WT α transcript is found in both normal mammary tissue and cancer tissue, the β transcript is only found to be expressed in breast cancer tissue and to a much lower extent (Xu et al., 1995). Methylation could potentially be involved in the mechanism switching the use from α- to the β-promoter. If methylation were to be involved in this switch, one might expect a setting where the α promoter was hypermethylated and the β.promoter unmethylated, but this was not the case. No prominent pattern in the methylation of the cell lines analyzed in this study support such a mechanism. Thus, no support for this hypothesis was found in this study.

For the cell line UACC-3199, the measured levels of *BRCA1* WT α mRNA were higher than *BRCA1* WT total, which is not logical. However, UACC-3199 is also the cell line with the highest standard deviation, and these uncertainties in the measurements might have affected the final concentrations measured. It is also important to stress that the relative calculations between qPCR assays are based on a theoretical approach, assuming perfect reaction efficacy, while the reaction efficacy may be slightly different between the assays.

With respect to the BRCA1 protein expression analysis, the cell lines that stand out are HCC38, MDA-MB 231 and MCF-7, where HCC38 and MDA-MB 231 show the highest expression and MCF-7 show the lowest expression of protein compared to the rest of the cell lines in the in the cell line panel studied. The cell line MDA-MB 231

had intermediate methylation frequency, while cell lines HCC38 and MCF-7 were found to have high and low methylation frequencies, respectively. The fact that HCC38 has high frequency of methylation as well as high protein expression, and MCF-7 has relatively low degree of methylation and low protein expression is directly opposite of what would be expected in a simple model where epigenetic regulation is directly linked to expression (Wu et al., 2016, Scott et al., 2016). HCC38 and MDA-MB 231 also have low mRNA expression levels, again, making it more difficult to link the increase in protein of these samples to the methylation pattern and mRNA levels, as well as to the data obtained for the rest of the breast cancer cell lines. Similarly, MCF7, has a low degree of methylation, medium high mRNA expression, but close to no protein expression. While this may seem counter-intuitive, there are several possible explanations including differences in the transcription- and translation factors expressed in the various cell lines, as well as differences in factors affecting mRNA stability and protein degradation. In sum, there might be a link between methylation and mRNA expression, but the two extremes of methylation frequency were not clearly reflected in the mRNA expression levels.

6.1.1 Methodical considerations for the experiments performed to link methylation frequency to expression data

A total of 20 successfully sequenced colonies were obtained for each cell line, which should be sufficient to establish the biological basis of the methylation pattern in the *BRCA1* promoter the in samples. Additionally, the samples are of low heterozygosity, which support the need of fewer numbers of individual molecules analyzed. The bisulfite conversion efficiency was found to be 95.63%, which is above the threshold (99.5%) set by the manufacturer of the kit. Furthermore, even though the aim of this part of the study was to search for potential links between DNA methylation, RNA and protein in cancer cell lines, it would have been very useful to have included a benign breast cell line, like the MCF10A, as a control sample, to be able to compare the methylation pattern, mRNA and protein expression from the cell lines harvested from breast cancer patients, with a non-cancer setting. However, even if the MCF10A cell line arise from normal breast tissue, the cells are immortalized and it is therefore uncertain whether they truly reflect normal breast tissue. Furthermore, western blot analysis without ghost bands and darker regions should ideally be obtained, but limiting

sample material of cell line UACC-3199 did not permit a new SDS-PAGE run to be performed. Aliquots of this cell line were collected and frozen in liquid nitrogen and could have thawed for propagation and harvesting for sample material, but as the cell line was extremely difficult to grow, this was not prioritized.

6.2 Modification of the site-specific methylation protocol

One of the sub aims of this study was to introduce methylation in specific positions within the *BRCA1* promoter and investigate if this would affect the promoter strength and ratio of α and β transcript by performing luciferase assay. However, the selected protocol for introduction of site-specific methylation turned out to be challenging to perform, and constructs with methylation in the wanted positions were never successfully obtained. The protocol was initially performed as described in the article "Site-specific reporter construct for functional analysis of DNA methylation" by Han and colleagues (Han et al., 2013), and additional information was obtained by personal correspondence with the author. However, agarose gel analysis of product after the second step of the protocol, which involved digestion of parental and un-ligated DNA, showed the presence of DNA before exonuclease treatment, but not after. These results indicated a problem with the ligation of the PCR product in the first step in the protocol.

Several modifications were tried to optimize the combined PCR and ligation reaction, including excess of ligase in the reaction mixture, two types of ligases along with polynucleotide kinase, as well as addition of ligase directly after the PCR-program was finished. DNA loss was minimized by reducing the numbers of clean up steps and different purification protocols were attempted, for selection of the protocol with the highest yield. Furthermore, the duration of exonuclease treatment was reduced from overnight to only 30 minutes. Nevertheless, none of these steps resulted in a visible product when analyzing post-exonuclease samples on agarose gel. The Taq DNA ligase, used both in the experiments for this thesis and in the experiments described in the original paper, is active at temperatures 45-65°C according to the manufacturer. However, PCR cycling includes much higher temperatures, and the enzyme would need to tolerate temperatures up to 98°C. Han and colleagues do not mention this in their description of the method, and seem to have got the reaction to work satisfactory, but we consider this to be a likely cause for the failure of the method.

6.3 Polymorphic variants affecting the expression of *BRCA1* assessed by Luciferase assay

Due to unsuccessful attempts to introduce site specific methylation, the focus was shifted to only assessing the effect of the polymorphisms rs71361504 and rs799905 in the *BRCA1* promoter on expression. The naturally existing variants, detected in a pooled blood sample from five healthy individuals, differed in two positions, thus intermediate variants were made to be able to determine the effect of each position separately. *BRCA1* promoter-pGL4.10[*luc2*] variant 1.1 and 2.1 exist naturally and were found to have equal expression of luciferase reporter vector, while variant 1.2 and 2.2, both generated in the lab, showed significantly lower luciferase expression. The two naturally occurring variants seem to counterbalance the effect of each other, resulting in an equal *BRCA1* expression. SNP rs799905 lies with in a CpG, if the G-allele is present, then a possible methylation site is deleted (figure 5.1) (Hansmann et al., 2012). This SNP is in addition located in close proximity to several TF binding sites, and thus, has the potential to affect transcription depending on whether the methylation site is present or not, as well as if it is methylated or not. Possible TF binding sites that can be affected by this SNP are SP1, AP1 and CREB, some of which is known to be affected by methylation (Choudhury et al., 2016).

Polymorphism rs71361504 is described in relation to endometriosis, but not in relation to breast cancer or methylation (Govatati et al., 2015). Furthermore, current literature has described transcription factor binding sites in the α and β promoter, while this polymorphism is located closer to the CpG Island, thus little was found regarding possible effects on transcription by the presence/absence of the trinucleotides AAC. However, when searching in the transcription factor binding site database JASPAR, three additional TF binding sites were found when the trinucleotides AAC were present in the sequence; namely SRY, SOX10 and NFIC. The existence of variants 1.2 and 2.2 in the population is not described in the literature. If these versions do not exist, a possible explanation could be that when these polymorphic variant combinations are present in the genome, the expression of *BRCA1* is too low, resulting in a non-viable offspring. However, if the version does exist, more knowledge would be needed to potentially link these to disease risk.

6.3.1 Methodical considerations for the assessment of polymorphic variants

In the original protocol for luciferase assay Dual-Glo® Luciferase Assay System, both firefly and Renilla luciferase luminescence signals are measured. However, in this study, eGFP was chosen for normalization as previous experiments in the lab have shown bleed-trough of the Renilla signal.

MCF7 was selected for this assay as transfection protocols were already established for this cell line in the lab. The strength of the findings would have increased if the experiments were repeated for several additional cell lines, however due to time limitations this was not feasible.

6.4 Long term treatment of SKBR3 with doxorubicin and olaparib for methylation status assessment

The aim of the third section of the presented work was to investigate the effect of long term treatment with olaparib and doxorubicin on the methylation status in the *BRCA1* promoter of the breast cancer cell line SKBR3. It was of interest to assess whether long term treatment with olaparib or doxorubicin, pushing the cell culture towards drug resistance, could potentially cause demethylation and subsequent upregulation of *BRCA1*, as a possible mechanism of drug resistance.

NGS analysis, performed on the selected harvests from the long-term treatment experiment, showed only minor effects on the *BRCA1* methylation pattern. A small decrease in methylation was detected over time, however, this slight trend was also observed for the control (DMSO) sample. Calculations of the ratio for the last harvesting versus the harvesting at time point zero show that the demethylation pattern of both backup lines look very similar to the DMSO control, while the experiment lines for both drugs showed a slightly more prominent demethylation pattern. As such, the overall impression from these experiments is that the minor changes observed are linked to general development of the cell cultures rather than drug specific effects. DMSO is also slightly toxic to the cells, and can potentially affect the methylation pattern over time.

Even though only a small difference is seen for experiment lines compared to backup lines and DMSO, it might be possible that this trend could be more evident if the cells had been treated with a higher dose and/or for a longer period of time. The first increase in concentration was done only after four weeks of incubation with the initial drug doses. The drug concentrations were then increased gradually until maximum dose were reached, detected when proliferation drastically decreased. A more slow and steady increase, as well as treatment over an even longer period could possibly have allowed a higher max concentration, and maybe, a more visible effect could have been detected. However, in this experiment, the differences are too small to suggest that the long term drug treatment causes demethylation.

SKBR3 was chosen as the model cell line for this experiment because it has previously been reported to be methylated in the promoter region of *BRCA1* (Cai et al., 2014). Ideally, the selection of an appropriate cell line should have been based on own findings. However, due to time limitations for the project, a cell line had to be chosen based on literature search, prior to own methylation-screen of cell lines. When the methylation data from the cloning experiment with the nine breast cancer cell lines were obtained, SKBR3 were found to be methylated only in the CpG Island region of the promoter. The relatively low frequency of methylation in this cell line in the first place, limited the observations to the CpG Island and approximately 20 CpG sites. This is a potential drawback of the experimental set up. If this methylation data (figure 5.2) had been obtained prior to starting the long-term experiment, the cell line UACC-3199 or HCC38 may have been used instead of SKBR3, as these were methylated in the whole region studied.

6.4.1 Methodical considerations for the long term cell culture experiments

The results of the NGS analysis performed by the use of Roche NimbleGen Seq Cap Epi Enrichment Systems with a custom-made probe design showed a bisulfite conversion rate of 99.45%. Although this is just below what is usually recommended as standard conversion rate (99.5%), it should not cause any ambiguity in the data. When comparing the methylation pattern of cell line SKBR3 for the cloning experiment and the NGS experiment (untreated sample), the two different approaches give the same results, strengthening the data of both experiments.

The duplication rate was below 10% for all samples, except one. This may seem like a high duplication rate compared to sequencing of unmodified DNA. However, for bisulfite converted samples, such numbers still indicate libraries of high quality. The sequencing data for the *BRCA1* promoter showed that the average capture target coverage for all samples, except one, were above 60x. In the ideal setting, one may always wish for a higher coverage, but the amount of data generated in this study is a compromise between the number of reads per nucleotide, size of target region and the time/cost issues. It is arguable that 60x is adequate coverage for this project since the data are collected from one cell line (low heterozygosity of the sample) and the aim was to investigate changes over time. On the other hand, the study focuses on methylation frequency, meaning that the results will be detected as continuous values and not merely negative, 50% or positive. To re-run the sample libraries to increase the resolution could be a future perspective.

Compared to all other samples, the sample named "Dox, week 3", had a much lower coverage and higher duplication rate, despite using recommended amount of input DNA in the library preparation. The sample was run twice, but the same results were obtained in both cases. The identical index (named 002) was used in both library preparations, in order to be able to combine the reads from the two runs in the bioinformatical pipeline after sequencing. When measuring the concentration of index sample 002 solution, it turned out to be much lower compared to other randomly selected indexes shown to provide adequate sequencing results. The low DNA concentration of this index most likely explains the low quality parameters observed for "Dox, week 3". Further, even though this sample does not meet all the quality criteria, the final heat map does not seem be affected by the lowered confidence of the results that this sample is based on.

In the context of methylation, mapping of the resulting reads after NGS is extra challenging because a target region could be fully methylated, partly methylated or unmethylated. An exponential relationship exists between the number of methylation sites and the theoretical possible combinations of methylations patterns. In addition, after the bisulphite conversion of the DNA the upper and lower strand are no longer complementary, this doubles the size of the genome and makes the mapping even more

complex and time consuming. The program Bismap used is this experiment, is among most accurate program in current use (Xi and Li, 2009).

It is important to keep in mind that several techniques excite for detection of methylation, as described in the inductions (section 1.4.2 and 1.4.3each with its own strength and weaknesses, and comparison of the resulting data can therefore be challenging. This includes methylation data obtained both in this experiment, as well as date data found in existing literature.

6.5 Conclusion

Being aware of the significant number of people that are affected by breast cancer worldwide, and in recognizing the huge effect epigenetics can have in disease development, it is of great interest to increase the understanding of *BRCA1* promoter methylation. This study aimed to investigate the effect of CpG methylation and polymorphic variants, in the promoter of *BRCA1,* on *BRCA1* expression, as well as the effect of long term drug treatment on methylation levels in the *BRCA1* promoter.

In this study, only a weak correlation could be found between the *BRCA1* methylation pattern and *BRCA1* mRNA expression, and no correlation was found between the methylation pattern and expressed protein, or between mRNA levels and protein expression. Furthermore, analysis of construct harboring site-specific methylation was never performed successfully, but the effects of two polymorphic variants rs71361504 and rs799905 were analyzed by luciferase assay. The two naturally occurring versions differing in two positions counterbalanced the effect of each other, both showing equal levels of *BRCA1* expression as measured by luciferase reporter construct. The two intermediate versions, generated in the lab, had significantly lower *BRCA1* expression. To our knowledge, these are not known to existing naturally. Finally, long term drug treatment was not linked to demethylation of the promoter region of *BRCA1*, and thus demethylation could not found to associated to the cells ability to survive drug treatment in the experiment setup tested in this study.

6.6 Future perspectives

Although *BRCA1* is one of the best characterized tumor suppressor genes, the vast majority of research efforts has been focused on mutations and hereditability of mutations in the gene. Much remains unknown regarding other mechanisms of *BRCA1* inactivation. As such, further research on *BRCA1* methylation is highly warranted, together with research on other potential inactivation mechanisms, such as translocations of the gene, deregulation of *BRCA1*, affecting microRNA etc.

Regarding the specific experiments performed in this thesis, there are several lines of future work that could be followed; the experiments performed in this study comparing *BRCA1* promoter methylation patterns to *BRCA1* expression showed that there might be a link between methylation of some CpGs and mRNA expression, but no clear relationship was observed in the chosen panel of breast cancer cell lines. A larger study including patient samples and healthy controls would be of interest to get a better understanding of which methylation sites are of high importance for mRNA (and protein) expression. In a potentially extended study, it would be of interest to include both normal tissue of relevance, and biopsy samples from breast or ovarian cancer patients (the two cancer forms strongest linked to *BRCA1* deficiency). Furthermore, it would be interesting to assess the degradation rate of the α and β transcripts by performing stability assays. This could be performed by addition of a transcription blockage and subsequent performance of qPCR measurements at various time points.

Studies based on site specific methylated construct is a very attractive model for studying of the impact of *BRCA1* promoter methylation. Therefore, in the future, more efforts should be put into making the site-specific methylation protocol to work for this promoter. A tempting next step would be to search for a more thermostable ligase known to withstand PCR conditions. Another solution could be to try alternative methods to generate vectors with site-specific methylation, including methods that are mentioned in the article form Han et al, for instance one protocol that used siRNA from yeast and plants. However, according to Han and collogues, these methods have lower precision and are more time consuming (Han et al., 2013). Recently the CRISPR/Cas9 method had been modified to be able to add or remove methyl groups at specific positions in the DNA (Liu et al., 2016). This could be an exciting approach worth trying,

since it would provide a model system where *BRCA1* may be expressed at levels close to the physiological ones, and one would not need to rely on transfections and overexpression experiments. Regarding the polymorphisms studied, only two of the four combinations were known to occur naturally, while the two others were made in the lab. If the two latter exist in nature, it would be of great interest to assess their function further in a high number of human samples and to test the correlation between these polymorphic variants and cancer risk.

The long term experiment did not show a strong link between treatment with olaparib or doxorubicin and demethylation of the *BRCA1* promoter region. In a potential new experiment, it would be interesting to use several additional cell lines, all with higher degree of methylation as well as a more empirical reasoned treatment plan and potentially increased drug doses. A goal would be to cover as much of the *BRCA1* promoter region as possible, as well as covering other regions potentially involved in *BRCA1* regulation. Additionally, the analysis could be designed to include other genes involved in HR repair. Moreover, an overview of methylation position and frequency from tumor material of patients that have undergone cancer treatment would be of great interest to select for CpGs of high impact, and potentially connect these to change in methylation pattern over time.

7 Referenses

Ali, A. B., Iau, P. T. & Sng, J. H. 2011. Cancer-specific methylation in the BRCA1 promoter in sporadic breast tumours. *Medical Oncology,* 28**,** 64-6.

Antoniou, A., Pharoah, P. D. P., Narod, S., Risch, H. A., Eyfjord, J. E., Hopper, J. L., Loman, N., Olsson, H., Johannsson, O., Borg, Å., Pasini, B., Radice, P., Manoukian, S., Eccles, D. M., Tang, N., Olah, E., Anton-Culver, H., Warner, E., Lubinski, J., Gronwald, J., Gorski, B., Tulinius, H., Thorlacius, S., Eerola, H., Nevanlinna, H., Syrjäkoski, K., Kallioniemi, O. P., Thompson, D., Evans, C., Peto, J., Lalloo, F., Evans, D. G. & Easton, D. F. 2003. Average Risks of Breast and Ovarian Cancer Associated with BRCA1 or BRCA2 Mutations Detected in Case Series Unselected for Family History: A Combined Analysis of 22 Studies. *The American Journal of Human Genetics,* 72**,** 1117-1130.

Birgisdottir, V., Stefansson, O. A., Bodvarsdottir, S. K., Hilmarsdottir, H., Jonasson, J. G. & Eyfjord, J. E. 2006. Epigenetic silencing and deletion of the BRCA1 gene in sporadic breast cancer. *Breast Cancer Research,* 8**,** R38-R38.

Brodie, K. M. & Henderson, B. R. 2010. Differential modulation of BRCA1 and BARD1 nuclear localisation and foci assembly by DNA damage. *Cell Signal,* 22**,** 291-302.

Buell, P. 1973. Changing incidence of breast cancer in Japanese-American women. *Journal of the National Cancer Institute,* 51**,** 1479-83.

Buyru, N., Altinisik, J., Ozdemir, F., Demokan, S. & Dalay, N. 2009. Methylation Profiles in Breast Cancer. *Cancer Investigation,* 27**,** 307-312.

Cai, F., Ge, I., Wang, M., Biskup, E., Lin, X. & Zhong, X. 2014. Pyrosequencing analysis of BRCA1 methylation level in breast cancer cells. *Tumor Biology,* 35**,** 3839-3844.

Catteau, A. & Morris, J. R. 2002. BRCA1 methylation: a significant role in tumour development? *Seminars in Cancer Biology,* 12**,** 359-371.

Chenevix-Trench, G., Healey, S., Lakhani, S., Waring, P., Cummings, M., Brinkworth, R., Deffenbaugh, A. M., Burbidge, L. A., Pruss, D., Judkins, T., Scholl, T., Bekessy, A., Marsh, A., Lovelock, P., Wong, M., Tesoriero, A., Renard, H., Southey, M., Hopper, J. L., Yannoukakos, K., Brown, M., Easton, D., Tavtigian, S. V., Goldgar, D. & Spurdle, A. B. 2006. Genetic and histopathologic evaluation of BRCA1 and BRCA2 DNA sequence variants of unknown clinical significance. *Cancer research,* 66**,** 2019.

Cho, Y. H., Yazici, H., Wu, H. C., Terry, M. B., Gonzalez, K., Qu, M., Dalay, N. & Santella, R. M. 2010. Aberrant promoter hypermethylation and genomic hypomethylation in tumor, adjacent normal tissues and blood from breast cancer patients. *Anticancer Res,* 30**,** 2489-96.

Choudhury, S. R., Cui, Y., Lubecka, K., Stefanska, B. & Irudayaraj, J. 2016. CRISPR-dCas9 mediated TET1 targeting for selective DNA demethylation at BRCA1 promoter. *Oncotarget,* 7**,** 46545-46556.

Curigliano, G., Romieu, G., Campone, M., Dorval, T., Duck, L., Canon, J. L., Roemer-Becuwe, C., Roselli, M., Neciosup, S., Burny, W., Callegaro, A., De Sousa Alves, P. M., Louahed, J., Brichard, V. & Lehmann, F. F. 2016. A phase I/II trial of the safety and clinical activity of a HER2-protein based immunotherapeutic for treating women with HER2-positive metastatic breast cancer. *Breast Cancer Research and Treatment,* 156**,** 301-10.

Davies, H., Glodzik, D., Morganella, S., Yates , L. R., Staaf, J., Zou, X., Ramakrishna, M., Martin, S., Boyault, S., Sieuwerts, A. M., Simpson, P. T., King, T. A., Raine, K., Jorunn, E. E., Gu, K., Åke, B., Ewan, B., Hendrik, G. S., Marc, J. V. D. V., Anne-Lise, B.-D., John, W. M. M., Paul, N. S., Sunil, R. L., Anne, V.-S., Christos, S., Andrew, T., Alastair, M. T., Steven Van, L., Andrea, L. R., Alain, V., Peter, J. C., Michael, R. S. & Serena, N.-Z. 2017. HRDetect is a predictor of BRCA1 and BRCA2 deficiency based on mutational signatures. *Nature Medicine*.

De Siervi, A., De Luca, P., Byun, J. S., Di, L. J., Fufa, T., Haggerty, C. M., Vazquez, E., Moiola, C., Longo, D. L. & Gardner, K. 2010. Transcriptional autoregulation by BRCA1. *Cancer research,* 70**,** 532.

Debora, N. M., David, I. R., Peter, J. A., Frances, P. O. M., Shiva, M. S., Weirong, X. & Trevor, K. A. 1998. CpG methylation within the 5′ regulatory region of the BRCA1 gene is tumor specific and includes a putative CREB binding site. *Oncogene,* 16**,** 1161.

Delpu, Y., Cordelier, P., Cho, W. C. & Torrisani, J. 2013. DNA methylation and cancer diagnosis. *International Journal of Molecular Science,* 14**,** 15029-58.

Desantis, C. E., Fedewa, S. A., Goding Sauer, A., Kramer, J. L., Smith, R. A. & Jemal, A. 2016. Breast cancer statistics, 2015: Convergence of incidence rates between black and white women. *CA: A Cancer Journal for Clinicians,* 66**,** 31-42.

Eden, A., Gaudet, F., Waghmare, A. & Jaenisch, R. 2003. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science (New York, N.Y.),* 300**,** 455.

Elstrodt, F., Hollestelle, A., Nagel, J. H., Gorin, M., Wasielewski, M., Van Den Ouweland, A., Merajver, S. D., Ethier, S. P. & Schutte, M. 2006. BRCA1 mutation analysis of 41 human breast cancer cell lines reveals three new deleterious mutants. *Cancer research,* 66**,** 41-5.

Esteller, M., Corn, P. G., Baylin, S. B. & Herman, J. G. 2001. A gene hypermethylation profile of human cancer. *Cancer research,* 61**,** 3225.

Esteller, M., Silva, J. M., Dominguez, G., Bonilla, F., Matias-Guiu, X., Lerma, E., Bussaglia, E., Prat, J., Harkes, I. C., Repasky, E. A., Gabrielson, E., Schutte, M., Baylin, S. B. & Herman, J. G. 2000. Promoter Hypermethylation and BRCA1 Inactivation in Sporadic Breast and Ovarian Tumors. *Journal of the National Cancer Institute,* 92**,** 564-569.

Ewald, I. P., Ribeiro, P. L., Palmero, E. I., Cossio, S. L., Giugliani, R. & Ashton-Prolla, P. 2009. Genomic rearrangements in BRCA1 and BRCA2: A literature review. *Genet Mol Biol,* 32**,** 437-46.

Fernandes, L., Costa, E., Vargas, F. & Moreira, M. 2014. Influence of estrogen and variations at the BRCA1 promoter region on transcription and translation. *An International Journal on Molecular and Cellular Biology,* 41**,** 489-495.

Fong, P. C., Yap, T. A., Boss, D. S., Carden, C. P., Mergui-Roelvink, M., Gourley, C., De Greve, J., Lubinski, J., Shanley, S., Messiou, C., A'hern, R., Tutt, A., Ashworth, A., Stone, J., Carmichael, J., Schellens, J. H., De Bono, J. S. & Kaye, S. B. 2010. Poly(ADP)-ribose polymerase inhibition: frequent durable responses in BRCA carrier ovarian cancer correlating with platinum-free interval. *Journal of Clinical Oncology,* 28**,** 2512-9.

Foo, J. & Michor, F. 2009. Evolution of resistance to targeted anti-cancer therapies during continuous and pulsed administration strategies. *PLOS Computational Biology,* 5**,** e1000557.

Forbes, S. A., Beare, D., Boutselakis, H., Bamford, S., Bindal, N., Tate, J., Cole, C. G., Ward, S., Dawson, E., Ponting, L., Stefancsik, R., Harsha, B., Kok, C. Y., Jia, M., Jubb, H., Sondka, Z., Thompson, S., De, T. & Campbell, P. J. 2017. COSMIC: somatic cancer genetics at high-resolution. *Nucleic Acids Res,* 45**,** D777-d783.

Fridlyand, J., Snijders, A. M., Ylstra, B., Li, H., Olshen, A., Segraves, R., Dairkee, S., Tokuyasu, T., Ljung, B. M. & Jain, A. N. 2006. Breast tumor copy number aberration phenotypes and genomic instability. *BMC cancer,* 6**,** 96.

Frommer, M., Mcdonald, L. E., Millar, D. S., Collis, C. M., Watt, F., Grigg, G. W., Molloy, P. L. & Paul, C. L. 1992. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proceedings of the National Academy of Science of the USA,* 89**,** 1827-31.

Gaal, Z. & Olah, E. 2014. [Epigenetic regulatory mechanisms and their disorders in leukemia]. *Magyar Onkology,* 58**,** 99-107.

Galizia, E., Giorgetti, G., Piccinini, G., Santinelli, A., Loretelli, C., Bianchi, F., Gagliardini, D., Carbonari, G., Pisa, E. & Belvederesi, L. 2010. BRCA1 expression in triple negative sporadic breast cancers. *Analytical and Quantitative Cytology and Histology,* 32**,** 24-29.

Gama-Sosa, M. A., Slagel, V. A., Trewyn, R. W., Oxenhandler, R., Kuo, K. C., Gehrke, C. W. & Ehrlich, M. 1983. The 5-methylcytosine content of DNA from human tumors. *Nucleic acids research,* 11**,** 6883.

Govatati, S., Challa, K., Reddy, S. B., Pramod, K., Deenadayal, M., Chakravarty, B., Shivaji, S. & Bhanoori, M. 2015. BRCA1 alterations are associated with endometriosis, but BRCA2 alterations show no detectable endometriosis risk: a study in Indian population. *Journal of Assisted Reproductian and Genetics,* 32**,** 277-85.

Greenman, C., Stephens, P., Smith, R., Dalgliesh, G. L., Hunter, C., Bignell, G., Davies, H., Teague, J., Butler, A., Stevens, C., Edkins, S., O'meara, S., Vastrik, I., Schmidt, E. E., Avis, T., Barthorpe, S., Bhamra, G., Buck, G., Choudhury, B., Clements, J., Cole, J., Dicks, E., Forbes, S., Gray, K., Halliday, K., Harrison, R., Hills, K., Hinton, J., Jenkinson, A., Jones, D., Menzies, A., Mironenko, T., Perry, J., Raine, K., Richardson, D., Shepherd, R., Small, A., Tofts, C., Varian, J., Webb, T., West, S., Widaa, S., Yates, A., Cahill, D. P., Louis, D. N., Goldstraw, P., Nicholson, A. G., Brasseur, F., Looijenga, L., Weber, B. L., Chiew, Y. E., Defazio, A., Greaves, M. F., Green, A. R., Campbell, P., Birney, E., Easton, D. F., Chenevix-Trench, G., Tan, M. H., Khoo, S. K., Teh, B. T., Yuen, S. T., Leung, S. Y., Wooster, R., Futreal, P. A. & Stratton, M. R. 2007. Patterns of somatic mutation in human cancer genomes. *Nature,* 446**,** 153-8.

Hall, J. M., Lee, M. K., Newman, B., Morrow, J. E., Anderson, L. A., Huey, B. & King, M.-C. 1990. Linkage of Early-Onset Familial Breast Cancer to Chromosome 17q21. *Science,* 250**,** 1684-1689.

Hampton, G. M., Mannermaa, A., Winquist, R., Alavaikko, M., Blanco, G., Taskinen, P. J., Kiviniemi, H., Newsham, I., Cavenee, W. K. & Evans, G. A. 1994. Loss of heterozygosity in sporadic human breast carcinoma: a common region between 11q22 and 11q23. 3. *Cancer research,* 54**,** 4586-4589.

Han, W., Shi, M. & Spivack, S. D. 2013. Site-specific methylated reporter constructs for functional analysis of DNA methylation. *Epigenetics,* 8**,** 1176-1187.

Hanahan, D. & Weinberg, R. A. 2000. The Hallmarks of Cancer. *Cell,* 100**,** 57-70.

Hanahan, D. & Weinberg, Robert a. 2011. Hallmarks of Cancer: The Next Generation. *Cell,* 144**,** 646-674.

Hansmann, T., Pliushch, G., Leubner, M., Kroll, P., Endt, D., Gehrig, A., Preisler-Adams, S., Wieacker, P. & Haaf, T. 2012. Constitutive promoter methylation of BRCA1 and RAD51C in patients with familial ovarian cancer and early-onset sporadic breast cancer. *Human molecular genetics,* 21**,** 4669.

Haoyang, L. 2013. DNA methylation, a hand behind neurodegenerative disease. *Frontiers in Aging Neuroscience,* 5**,** NA-NA.

Hasan, T. N., Leena Grace, B., Shafi, G. & Syed, R. 2013. Association of BRCA1 promoter methylation with rs11655505 (c.2265C>T) variants and decreased gene expression in sporadic breast cancer. *Clinical Translational Oncology,* 15**,** 555-62.

Hayatsu, H. 2008. Discovery of bisulfite-mediated cytosine conversion to uracil, the key reaction for DNA methylation analysis-A personal account. *Proceedings of the Japan Academy Ser. B, Physical and Biological Science.*

Hockings, J. K., Degner, S. C., Morgan, S. S., Kemp, M. Q. & Romagnolo, D. F. 2008. Involvement of a specificity proteins-binding element in regulation of basal and estrogen-induced transcription activity of the BRCA1 gene. *Breast Cancer Research,* 10**,** R29-R29.

Hollestelle, A., Nagel, J. H., Smid, M., Lam, S., Elstrodt, F., Wasielewski, M., Ng, S. S., French, P. J., Peeters, J. K., Rozendaal, M. J., Riaz, M., Koopman, D. G., Ten Hagen, T. L., De Leeuw, B. H., Zwarthoff, E. C., Teunisse, A., Van Der Spek, P. J., Klijn, J. G., Dinjens, W. N., Ethier, S. P., Clevers, H., Jochemsen, A. G., Den Bakker, M. A., Foekens, J. A., Martens, J. W. & Schutte, M. 2010. Distinct gene mutation profiles among luminal-type and basal-type breast cancer cell lines. *Breast Cancer Research and Treatment,* 121**,** 53-64.

Holliday, D. L. & Speirs, V. 2011. Choosing the right cell line for breast cancer research. *Breast Cancer Research,* 13**,** 215.

Hosny, M. M., Sabek, N. A., El-Abaseri, T. B., Hassan, F. M. & Farrag, S. H. 2016. Promoter Methylation Status of Breast Cancer Susceptibility Gene 1 and 17 Beta Hydroxysteroid Dehydrogenase Type 1 Gene in Sporadic Breast Cancer Patients. *International Journal of Breast Cancer,* 2016.

Iarc, W. G. 2012. Estimated cancer incidence, mortality and prevalence worldwide in 2012. 2012. *Reference Source.*

Iglehart, J. D. & Silver, D. P. 2009. Synthetic lethality--a new direction in cancer-drug development. *New England Journal of Medicine,* 361**,** 189-91.

Ignatov, T., Poehlmann, A., Ignatov, A., Schinlauer, A., Costa, S., Roessner, A., Kalinski, T. & Bischoff, J. 2013. BRCA1 promoter methylation is a marker of better response to anthracycline-based therapy in sporadic TNBC. *Breast Cancer Research and Treatment,* 141**,** 205-212.

Jeffy, B. D., Hockings, J. K., Kemp, M. Q., Morgan, S. S., Hager, J. A., Beliakoff, J., Whitesell, L. J., Bowden, G. T. & Romagnolo, D. F. 2005. An estrogen receptor-alpha/p300 complex activates the BRCA-1 promoter at an AP-1 site that binds Jun/Fos transcription factors: repressive effects of p53 on BRCA-1 transcription. *Neoplasia (New York, N.Y.),* 7**,** 873.

Jones, P. A. 2012. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature Reviews Genetics,* 13**,** 484.

Kloten, V., Becker, B., Winner, K., Schrauder, M. G., Fasching, P. A., Anzeneder, T., Veeck, J., Hartmann, A., Knuchel, R. & Dahl, E. 2013. Promoter hypermethylation of the tumor-suppressor genes ITIH5, DKK3, and RASSF1A as novel biomarkers for blood-based breast cancer screening. *Breast Cancer Research,* 15**,** R4.

Kreftforeningen.No. Available: https://kreftforeningen.no/ [Accessed 15.05.17 2017].

Kreftregisteret.No. 2017. *Cancer in Norway 2015* [Online]. Elisabeth Jakobsen. Available: https://www.kreftregisteret.no/Registrene/Kreftstatistikk/ [Accessed 12.02.17 2017].

Kulis, M. & Esteller, M. 2010. DNA methylation and cancer. *Adv Genet,* 70**,** 27-56.

Li, E., Bestor, T. H. & Jaenisch, R. 1992. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell,* 69**,** 915-926.

Ling, B., Watt, K., Banerjee, S., Newsted, D., Truesdell, P., Adams, J., Sidhu, S. S. & Craig, A. W. 2017. A novel immunotherapy targeting MMP-14 limits hypoxia, immune suppression and metastasis in triple-negative breast cancer models. *Oncotarget*.

Lips, E. H., Mulder, L., Oonk, A., Van Der Kolk, L. E., Hogervorst, F. B., Imholz, A. L., Wesseling, J., Rodenhuis, S. & Nederlof, P. M. 2013. Triple-negative breast cancer: BRCAness and concordance of clinical features with BRCA1-mutation carriers. *Br J Cancer,* 108**,** 2172-7.

Liu, X. S., Wu, H., Ji, X., Stelzer, Y., Wu, X., Czauderna, S., Shu, J., Dadon, D., Young, R. A. & Jaenisch, R. 2016. Editing DNA Methylation in the Mammalian Genome. *Cell,* 167**,** 233-247.e17.

Lonning, P. E. & Knappskog, S. 2013. Mapping genetic alterations causing chemoresistance in cancer: identifying the roads by tracking the drivers. *Oncogene,* 32**,** 5315-30.

Ma, J. & Jemal, A. 2013. Breast cancer statistics. *Breast Cancer Metastasis and Drug Resistance.* Springer.

Mancini, D. N., Rodenhiser, D. I., Ainsworth, P. J., O'malley, F. P., Singh, S. M., Xing, W. & Archer, T. K. 1998. CpG methylation within the 5' regulatory region of the BRCA1 gene is tumor specific and includes a putative CREB binding site. *Oncogene,* 16**,** 1161-9.

Miki, Y., Swenson, J., Yakumo, K., Lewis, C., Neuhausen, S. & Goldgar, D. 1994. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science,* 266**,** 66-71.

Miller, R. E. & Ledermann, J. A. 2016. The status of poly(adenosine diphosphate-ribose) polymerase (PARP) inhibitors in ovarian cancer, part 1: olaparib. *Clinical Advances in Hematology and Oncology,* 14**,** 619-27.

Mueller, C. R. & Roskelley, C. D. 2003. Regulation of BRCA1 expression and its relationship to sporadic breast cancer. *Breast Cancer Research,* 5**,** 45-52.

Murata, S., Zhang, C., Finch, N., Zhang, K., Campo, L. & Breuer, E. K. 2016. Predictors and Modulators of Synthetic Lethality: An Update on PARP Inhibitors and Personalized Medicine. *Biomed Res Int,* 2016**,** 2346585.

Nguyen, A., Rauch, T. A., Pfeifer, G. P. & Hu, V. W. 2010. Global methylation profiling of lymphoblastoid cell lines reveals epigenetic contributions to autism spectrum disorders and a novel autism candidate gene, RORA, whose protein product is reduced in autistic brain. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* 24**,** 3036.

Niethammer, D. & Bruchelt, G. 1998. Anthracycline-derived chemotherapeutics in apoptosis and free radical cytotoxicity (Review). *International journal of molecular medicine,* 1**,** 491-494.

Perou, C., Sorlie, T., Eisen, M. & Van De Rijn, M. 2000. Molecular portraits of human breast tumours. *Nature,* 406**,** 747-52.

Pettigrew, C. A., French, J. D., Saunus, J. M., Edwards, S. L., Sauer, A. V., Smart, C. E., Lundstrom, T., Wiesner, C., Spurdle, A. B., Rothnagel, J. A. & Brown, M. A. 2010. Identification and functional analysis of novel BRCA1 transcripts, including mouse Brca1-Iris and human pseudo-BRCA1. *Breast Cancer Research and Treatment,* 119**,** 239-47.

Rafii, S., Gourley, C., Kumar, R., Geuna, E., Ang, J. E., Rye, T., Chen, L. M., Shapira-Frommer, R., Friedlander, M., Matulonis, U., De Greve, J., Oza, A. M., Banerjee, S., Molife, L. R., Gore, M. E., Kaye, S. B. & Yap, T. A. 2017. Baseline clinical predictors of antitumor response to the PARP inhibitor olaparib in germline BRCA1/2 mutated patients with advanced ovarian cancer. *Oncotarget*.

Rahman, N. & Stratton, M. R. 1998. The genetics of breast cancer susceptibility. *Annual review of genetics,* 32**,** 95-121.

Rice, J., C., Massey-Brown, K., S. & Futscher, B., W. 1998. Aberrant methylation of the BRCA1 CpG island promoter is associated with decreased BRCA1 mRNA in sporadic breast cancer cells. *Oncogene,* 17**,** 1807.

Rice, J. C., Ozcelik, H., Maxeiner, P., Andrulis, I. & Futscher, B. W. 2000. Methylation of the BRCA1 promoter is associated with decreased BRCA1 mRNA levels in clinical breast cancer specimens. *Carcinogenesis,* 21**,** 1761-1765.

Riggs, A. D. 1975. X inactivation, differentiation, and DNA methylation. *Cytogenetic and Genome Research,* 14**,** 9-25.

Rohini, R., Jarin, C. & Simon, N. P. 2011. BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nature Reviews Cancer,* 12**,** 68.

Ronit, I. Y., Sherly, P.-R., Magda, S., Kenneth, H. C. & Lawrence, C. B. 2002. BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage. *Nature Genetics,* 30**,** 285.

Savage, K. I. & Harkin, D. P. 2015. BRCA 1, a 'complex' protein involved in the maintenance of genomic stability.

Scott, C. M., Joo, J. E., O'callaghan, N., Buchanan, D. D., Clendenning, M., Giles, G. G., Hopper, J. L., Wong, E. M. & Southey, M. C. 2016. Methylation of Breast Cancer Predisposition Genes in Early-Onset Breast Cancer: Australian Breast Cancer Family Registry.(Research Article). *PLoS One,* 11**,** e0165436.

Scully, R., Chen, J., Ochs, R. L., Keegan, K., Hoekstra, M., Feunteun, J. & Livingston, D. M. 1997. Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. *Cell,* 90**,** 425-35.

Scully, R. & Livingston, D. M. 2000. In search of the tumour-suppressor functions of BRCA1 and BRCA2. *Nature,* 408**,** 429-32.

Senkus, E., Kyriakides, S., Penault-Llorca, F., Poortmans, P., Thompson, A., Zackrisson, S. & Cardoso, F. 2013. Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of Oncology,* 24**,** vi7-vi23.

Sharma, P., Stecklein, S. R., Kimler, B. F., Sethi, G., Petroff, B. K., Phillips, T. A., Tawfik, O. W., Godwin, A. K. & Jensen, R. A. 2014. The prognostic value of BRCA1 promoter methylation in early stage triple negative breast cancer. *Journal of Cancer Therapeutics and Research,* 3**,** 1-11.

Shin, S. Y., Kim, C. G. & Lee, Y. H. 2013. Egr-1 regulates the transcription of the BRCA1 gene by etoposide. *BMB Rep,* 46**,** 92-6.

Shukrun, M., Jabareen, A., Abou-Kandil, A., Chamias, R., Aboud, M. & Huleihel, M. 2014. HTLV-1 Tax Oncoprotein Inhibits the Estrogen-Induced-ER [alpha]-Mediated BRCA1 Expression by Interaction with CBP/p300 Cofactors. *PLoS One,* 9.

Simpson, J. T., Workman, R. E., Zuzarte, P. C., David, M., Dursi, L. J. & Timp, W. 2017. Detecting DNA cytosine methylation using nanopore sequencing. *Nat Methods,* 14**,** 407-410.

Sjoblom, T., Jones, S., Wood, L. D., Parsons, D. W., Lin, J., Barber, T. D., Mandelker, D., Leary, R. J., Ptak, J., Silliman, N., Szabo, S., Buckhaults, P., Farrell, C., Meeh, P., Markowitz, S. D., Willis, J., Dawson, D., Willson, J. K., Gazdar, A. F., Hartigan, J., Wu, L., Liu, C., Parmigiani, G., Park, B. H., Bachman, K. E., Papadopoulos, N., Vogelstein, B., Kinzler, K. W. & Velculescu, V. E. 2006. The consensus coding sequences of human breast and colorectal cancers. *Science,* 314**,** 268-74.

Sobczak, K. & Krzyzosiak, W. J. 2002. Structural determinants of BRCA1 translational regulation. *Journal of Biological Chemistry,* 277**,** 17349-58.

Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., Van De Rijn, M., Jeffrey, S. S., Thorsen, T., Quist, H., Matese, J. C., Brown, P. O., Botstein, D., Lonning, P. E. & Borresen-Dale, A. L. 2001. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proceedings of the National Academy of Sciences of the USA,* 98**,** 10869-74.

Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J. S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S., Demeter, J., Perou, C. M., Lonning, P. E., Brown, P. O., Borresen-Dale, A. L. &

Botstein, D. 2003. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proceedings of the National Academy of Sciences of the USA,* 100**,** 8418-23.

Stewart, B. W. 2014. *World Cancer Report 2014,* Lyon, Lyon, FRA: International Agency for Research on Cancer.

Stratton, M. R., Campbell, P. J. & Futreal, P. A. 2009. The cancer genome. *Nature,* 458**,** 719-24.

Tapia, T., Smalley, S. V., Kohen, P., Muñoz, A., Solis, L. M., Corvalan, A., Faundez, P., Devoto, L., Camus, M., Alvarez, M. & Carvallo, P. 2008. Promoter hypermethylation of BRCA1 correlates with absence of expression in hereditary breast cancer tumors. *Epigenetics,* 3**,** 157.

Thompson, M. E., Roy, A. J., Patrice, S. O., David, L. P. & Jeffrey, T. H. 1995. Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. *Nature Genetics,* 9**,** 444.

Tubio, J. M. C., Li, Y., Ju, Y. S., Martincorena, I., Cooke, S. L., Tojo, M., Gundem, G., Pipinikas, C. P., Zamora, J., Raine, K., Menzies, A., Roman-Garcia, P., Fullam, A., Gerstung, M., Shlien, A., Tarpey, P. S., Papaemmanuil, E., Knappskog, S., Van Loo, P., Ramakrishna, M., Davies, H. R., Marshall, J., Wedge, D. C., Teague, J. W., Butler, A. P., Nik-Zainal, S., Alexandrov, L., Behjati, S., Yates, L. R., Bolli, N., Mudie, L., Hardy, C., Martin, S., Mclaren, S., O'meara, S., Anderson, E., Maddison, M., Gamble, S., Foster, C., Warren, A. Y., Whitaker, H., Brewer, D., Eeles, R., Cooper, C., Neal, D., Lynch, A. G., Visakorpi, T., Isaacs, W. B., Van't Veer, L., Caldas, C., Desmedt, C., Sotiriou, C., Aparicio, S., Foekens, J. A., Eyfjörd, J. E., Lakhani, S. R., Thomas, G., Myklebost, O., Span, P. N., Børresen-Dale, A.-L., Richardson, A. L., Van De Vijver, M., Vincent-Salomon, A., Van Den Eynden, G. G., Flanagan, A. M., Futreal, P. A., Janes, S. M., Bova, G. S., Stratton, M. R., Mcdermott, U. & Campbell, P. J. 2014. Mobile DNA in cancer. Extensive transduction of nonrepetitive DNA mediated by L1 retrotransposition in cancer genomes. *Science (New York, N.Y.),* 345**,** 1251343.

Turner, N., Tutt, A. & Ashworth, A. 2004. Hallmarks of 'BRCAness' in sporadic cancers. *Nature Reviews Cancer,* 4**,** 814-9.

Van 'T Veer, L. J., Dai, H., Van De Vijver, M. J., He, Y. D., Hart, A. a. M., Mao, M., Peterse, H. L., Van Der Kooy, K., Marton, M. J., Witteveen, A. T., Schreiber, G. J., Kerkhoven, R. M., Roberts, C., Linsley, P. S., Bernards, R. & Friend, S. H. 2002. Gene expression profiling predicts clinical outcome of breast cancer. *Nature,* 415**,** 530-536.

Van De Vijver, M. J., He, Y. D., Van 'T Veer, L. J., Dai, H., Hart, A. a. M., Voskuil, D. W., Schreiber, G. J., Peterse, J. L., Roberts, C., Marton, M. J., Parrish, M., Atsma, D., Witteveen, A., Glas, A., Delahaye, L., Van Der Velde, T., Bartelink, H., Rodenhuis, S., Rutgers, E. T., Friend, S. H. & Bernards, R. 2002. A Gene-Expression Signature as a Predictor of Survival in Breast Cancer. *The New England Journal of Medicine,* 347**,** 1999-2009.

Vardhman, K. R., Thomas, A. D., David, J. B. & Stephan, B. 2011. Epigenome-wide association studies for common human diseases. *Nature Reviews Genetics,* 12**,** 529.

Veeck, J., Ropero, S., Setien, F., Gonzalez-Suarez, E., Osorio, A., Benitez, J., Herman, J. G. & Esteller, M. 2010. BRCA1 CpG island hypermethylation predicts sensitivity to poly(adenosine diphosphate)-ribose polymerase inhibitors. *Journal of Clinical Oncology,* 28**,** e563-4; author reply e565-6.

Wang, R. Y., Gehrke, C. W. & Ehrlich, M. 1980. Comparison of bisulfite modification of 5-methyldeoxycytidine and deoxycytidine residues. *Nucleic acids research,* 8**,** 4777.

Wilcox, C. B., Baysal, B. E., Gallion, H. H., Strange, M. A. & Deloia, J. A. 2005. High-resolution methylation analysis of the BRCA1 promoter in ovarian tumors. *Cancer Genetics and Cytogenetics,* 159**,** 114-122.

Wolf, R., Andrew, C., Michael, L. N., Sheila, C. B. & Surani, M. A. 1987. Genomic imprinting determines methylation of parental alleles in transgenic mice. *Nature,* 328**,** 248.

Wolf, S. F., Jolly, D. J., Lunnen, K. D., Friedmann, T. & Migeon, B. R. 1984. Methylation of the hypoxanthine phosphoribosyltransferase locus on the human X chromosome: implications for X-chromosome inactivation. *Proceedings of the National Academy of Sciences of the United States of America,* 81**,** 2806.

Wu, L., Shen, Y., Peng, X., Zhang, S., Wang, M., Xu, G., Zheng, X., Wang, J. & Lu, C. 2016. Aberrant promoter methylation of cancer-related genes in human breast cancer. *Oncology Letters,* 12**,** 5145-5155.

Wu, L., Wang, F., Xu, R., Zhang, S., Peng, X., Feng, Y., Wang, J. & Lu, C. 2013. Promoter methylation of BRCA1 in the prognosis of breast cancer: a meta-analysis.(EPIDEMIOLOGY). *Breast Cancer Research and Treatment,* 142**,** 619.

Www.Arup.Utah.Edu. 2017. *The University of Utah* [Online]. Available: www.arup.utah.edu/database/BRCA/database/BRCA/Home/BRCA1_Landing [Accessed 23.05.2017].

Xi, Y. & Li, W. 2009. BSMAP: whole genome bisulfite sequence MAPping program. *BMC Bioinformatics,* 10**,** 232.

Xu, C., Brown, M. A., Chambers, J., Griffiths, B., Nicolai, H. & Solomon, E. 1995. DISTINCT TRANSCRIPTION START SITES GENERATE 2 FORMS OF BRCA1 MESSENGER-RNA. *Human molecular genetics,* 4**,** 2259-2264.

Xu, C. F., Chambers, J. A. & Solomon, E. 1997. Complex regulation of the BRCA1 gene. *The Journal of biological chemistry,* 272**,** 20994.

Xu, J., Huo, D., Chen, Y., Nwachukwu, C., Collins, C., Rowell, J., Slamon, D. & Olopade, O. 2010. CpG island methylation affects accessibility of the proximal BRCA1 promoter to transcription factors. *Breast Cancer Research and Treatment,* 120**,** 593-601.

Zhang, L. & Long, X. 2015. Association of BRCA1 promoter methylation with sporadic breast cancers: Evidence from 40 studies. *Scientific Reports (Nature Publisher Group),* 5**,** 17869.