Qualitative and Quantitative Identification of DNA Methylation Changes in Blood of the Breast Cancer patients

A Thesis Submitted to the Council of

Faculty of Science and Science Education

School of Science at the University of Sulaimani

in partial fulfillment of the requirements for the degree of

Master of Science in Biology

(Molecular Biology)

By

Harem Othman Smail

B.Sc. Biology (2011), University of Koya

Supervised By

Dr. Dlnya Asad Mohamad

Assistant Professor

Rebandan 2715 February 2016

Supervisor's certification

I herby certify that this thesis titled "**Qualitative and Quantitative Identification of DNA Methylation Changes in Blood of the Breast Cancer patients**" prepared by (Harem Othman Smail), at the University of Sulaimani, Faculty of Science and Science Education and do hereby recommend it to be accepted as a partial fulfillment of the requirements for the degree of Master of Science in Biology "Molecular Biology ".

Signature:

Name: **Dlnya A. Mohamad**

Title: Assistant Professor

Date: / / 2016

In view of the available recommendations, I forward the thesis for debate by the examining committee.

Signature

Name: Dr. **Huner Hiwa Arid**

Head of Biology Department.

Title: Lecture

Date: / / 2016

Linguistic Evaluation Certification

I herby certify that this thesis titled "**Qualitative and Quantitative Identification of DNA Methylation Changes in Blood of the Breast Cancer patients**" prepared by (Harem Othman Smail), has been read and checked and after indicating all the grammatical and spelling mistakes; the thesis was given again to the candidate to make the adequate corrections. After the second reading, I found that the candidate corrected the indicated mistakes. Therefore, I certify that this thesis is free from mistakes.

Signature

Name: **Jutyar O. Salib**

Position: **English Department, School of Languages, University of Sulaimani**

Date: / / 2016

Examining Committee Certification

We certify that we have read this thesis entitled "**Qualitative and Quantitative Identification of DNA Methylation Changes in Blood of the Breast Cancer patients**" prepared by (Harem Othman Smail), and as Examining Committee, examined the student in its content and in what is connected with it, and in our opinion it meets the basic requirements toward the degree of Master of Science in Biology "Molecular Biology ".

Signature
Name: **Dr. Beston F. Nore**
Title: **Professor**
Date / / 2016
(Chairman)

Signature
Name: **Dr. Kawa A. M. Amin**
Title: **Assistant Professor**
Date / / 2016
(Member)

Signature
Name: **Dr. Tara K. Raouf**
Title: **Lecture**
Date: / / 2016
(Member)

Signature
Name: **Dr. Dlnya A. Mohamad**
Title: **Assistant Professor**
Date / / 2016
(Supervisor -member)

Approved by the Dean of the Faculty of Science and Science Education.

Signature
Name: **Dr. Bakhtiar Q. Aziz**
Title: **Professor**
Date: / / 2016

Dedication

I dedicate this project to those who sacrificed their lives for Kurdistan.

To my wife; (Sheelan)

To my sisters ;(Qumry and zoohry)

To my brother ;(Naseh)

Harem

Acknowledgements

Thanks go to my supervisor Dr. Dlnya Asad Mohamad and evryone for who helped me for success in my research.

I would like to express my thanks to the staff of Hiwa hospital and Genome center at koya University especially Dr. Shwan Rashid, Mr. Muhammad, and Mr Shwan. Also I would like to express my thanks to Mrs. Lina Mrs .Banaz, Mr. Shkar and Dr. Karzan.

Harem

Abstract

Recently , DNA methylation is a main epigenetic modification in human breast cancer are discovered as a promising biomarker in early detection of breast cancer.This study was carried out to identification of DNA methylation changes in the promoter region of three different genes (ESR2, OPN, and BRCA1) in breast cancer. Many genes have been reported to be a hypermethylation in women breast cancer in the diagnosis of breast cancer, but there has been a little comprehensive study on the clinical usefulness of these genes in breast cancer.

In total, 30 cases of in women breast cancer and 8 cases of healthy blood were studied. DNA extracted from blood samples, and bisulfate conversion reaction was used to unmethylated conversion cytosine to Uralic and ethylated cytosine not affected. Promoter methylation pattern was determined by using bisulfite sequencing. BiQ Analyzer software was used for data analysis for determining bisulfite conversion rate, the percentage of methylation, percentage unmethylated, errors during sequencing and alignment of the gene sequence.

Based on the sequence data analysis by BiQ Analyzer software the results for ESR2 are (4.16 %)from CPG from the healthy sample was methylated while (12.39%) CPG from cancer sample was methylated, and Also unmethylated CPG were difference between healthy and cancer sample. Total unmethylated CPG from the healthy sample (87.5%). While from cancer samples (72.5%) Total CpGs, not present not from cancer samples (14.99%) while from healthy specimens (8.31 %).OPN total CPG methylation from cancer sample (57.57 %) while from healthy sample(0%)but unmethylated CPG from cancer sample (39.39%) while form healthy sample it was (88.8%).A minimum CPG not presents from cancer sample (3.03%) while from healthy samples it was increased to (11.11%). The CPG

percentage methylation for BRCA1 in cancer sample was (63.63%) but in healthy specimens it was (36.36%). Minimum unmethylated from CPG was (3.03%) present from cancer sample but in healthy samples (%9.09) is present .Maximun CPG not present from both healthy and cancer samples was (33.6%) but for healthy samples it was (54.54 %).

This study demonstrates that DNA methylation changes in breast cancer for three different genes(ESR2, OPN, and BRCA1) and it can be use as a potential biomarker for detection of breast cancer and statistically significant between cancer and healthy samples for all three genes at P- values < 0.05.The grade 1 and grade 2 samples of ESR2 and BRCA1, statistically are significant but not for OPN at P- values < 0.05.

CONTENTS

Chapter Three: Materials and methods

Chapter four: Results and Discussion

Chapter five: Conclusions and Recommendations

List of Tables

List of Figures

List of abbreviations

Symbols	Description
°C	Celsius degree
5mC	5-methylcytosine
ATM	Ataxia-telangiectasia
BiQ	Bisulfite sequence
bp	base pairs
BRCA1	Breast Cancer Gene 1
BRCA2	Breast Cancer Gene 2
BRCT	BRCA1 C Terminus
BRIP1	BRCA1 Interacting Protein C-Terminal Helicase
BS-Seq	bisulfite sequencing
BstUI	Bacillus stearothermophilus U458
CCNDI	cyclin D1 gene
CDH1	Cadherin-1
CHEK2	checkpoint kinase 2

C-myc	C-my myelocytomatosis viral oncogene homolog
CoBRA	Combined bisulphite restriction analysis
CpG	Cytosine-phospho-guanine
DCIS	Ductal carcinoma in situ
ddH2O	Double-distilled water
DDR	DNA damage response
DNA DSB	DNA double-strand break
DNMT1	DNA-cytosine methyltransferase-1
DNMT3A	DNA-cytosine methyltransferase-3A
DNMT3B	DNA-cytosine methyltransferase-3B
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERBB2	Receptor tyrosine-protein kinase erbB-2
ESR1	Estrogen Receptor 1

ESR2	Estrogen Receptor 2
F-actin	Filamentous actin
FGFR1	Fibroblast growth factor receptor 1
g	Gravity
GADD45	Growth arrest and DNA damage
HER2	Human epidermal growth factor receptor 2
HhaI	Haemophilus haemolyticus I
HpaII	Haemophilus haemolyticus
HPV	Papillomavirus II
IHC	Immunohistochemistry
kb	kilobase
M	Molarity
MBD	Methyl-CpG binding domain
MDM2	Mouse double minute 2 homolog
Met	Methylated
miRNA	microRNA

MSP	Methylation-specific PCR
MW	Molecular Weight
Na2EDTA	Disodium salt of ethylenediaminetetraacetic acid
ng	nanogram
OPN	Osteopontin
p21	Protein 21
P53	Protein 53
PALB2	partner and localizer of BRCA2
PCD	Programmed cell death
Pg	Picogram
PIK3CA	phosphatidylinositol-4,5-bisphosphate3-kinase, catalytic subunit alpha
PR	progesterone receptor
PTEN	Phosphatase and tensin homolog
RAD51D	Reca archeal RADA51D
RISC	RNA-induced silencing complex
RPM	Round per minute

SAH	S-adenosyl homocysteine
SAM	S-adenosyl-L-methionine
STK11	serine/threonine kinase 11
TAE	Tris-acetate-EDTA
TBE	Tris/Borate/EDTA
TE	Tris-EDTA
TNBC	Triple-negative breast cancer
TSGs	Tumor suppressor genes
TSS	Transcription start site
U	Unmethylatd
UV	Ultraviolet
WHO	World Health Organization
µl	Microliter

Chapter one

Introduction

Introduction

Breast cancer is the most well-known malignant tumor in women worldwide. According to estimations, via the World Health Organization (WHO) breast cancer leads to about 519,000 deaths per year in the world and is among the foremost ten mortal diseases[61]. In Iraqi kurdistan region , breast cancer is mainly a disease of pre-menopausal women having multiple pregnancies[207]. For younger patients, breast cancer frequency was smilar to the West and maybe higher than many Middle-Eastern countries, but dissimilar to the West, the estimated rates declined markedly in the elderly. The familial breast cancer risk for both older and younger women was inside the common population risk of Western countries. Clinical stages were advanced and indicated postponement in diagnosis that were unrelated to patient age [116].

Recently , the function of epigenetic modification as a well-defined and vital mechanism to silence a variety of methylated tissue specific and imprinted genes has emerged in many cancer types[55].This epigenetic change in DNA is heritable that cannot be explained via changes in the primary structure of DNA. Also, indifference to genetic changes epigenetic modifications are potentially reversible [50, 8].

DNA methylation is the major epigenetic alteration in human breast cancer and is found early during carcinogenesis It most usually occurs at CpG dinucleotides in mammals [78]. In the normal somatic cell, most CPG dinucleotides are methylated, but those in CPG islands associated with promoter regions are in general unmethylated. CPG island related to promoter regions of many genes and their methylation in the gene becoming silencing [13]. The use of methylation changes as a biomarker has some advantages compared to other approaches. Two of these

benefits should underline: First, there is strong evidence that a methylation is an early stage event in carcinogenesis, a characteristic highly desired in cancer biomarkers. Second, the DNA containing the methylation information is highly stable and can be easily isolated from most body fluids, as well as from archived fixed tissues. These include early detection, chemoprevention, and disease monitoring [176].

Aims of the study

The aim of this study is to identify DNA methylation changes occurance in the promoter region of the three different genes including (ESR2, OPN and BRCA1) in breast cancer patients and to compare DNA methylation changes between healthy and breast cancer patients.

Chapter two

Literature review

Literature review

2.1. Cancer

Cancer is a collection of diseases discriminate by the abandoned progress and spread of abnormal cells; it is the second leading cause of death after cardiovascular diseases [2].

2.2. Epidemiology of cancer

Approximately, 7.8 million cases of cancer were diagnosed worldwide in 1990. The number of new cases doubled between 1970 and 2001 [24]. According to the estimates published by the International Agency for Research on Cancer (IARC), 12.7 million new cancer cases reported worldwide in 2008. Out of 5.6 million occurred in economically developed countries, and 7.1 million new cases reported in economically developing countries.

Other estimates reported 7.6 cancer deaths in 2008. In economically developed countries and economically developing countries the rates were 2.8 and 4.8 million respectively The number of new cancers cases is expected to grow to 21.4 million and 13.2 million cancer deaths by 2030 [3].

Cancer as a group account for about 13% of deaths/ year. The most common being: lung cancer 1.3million, stomach cancer 803000, colorectal cancer 639000, liver cancer 610000, and breast cancer 519000 deaths [195].

Approximately, 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012. Estimates of cancer prevalence in 2012 showed that there were 32.6 million people (over the age of 15 years) alive who had cancer diagnosed in the last five years. The number of new cases is expected to rise by about 70% over the next two decades. The five most diagnosed cancers in 2012 among men were lung, prostate, colorectum, stomach, and liver cancer. The five

most diagnosed cancers among women were breast, colorectum, lung, cervix, and stomach cancer [197].

David in 2013 reported that the most commonly diagnosed cancers worldwide were those of the lung (1.8 million, 13.0%), breast (1.7 million, 11.9%), and colorectum (1.4 million, 9.7%). The most common causes of cancer death were cancers of the lung (1.6 million, 19.4%), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%).

2.3. Causes of cancer

Cancers are caused by combining genetic and non-genetic changes induced by environmental factors that stimulate inappropriate activation or inactivation of specific genes leading to neoplastic transformations, or abnormal cell growth. There is a lack of information about key cellular events that occur in early stages of cancer development as well as environmental factors and internal cause that trigger these changes [91,181].

Environmental factors includes chemicals, drinking overload alcohol, environmental toxins, for example, certain toxic mushrooms and a kind of poison that can grow on peanut plants (aflatoxins), extreme sunlight exposure, obesity, radiation and viruses, and infectious organisms. Genetic factors include inherited mutations, hormones, immune conditions, and mutations that occur from metabolism [129].

2.4. Biology of Cancer

There are over 100 types of cancers consisting of multiple subtypes able to form in a single organ or tissue type, the majority of cancers can be classified, on a very general level, based on their tissue site of origin. The most common form of cancer, accounting for over 80% of all cancers, arises in epithelial cells; these

cancers are termed carcinomas. The majority of carcinomas may be split into squamous cell carcinomas or adenocarcinomas depending on whether the epithelial cells of origin are part of the protective epithelial layer (squamous cell carcinomas) or have secretory properties (adenocarcinomas) [194].

Examples of squamous cell carcinomas include squamous cell carcinoma of the skin and squamous cell carcinoma of the cervix, while adenocarcinomas include adenocarcinomas of the breast and colon. Although some carcinomas can be defined as pure squamous cell carcinomas or adenocarcinomas, many carcinomas are located where both cell types co-exist. After carcinomas, the majority of malignancies are either sarcoma, hematological or arise within the central or peripheral nervous system [76].

Sarcomas arise from mesenchymal cells such as connective tissue, adipocytes or osteoblasts. Examples include bone cancers, such as osteosarcomas and chondrosarcomas, and muscle tissue based malignancies, such as rhabdomyosarcomas. Haematological malignancies can be split into leukemias and lymphomas; leukemias arising from various lineages of white blood cells and lymphomas arising from lymphoid lineages that then go on to form solid tumor masses.Central and peripheral nervous system tumors include gliomas, neuroblastomas, and medulloblastomas, among others. There are a small number of other cancers, such as melanomas, that do not fit into any of the above categories [194].

Despite the site of origin of malignancies, cells must acquire various biological traits to form a malignant tumor. Reviewed in 2000 [71] ,these biological attributes include:

i. Self-sufficiency in growth i.e. does not require external growth signals to proliferate.

ii. Insensitivity to antigrowth signals i.e. becoming able to block the inhibition of proliferation initiated by external growth signals.

iii. The ability to evade apoptosis – the majority of cancer cells has acquired some mechanism of resistance to apoptosis.

iv. Potentially limitless replication – overcoming the intrinsic switch to senescence after numerous cell divisions.

v. Sustained angiogenesis – angiogenic properties are normally tightly regulated but essential for transition from an aberrant proliferative lesion to a larger malignant tumor entity.

vi. Ability to invade surrounding tissue and metastasize – tumors only become malignant upon invasion of surrounding tissue.

To be able to acquire these biological properties that enable malignant tumor growth a multitude of genetic changes must occur within the cell.

2.4.1. Apoptosis

The term apoptosis (a-po-toe-sis) was first used by Kerry et al., in 1972 to describe a morphologically distinct form of cell death [43]. Apoptosis is a highly complex and tightly regulated process of cell death, which deprives the proliferating cellular pool and allows the elimination of genetically damaged cells after their division. It is also a protective cellular mechanism against malignant transformation [127].

Programmed cell death (PCD) involves an orchestrated series of biochemical events leading to a characteristic cell morphology and death. In more concrete terms, a series of biochemical events that lead to a variety of morphological changes, including blebbing, changes to the cell membrane such as loss of

membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation.

There are two cell death types: Programmed cell death (PCD) and Necrosis. PCD has classified into two main types: (1) Apoptosis (or Type I cell death), is a particular form of programmed cell death. (2) Autophagic (cytoplasmic, or Type II) cell death, characterized by the formation of large vacuoles that eat away organelles in a particular sequence before the nucleus destroyed [123]

2.4.2. Tumorigenesis

The process of formation of cancer called as oncogenesis or tumorigenesis is caused by the interplay between genetic/epigenetic and environmental factors. There are specific traits that are called hallmarks that convert normal cells into cancerous cells [169].

2.4.3. Metastasis of cancer

Cancer cells can invade the other tissues and spread to different locations. Cells become cancerous due to DNA damage. Normally ,DNA damages are repaired by various mechanisms involved in maintaining tissue homeostasis, however, in the case of cancer cells, DNA damage is not repaired, and the unwanted cells proliferate instead of being removed. Sometimes the DNA damage is inherited, but mostly it is due to the change in normal cells and the environmental factors. So, cancer is a multifactor disease caused due to molecular alterations in the genome of somatic cells [169].

Although the exact mechanisms are not yet understood, the newly acquired resistance (both chemical/drug and radio-resistant) appears to coincide with increased invasiveness. There is growing evidence that there are significant

similarities between drug-resistant and metastatic cancer cells; particularly, regarding profound resistance to apoptosis and enhanced invasivenes [53].

Metastasis is a complex process in which tumor cells acquire the ability to spread to other tissues via lymphatics or blood vessels. Invading cancer cells form filamentous actin (F-actin)-based membrane protrusions called invadopodia, whose extracellular matrix (ECM) degrading activity allows them to invade through basement membranes and migrate toward blood vessels [26].

Metastasis is a complex process in which cancer cells break away from the primary tumor and circulate through the bloodstream or lymphatic system to other sites in the body. At new sites, the cells continue to multiply and eventually form additional tumors comprised of cells that reflect the tissue of origin. The ability of tumors, such as pancreatic cancer and uveal cancers, to metastasize contributes greatly to their lethality. Many fundamental questions remain about the clonal structures of metastatic tumors, phylogenetic relationships among metastases, the scale of ongoing parallel evolution in metastatic and primary sites, how the tumor disseminates, and the role that the tumor microenvironment plays in the determination of the metastatic site [82].

2.4.4. Angiogenesis of cancer

Angiogenesis is a complex multi-step process, consisting of coordinated, sequential and interdependent steps leading to the formation of new blood vessels from pre-existing vascular networks. It is a highly restricted process in normal human adult tissues, and to start it, a tumor must switch to the angiogenic phenotype [127].

2.5. Types of tumor

1- Benign tumors

Benign tumors are noncancerous growths in the body. Unlike cancerous tumors, they do not spread to other parts of the body. Benign tumors can form anywhere in the body. Discovering a lump or mass in the body that can be felt from the outside, it is normal to assume it is cancerous. For instance, women who find lumps in their breasts during self-examinations may become alarmed. However, most breast growths are benign. Moreover, in fact, many growths throughout the body are benign [98].

2- Malignant tumors

A malignant tumor is often used synonymously with the word "dangerous" in medicine. While it usually refers to a cancerous tumor, it may be used to describe other medical conditions. For example, malignant hypertension (malignant high blood pressure) refers to blood pressure that is dangerously high, and malignant tumors (cancerous tumors) are those that have the ability to spread to other regions of the body either locally, via the bloodstream, or through the lymphatic system[119].

2.6. Cancer genetics

All tumors accumulate somatic mutations during their development. Most common cancers are associated with diverse cancer genes that are mutated at a low frequency. One of the most striking observations from large cancer databases is the genetic heterogeneity of cancers and even within individual cancer types [42].

However, it appears that a limited number of cellular pathways are central to tumor cell biology, Comprehensive catalogs of somatic mutations are being

compiled for various cancer types to understand better the mechanisms that underlie this disease [41].

Many alterations within the genome, both germline and somatic, can influence the process of tumorigenesis. Mutations, genome rearrangements, amplifications, deletions, genome instability and epigenetic changes, such as DNA methylation and histone modifications, can all contribute to the tumorigenic process [76].

Somatic mutations occur in the genomes of all dividing cells, both normal and neoplastic. They may occur as a result of misincorporation of DNA replication or through exposure to exogenous or endogenous mutagens. Cancer genomes carry two biological classes of a somatic mutation arising from these various processes. 'Driver' mutations confer a growth advantage on the cell in which they occur, and are causally implicated in cancer development and have therefore been positively selected. By definition, these mutations are in 'cancer genes'. Conversely, 'passenger' mutations have not been subject to selection. They were present in the cell that was the progenitor of the final clonal expansion of cancer, They also biologically neutral and do not confer a growth advantage [68].

Genetic changes that occur in metastatic cells have been studied at the level of individual genes, tissue-specific profiles, and the whole genome approaches. In general, metastases and primary cancers have exhibited very similar expression signatures. The resemblance between primary and secondly metastasis lymph nodes provide evidence that the fundamental biological processes that shape the emergence of the metastatic phenotype have some underlying homologies [53].

2.7. Breast cancer

In women, breast cancer is the most common class of cancer worldwide with more than one million cases diagnosed annually, followed by cancer of the lung and colon, making it the leading cause of cancer deaths in women with >400,000 deaths per year [65].

Breast cancer has different histological, molecular and clinical characteristics, including tumor growth patterns, with at least 17 different histological types recently described [186].

The majority of breast cancer cases occurs in women aged 50 years or older (130). According to estimations by the World Health Organization (WHO), breast cancer leads to about 519,000 deaths per year in the world and belongs to the top ten mortal diseases. Besides a large number of cases, breast carcinoma is a very heterogeneous cancer regarding histopathological and clinical classification, genetic and genomic background of the tumor mass and health outcomes [152]. Just in 2010 with almost 1.5 million women in the world diagnosed [40].

Depending on the American Cancer Society report in 2011, breast cancer is the second leading cause of cancer death in women and accounts for nearly 1 in 3 cancers diagnosed among women in the United States. Approximately 230,000 new cases of invasive breast cancer along with nearly 60,000 non-invasive cases will be diagnosed in US women this year [3].

Breast cancer metastasis is an important cause of the failure of treatment in women, making it crucial to identify proteins and signaling pathways involved in tumor cell dissemination. During the metastasis process, the oncogenes are activated, and the tumor suppressor genes are inactivated, which may handle breast

cancer metastasis. Promoter hypermethylation of tumor suppressor genes is one of the ways to suppress its expression during the breast cancer metastasis [162].

Every year more than one million women are diagnosed with breast cancer, and approximately 400,000 die [59]. Global cancer statistics identifies breast cancer as the most frequently diagnosed cancer (23%) and leading cause of cancer-related death (14%) in females [118].

The lifetime risk of developing breast cancer is about one in nine for women with around 1,41,000 new cases being diagnosed in the worldwide each year. This has been proved recently as the worst and fatal breed of cancers in females. [183].

Breast cancer remains the most frequently diagnosed cancer and the leading cause of cancer deaths in European women. According to the World Health Organization (WHO), more than 449,000 women in Europe will be diagnosed yearly with breast cancer, comprising approximately 28% of all cancers in female patients. Localized, early-stage breast cancer has a favorable prognosis, with a 5-year survival rate of up to 98% [94].

At 28% of all new cancers, breast cancer is the most common and the second deadliest (15%). Except lung cancer, it is the only cancer type that has increased in incidence over the last 35 years. Furthermore, it is expensive to treat, with total treatment expenditures nearing $14 billion in 2006—the most expensive of any single cancer [129].

Breast cancer is a devastating illness that affects tens of thousands of American women each year. In 2008, it was estimated that 182,460 women will be diagnosed, and 40,480 women will die of this disease [200].

According to estimates in 2008, there were 1,383,000 new cases of breast cancer diagnosed, 458,000 deaths caused by breast cancer, and more than 4.4 million women living with breast cancer worldwide [36].

In 2002, the estimated number of deaths was about 411,000 (14% of female cancer deaths) [127]. For 2009, estimates indicated that 192,370 women were being diagnosed with breast cancer. The median age at diagnosis was 61 years of age, and women that are 55 to 64 years of age are in the highest risk group, at 23.7%, with values declining with either increase or decrease of age.White women are also more likely to be diagnosed that other races. There has also been a notable change in the incidence of breast cancer over time. From 1980 to 1987, breast cancer incidence went up nearly 4%, and there was a second surge from 1992 to 1999 with an increase in the impact of approximately 1.6% [11].

Epidemiologic studies reported that women who are BRCA1 mutation carriers have a 45–60% cumulative risk to develop breast cancer before age 35–40, and the average cumulative risk in BRCA1- mutation carriers by age 70 years is 65% (95% confidence interval 44–78%) whereas the corresponding risk to develop this neoplasm for BRCA2 mutation carriers is estimated to be 25–40% and 45% (31–56%), respectively [64].

2.7.1. Causes of breast cancer

There are two main factors that cause breast cancer and they are either environmental or genetic factors. Like other cancers, mixed between two of these factors genetic, environmental also with and epigenetic alterations of multiple cancer genes including oncogenes and tumor suppressor genes (TSGs) [201].

Breast cancer development and progression are influenced by intrinsic properties of the tumor cells, as well as by macro-environmental factors. There is

an extensive interplay between tumor cells and signaling molecules such as chemokines. Chemokine receptors and growth factors have been extensively implicated in the metastatic process of breast cancer [147].

Also, there are many anthropometric factors such as height, weight, body mass and fat distribution all have been shown to influence breast cancer risk [19].

2.7.1.1. Environmental factors and life style

Much carcinogenic substance has roles in breast cancer as all of the cancers such as toxins, free radicals, UV exposure and some viruses such as human papillomavirus (HPV) that cause functional mutations in DNA, also, there are many risk factors such as smoking, drinking alcohol, chest radiation, and overweight that has roles in increasing of the breast cancer [152, 11, 127, 130].

2.7.1.2. Genetic factors

Many alterations within the genome, both germline and somatic, can influence the process of tumourigenesis. Mutations, genome rearrangements, amplifications, deletions, Genome instability and epigenetic changes, such as DNA methylation and histone modifications, can all contribute to the tumorigenic process. The view of cancer genetics has changed dramatically over the past 100 years and provides an almost unending wealth of knowledge that could be discussed [76].

BRCA1 and BRCA2 genes handle approximately 20-40% of inherited breast cancer [17].

There are over ten genes causing hereditary forms of breast cancer. However, only BRCA1- and BRCA2-related disease has been studied with sufficient level of comprehension. It is commonly stated that BRCA-driven malignancies are triggered by somatic inactivation of the remaining (wild-type) BRCA allele, thus providing a unique opportunity for a tumor-specific therapy [85].

The BRCA1 germline mutation (BRCA1+) is a well-known genetic predisposition for inherited breast cancer [202].

Several rare inactivating mutations in other high penetrance genes have been described to contribute to an increased breast cancer risk, such as TP53, CDH1, PTEN, STK11, RAD51C, and RAD51D and in the low/moderate penetrance genes ATM, CHEK2, BRIP1, and PALB2 among others [106].

2.8. Breast cancer genetics

Cancer genes can be divided into three categories: tumor suppressor genes, oncogenes and genome stability maintenance genes.

2.8.1. Tumor Suppressor Genes

Tumor suppressor genes are genes that promote tumor growth when inactivated and these genes play a role in regulating growth factors, cell proliferation, DNA damage response, cell cycle arrest and apoptosis. Hence, tumor suppressors are considered to be the "gatekeepers" of the genome [130].

In a normal cellular context, tumor suppressor genes handle keeping cell behavior under control. When inactivated, associated normal cell functions can spiral out of control causing, or adding to, the tumorigenic process. In most cases, both copies of a tumor suppressor must be inactivated to lead to cancer formation [76].

Several tumor suppressor genes have been implicated in breast carcinogenesis; mutations in genes such as BRCA1, BRCA2, TP53, PTEN and ATM [127].

2.8.1.1. BRCA1 and BRCA2

BRCA1 and BRCA2 genes are two well-known tumor suppressor genes, which prevent cancer [169]. BRCA1 and BRCA2 are clinically the most important genes associated with breast cancer susceptibility. Depending on the studied population,

heterozygous mutations in these two high-penetrance cancer genes handle 20-30% of familial breast cancer cases [70].

Earlier estimates suggested that BRCA1 and BRCA2 mutations handled 75% of site-specific breast cancer families and the majority of breast and ovarian cancer families, however, these percentages may have been overestimated and that the proportion of families classified caused by mutations in BRCA1 or BRCA2 is much lower and strongly depends on the population analyzed and the particular characteristics of the selected families [136].

The most common inherited mutations are in the Breast Cancer (BRCA) tumor suppressor genes, BRCA1, and BRCA2. Women born with a mutation in either of these genes have up to an 80% chance of developing breast cancer in their lifetime [11].

BRCA1 and BRCA2 genes handle approximately 20-40% of inherited breast cancer. Prevalence of BRCA1 or BRCA2 germline mutations varies considerably among ethnic groups, and in some countries, founder mutations handle a significant proportion of breast cancer cases. Specific mutations have been described, for example, among Ashkenazi Jews, in Iceland, and in several other countries where isolated populations exist [17].

Mutations in BRCA1 and BRCA2 confer a high lifetime risk of breast cancer. Many different BRCA1 and BRCA2 mutations have been described in families with early-onset breast cancer. The presence of recurrent mutations in BRCA1 suggests the presence of founder effects; this was first confirmed in the Ashkenazi Jewish population. Founder mutations in other populations have also been described [88].

The breast cancer risks in BRCA1 and BRCA2 mutation carriers have also been found to vary by the age of diagnosis and the type of cancer (unilateral breast cancer, contralateral breast cancer,) in the index patient. Some studies have evaluated associations between genetic variants and breast cancer risk in BRCA1 and BRCA2 mutation carriers [170].

It is commonly stated that BRCA-driven malignancies are triggered by somatic inactivation of the remaining (wild-type) BRCA allele, thus providing a unique opportunity for a tumor-specific therapy. Indeed, while normal tissues of BRCA mutation carriers retain a non-altered copy of the gene, the transformed cells are characterized by complete loss of BRCA function. The absence of the BRCA1 or BRCA2 compromises DNA repair and increases the sensitivity of the cell to particular DNA damaging agents [85].

Genetic testing for BRCA1 and BRCA2 is expensive and time-consuming because of the large size of both genes, the absence of hot spots for mutations throughout their entire coding region, and the small percentage of mutated cases. It is, therefore, important to find clinical or pathological factors that could suggest or exclude the presence of BRCA1 or BRCA2 mutations in a given patient [136].

The identification of the breast cancer susceptibility genes BRCA1 and BRCA2 in the past decade has permitted identification of presymptomatic subjects at risk of developing breast/ovarian cancer using a genetic test [34].

The primary function of BRCA1 involved in the control of cell cycle progression and DNA double-strand break (DSB) repair. The vast majority of breast tumors developing in BRCA1 carriers shows inactivation of the wild-type allele by either somatic genetic (mutations or loss of heterozygosity or epigenetic (promoter hypermethylation) changes [154 ,179]. Due to its important role in

genome stability, the complete loss of function of BRCA1 in breast epithelial cells has been suggested as an accelerator of proliferation and tumor progression [179].

The Breast Cancer 1, Early onset gene (BRCA1) located on chromosome 17q21.31, [54,130]. Encodes a tumor suppressor that plays a critical role in the DNA damage response and repair pathways. Germline variants in the open reading- frame of BRCA1 confer a mean risk of 54% and 39% for developing hereditary breast and ovarian cancer (respectively) by age 70 [40].

BRCA1 may have a significant role in cellular differentiation and proliferation [114]. BRCA1 maintains genome stability through repairing double-strand DNA damage and other mechanisms.The BRCA1 germline mutation (BRCA1+) is a well-known genetic predisposition for inherited breast cancer. Women who inherited BRCA1+ have a 60-80% risk of developing breast cancer by the age of 70 [202]. The BRCA1 is activated by the protein kinase ATM, which initiates cell cycle changes after DNA damage [81].

Inheritance of a germline mutation in the BRCA1 gene are associated with an increased risk of developing breast cancer. However, there is also substantial variability in the ages at which breast cancers is diagnosed in BRCA1 mutation carriers [149].

BRCA1 comprises 24 exons encoding a protein of 1863 amino acids. Exon 11 is unusually large and, except the highly conserved domains located at the terminal regions of the protein, sequence conservation is weak [130]. BRCA1 contains two important domains, a RING domain at the N-terminus and two BRCT domains at the C-terminus and a coiled-coil domain upstream of the two BRCT domains.

The N-terminal ring finger domain of BRCA1 interacts with another ring finger protein, BARD1 (BRCA1-associated ring domain protein 1); and the

BRCA1: - BARD1 complex can function as an E3 ubiquitin ligase, an activity that may be important for tumor suppression. The BRCA1 carboxy-terminal transcriptional activation domain contains a tandem repeat of 95 amino acids called a BRCA1-associated carboxy-terminal domain (BRCT) that is homologous to similar domains found in various DNA repair and cell cycle checkpoint proteins. The BRCA1 protein is a 220 kDa nuclear phosphoprotein that is expressed and phosphorylated during the cell cycle, with maximum expression and phosphorylation in late G1 and early to mid-S-phase [159].

Breast cancers associated with BRCA1 mutations often show characteristic histological features including high-grade, high mitotic count, solid architecture and prominent lymphocytic infiltrates, all the characteristics resembling so-called medullary cancer [171]. BRCA1 tumors are frequently grade 3, estrogen receptor (ER) negative, progesterone receptor (PR) negative and HER2-negative (triple-negative), while the majority of BRCA2 tumors are grade 2/3, ER-positive, and HER2-negative Contrary, familial non-BRCA1/2 breast cancers have been shown to be a very heterogeneous group with varied histopathologic features [106].

BRCA2 is a large gene with 27 exons encoding a 3418 amino acid protein, with exon 11 being the largest. BRCA2 contains eight BRC repeats and one DNA binding domain that includes a helical motif, three oligonucleotide binding folds and a tower domain at the C-terminus structure BRCA2 also showing the binding sites of its interacting proteins is presented in [130]. BRCA2 has imperative functions in DDR since its protein product facilitates HR and is involved in DNA DSB repair. One of these functions is the regulation of RAD51 loading to DSBs. RAD51 is a crucial protein that covers appropriate sites of single-stranded DNA, thus preventing it from binding to double-stranded DNA and, therefore, activates strand invasion in HR[122].

The main indicators of a hereditary disease predisposition are familial clustering of breast cancers, early disease onset (under 50 years), and the occurrence of multiple primary tumors in the same individual. BRCA1 and BRCA2 are the two main breast cancer predisposing genes [130]. In the majority (90%) of sporadic breast cancers, BRCA2 is not mutated [150].

Sixty-four to 90% of breast cancers that occur in BRCA1 mutation carriers (BRCA1 carriers) are estrogen receptor negative (ER-), progesterone receptor negative (PR-) and lack HER2 protein overexpression and gene amplification so-called "triple-negative" breast cancer [184].

Although inherited cancer syndromes are rare, the genes accounting for them are believed to play a major role in sporadic cancer. It was anticipated; Therefore, that somatic BRCA mutations would be found to contribute to sporadic breast carcinogenesis. Surprisingly, somatic BRCA gene mutation have not been found in sporadic breast tumors. On the other hand, allelic imbalance (AI) at the BRCA loci, an indicator for loss of heterozygosity, is known to be a relatively common event in breast cancer [16].

Most hereditary cases of breast cancers are due to inherited mutations in the 2 identified breast cancer susceptibility genes, BRCA1 or BRCA2.1 Women, who inherit a BRCA1 mutation, for example, have more than an 80% chance of developing breast cancer in their lifetime [23].

BRCA2 was identified on chromosome 13 by focusing on families with a high incidence of breast cancer in both male and female members. Mutations in BRCA2 are thought to account for breast cancer in about 35 percent of families with a high incidence of early-onset breast cancer [128].

Among Ashkenazy Jewish population, three founder mutations have been found: these are BRCA1-185delAG, with a frequency of 1%, the 5382insC BRCA1 mutation, with a frequency of 0.13%, and 6174delT in the BRCA2 gene, with a frequency of 1.52%. The overall rate of these three founder mutations is 2.6% (1/40) compared with the rate of 0.2% (1/500) of BRCA1/2 mutation carriers in the general population [64].

Founder mutations have also been found in several European countries, the majority of which are high penetrance. Eleven recurrent mutations with a founder effect have been reported in the Finnish population and represent 84% of all the mutations found in the BRCA1/2 genes. Two other founder mutations were reported in Iceland: BRCA1 G5193A, and BRCA2 999del5; the latter is the most common mutation with a founder effect in this population [64].

2.8.1.2. TP53

The TP53 gene is the most frequently mutated gene in breast cancer and other human cancers. About 25% of breast cancers have somatic TP53 mutations, and 30-50% of breast tumors have an overexpression of p53 protein [127]. The TP53 gene helps in making the p53 proteins and stops the abnormal cell growth (169).

Four other high-risk breast cancer genes have been validated to date, of which TP53 confers the highest risk for mutations in this gene increase the breast cancer risk by 18- to 60-fold by the age of 45 years when compared to the general population [130].

Some of these genes, such as GADD45, p21, and MDM2, are transcriptionally activated by p53 whereas p53 represses genes such as c-myc and c-fos. The transcriptional activation of p21 during the G1 phase leads to cell cycle arrest and prevents cells with damaged DNA from entering the cell cycle phases of DNA

synthesis and replication. Also, the p53 gene transcriptionally activates Bax, a pro-apoptotic gene and downregulates transcription of bcl-2 that is a powerful antiapoptotic proto-oncogene [127].

2.8.1.3. E-cadherin

E-cadherin, encoded by the tumor suppressor gene CDH1, is a homophilic cell-to-cell adhesion protein localized to the adherens junctions of all epithelial cells. Its cytoplasmic domain effectively creates a bridge between the cytoskeletons of adjacent cells by interacting with both cortical actin filaments and the microtubule network. In both LBC and DGC, CDH1 are inactivated [27].

2.8.2. Oncogenes

Oncogenes are genes that contribute to oncogenesis when mutationally activated or activated under conditions in which the wild-type gene is not [130,162].

They act in a dominant fashion, i.e. mutation of one copy of the gene suffices for activation. Oncogenes typically play a role in cell survival, cell proliferation, and growth-related processes ; therefore, an activating somatic change in one allele of an oncogene is usually sufficient to confer a selective growth advantage to the cell [130]. Oncogenes usually promote cell division and/or inhibit apoptosis, and they may exert their effect through many different mechanisms, including growth factor receptors, signal transducers, transcription factors, protein tyrosine kinases, enzymes, and apoptosis repressors. Since oncogenic activation represents an increase in expression levels and/or activity, conversion of proto-oncogenes to oncogenes is considered a gain of function [11].

Several significant targetable oncogenes are known and relatively well described in female breast cancer (FBC). The most frequent gain of function

mutations is seen in phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha 9 (PIK3CA) which forms one of the catalytic subunits of the phosphatidylinositol 3-kinase (PI3K) holoenzyme [35]. In breast cancer, oncogene amplification is a common mechanism and is an important mechanism for oncogene overexpression. The HER2, EGFR, c-myc, CCNDI, FGFR1, ESR1, and MDM2 are among the frequently amplified oncogenes. Coamplifications (HER2/c-myc or CCND1/FGFR1) have also been reported [127].

Oncogenes were first described in 1976, with the discovery that alteration of the normal activity of certain genes resulted in the oncogenic transformation of cells. A good example of an oncogene is the PIK3CA (phosphoinositide-3-kinase, catalytic, alpha polypeptide) gene. It codes for the p110α protein, a class I PI 3-kinase catalytic substrate. PIK3CA mutations have observed in numerous cancer types and, to date, PIK3CA represents the most frequently somatically mutated gene in breast cancer [76].

Oncogenes are genes whose products drive cancer progression. Before activation, oncogenes exist as precursors, known as proto-oncogenes, which have normal cellular functions and are usually regulated by internal and/or external cell signaling [11].

2.8.2.1. HER2/neu gene

The HER2/neu proto-oncogene amplified in 15-30% of breast cancer. HER2 (also known as new, c-erbB-2 or human epidermal growth factor 2) is a transmembrane protein with tyrosine kinase activity. HER2 has implicated in breast carcinogenesis and plays a significant role in the development and progression of cancer. HER2 overexpression has been reported in 10-44% of human breast cancers. Overexpression in Breast carcinoma occurs through either

amplification of the gene or mRNA overexpression [127]. HER2/neu is considered as the most important biomarker for diagnosis of breast cancer [5].

2.8.2.2. EGFR gene

EGFR is another member of the tyrosine kinase family of receptors that are transmembrane proteins regulating major cellular events such as cell proliferation, differentiation, apoptosis, and adhesion and cell migration [127].

2.8.2.3. C-myc gene

C-myc amplification is relatively common (8-37%) in breast cancer and may provide independent prognostic information. It encodes for a helix-loop/leucine zipper protein, and myc responsive genes including those whose protein products regulate cell proliferation and apoptosis [127].

2.8.2.4. CCNDI gene

The CCNDI gene located on chromosome 11q3 and coding for the G1-cyclin protein (cyclin D1) involved in regulation of the cell cycle has been found amplified in 10-27% of breast cancers. CCNDI amplifications associated with ER and PR positivity, but studies on prognostic significance are still controversial [127].

2.8.2.5. MDM2 gene

The MDM2 gene protein product downregulates the TP53 tumor suppressor gene and is amplified in 4-7.7% of breast cancers and has been associated with poor prognosis in some studies [127].

2.8.2.6. Osteopontin

Osteopontin is a secreted acidic glycoprotein that interacts with a variety integrins to promote cell adhesion, migration, and invasion. It is variably phosphorylated on up to 28 sites that are distributed throughout the molecule. It

can be cleaved by thrombin resulting in an enhancement of its function in promoting cell adhesion and migration. There is a great deal of evidence for a role of osteopontin in tumor progression. Transfection of cells with osteopontin results in an increase in the malignant phenotype, while down-regulation causes a decrease in malignancy [159].

2.8.3. Genomic Stability Maintenance Genes

The third class of cancer genes consists of genomic stability maintenance genes, also called caretakers, which include the MMR, NER and BER genes responsible for repairing mistakes during normal replication or induced by mutagenesis exposure. Other stability maintenance genes, like BRCA1, BLM, and ATM, handle mitotic recombination and chromosomal segregation. Altogether, normally functioning, stability genes keep genetic alterations to a minimum [130].

Genomic stability maintenance genes usually involved in DNA metabolism and repair. Mutations in DNA repair genes compromise the ability of a cell to deal with genotoxic agents, allowing the persistence of DNA damage that can then be fixed in the genome as mutations during replication [90].

Regard of all of these three types of genes in breast cancer, regions of chromosomal loss and gain are also a common feature of cancer genomes, often termed copy number alterations. Both large regional aberrations and more focal aberrations are common and are capable of affecting well-known tumor suppressor genes and oncogenes [76]. DNA aneuploidy is a manifestation of chromosomal instability that is recognized as an early feature of malignant transformation and found to be an indicator of prognosis in breast cancer [127].

2.9. Hormones of breast cancer

During puberty, the ovaries begin to produce hormones that promote breast development. Estrogen production encourages the formation of the periductal stroma and expansion of the mammary ducts, and progesterone production accelerates the formation of terminal ductal lobular units These lobular units are specialized cutaneous glands similar to the sweat gland. During pregnancy, progesterone levels become elevated, resulting in rapid growth of terminal ducts and lobules, and additional hormones, such as follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin, oxytocin, and human placental lactogen (HPL) play a role in stimulating the mammary glands [11].

Breast cancers are characterized by their estrogen receptor (ER) and progesterone receptor (PR) status, and it is established that ER expression (ER-positive) identifies a tumor phenotype with improved near/mid-term prognosis and likely benefits from adjuvant endocrine therapy when compared with ER-negative tumors [48]. The presence of hormone receptor identifies a subset of patients responsive to endocrine therapy and with a better prognosis, but even in this group the clinical outcome can be heterogeneous [139].

The presence of estrogen receptor (ER) and/or progesterone receptor (PR) is an important diagnostic feature of breast cancer, reflective of disease etiology and predictive of response to treatment with the antiestrogen tamoxifen [196].

2.9.1. Estrogen

The estrogen receptor (ER) is an intracellular steroid hormone receptor responsible for a cell's response to the effects of estrogens. Estrogen is a potent mitogen in the normal breast. When combined with the action of anterior pituitary hormones, It is also responsible for breast development. Systemically, estrogen

stimulates the release of growth hormone [90**].** Estrogen and its receptor have a significant role in the pathogenesis of breast cancer [87].

Downregulation of the estrogen receptor number or function has historically been the single most effective adjuvant therapy for the treatment of sex steroid receptor positive breast cancers in women [159].

Loss of estrogen receptor expression is an important means of hormone resistance, but the mechanisms involved are poorly understood. Both the initiation and progression of breast carcinoma profoundly influenced by hormonal factors. The molecular mechanisms underlying the clinical problem of hormone resistance are poorly understood. Hormone resistance clearly can develop in some breast cancers, occasionally through mutant receptors or more commonly through changes distal to the ER. However, one-third of breast cancers are ER-negative, and de novo or acquired loss of ER gene expression at the transcriptional level is a likely mechanism for hormone resistance [135].

2.9.2. Progesterone Receptor

Like the estrogen receptor, the progesterone receptor mediates the body's response to a steroid hormone through dimerization, translocation to the site of action, and action as a transcription factor. Like many other factors, including peptides and hormones (inhibin and TGFß, etc.), progesterone can contribute to the lobuloalveolar development of the breast. This action of progesterone requires the presence of the hormone prolactin, and can only take place when preceded by or concurrent with estrogen [90].

While its role in breast carcinogenesis is less well-understood than ER, it can also be used as a prognostic factor. Overall, 66.5% of patients have PR + tumors. Patients whose tumors have both ER + and PR + have decreased mortality when

they are treated with endocrine-based therapies. However, patients whose tumors are ER + and PR - have a worse outcome, and do not have the same decrease in mortality with endocrine-based therapies. Because progesterone receptor is a downstream target of ER, its lack of response to the signals through ER is considered to be a sign of aberrant growth factor signaling within the cell [90].

2.10. Cancer Epigenetics

Epigenetics refers to the study of heritable changes that cannot be explained by changes in the DNA sequence[113, 138]. The term ‘‘epigenetics’’ was first coined in the 1940s to describe the interaction between genes and environment in the development of specific phenotypical traits [187**].** The word is epigenetic-derived from the Greek word (“epi” in classical Greek means “on top”) [134].

The epigenetics-based mechanism leading to carcinogenesis can be alienated into three different categories. First and is the repression of normally active genes. Second is the activation of normally repressed genes. The last is the substitution of core histones by specifically modified histone variants [137]. Epigenetics is an important intracellular procedure that can change the genetic information of the cells that is transmitted during cell division without changing the sequences of the DNA bases. The mechanisms of epigenetics, methylation of DNA and histone alteration are related to carcinogenesis [28].

There are two major epigenetic mechanisms that have a role in cancer; histone modifications and DNA methylation [138, 49].

2.10.1 Histone modifications

There are currently nine types of histone modifications known as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, deimination, proline isomerism and propionylation [164].

In a normal cell, histone modifications function primarily in chromatin remodeling, various modifications or groups of modifications being able to adjust the chromatin from a less accessible heterochromatin form to a more accessible euchromatin form and vice-versa Histone modifications are therefore able to have profound effects on gene transcription, DNA repair, and DNA replication. Histone acetylation and methylation are the most commonly associated modifications associated with cancer[146].

Histone acetylation occurs at lysine residues. By removing the histone tail's positive charge, lysine acetylation of histones results in a weakening of nucleosome-nucleosome and nucleosome-DNA interactions, resulting in changes in chromatin conformation that render DNA more accessible. Acetylation of histones is achieved by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs). Both HATs and HDACs have been demonstrated to be involved in cancer. Two HATs, *p300* and *CBP*, are considered tumor suppressor genes and both proteins are involved in fusion proteins in leukemias [174,84,63]and loss of heterozygosity (LOH) at the p300 locus is associated with hyperacetylation in numerous cancers [180, 97]. Aberrant expression of HDACs has been shown in multiple cancers; upregulation of HDAC2, 3, 8, 6 and 7 has been observed in childhood ALL (124), HDAC1, 2 and 3 upregulation has been observed in ovarian cancer [72], upregulation of HDAC6 has been observed in breast cancer and oral squamous cell carcinoma and HDAC2 upregulation has been observed in numerous cancers .Due to the overexpression of numerous HDACs in cancer, they have become useful therapeutic targets[89].

Histone methylation can occur on arginine or lysine residues and is catalyzed by different enzymes depending on where the methylation occurs. Arginine residues can be mono- or dimethylated while lysine residues can also be tri-

methylated The effects of histone methylation on chromatin conformation are dependent on the context, aiding either a euchromatin or heterochromatin conformation. *LSD1* (*lysine (K)-specific demethylase 1A*), a histone demethylase, has been shown to be aberrantly expressed in cancer with overexpression observed in bladder cancer estrogen receptor negative breast cancer and neuroblastoma Recent genome-wide[164].

2.10.2. DNA methylation

DNA methylation is a covalent chemical modification, resulting in the addition of a methyl (CH3) group at the carbon five position of the cytosine ring [183]**.** Methylation of cytosine located 5‘ to a guanosine can occur across the genome, but most notably within 0.5-4kb CpG dinucleotide rich regions, known as CpG islands Under normal conditions, the vast majority of CpG sites in the genome are methylated, with the exception of CpG islands located 5‘ to the promoter and exon 1 of more than 50% of genes [21] DNA methylation is reversible because it does not alter the DNA sequence; however, it is heritable from cell to cell [47] **.**

About 3–6% of cytosines are methylated in mammals. Approximately 70–80% of CpG sites in the human genome are methylated. Cytosine residues in newly synthesized DNA are methylated by DNA-cytosine methyltransferase-1(DNMT1 [206]. CpG islands, which are areas that show a high density of CpG sites,and are typically associated with active transcription, contain largely unmethylated CpGs Approximately 60% of genes are estimated to be involved in a CpG island in their promoter regions [138].

In the human genome, the most frequently methylated nucleotide is a cytosine that is followed by the N6 position of guanine, giving rise to a CpG dinucleotide. The methylation of cytosine occurs in the C-5 position by a family of DNA

(cytosine-5) methyltransferases (DNMTs) which transfers the methyl group from the universal methyl donor S-adenosyl- L-methionine (SAM / AdoMet) [67].

CpG "islands" are called islands because they are usually found in stretches of DNA that have very high frequencies of C-G dinucleotide repeats. They also occupy approximately 60% of human gene promoters [144]. The term "CpG island" was coined in the late 1970s after a characteristic appearance of DNA restriction fragment on an agarose gel. The Bird group first detected such "islands" of DNA with an average size of 1kb when genomic DNA from a variety of vertebrates was digested with HpaII and HhaI restriction enzymes [90].

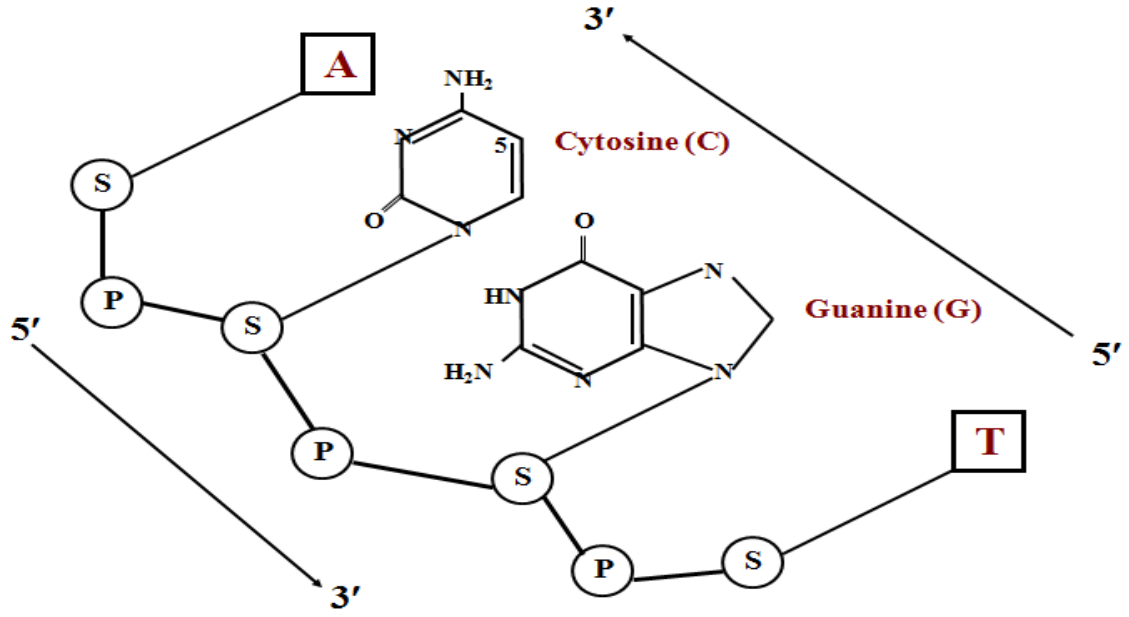


Figure 2.1: The –CpG– dinucleotide of DNA [140]

2.10.2.1. Functions of DNA methylation

DNA methylation has various functions in both normal conditions and roles in disease, especially in carcinogens.DNA methylation plays an essential role in development, chromosomal stability, and for maintaining gene expression States [79] During the developmental process, DNA methylation plays an essential role in

X chromosome inactivation, [206,115] in female somatic cells and in the monoallelic silencing of parentally imprinted genes Once these DNA methylation patterns are acquired in the early embryo stage these patterns are inherited and maintained over successive cell generations [178].

2.10.2.2. CpG islands

The accepted definition of a CpG island is a region having a GC content greater than 50% and an observed CpG to expected CpG ratio of greater than 60% [58].

The overall G/C content within CpG islands is approximately 60%, and the number of CpG dinucleotides can be ten times greater than any other similarly sized region of the genome. There are estimated to be approximately 27 000 CpG islands within the human genome [104] with approximately 50-60% of genes having at least one CpG island associated with them, a large number of which are important housekeeping genes [192, 105].

Similarly, there are about 15 500 CpG islands within the mouse genome [125]. Often, but not exclusively, located at the 5' end of genes and in some cases extending over hundreds of nucleotides, the methylation status of CpG islands can have a large impact on gene expression, mainly via chromatin modifications. In general, it is thought that CpGs in CpG islands are much less likely to be methylated than other CpGs throughout the genome due to the selective pressure for them to remain unmethylated and active [14]. Although it has now been shown that CpG islands can be methylated in normal tissues [193], this may relate to different types of cell differentiation and activity. Also, a study identified regions of DNA that show differential methylation levels between different tissue types that are up to 2kb away from CpG islands, known as CpG shores (. These regions have a median size of 255bp and have been suggested to be responsible for the

majority of tissue differential methylation, rather than CpG islands . Differential methylation at these regions has also been implicated in cancer [86].

2.10.2.3 DNA methylation machinery

DNA methylation is laid down by two classes of DNA methyltransferase enzymes, *DNMT1* (*DNA methyltransferase 1*) and *DNMT3a* and *3b* (*DNA methyltransferase 3a and 3b* respectively). Originally named *DNMT1* and *DNMT3* because of an additional molecule, *DNMT2*, discovered at the same time with high levels of similarity. However, little evidence for the ability of *DNMT2* to methylate cytosine was discovered and it has since been determined that *DNMT2* catalyze the methylation of position 38 in the aspartic acid of tRNA [66].

It is now also referred to as *TRDMT1* (*tRNA aspartic methyltransferase 1*).While both *DNMT1* and *DNMT3a/b* use the same methyl donor, S-adenosyl methionine, the two classes of enzyme target different states of DNA to methylate.

DNMT1 is often known as maintenance methyltransferase since it primarily methylates hemimethylated DNA on the nascent strand of DNA following DNA replication [12], thus providing a mechanism by which methylation status of DNA is maintained in daughter cells. *DNMT1* has been shown to be essential for maintaining DNA methylation patterns in In mammals, promoter methylation at CpG dinucleotides is a like histone modifications, DNA methylation does not impact genomic DNA sequence itself [37] but adds a methyl (CH3) group on cytosines of CG dinucleotides.This reaction catalyzed by a DNA methyltransferase enzyme families [7,175,37].

DNMTs have commonly been classified as either “maintenance” (DNMT1) or “de novo” (DNMT3) methyltransferases. This classification is based on the observation that Dnmt1 interacts with proliferating cell nuclear antigen (PCNA),

an auxiliary component of the DNA replication complex, and localizes to replication foci (188). DNMT3 are classified into two type's methylation than DNMT3a and DNMT3b [90, 76 ,183].

DNMTs are involved in the downregulation of tumor suppressor genes and the stimulation of proto-oncogenes Hypo- and hypermethylation when gene expression altered due to DNA methylation. It is usually categorized as due to hypomethylation or hypermethylation [1].

ADNMTgenerally comprises two domains: a well-conserved catalytic domain in the carboxy-terminal part of the protein and a more variable regulatory domain in the amino-terminal region [188].

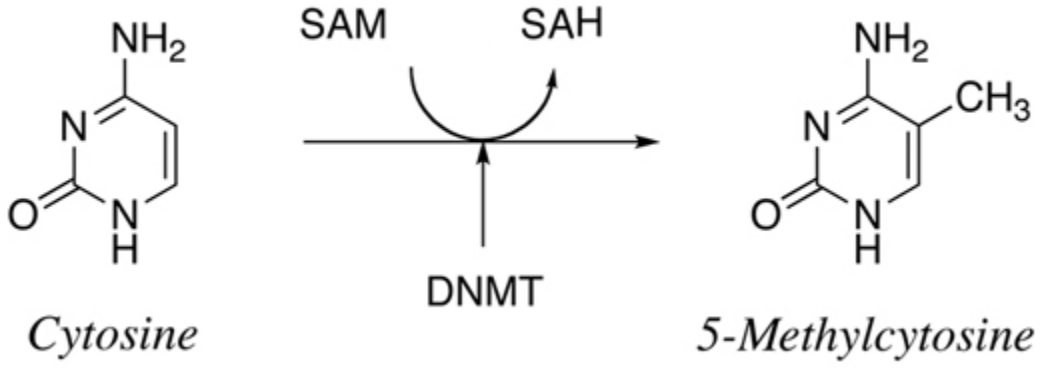


Figure 2.2: Methylation of cytosine by DNMTs [190]

A methyl group covalently bonded to the 5-carbon on the cytosine base. This process is mediated by one or more of a panel of enzymes known as DNA methyltransferases. The methyl group is provided by S-adenosyl methionine (SAM), and this converted to S-adenosyl-homocysteine (SAH) in the process. This recycled back to SAM in a folate- and cobalamin-dependant pathway. Biologic methylation in vertebrates occurs only on the cytosine bases, and further only on

those linked directly to a guanine by the phosphodiesterase link, forming a CpG dinucleotide pair [189]. Cytosine residues in newly synthesized DNA are methylated by DNA-cytosine methyltransferase-1(DNMT1).This enzyme transfers a methyl group from the methyl donor, S-adenosylmethionine, to nascent DNA using a hemimethylated DNA template to maintain DNA methylation patterns during cell division in mammals[206].

The mechanism of methylating DNA involves the DNMT catalytic domain to induce base flipping, everything the C residue out of the helix to insert into the active site of the enzyme. This allows association of the base with the methyl donor, S-adenosyl-L-methionine (SAM), and the formation of 5-methylcytosine Although the mechanism is similar for different DNMTs, they appear to have different responsibilities in various developmental stages within organisms [144].

2.10.2.4 Types of DNA methyltransferases

2.10.2.4.1 DNMT1

DNMT1 plays a significant role in the development of breast cancer.However, expression levels of DNMT1 in breast cancer tissues have been a matter of debate, and the underlying mechanism is still not entirely clear. It handles the exact copying of the DNA methylation pattern on the neo-synthesized strand during DNA replication. Therefore, it principally localizes to the DNA replication fork [166, 37]. The DNMT1 gene in human spans more than 60kb in the genome, composing at least of 40 exons and 39 introns, and its single canonical transcript spreads about 5.2 kb long. The protein DNMT1 is predominantly expressed in somatic tissues and proliferating cells and contains 1616 amino acid residues with a molecular mass of about 190 kDa.DNMT1 has specificity for hemimethylated double-stranded DNA as compared to unmethylated double-stranded DNA [67].

2.10.2.4.2. DNMT3a and DNMT3b

DNA methyltransferase 1, 3a, and 3b affect DNA methylation, and it is thought to play an important role in the malignant transformation of various cancers. DNMT3a and DNMT3b are mainly involved in the de novo establishment of methylation patterns during embryogenesis [10,76]. The methyltransferase DNMT1 maintains De novo methyltransferases DNMT3a and DNMT3b, Overexpression of Dnmt1, 3a and 3b has been demonstrated in a variety of tumor types including bladder, colon, kidney and pancreas [23]. Both DNMT3a and DNMT3b expressed in ES cells; they both undergo a decrease in expression of differentiation [157].

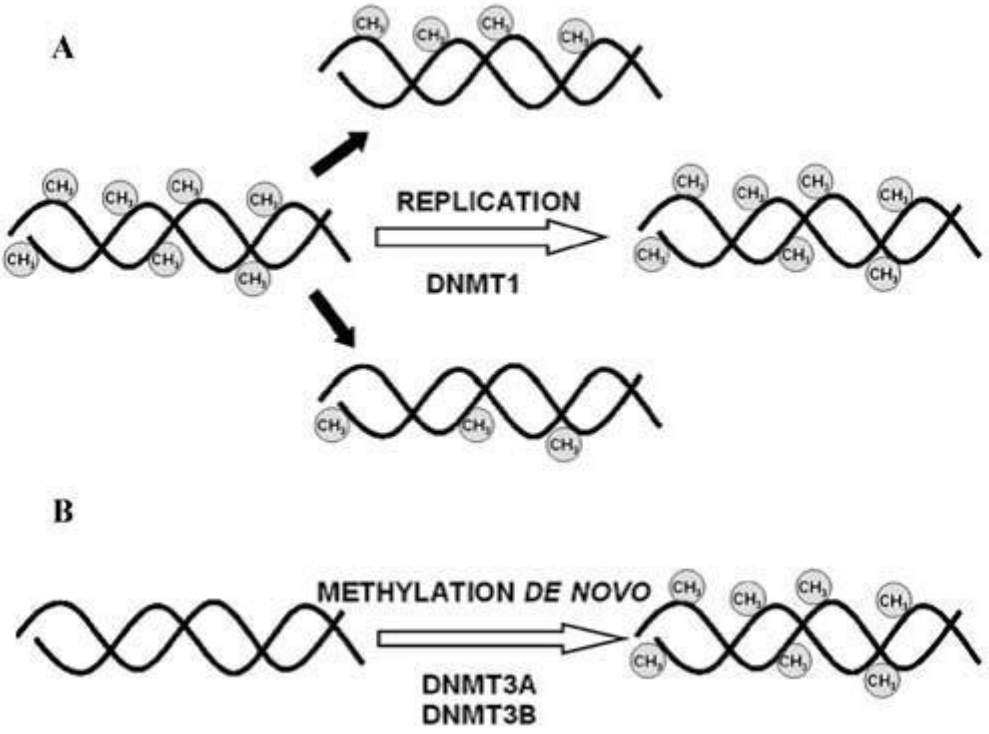


Figure 2.3: (A) Maintenance and (B) de novo DNMTS methylate DNA. DNMT1 binds methyl groups to the hemimethylated DNA during replication, whereas DNMT3A and DNMT3B can add methyl groups to CpG dinucleotides of unmethylated DNA [183].

2.10.2.4.3 DNA methyltransferases and cancer

A study in (2007) demonstrated the necessity of cancer cells for functioning *DNMT1*. A conditional *DNMT1* knockout colorectal cancer cell line, HCT116, was made that demonstrated a reduction of CpG-CpG dyads within the genome and arrest at the G2 phase of the cell cycle, suggesting *DNMT1* is essential for maintenance of methylation in cancer cells and required for proliferation and survival [27]. Whilst inhibition of *DNMT3a* and *3b* appear to have a lesser effect on cancer cells [151, 156], downregulation of the miRNA (miR)-29 family (29a, 29b, and 29c), which have complementary sites within the 3'-UTRs of *DNMT3a* and *DNMT3b*, in lung cancers have shown upregulation of DNMT3a and 3b, associating with worse prognosis [46]. upon re-expression of the miRNAs, normal patterns of DNA methylation were restored, as was the expression of some methylation-silenced tumor suppressor genes [46]. A recent study identified mutations in *DNMT3a* present in 22.1% of acute myeloid leukemia patients [109]. While mutations were associated with worse overall survival, no significant differences in overall 5mC levels were observed in patients with mutations. However, it was noted that some regions showed significantly less methylation in a fraction of patients [109]. Since this study, mutations in *DNMT3a* have also been identified in acute monocytic leukemia [205] and myelodysplastic syndromes [191].

2.10.2.4.4 DNA methyltransferase inhibitors

DNMTis are used to prevent DNA re-methylation after cell division and can be classified as nucleoside analogs and non-nucleoside analogs [49]. First, the nucleoside analog 5-aza-CdR, which incorporated into DNA during replication following its phosphorylation to the trinucleotide form and trapped the DNA methyltransferase as it moves along with the replication fork. As the replication

fork is progressing, nascent DNA is synthesized in the absence of DNA methylation. A second inhibitor is an antisense oligonucleotide inhibitor of DNMT1, which knocks down DNMT1 protein levels [64].

During DNA replication, incorporated 5-azacytidine irreversibly binds DNA methyltransferase (DNMT)1, which prevents maintenance methylation on site while depleting available cellular DNMT1, and creates cytotoxic DNA adducts. Depletion of DNMT1 causes DNA replication to proceed, with progressive loss of methylation. 5-Azacytidine-induced hypomethylation affects global DNA as well as CpG islands in gene promoters, where it often associated with transcriptional upregulation [160].

The nucleoside analogues 5-azacytidine and 5-azadeoxycytidine are potent demethylating agents that have been used to investigate methylation experimentally for years [90, 157,1, 11 , 76 , 141, 2011 ,115] and Two drugs,(non- nucleoside) hydralazine and procainamide, are currently in use clinically to treat different disorders[90].

2.11 DNA methylation in breast cancer

DNA methylation exhibits different patterns in different cancers. DNA methylation plays an important role in carcinogenesis and cancer progression through hypermethylation to turn off the expression of tumor suppressors and hypomethylation to activate the expression of oncogenes [145]. The degree of DNA methylation in the promoter region of tumor suppressor genes, transcription factors, and drug response genes may play a role in the initiation of cancer, tumor progression and response to treatment [93].

2.11.1. Aberrant DNA Methylation in breast cancer

Two obvious theories can postulate for this aberrant de novo methylation. First, the cancer methylation spreads from normal methylation-centers surrounding the methylation-free CpG island, for example from Alu regions. Second, a `seeding' of methylation exists, and certain single CpG dinucleotides in the island became methylated and subsequently this `attracts' now more methylation. This process has a positive, cooperative effect until hypermethylation achieved [45].

Epigenetic alterations are one of the most common molecular changes in human cancer [92]. In cancer cells, aberrant hypermethylation of these promoter regions is associated with transcriptional silencing. Hypermethylation is an alternative mechanism for inactivation of tumor suppressor genes [201,59]. The contribution of aberrant DNA hypermethylation of cancer-related genes to the transcriptional silencing and carcinogenesis has been demonstrated in different diseases including different cancer types [6].

Promoter hypermethylation causes gene silencing and is thought to be an early event in carcinogenesis. Along with genetic alterations, epigenetic events are important in cancer development and progression. Hypermethylation of CpG islands in promoter regions is the well-characterized epigenetic change and is a common mechanism for silencing tumor suppressor genes [96]. Hypermethylation of CpG islands affects genes involved in cell cycle control, DNA repair, cell adhesion, signal transduction, apoptosis and cell differentiation [79].

2.11.1.1. DNA hypermethylation in breast cancer

Hypermethylation of the cytosine-phospho-guanine (CpG) islands of gene promoters is an important epigenetic mechanism for gene silencing, which may confer a growth advantage to tumor cells [10].

Hypermethylation of CpG dinucleotides near the transcriptional regulatory region may initiate the recruitment of the methyl-CpG binding domain (MBD) family proteins that mediate silencing of genes via facilitation of a repressive chromatin environment. At least five methyl-CpG binding proteins, including MeCP2, MBD1, MBD2, MBD3, and MBD4, have been identified in vertebrates [108].

2.11.1.1.1 Tumor suppressor-DNA methylation in breast cancer

Epigenetic silencing of TSGs is frequently involved in the pathogenesis of multiple cancers. Aberrant methylation of promoter CpG islands (CGI) is an important epigenetic inactivation, leading to the binding of transcription repressors, compressed chromatin and transcription silencing [201]. Methylation of cytosines in promoter region CpG dinucleotides is a common mechanism for silencing tumor suppressor genes (TSG) and frequently occurs in breast cancer as well as in many other cancers [108].

Tumor suppressor genes are the key targets of hypermethylation in breast cancer and, therefore,they may lead to malignancy by deregulation of cell growth and division [24]. There are numerous examples of hypermethylation of promoter region in the aberrant CpG islands of the tumor suppressor genes, genes that are involved in cell-cell adhesion and genes that are involved in DNA repair [161]. Some genes commonly hypermethylated in breast cancers are involved in evasion of apoptosis (RASSF1, HOXA5, TWIST1) and cellular senescence (CCND2, CDKN2A) while others regulate DNA repair (BRCA1), cell growth (ESR1, PGR), and tissue invasion (CDH1). DNA methylation is the most studied epigenetic event in cancer [80]**.** These hypermethylated promoters are very rarely found in normal breast [210].

2.11.1.1.2 DNA hypermethylation as Biomarker

Hypermethylation events have also been shown to serve as biomarkers in human cancers, for early detection in blood and other bodily fluids, prognosis or prediction of response to therapy, and to monitor cancer recurrence [36]. Although the list of hypermethylated genes in breast cancer is growing, only a few show promise as biomarkers for early detection and risk assessment [77]. Methylated genes can serve as biomarkers for early detection of cancer [47].

2.11.1.2 DNA hypomethylation in breast cancer

Loss of DNA methylation was the first epigenetic alteration identified in cancer cells. Global genomic hypomethylation is due in large part to the loss of methylation in repetitive DNA sequences .the degree of hypomethylation has associated with disease severity and metastatic potential [53]. There are many functional implications of global DNA hypomethylation as it relates to cancer. By weakening transcriptional repression, DNA hypomethylation can facilitate chromosomal instability, which is another hallmark of tumor cells [138].

Hypomethylation at DNA repetitive elements leads to genomic instability and might also induce overexpression of oncogenes. Aberrations leading to decreased methylation of CpG islands (hypomethylation) of oncogenes are known to occur [113)].

Global hypomethylation of the cancer genome was initially shown to cause genomewide allelic instability [120, 9 ,199]. It is seen in some cancers, such as breast, cervical and brain, showing a progressive increase in proficiency of malignancy [204]. Hypomethylation of DNA plays a significant role in the activation of certain genes, particularly oncogenes [204]. Normally unmethylated promoters may become densely methylated, and this results in the silencing of

critical genes such as tumor suppressor genes. Other sequences become instead hypomethylated in tumors, leading to the aberrant activation of genes that are normally repressed by DNA methylation [36].

In some cancers, both hypermethylation and hypomethylation are observed. Hypomethylation increases progressively with increasing malignancy grade in breast, ovarian, cervical, and brain cancers. Breast and colorectal cancers are malignancies commonly caused by regional hypermutability or global hypomethylation. Global hypomethylation could be a mechanism for late stages of breast cancer while local hypermethylation is plausible for early stages of breast cancer [1].

Cancer cells of many different human neoplasms exhibit aberrant DNA methylation patterns, with global hypomethylation [155]. The methylation patterns of virtually all types of cancer, including breast carcinoma, have been found to differ extensively from that of the corresponding normal tissue. These alterations are cancer-type concrete and include global genomic hypomethylation as well as non-random hypermethylation of normally unmethylated CpG-island promoters [16].

2.11.1.2.1 Hypomethylation and gene activation

The global hypomethylation seen in some cancers, such as breast, cervical, and brain, shows a progressive increase with the grade of malignancy. This hypomethylation occurs mainly in the body of genes (coding regions and introns), as well as in pericentromeric regions of chromosomes rich in repetitive DNA sequences. The principal mechanisms put forward in attempting to explain cancer causation by hypomethylation include chromosome instability and reactivation of transposable elements and/or inappropriate gene activation,Oncogenes such as cMYC and H-RAS75 [183]. Global hypomethylation of genomic DNA in the

regulatory sequences is observed in numerous tumor cells and is responsible for transcriptional activation and overexpression of proto-oncogene, retrotransposons, growth factors and genes which via their protein products are involved in genomic instability and malignant cell proliferation, invasion, and metastasis [134].

2.12 Approaches for DNA Methylation Studies

Traditionally, methylation analysis has been carried out by Southern hybridization, which assesses a few methylation-sensitive restriction sites within CpG islands of known genes. Further development of sensitive assays, such as bisulfite DNA sequencing and methylation-specific PCR, has allowed a detailed analysis of multiple CpG sites across a CpG island of interest [83].

2.12.1 Bisulfate conversion

Sodium bisulfite treatment of genomic DNA revolutionized DNA methylation studies [37].

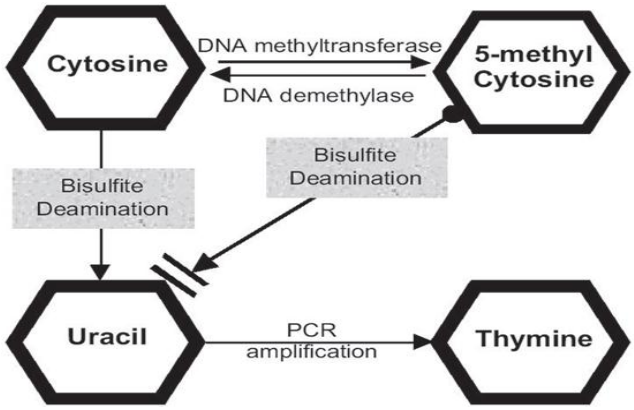


Figure 2.4: Biochemical reaction pathways of cytosine in vivo and in-vitro. Sodium bisulfite can convert cytosine into uracil through the steps of sulfonation, hydrolytic deamination, and subsequent desulfonation with alkali. 5-Methylcytosine is, however, protected from this bisulfite reaction owing to the presence of the methyl group, which blocks the sulfonation by bisulfate [182].

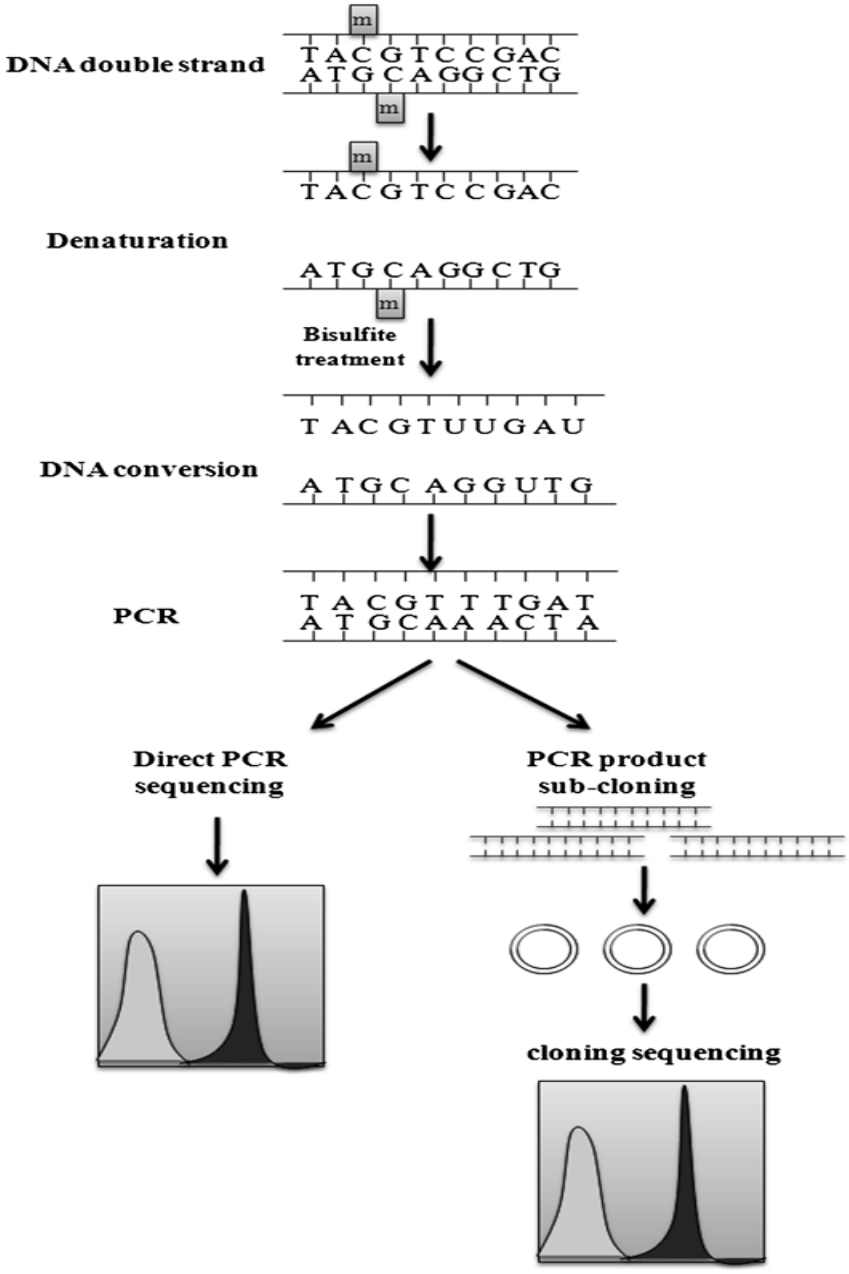


Figure 2.5: Principles of methylation analysis using bisulfite genomic sequencing. After treatment with sodium bisulfite, unmethylated cytosine residues are converted to uracil whereas 5-methylcytosine (5mC) remains unaffected. After PCR amplification, uracil residues are converted to thymine. DNA methylation status can determine by direct PCR sequencing or cloning sequencing [182].

2.12.2 CoBRA (combined bisulphite restriction analysis)

CoBRA (combined bisulphite restriction analysis) developed as a rapid way of detecting methylation in bisulphite modified DNA. Following bisulphite conversion, PCR amplification is performed in the region of interest and subsequently digested with an enzyme that will only cleave DNA if methylation was present in the original unmodified DNA. The two most commonly used enzymes are BstUI and TaqαI [182 ,76].

2.12.3 Methylation-specific PCR (MSP)

Methylation-specific PCR (MSP) is another commonly used PCR-based technique on bisulphite modified DNA. It is based on designing primers that are capable of specifically amplifying either methylated DNA or unmethylated DNA [108,182 ,52,76,37].

2.12.4 Pyrosequencing

Pyrosequencing of methylated DNA developed a sensitive and quantitative method for analyzing the methylation status of CpGs. The basic pyrosequencing technique based on incorporation of other dinucleotides to a template that are released in a predetermined order; when the correct nucleotide released, it binds to the template strand, releasing pyrophosphate [76].

2.12.5 MethyLight™

The MethyLight™ method uses quantitative PCR techniques of the TaqMan™ system, which utilizes a sequence-specific probe, designed to anneal to PCR products, emitting a fluorescent signal when binding occurs which can accurately measure throughout the PCR reaction [76].

Chapter three

Materials and Methods

Materials and Methods

3.1. Materials

3.1.1. Apparatus and instruments

The followings are apparatus and instruments that use of in this study:

Table 3.1 Laboratory instruments

No.	**Apparatus and instruments**	**Company name**
1	Oven	Lab Tech
2	Balance	AND
3	Autoclave	LGAGS
4	Centrifuge	Through scientific
5	Gel apparatus	Bio-Rad
6	PH meter	EUtech
7	Vortex	Lab Tech
8	Shaker	Cale farmer
9	Microwave	JEC
10	Refrigerator	Concord
11	Deep freeze	GFL
12	Nanodrops	Janeway
13	Water bath	Mememrt
14	PCR	Corbet research
15	Ice maker	Lab Tech
16	Accuflow Mini Centrifuge	Lab Tech
17	Gel documentation	Lab Tech
19	Water distillation	T and M (Japan).
20	genetic analyzer	Applied Biosystems

21	1.5 ml Microcentrifuge tubes	Promega
22	Different size of pipette	Lab Tech
23	Pipette tips with aerosol barrier	Promega

Table 3.2: Chemical materials

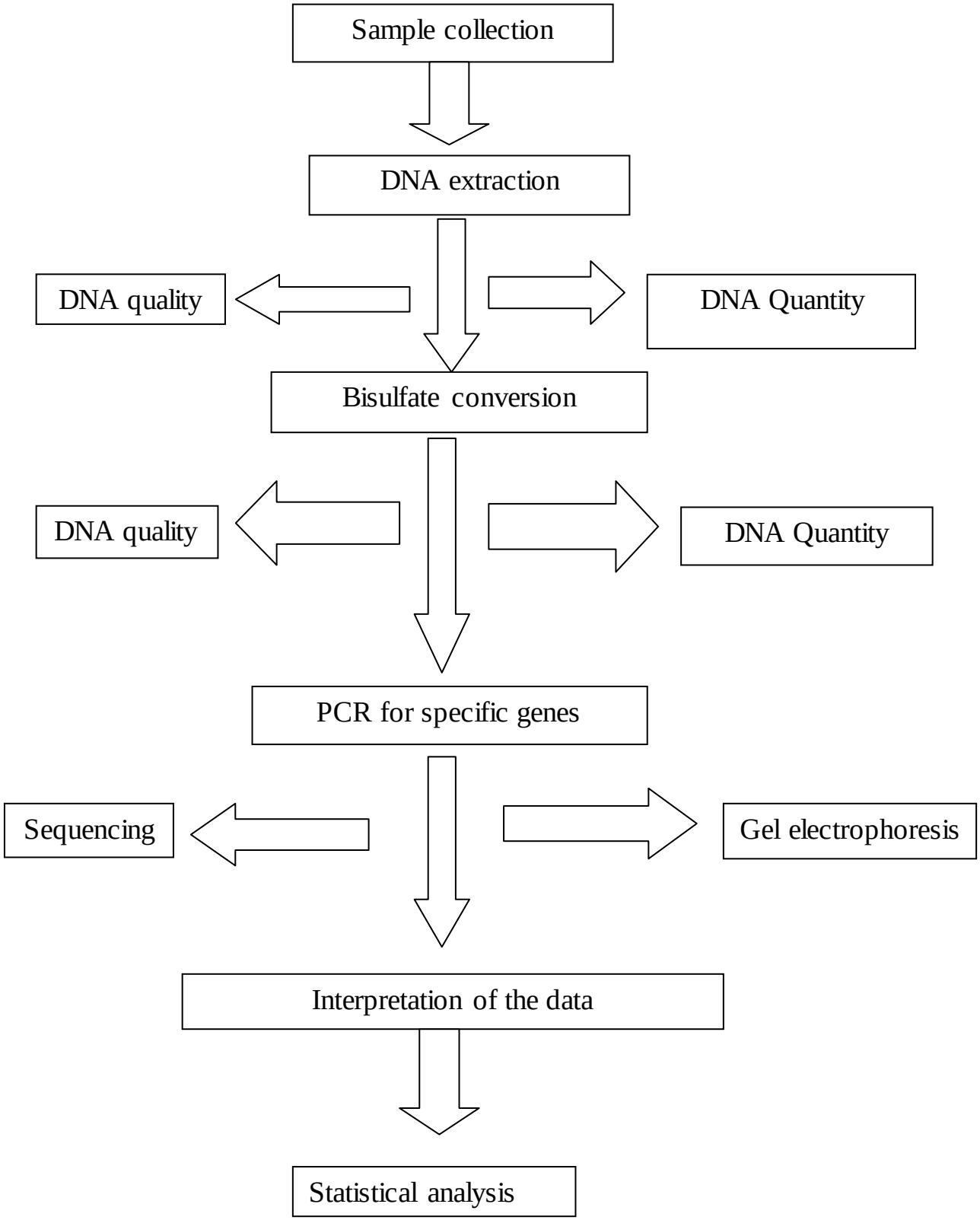
NO	**Materials**	**Company name**
1	Ethanol (96–100%)	MERCK
2	EDTA	Sigma
3	NaOH	MERCK
4	Tris base	Schedule
5	Boric acid	Tomas Baker
6	Glacial Acetic Acid	MERCK
7	Acetic Acid	MERCK
8	Ethidium bromide	Promega
9	Loading dyes	Promega
10	Agarose	Genet bio
11	Sodium acetate	MERCK

3.1.1 .Kits

Table 3.3: kits used in this research

No	**Kits**	**Company**	**Cat number**
1	DNA extraction	QIAGEN	51304
2	Diamond™ Nucleic Acid Dye	Promega	H1181
3	MethylEdge™ Bisulfite Conversion System	Promega	N1301
4	Converted Methylated Human Control	Promega	N1221
5	Methylated Human Control	Promega	N1231
6	GoTaq Green Master Mix	Promega	M7122
7	DNA ladder 100 bp	Genet bio	M-1000
8	PCR purification	Gene aid	QAIC/TW/50077
9	Hi-Di™ Formamide sequencing	Applied Biosystems	4311320
10	BigDye Terminator v3. 1 Cycle Sequencing Kit	Applied Biosystems	4336935

3.1.2. Research design



3.2. Methods

3.2.1. Sample collection

A total of 38 blood samples were collected from women with different grades of breast cancer in Hiwa hospital via sterile disposable syringe from an arm vein as these vessels were typically large. All blood samples were placed in a test tube containing anticoagulant ethylene diamine tetraacetic acid (EDTA) to prevent clotting of the blood. Blood samples were divided into three groups, 13, 14 and 11 blood samples for ESR2, OPN and BRCA1 genes respectively (**Tables 3.4, 3.5 and 3.6**).

Based on Table 3.4, a total of thirteen sample for ESR2 gene were collected (3) 27.07 %healthy. Ten samples 76.92 %had breast cancer, (7) 70% from breast cancer hade grade 1 and (3) 30% grade 2.

Based on Table 3.5, fourteen samples were collected for OPN gene (3) 21.42 %healthy, (11) 78.57 %had breast cancer. From thes 11 sample ,(7) 63.63% had grade 1 and 36.36% had grade 2.

Based on Table 3.6, eleven samples were collected for BRCA1 gene (2) 18.18% healthy, (9)%81.81% had breast cancer. 9 samples had breast cancer (5)55.55 % had grade 1 and(4)%44.44 had grade 2.

Table 3.4: sample of ESR2 gene

NO	**Types of sample**	**Grades of breast cancer**	**Age**
1	Breast cancer	2	43
2	Healthy		58
3	Breast Cancer	1	64
4	Breast Cancer	1	35
5	Breast Cancer	1	55
6	Breast Cancer	2	48
7	Breast Cancer	1	61
8	Healthy		44
9	Healthy		53
10	Breast Cancer	1	35
11	Breast Cancer	1	41
12	Breast cancer	1	64
13	Breast cancer	2	49

Table 3.5: samples for OPN genes

NO	**Types of sample**	**Grades of breast cancer**	**Age**
14	Breast cancer	1	44
15	Breast cancer	1	35
16	Breast cancer	1	39
17	Breast cancer	1	46
18	Breast cancer	2	53
19	Healthy		64
20	Breast cancer	1	44
21	Breast cancer	1	43
22	Breast cancer	1	47
23	Breast cancer	2	70
24	Breast cancer	2	47
25	Breast cancer	2	55
26	Healthy		50
27	Healthy		47

Table 3.6: samples for BRCA1 gene

NO	Types of sample	Grades of breast cancer	Age
28	Breast cancer	1	53
29	Breast cancer	1	34
30	Healthy		54
31	Breast cancer	2	47
32	Breast cancer	1	35
32	Breast cancer	2	40
34	Breast cancer	2	50
35	Breast cancer	2	80
36	Breast cancer	1	43
37	Healthy		53
38	Breast cancer	1	52

3.3 .DNA extraction

3.3.1. Preparation of reagents

3.3.1.2. proteinase K

Pipette 1.2 ml proteinase K solvent into the vial containing lyophilized proteinase K, mixed by vortexing and store at -2-8°C in the refrigerator.

3.3.1.3. Buffer AL

Mix Buffer AL thoroughly by shaking before use. Buffer AL is stable for one year when stored at room temperature (store at room temperature, 15–25°C).

3.3.1.4 .Buffer AW1

Buffer AW1 was complete as a concentrate. Before using it for the first time, added the appropriate amount of ethanol was added (96–100%) as indicated on the bottle. Buffer AW1 is stable for one year when stored closed at room temperature (stored at room temperature, 15–25°C).

3.3.1.5 .Buffer AW2

Buffer AW2 was full as a concentrate. Before used for the first time, add the appropriate amount of ethanol (96–100%) to Buffer AW2 concentrate as indicated on the bottle. Buffer AW2 is stable for one year when stored closed at room temperature (stored at room temperature, 15–25°C).

3.3.2. Procedure

1. Twenty µl of Qiagen proteinase K was Pipetted onto the bottom of a 1.5 ml microcentrifuge tube.

2. Two hundred microliters of samples were added to the microcentrifuge tube.

3. Two hundred microliters of Buffer AL were added to the sample, and then mixed with pulse vortexing for 15 seconds. For guarantee efficient lysis, It is vital that the sample and Buffer AL be mixed thoroughly to yield a homogeneous solution.

4. The mixture incubated at 56°C for 10 min. DNA yield reaches a maximum after a loss for 10 minutes at 56°C.

5. A Brief centrifugation was required for the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

6. Two hundred microliters of ethanol (96–100%) were added to the sample and mixed again by pulse vortexing for 15 seconds. After mixing, brief centrifugation

applied to the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

7. The mixture from step 6 carefully applied to the QIAamp Mini Spin Column (in a 2 ml collection tube) without wetting the rim. The cap closed, and centrifuged at 6000x g for 1 min. The QIAamp Mini spin column placed in a clean 2 ml collection tube (provided), and the tube containing the filtrate was discarded. Spin column was closed to avoid aerosol formation during centrifugation.

8. The QIAamp Mini Spin column was opened and added 500 µl Buffer AW1 without wetting the rim. The cap was closed and centrifuged at 6000x g for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube (provided), and the collection tube containing the filtrate was discarded.

9. The QIAamp Mini Spin Column carefully opened and added 500 µl Buffer AW2 without wetting the rim. The cap closed and centrifuged at full speed; 20000x g for 3 minutes.

10. The QIAamp Mini spin column was placed in a new 2 ml collection tube, and the old collection tube with the filtrate was discarded. Centrifuge at full speed for 1 minutes.

11. The QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube, and the collection tube containing the filtrate was discarded. The QIAamp Mini Spin Column carefully opened and 200 µl Buffer AE or distilled water was added and then Incubated at room temperature (15–25°C) for 1 minutes, there after centrifuged at 6000x g for 1 minute and again incubated, the QIAamp Mini Spin column, loaded with Buffer AE or water for 5 min at room temperature before centrifugation to increase DNA yield.

3.3.3. Determination of DNA quantity

3.3.3.1 .Procedure

1. To begin, the upper and lower optical surfaces of the microvolume nano drops sample retention system were cleaned by pipetting of three μL of clean deionized water onto the lower optical surface.

2. The lever arm was closed, ensuring that the upper pedestal comes in contact with the deionized water. The lever arm was left and wiped off both optical surfaces with a clean, dry, lint-free lab wipe.

3. The NanoDrop software was opened, and the Nucleic Acid application was selected. A small-volume was used, calibrated Pipettor to Perform a blank measurement by dispensing 1 μL of buffer onto the lower optical surface.

4. Once the blank measurement was completed, both optical surfaces were cleaned with a clean, dry, lint-free lab wipe.

5. The appropriate constant for the sample that is to be measured was selected.

6. 1 μL of the nucleic acid of the sample dispensed onto the lower optical pedestal and closed the lever arm. Because the measurement was volume independent, the sample only needed to bridge the gap between the two optical surfaces for a measurement make.

7. The software automatically calculates the nucleic acid concentration and purity ratios.

Table 3.7: Typical nucleic acid concentration ranges for direct A280 absorbance measurements using a nanoDrop

Sample Type	Select Option	Constant Used to calculate Concentration
dsDNA	DNA-50	50
RNA	RNA-40	40

3.3.3.2 .Determination of DNA quality

3.3.3.2.1. 0.5 M EDTA

EDTA (93,05 gram) was dissolved in 400 ml of deionized water by using magnetic stirrer. NaOH was added until the solution became clear. The volume was adjusted to 500 ml with deionized water [132].

3.3.3.2.2. TBE 10x

Tris (108gm) and 55gm of Boric acid were dissolved by a magnetic stirrer in 800 ml distilled water, and then 40 ml 0.5 M Na_2EDTA (pH 8.0) (alternatively use 9.3 grams Na_2EDTA) was added to the solution, after that the volume adjusted to 1000ml and stored at room temperature. For agarose gel electrophoresis, working solution of TBE was prepared at a concentration ratio of (1:10 dilution of the concentrated stock solution) [142].

3.3.3.2.3. 50X TAE

Tris base (242 gm) (MW=121. 1) was dissolved in 750ml ddH_2O, and then (57.1 ml) Glacial Acetic Acid and (100 ml) 0.5 M EDTA was added and mixed in a stir bar. The final volume was adjusted to 1L with ddH_2O and Stored at room temperature [142].

3.3.3.2.4 6X DNA Loading Dye

Loading Dye 6X purchased from Promega /USA is a convenient marker dye containing 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10 mM Tris-HCl (pH 7.5) and 50mM EDTA (pH 8.0).

3.3.3.2.5. Ethidium bromide solution (1000× stock solution, 0.5 mg/ml)

Ethidium bromide (50mg) was added to 100 ml H_2O and mixed well. The stock solution diluted (1:1000), stored and protected from light in the refrigerator.

3.3.3.2.6. Diamond™ Nucleic Acid Dye

Diamond™ Nucleic Acid Dye (a) is a sensitive fluorescent dye that binds to single-stranded DNA, double-stranded DNA, can be used to stain and visualize nucleic acids in gels. Dys 10000 x and stored at room temperature for 90 days or -20 for long times 1 x dye used just for three gels and stable for three days.

3.3.3.2.7. Preparation of 1X Diamond™ Nucleic Acid Dye

Diamond™ Nucleic Acid Dye was thawed completely at room temperature (22°–25°C) protected from light, and then vortexed briefly. The thawed dye was diluted (1:10,000) in a 1X of TBE or TAE buffer. For best results, the buffer used to dilute the dye should be the same as the buffer used to cast the gel.

3.3.3.2.8 Agarose gel preparation

Agarose (1.5 gm) was added to 100 ml of TAE or TBE buffers in a conical flask. The solution was melted in a microwave oven and swirled to ensure even mixing.

3.3.3.2.9. Loading and running the gel

After melted agarose had solidified, the comb removed accurately. Loading dye 1µl was mixed with 5µl of the DNA sample. Moreover, then the mixture was put in the gel casting tray to the electrophoresis tank, and adequate amount running

buffer was added. The electrophoresis power supply was set on 80V, 5W, and 76A for 60 minutes.

3.3.3.2.10. Photography of DNA

DNA photographed in agarose gels stained with ethidium bromide or Diamond™ Nucleic Acid Dye by illumination with UV light.

3.4. MethylEdge™ Bisulfite Conversion System

The principle of bisulfite converted reaction based on the unmethylated cytosine converted to uracil while methylated cytosine unaffected.

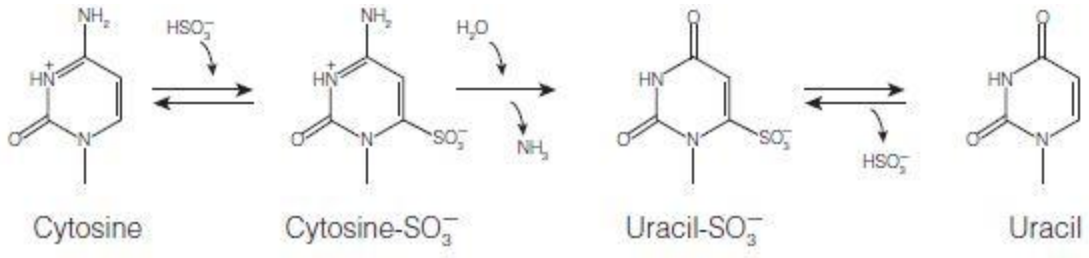


Figure 3.1 Bisulfite conversions of unmethylated cytosines to uracil.

3.4.1. Prepare 1X ME Wash Buffer

Ethanol (24ml) of 95–100% was added to a bottle containing 6ml of the concentrated ME Wash Solution.

3.4.2. DNA samples preparation

1.20µl of purified DNA was prepared; the optimal range was 200–500ng.

2. Control reactions were prepared by using pre-qualified methylated and unmethylated DNA from a source that is similar to the source of the experimental samples.

3.4.3. Bisulfite Conversion

1. DNA samples (20µl) of each were placed in a 200µl PCR tube.

2. Bisulfite ME Conversion Reagent (130µl) was added to each DNA sample, and pipette gently to mix. Brief centrifugation collected the samples.

3. Thermocycler programming was at 8 minutes at 98°C, 60 minutes at 54°C moreover, hold at 4°C.

4. Samples stored at 4°C or on the ice, protected from light for up to 20 hours until ready to proceed to, DNA Desulfonation and Cleanup.

3.4.4 .DNA Desulfonation and Cleanup

1. For each sample to be processed, a ME Spin Column was placed into one of the provided collection tubes.

2. ME Binding Buffer (600µl) was added to the ME Spin column. The entire bisulfite-treated sample transferred to the column, the cap closed and mixed by inverting the tube several times.

3. The tubes spin at maximum speed (10, 000 × g) for 30 seconds. Discard the flow through and re-inserted the ME Spin column into the same collection tube

4. ME Wash Buffer (100µl of 1X) was added, and spin at maximum speed (10, 000 × g) for 30 seconds.

5. ME Desulfonation Buffer (200µl) was added to each ME spin column, the caps closed and incubated for 15 minutes at room temperature.

6. The tubes were centrifuged at maximum (10, 000 × g) for 30 seconds.

7. ME wash buffer (200µl) was added and then spin at top speed for 30 seconds. The wash step was repeated once more.

8. ME spin column was placed into a clean 1.5 ml microcentrifuge tube.

9. ME elution buffer (10µl) was added and spin at maximum speed (10, 000 × g) for 30 seconds.

10. ME spin column was removed and discarded and stored at -20.

3.4.5. Assessing DNA Quality Following Bisulfite Conversion

Nanodrops was used for determining quantities of bisulfite-converted DNA, and the sample type was set to RNA-40 because bisulfite converted DNA contains uracil and is widely single-stranded. DNA samples (100ng of each sample) were run on the 1% agarose gel and stained with Diamond Nucleic™ Acid Dye To evaluate the level of fragmentation following conversion.

3.4.6. Converted methylated human control

When studied DNA methylation using bisulfite conversion, it is essential that control reactions be run at every step in the procedure because the presence of a cytosine following bisulfite conversion indicates methylation.

Converted Methylated Human Control that has been bisulfite-converted. High percent methylation of CpG sites as determined by DNA sequencing. Significant percent bisulfite conversion of unmethylated cytosines as determined by DNA sequencing.

3.4.6.1 Amplification of bisulfite-converted DNA

Bisulfite-Converted DNA was amplified by using GoTaq Hot Start Green Master Mix . The PCR mixture inculding the DNA template 2x PCR matser mix, forward and reverse primers and the volumes of each componnet are shown in table **3.8** .

The samples were placed in DNA thermal cycler and under the following reaction conditions (**table 3.9**).

Table 3.8: PCR master mix for amplification of bisulfite-converted DNA

Component	**Volume**	**Final Concentration**
2X PCR master Mix	12.5 µl	1X
Upstream primer	0.9 µl	10 picomole
Downstream primer	0.9 µl	10 picomole
DNA template	3µl	20–50ng
Deionized water	6.7 µl	The final volume of 25µl

Table 3.9: Cycling conditions for endpoint PCR of bisulfite-converted DNA.

Steps	Temperature	Time	Number of cycles
Enzyme inactivation	95 °C	5 minutes	1
Denaturation	95°C	30 seconds	40
Annealing	61, 58, 57	60 seconds	40
Extension	72°C	60 seconds	40
Final extension	72°C	5 minutes	1

3.4.7. Methylated human control

Methylated Human Control DNA used as positive control purified from a male human source. CpG sites in the DNA were enzymatically methylated by M. SssI methyltransferase to provide a high percentage of methylated CpG motifs.

3.4.7.1. Amplification of bisulfite-converted DNA

Amplifying 2µl of converted methylated human control DNA per reaction in parallel with experimental samples.

Table 3.10: PCR master mix for amplification methylated human Control.

Component	Volume	Final Concentration
2X PCR master Mix	12.5µ	1X
Upstream primer	0.9 µl	10 picomole
Downstream primer	0.9µl	10 picomole
DNA template	2µl	20–50ng
Nuclease-Free Water	8.7µl	the final volume of 25µl

Table 3.11: Cycling conditions for endpoint PCR of methylated Human Control.

Steps	Temperature	Time	Number of cycles
Enzyme inactivation	95 °C	5 minutes	1
Denaturation	95°C	30 seconds	40
Annealing	61, 58, 57	60 seconds	40
Extension	72°C	60 seconds	40
Final extension	72°C	5 minutes	1

3.5 .Gene Amplification

3.5.1. Finding promoter regions

3.5.1.1. ESR2 gene

3.5.1.1.1. Location: Chromosome 14

3.5.1.1.2. Ensembl reference sequence: ENSG00000140009

3.5.1.1.3. ESRE2 promoter finding

Promoter sequences are usually the sequence immediately upstream the transcription start site (TSS) or first exon. If the TSS of a gene is known, the promoter could be known without experimental characterization. For many organisms, such as human, the genome is well annotated, and TSS well defined. The major genome browsers: NCBI, Ensembl, and UCSC, For our purpose, Ensembl provides the most convenient interface by.

1-Going to Ensemble website: http://www.ensembl.org/index.html

2-Choosing an organism such as human http://www.ensembl.o...iens/Info/Index

3-Searching gene such as ESR2 http://www.ensembl.org/Homo_sapiens/Search/Results?q=ESR2;site=ensembl;facet_species=Human

4- Clicking the right hit on the search result page, and it will bring to the gene summary page http://asia.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000140009;r=14:64084232-64338112

5-On the left, under "Gene Summary", click "Sequence", the sequence of the gene, including 5 flanking, exons, introns and flanking region will be displayed.

6-The exons highlighted with the pink background, and red text, the sequence in front of the first exon is the promoter sequence.

7-By default, 600 bp 5-flanking sequences (promoter) are displayed.

8-Sometimes there were discrepancies between Ensembl and UCSC annotation regarding TSS. Copy the promoter sequence to make sure the first exon given by the ensemble is right.

9-Go to UCSC BLAST search at http://genome.ucsc.e...t?command=start and choose the right genome (e.g., human), paste the sequence there. On the result page, click browse of the first hit, this will bring to the genome browser Page.

3.5.1.1.4. Promoter of ESR2

CGGACGGGGCGGCCTCGCGGGGGGTGGACGGGGCGGTCTGCGGGGAGGGGGGGCGGTCTG

CGGGGAGGGGGACGGGGCGGCCTCGCGGGAGGGAGGACAGTTTCGCGGGTTCGGGCGGCG

AGTCTCCCGGATGCTCCTCAGCTCTGGGGACGCGGTGCAGAAGTGTGAGGGCGCCCGGCT

TCCAGGCAGTAATGGGCGGGTCCCTGCGCGGGAGCGTGGCGGGCGCTGGACTCTACAGCA

GATGTGGAACTGGAGAGCTTGGCGCGCCTTCCGACTTTGTCACACACCTGCGCCGCCAGA

CTGGGGTCGGGCCCCTCCGCGTTCTGCTCTGGAGTGCCTGGGTCTGGGCCCAGCACCGCG

CTTTTAGAATCTCCTCAGCTGAATCTGACGCTCAGCAGTGGGTGAAGCGCAGCCCCCTGT

TTCAGGCCCTGCCGAGCTGGAAGGAGTGTCAGAGCTGGAGCGCGCGTGGCCCCCTCTGTG

TTGGGGTCACCCCGGGGTTGCCAGGGCTCAGGGAGGGTCGTAGTCTGGATTTTGTCACCC

GCACGTCCCCACCCCCCAGCAGGTCTGGGGTTGGAGAATCCACGCGGGCTTCATAAGCTA

3.5.1.2. OPN gene

3.5.1.2.1. Location: chromosome 4

3.5.1.2.2. Ensembl reference sequence: ENSG00000118785

3.5.1.2.3. Promoter of OPN

GAACCACGGTCTGGCTCCTGAAGCAGCCCTCTCAAGCAGTCATCCTGCTCTCAGTCAGAA

ACTGCTTTACTTCTGCAACATCTAGAATAAATTACCATTCTTCTATTTCATATAGAATTT

TATATTTTAATGTCACTAGTGCCATTTGTCTAAGTAACAAGCTACTGCATACTCGAAATC

ACAAAGCTAAGCTTGAGTAGTAAAGGACAGAGGCAAGTTTTCTGAACTCCTTGCAGGCTT

GAACAATAGCCTTCTGGCTCTTCAATAAGTACAATCATACAGGCAAGAGTGGTTGCAGAT

ATTACCTTTATGTTACTTAAACCGAAAGAAACAAAAATCCATTCTATTTAATTTTACATT

AATGTTTTTCCCTACTTTCTCCCTTTTTCATGGGATCCCTAAGTGCTCTTCCTGGATGCT

GAATGCCCATCCCGTAAATGAAAAAGCTAGTTAATGATATTGTACATAAGTAATGTTTTA

ACTGTAGATTGTGTGTGTGCGTTTTTGTTTTTTTTTGTTTTAACCACAAAACCAGAGGGG

GAAGTGTGGGAGCAGGTGGGCTGGGCAGTGGCAGAAAACCTCATGACACAATCTCTCCGC

3.5.1.3. BRCA1 gene

3.5.1.3.1. Location: chromosome 17

3.5.1.3.2 .Ensembl reference requence: ENSG00000012048

3.5.1.3.3 Promoter of BRCA1

ACTCCTGGGGCTGGATGGGAATTGTAGTCTCCCTAAAGAGTTGTACGTATCTTTTTAAGG

CCTAGTTTCTGCTTTCAAAATACGAAAACATAACACTCCAGTCCATAACTGTTGACAAGT

ACAAGCGCGCACAGGTCTCCAATCTATCCACTGGATTTCCGTGAGAATTGTGCCCGCTCT

GGTATTGGATGTTCCTCTCCATAAGACTACAGTTTCTAAGGAACACTGTGGCGAAGACCT

TTCATTCCGCAACGCATGCTGGAAATAATTATTTCCCTCCACCCCCCCAACAATCCTTAT

TACTTATATTTACCGAAACTGGAGACCTCCATTAGGGCGGAAAGAGTGGGGGATTGGGAC

CTCTTCTTACGACTGCTTTGGACAATAGGTAGCGATTCTGACCTTCGTACAGCAATTACT

GTGATGCAATAAGCCGCAACTGGAAGAGTAGAGGCTAGAGGGCAGGCACTTTATGGCAAA

CTCAGGTAGAATTCTTCCTCTTCCGTCTCTTTCCTTTTACGTCATCCGGGGGCAGACTGG

GTGGCCAATCCAGAGCCCCGAGAGACGCTTGGCTCTTTCTGTCCCTCCCATCCTCTGATT

3.5.2. Primer design

3.5.2.1. Design of primers based on the bisulfate conversion

When designing the primers from the identified DNA sequence, it is a good exercise to ‘convert’ the DNA first changing all of the most nonCpG cytosines to uracil.

The opening primer should be designed to base-pair with this converted sequence (normally this would be consideration of like the reverse primer). The next (upstream) primer must be designed to base-pair to the extension of the first primer and not the opposite strand like in traditional PCR. Primers for bisulfite

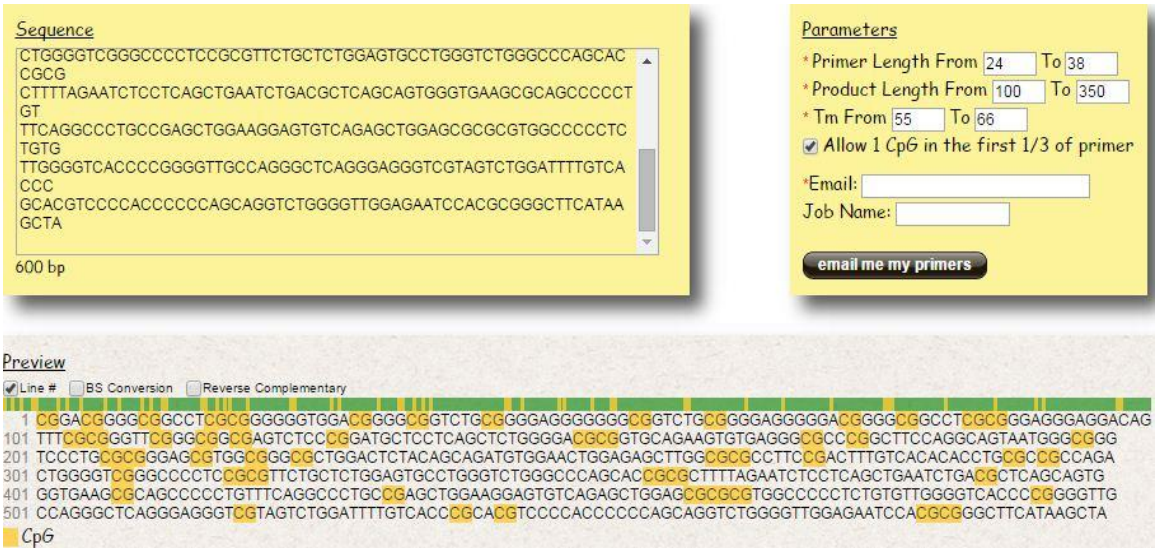
PCR usually ranges from 24-38 bases, with amplicon sizes commonly ranging from 350 for BRCA1, 343 for ESR2 and 324 for OPN bp

3.5.2.1. Bisulfate primer seeker

The free program recommended multiple options for both forward and reversed primer and choice on of them for each one.

www.zymoresearch.com/tools/bisulfite-primer-seeker

3.5.2.2. ESR2 primer



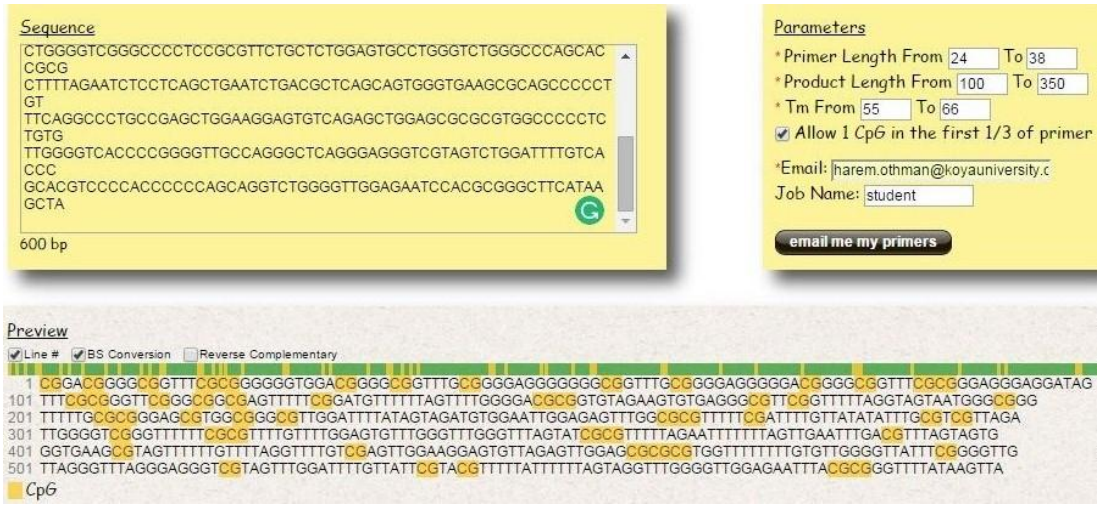


Table 3.12: ESR2 primer design by bisulfate primer seeker based on bisulfite conversion

Primer	Length	Start point	Endpoint	Amplicon CpGs	Product size	Tm	Sequence (5'->3'
Forward	30	119	148	25	343	59.1	GAGTTTTTAGGATGTTTTTTAGTTTTGGGG
Reverse	29	138	166	25	343	63.4	CGC TCC AAC TCT AAC ACT CCT TCC AAC TC

3.5.2.3. OPN primer

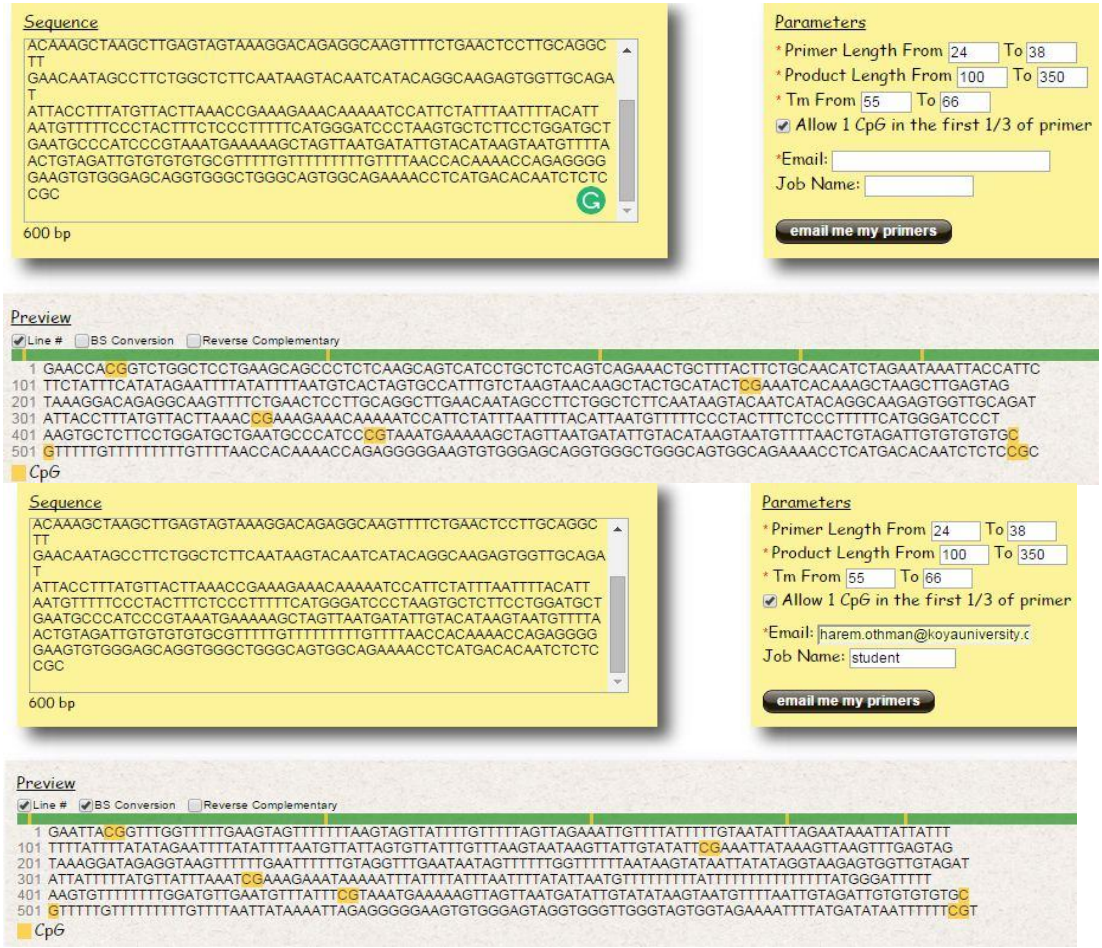


Table 3.13: OPN primer design by bisulfate primer seeker based on bisulfite conversion

Primer	Length	Start point	End point	Amplicon CpGs	Product size	Tm	Sequence(5'->3'
Forward	29	188	216	3	324	55.3	AAGTTTGAGTAGTAAAGGATAGAGGTAAG
Reverse	38	88	123	3	324	61.3	AAAAACAAAAACCCACACACACAATCTACAATTAAAAC

3.5.2.4. BRCA1 primer

Table 3.14: BRCA1 primer design by bisulfate primer seeker based on bisulfite conversion

Primer	Length	Start point	Endpoint	Amplicon CpGs	Product size	Tm	Sequence (5'->3'
Forward	31	12	42	11	350	57	GGATGGGAATTGTAGTTTTTTTAAAGAGTTG
Reverse	24	238	261	11	350	60	AAATCCCAATCCCCCACTCTTTCC

3.5.3. Primer synthesis

All of the primer sequence combination are suplied from MACRO GEN Company of the Republic of Korea.

3.5.4. Primer preparation 100 picomole / µl

The Preparation of all these primers are based on the synthesis by the business of MACRO GEN.

Table 3.15:ESRE2 _F primer preparation 100 picomole/ µl

Primer	**ESR2 _F**
SEQ	GAGTTTTTAGGATGTTTTTTAGTTTTGGGG (30 bp)
GC%	
36.67	
Vol. for 100 picomole / µl	
291.0	

Table 3.16: ESR2 _R primer preparation 100 picomole/ µl

Primer	**ESR2 _R**
SEQ	CGC TCC AAC TCT AAC ACT CCT TCC AAC TC (29 bp)
GC%	
51.72	
Vol. for 100 picomole / µl	
278	

Table 3.17:OPN_F primer preparation 100 picomole/ µl

Primer	OPN_F
SEQ	AAGTTTGAGTAGTAAAGGATAGAGGTAAG (29 bp)
GC%	
34.48	
Vol. for 100 picomole / µl	
299.0	

Table 3.18: OPN_R primer preparation 100 picomole/ul

Primer	OPN_R
SEQ	AAAAACAAAAACCCACACACACAATCTACAATTAAAAC (38 bp)
GC%	
28.95	
Vol. for 100 picomole / µl	
276	

Table 3.19: BRCA1_F primer preparation 100 picomole/ul

Primer	BRCA1 _F
SEQ	GGATGGGAATTGTAGTTTTTTTAAAGAGTTG (31 bp)
GC%	
32.26	
Vol. for 100 picomole / µl	
288	

Table 3.20:BRCA1_R primer preparation 100 picomole/ul

Primer	BRCA1 _R
SEQ	AAATCCCAATCCCCCACTCTTTCC (29 bp)
GC%	
50.0	
Vol. for 100 picomole / µl	
288	

3.6. GoTaq® Green Master Mix

GoTaq Green Master Mix is a premixed, ready-to-use solution containing derived Taq DNA polymerase, dNTPs, MgCl2 and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. GoTaq Green Master Mix contains two dyes (blue and yellow) that allow monitoring of progress during electrophoresis.

3.6.1. GoTaq® Green Master Mix, 2X

GoTaq DNA Polymerase is supplied in 2X Green GoTaq Reaction Buffer , 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM MgCl2. Green GoTaq Reaction Buffer was a proprietary buffer containing a compound that increases sample density, and yellow and blue dyes, which function as loading dyes when agarose gel electrophoresis analyzes reaction products.

Table 3.21 Preparation 25µl reaction volume for the PCR

Component	Volume	Final Concentration
2X PCR Master Mix	12.5µ	1X
Upstream primer	1 µl	10 picomole
Downstream primer	1 µl	10 picomole
DNA template	7 µl	<250ng
Deionized water	4.5	the final volume of 25µl

3.6.2. General steps for Amplification by PCR

1-Denaturation

Initial denaturation (5 min) at 95°C is sufficient. Subsequent denaturation steps will be 30 seconds.

2- Annealing

Typically annealing temperature for the genes was 61 °C for ESR2, 58 °C for OPN, and 57 °C for BRC1. The annealing step is typically 1 minute for ESR2, OPN, and BRCA1.

3- Extension

The extension reaction typically performed at the optimal temperature for Taq DNA polymerase, which is 72°C for five minutes.

3.7. PCR Clean Up including four steps

A- Sample Preparation

Up to 15 to 20µl of the reaction product was transferred to a 1.5 microcentrifuge tube, and then five volumes of DF buffer were added to 1 volume of the sample and mixed by the vortex.

B- DNA Binding

A DF column was placed in a 2 ml collection tube, and then the sample mixture was transferred to the DF column for centrifugation at 14-16,000 x g for 30 seconds. The flow-through was discarded, and the DF column was placed back in the 2 ml Collection Tube.

C- Wash

Wash buffer (600 µl) was added to the center of the DF column, left for 1 min. At room temperature to stand and then after centrifuged at 14-16,000 x g for 30 seconds. The flow-through discarded and the DF placed Column back in the 2 ml collection tube.

D- DNA Elution

Dried DF column was transferred to a new 1.5 ml microcentrifuge tube. 20-50 µl of Elution Buffer or TE was added to the center of the column matrix and then left for at least 2 minutes to ensure the Elution Buffer completely absorbed. After that centrifuged for 2 minutes at 14-16,000 x g to elute the purified DNA.

3.8. Sequencing

Table 3.22: Preparation of 20 µl PCR for sequencing

Component	**Volume**	**Concentration**
Ready reaction premix	4 µl	2.5X
Primer (forward or reverse	1.5 µl	3.2 picomole
DNA template	1 µl	3–10 ng
Buffer big dye terminator	4 µl	5X
Deionized water	9.5 µl	

Table 3.23: PCR condition for sequencing

Steps	**Temperature**	**Time**	**Number of cycles**
Enzyme inactivation	96°C	1 minute	1
Denaturation	96°C	10 seconds	25
Annealing	53°C	10 seconds	25
Extension	60°C	4 minutes	25
Final extension	60°C	2 minutes	1

3.9. Procedure for Sequencing after PCR

1- EDTA (1.35 ml 0.1 M) was added to stop the reaction, and the TE buffer 80 µl, sodium acetate (3M) (10)µl and absolute ethanol 330 µl was added respectively.

2- The mixture centrifuged for 10 minutes at 13000 RPM, and the supernatant was discarded. Moreover, then washed by 500 µL 70% ethanol, centrifuged again for 5 minutes at 13000 RPM and dried.

3- Formamide 20 µl was added for sequencing.

3.10 .Interpretations of the data

BiQ Analyzer, a software tool for DNA methylation analysis was used; this program developed in cooperation between the Max-Planck-Institute for Informatics and Saarland University, both situated in Saarbrücken, Germany [18].

3.11. Statistical analysis

The Fisher exact test was used to analyze the relationship between cancer samples and healthy samples for both methylated and unmethylated CPG islands and also for both grade 1 and 2 in breast cancer samples. P- values < 0.05 were considered to be statistically significant. All calculations were performed by using SPSS version 19 for Windows 7[173].

Chapter four

Results and Discussions

Results and Discussions

4.1. Sample Collection

Blood samples were collected randomly from 38 women who had different grades of breast cancer and also from 11 healthy peoples as a control group. Control samples may represent various levels of DNA methylation. The high GC content of these regions can strongly influence the denaturing and annealing behavior of DNA, which subsequently affects the PCR performance.

4.2. Determination of DNA quantity

Following the isolation of DNA from blood samples, the concentration of DNA samples was measured by Nanodrop. Nanodrop reads both absorbance and concentration of DNA automatically in ng/ul [38]. Usually, DNA concentration increased when absorbance increased and vice versa. The percentage of absorbance at 260 nm and 280 nm was used to assess the purity of DNA. A ratio of ~1.8 was normally accepted as pure for DNA [198].

The average absorbance of DNA for 13 samples selected for ESR2 gene was 1.66 and mean concentration was 20.5 ng /ul. (**Table 4.1**).

Table 4.1: DNA quantity of ESR2 gene samples

Sample number	Absorbance	Concentration ng/ul
1	1.8	23.1
2	2.61	32.81
3	1.98	28.39
4	0.8	10.17
5	1.17	14.04
6	0.9	10.84
7	2.07	25.2
8	2.76	33.01
9	1.23	14.14
10	1.35	16.98
11	1.8	21.45
12	1.82	21.51
13	1.3	15.34

The average absorbance of 14 samples selected for OPN gene was 1.9 and DNA concentration was 25.3 ng/ul (**Table 4.2**).

Table 4.2: DNA quantity of OPN gene samples

Sample number	Absorbance	Concentration ng/ul
14	0.91	11.35
15	1.71	26.5
16	3.06	38.85
17	1.98	25.77
18	1.99	25.82
19	2.07	26.07
20	1.8	24.33
21	3.33	42.6
22	1.86	24.52
23	1.53	19.117
24	1.26	16.096
25	2.03	30.1
26	1.58	23.44
27	1.53	19.6

The average DNA absorbance of the 11 samples selected for BRCA1 gene was 1.6, and DNA concentration was 22.06 ng/ul (**Table 4.3**).

Table 4.3: DNA quantity of BRCA1 gene samples

Sample number	Absorbance	Concentration ng/ul
28	2.04	26.48
29	1.19	15.19
30	1.78	22.75
31	1.7	22.3
32	1.35	16.59
33	2.77	41.54
34	2.34	30.56
35	1.72	23.1
36	1.63	20.7
37	1	12.45
38	0.9	11.1

4.3. DNA Quality

To confirm and identify the quality of the extracted DNA, agarose gel electrophoresis was conducted (Figure 4.1). DNA isolated from fresh blood sample exhibitedgood concentration and quality, suggesting little degradation and preserved structure [126].

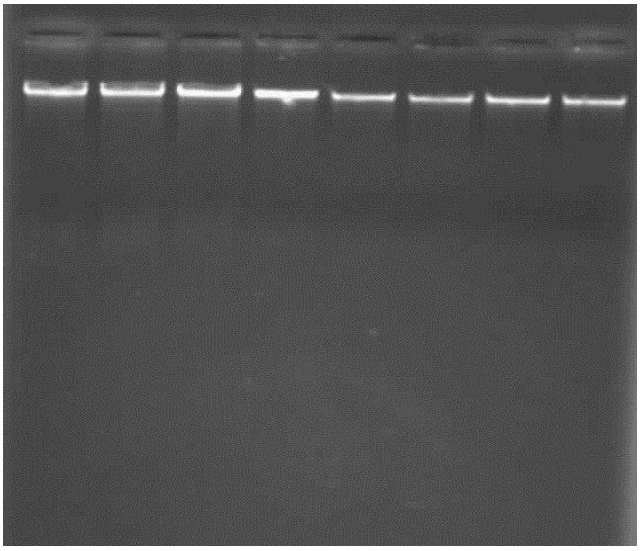


Figure 4.1: Quality of DNA extraction before bisulfite conversion.
Agarose gel (1%) electrophoresis showing DNA ioslated from fresh blood samples . DNA concentration was evaluated using Nanodrops.

4.4. Bisulfite conversion

4.4.1. DNA quantity of bisulfite converted samples

Because of the bisulfite converted DNA contains mostly uracil and single DNA strand, nanodrop was set at RNA-40.

The average absorbance of DNA for 13 samples selected for ESR2 gene was (1.2) and mean concentration was (5.6 **ng** /ul). (**Table 4.4**).

The mechanism by which this process is driven begins with the nucleophilic addition of bisulfite to the C-6 position of cytosine, which allow the rapid deamination of cytosine into 5,6-dihydrouracil-6-sulfonate [165,73]. Following treatment with an alkaline solution swiftly eliminates the sulfonate group and regenerate the double bond, yielding uracil [74,4]. When cytosines methylated, this reaction blocked due to the very low reaction rates for the creation of 5-methyl-6-dihydrocytosine-6-sulfonate and they are as a result not converted to uracil [57].

Table 4.4: DNA quantity of ESR2 gene samples after bisulfite conversion

Sample number	Absorbance	Concentration ng/ul
1	1.3	6.11
2	1.9	8.6
3	1.4	7.4
4	0.6	2.6
5	0.8	3.7
6	0.6	2.8
7	1.5	6.6
8	2	8.7
9	0.9	3.7
10	0.9	4.4
11	1.3	5.6
12	1.3	5.6
13	1.5	7.9

The average absorbance of DNA for 14 samples selected for OPN gene was (1.3) and mean concentration was (6.1 ng /ul). (**Table 4.5**).

Table 4.5: DNA quantity of OPN gene sample after bisulfite conversion

Sample number	Absorbance	Concentration ng/ul
14	2.3	10
15	1.7	8.01
16	1.1	6.1
17	1.5	6.9
18	0.6	2.88
19	1.3	6.4
20	0.9	3.9
21	1.3	6.1
22	1.4	6.7
23	2.5	11.2
24	1.3	6.4
25	1.2	6.1
26	0.9	3.8
27	0.6	2

The average absorbance of DNA for 11 samples selected for BRCA1 gene was (0.9) and mean concentration was (4.3 ng /ul). (**Table 4.6**).

Table 4.6: DNA quantity of BRCA1 gene sample after bisulfite conversion

Sample number	Absorbance	Concentration ng/ul
28	1.3	6.4
29	1.2	6.1
30	0.9	3.8
31	0.6	2
32	0.9	4.3
33	0.8	3.5
34	1.3	5.9
35	0.9	4.2
36	1.1	5.02
37	0.61	2.1
38	0.9	4.2

4.4.2. DNA Quantity of Converted Methylated Human Control

To determine and ensure that the DNA was bisulfite converted, methylated human control was run in the agarose gel in parallel with some samples before PCR. The absorbancy of the methylated human control was 0.974, and DNA concentration was 13.4 ng/ul.

4.4.3. DNA quality after bisulfite conversion

All the bisulfite converted DNA was run, with control, to prove that the DNA was converted before PCR. Diamond™ Nucleic Acid Dye was used instead of ethidium bromide because of the sensitivity of this dye for staining of bisulfite

converted DNA. Aside from the improved safety , several of these agents show better sensitivity when used to stain nucleic acids in gels.

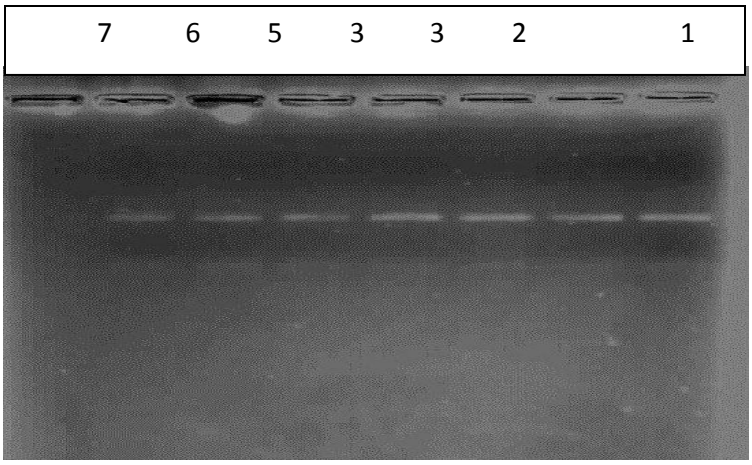


Figure 4.2: Bisulfite converted DNA

***lane 1: Human control, lane 2- 7 Bisulfite converted DNA**

agarose gel(1%) electrophoresis showing bisulfite converted DNA samples before PCR amplification.

4.5. Amplification of the bisulfite converted DNA:

4.5.1. Amplification of ESR2 gene promoter region:

Out of 38 blood samples, 13 were subjected to amplification by polymerase chain reaction including (7) grade 1, (3) grade 2 and (3) healthy, the amplified product size was 343bp (**figure 4.3, 4.4 and 4.5**).

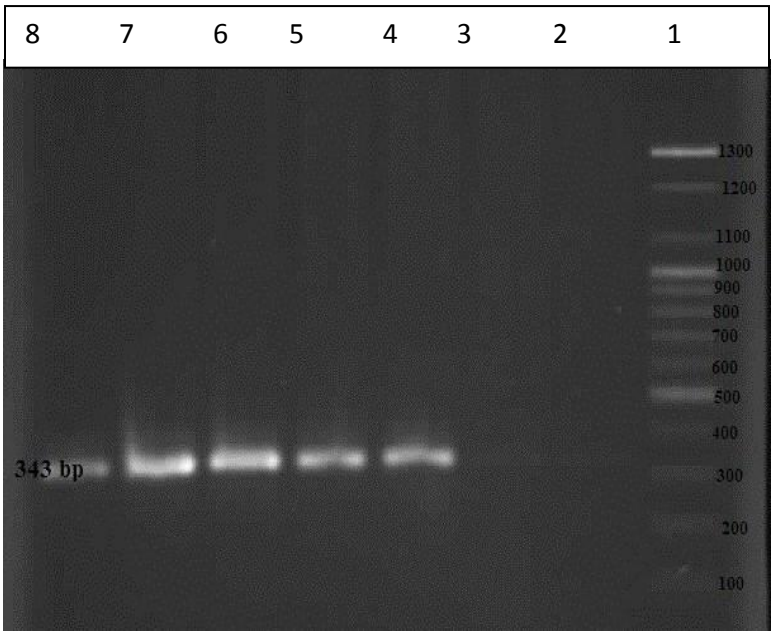


Figure 4.3: PCR-amplified promoter region of ESR2 gene.

***Lane 1: 100bp DNA ladder, lane (2-8): 343 bp methylated ESR2 promoter of samples 1, 2, 3, 4, 5, 6 and 7.**

Aagarose gel (1.5%) electrophoresis showing PCR products of ESR2 gene promoter region after amplification. The expect product size were 343 bp.

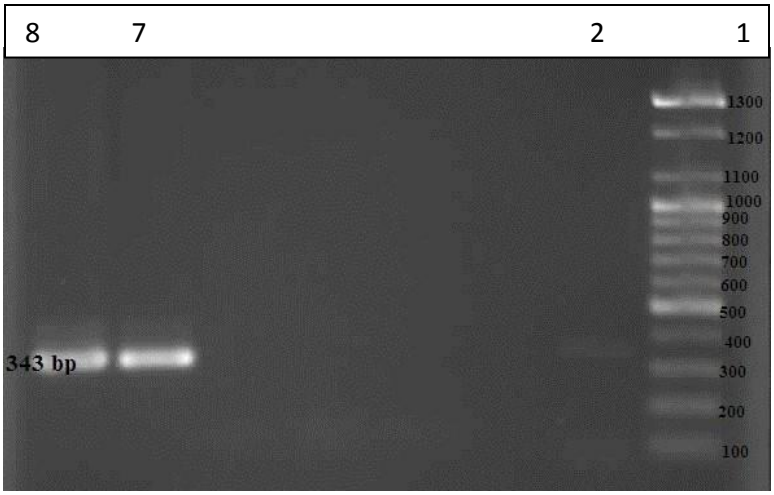


Figure 4.4: PCR-amplified promoter region of ESR2 gene.

***Lane 1: 100bp DNA ladder, lane (2, 7 and 8): 343 bp methylated ESR2 promoter of samples 8, 9and 10.**

Aagarose gel (1.5%) electrophoresis showing PCR products of ESR2 gene promoter region after amplification. The expect product size were 343 bp.

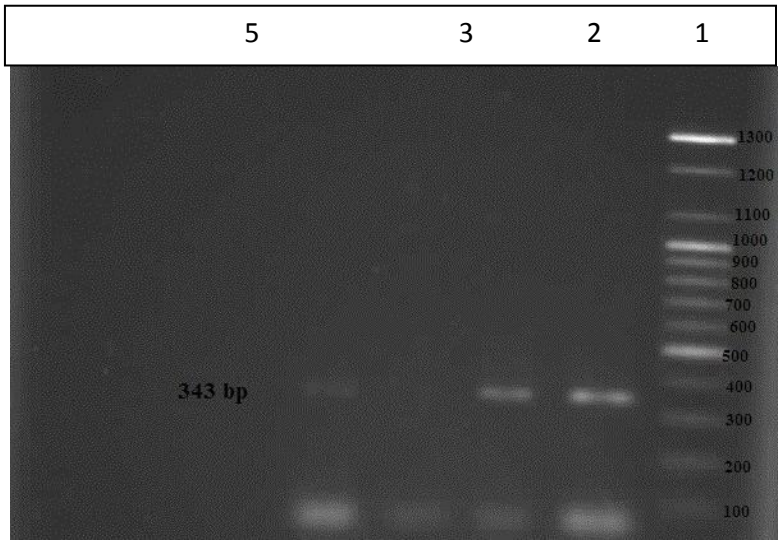


Figure 4.5: PCR-amplified promoter region of ESR2 gene.

***Lane 1:100bp DNA ladder, lane (2, 3 and 5): 343 bp methylated ESR2 promoter of samples 11, 12 and 13.**

Aagarose gel (1.5%) electrophoresis showing PCR products of ESR2 gene promoter region after amplification. The expect product size were 343 bp.

4.5.2. Amplification of OPN gene promoter region:

Total of 14 samples were selected for polymerase chain reaction amplification including breast cancer and healthy samples, (11) grades 1, (2) grade 2 and (3) healthy. The amplified PCR product was 324 bp (**Figures 4.6, 4.7, 4.8, 4.9, and 4.10**).

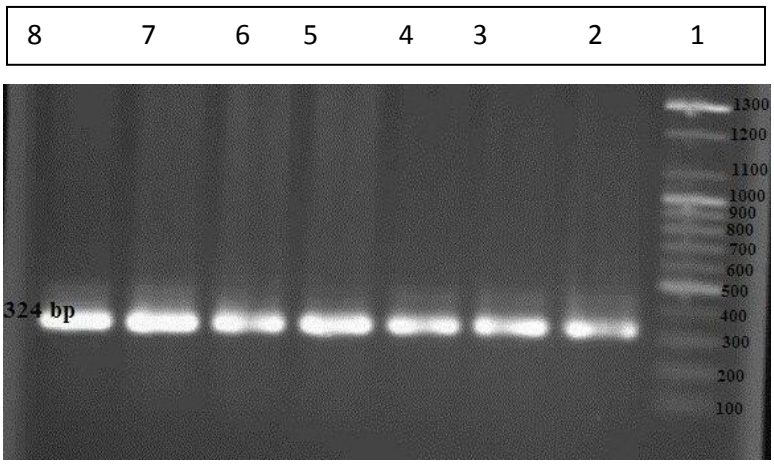


Figure 4.6: PCR-amplified promoter region of OPN gene.

***Lane 1: 100bp DNA ladder, lane (2-8): 324 bp methylated OPN promoter of samples 14, 15, 16, 17, 18, 19 and 20.**

Aagarose gel (1.5%) electrophoresis showing PCR products of OPN gene promoter region after amplification. The expect product size were 324 bp.

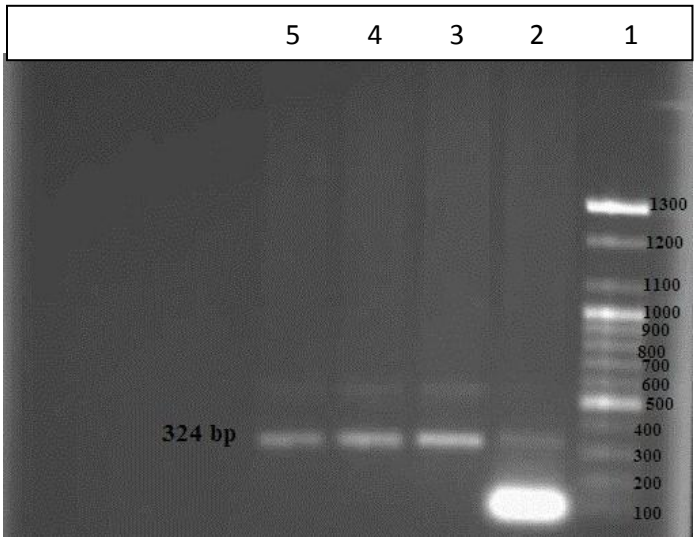


Figure 4.7: PCR-amplified promoter region of OPN gene.

***Lane 1: 100bp DNA ladder, lane (2-5): 324 bp methylated OPN promoter of samples 21, 22, 23, and 24.**

Aagarose gel (1.5%) electrophoresis showing PCR products of OPN gene promoter region after amplification. The expect product size were 324 bp.

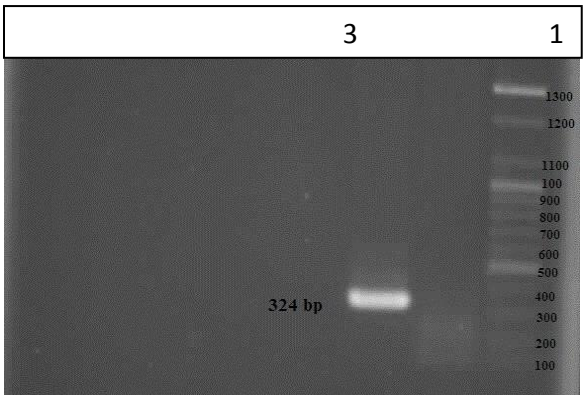


Figure 4.8: PCR-amplified promoter region of OPN gene.

***Lane 1: 100bp DNA ladder, lane (3): 324 bp methylated OPN promoter of samples 25.**

Aagarose gel (1.5%) electrophoresis showing PCR products of OPN gene promoter region after amplification. The expect product size were 324 bp.

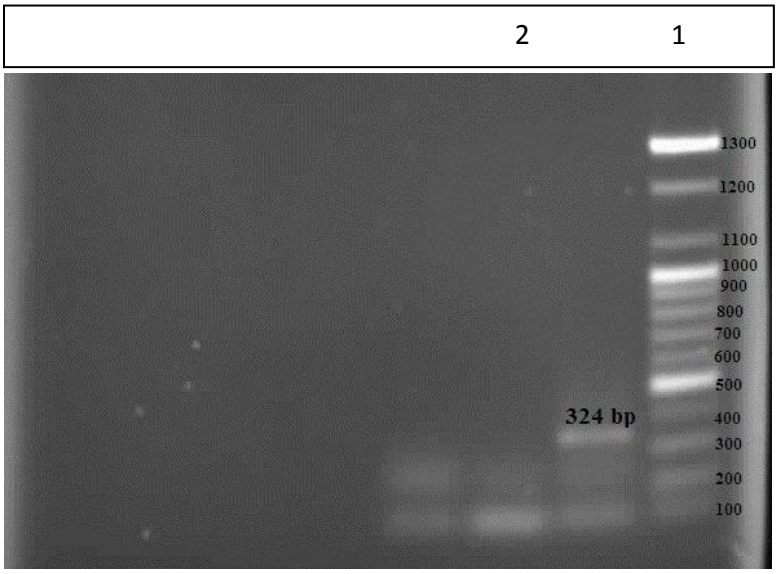


Figure 4.9: PCR-amplified promoter region of OPN gene.

***Lane 1: 100bp DNA ladder, lane (2): 324 bp methylated OPN promoter of samples 26.**

Aagarose gel (1.5%) electrophoresis showing PCR products of OPN gene promoter region after amplification. The expect product size were 324 bp.

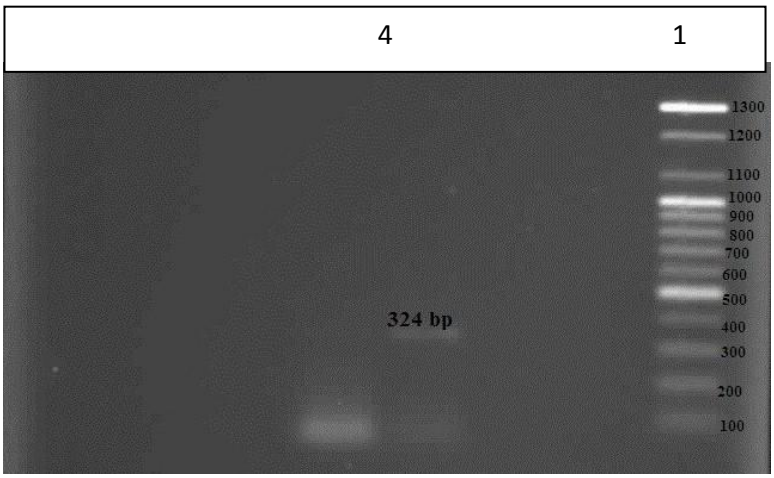


Figure 4.10: PCR-amplified promoter region of OPN gene.

***Lane 1: 100bp DNA ladder, lane (4): 324 bp methylated OPN promoter of samples 27.**

Aagarose gel (1.5%) electrophoresis showing PCR products of OPN gene promoter region after amplification. The expect product size were 324 bp.

4.5.3 .Qualitative identification of BRCA1 gene:

Totally 11 DNA samples amplified by PCR from breast cancer and healthy samples, the product size was 350 bp(**Figures 4.11 and 4.12**).

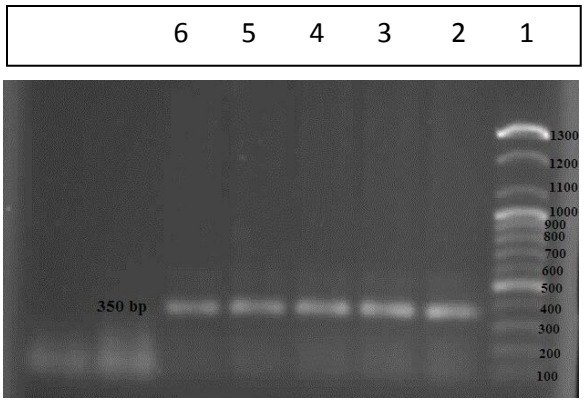


Figure 4.11: PCR-amplified promoter region of BRCA1 gene.

***Lane 1: 100bp DNA ladder, lane (2- 6): 350 bp methylated BRCA1 gene of samples 28, 29, 30, 31, and 32.**

Aagarose gel (1.5%) electrophoresis showing PCR products of BRCA1 gene promoter region after amplification. The expect product size were 350 bp.

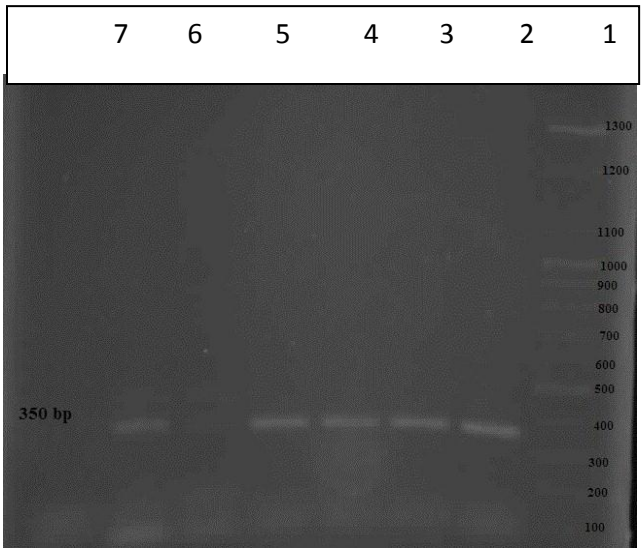


Figure 4.12: PCR amplified products of BRCA1 gene promoter region.

***Lane 1: 100bp DNA ladder, lane (2- 7): 350 bp methylated BRCA1 gene of samples 33, 34, 35, 36, 37, and 38.**

Aagarose gel (1.5%) electrophoresis showing PCR products of BRCA1 gene promoter region after amplification. The expect product size were 350 bp.

4.5.4. Qualitative identification of bisulfite converted control of BRCA1, OPN, and ESR2

To compare the obtained data with control, the human bisulfite converted control of BRCA1, OPN, and ESR2 genes were run separately on agarose gel electrophoresis. The results showed 350bp, 324bp and 343bp amplified a product of BRCA1, OPN and ESR2 genes respectively (**Figure 4.13**).

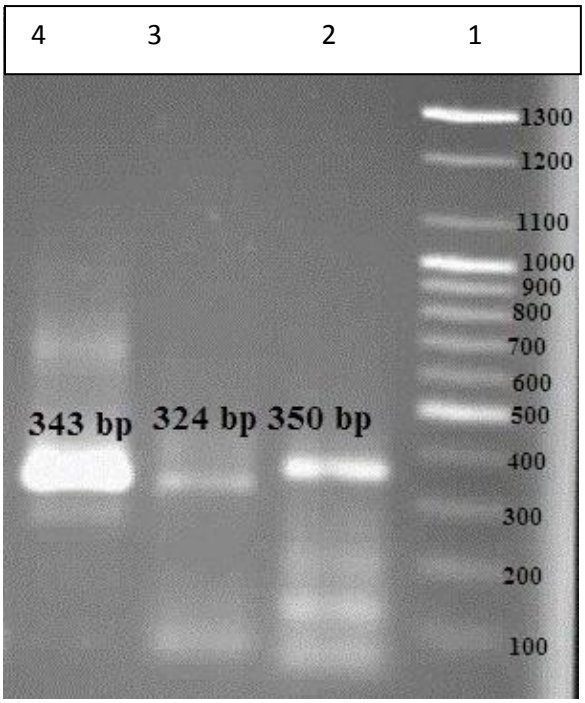


Figure 4.13 PCR amplified products of bisulfite converted control of BRCA1, OPN, and ESR2 genes promoter regions.

***Lane 1: 100bp DNA ladder, line 2: BRCA1 (350)bp, lane 3: OPN (324) bp and lane 4: ESR2 (343) bp.**

4.5.5. Qualitative identification of methylated control of ESR2, BRCA1and OPN gene:

Methylated human control also runs in separate on agarose for the purpose of confirming the obtained previously amplified product of the three genes in the subject.

The results indicated that the PCR-amplified product of the methylated BRCA1, OPN, and ESR2 genes were 350, 324 and 343 bps respectively (**Figure 4.14 and 4.15**).

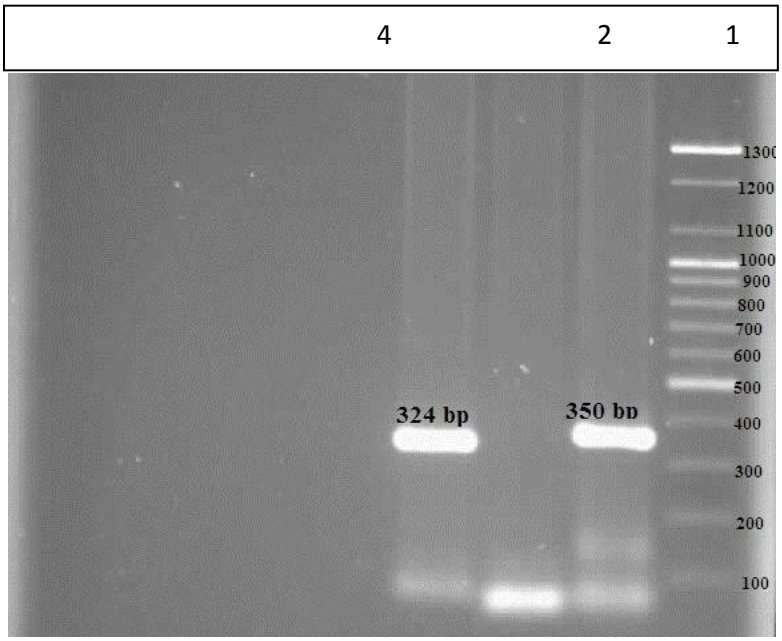


Figure 4.14: PCR amplified products of methylated control of BRCA1, OPN genes promoter region.
***Lane 1: 100bp DNA ladder, lane 2: BRCA1 (350) bp, lane 4: OPN (324) bp.**

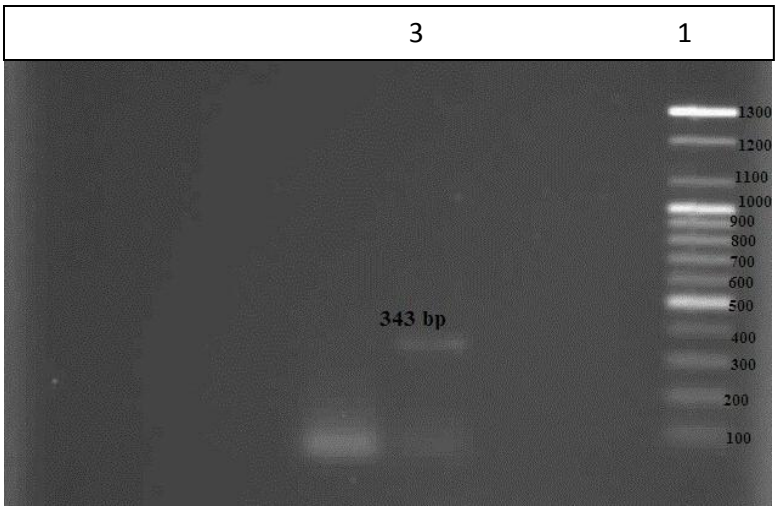


Figure 4.15: PCR amplified product of methylated control of ESR2 gene promoter region.

***Lane 1: 100bp DNA ladder, lane 3: ESR2 (343) bp.**

4.6 Quantitative identification of DNA methylation changes in breast cancer for ESR2, OPN, and BRCA1genes

4.6.1. Quantitative Identification of CPG methylation of ESR2 gene

Out of (thirtten) PCR amplified product of ESR2 gene promoter region, (nine) were subjected to DNA sequencing. Six samples have different grades of breast cancer (four) grade1and (two) grade 2; the remaining were healthy .

The results indicated that the ratio of DNA methylation of ESR2 promoter region among breast cancer patients was (12.39 %), (8.33 %) for grade1 patients and (20.5 %) for grade 2 (**Table 4.9 and 4.10), figure 4.16 and 4.17**).

In forward strand were cytosines converted to thymine, but in reverse strand guanine was converted to adenine.There were different rates of cytosine conversion that could be seen based on the sequence analysis by the BiQ Analyzer tool (**Table 4.8**).

Table 4.7: Cytosine conversion rates among ESR2 gene

Sample number	conversion rates in the final pileup	Percent of conversion%
1	(58 out of 68) forward strand	85
2	(64 out of 66) forward strand	97
3	(66 out of 68) forward strand	97
4	(63 out of 67) forward strand	94
5	(62 out of 66) forward strand	94
6	(46 out of 47) forward strand	98
7	(73 out of 74) reverse strand	99
8	(74 out of 75) reverse strand	99
9	(62 out of 62) reverse strand	100
Positive Control	(62 out of 63) forward strand	98
Positive Control	(59 out of 68) reverse strand	87

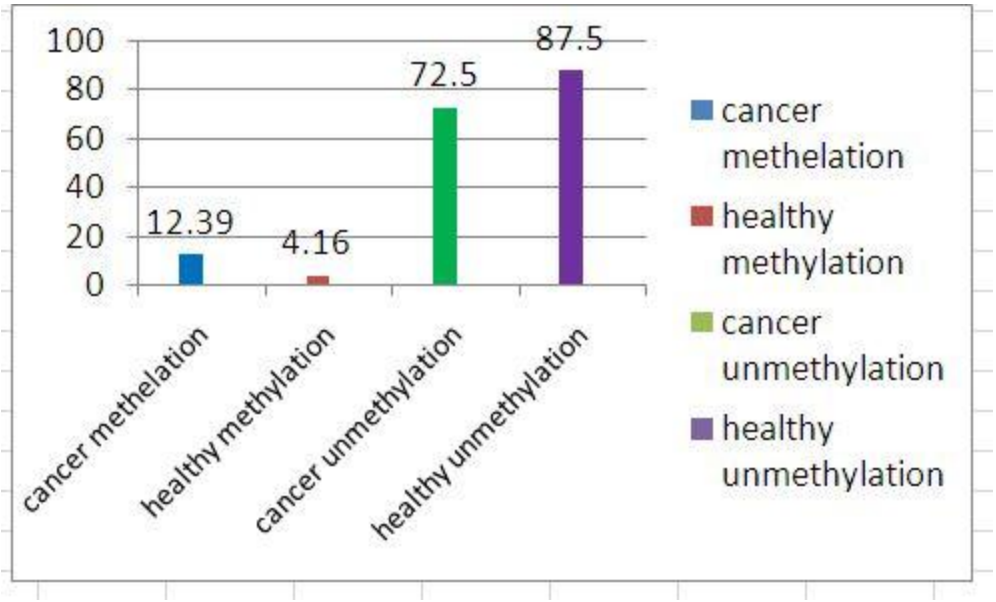


Figure 4.16: Total methylation and unmethylation between cancer and healthy samples of ESR2 gene.

Percentage of methylated and unmethylated CpG from the healthy and breast cancer samples were concluded as shown in table 4.9

Level of methylation for the healthy samples 2, 8 and 9 were 8.33%, 4.16, and 0%, respectivly .

However , the methylation level fpr six breast cancer pateints(1,3,4,5,6,7) were 12.5%, 8.33%, 8.33%, 12.5%, 28.57%, and 4.16% , respectivly .

Total unmethylated CPG from the healthy sample (87.5%),while from cancer samples (72.5%) Total CpGs, not present % not from breast cancer samples (15.07%) while from healthy samples (8.16%) (**Table 4.8)**

Table 4.8: Percent of DNA methylation in ESR2 gene

Sample number	Unmethylated CpGs %	Methylated CpGs%	CpGs, not present%
1	(17 cases) 70.83%	(3 cases) 12.5%	(4 cases) 16.66%
2	(20 cases) 83.33%	(2 cases) 8.33%	(2cases) 8.33%
3	(20 cases) 83.33%	(2 cases) 8.33%	(2cases) 8.33%
4	(18 cases) 75%	(2 cases) 8.33%	(4cases) 16.66%
5	(17 cases) 70.83%	(3 cases) 12.5%	(4cases) 16.66%
6	(10 cases) 47.61%	(6cases) 28.57%	(5cases) 23.80%
7	(21 cases) 87.5%	(1 cases) 4.16%	(2cases) 8.33%
8	(23 cases) 95.83%	(1 cases) 4.16%	(0cases) 0%
9	(20 cases) 83.33%	(0 cases) 0%	(4cases) 16.16%

Table 4.9: Percent of DNA methylation in ESR2 gene of grades 1 and 2 in breast cancer samples

Samples	Grades	Percent methylation	Percent unmethylation	Total methylation	Total unmethylation
3	1	8.33	83.33	8.33	79.16
4	1	8.33	75		
5	1	12.5	70.83		
7	1	4.16	87.5		
1	2	12.5	70.83	20.5	59.22
6	2	28.57	47.61		

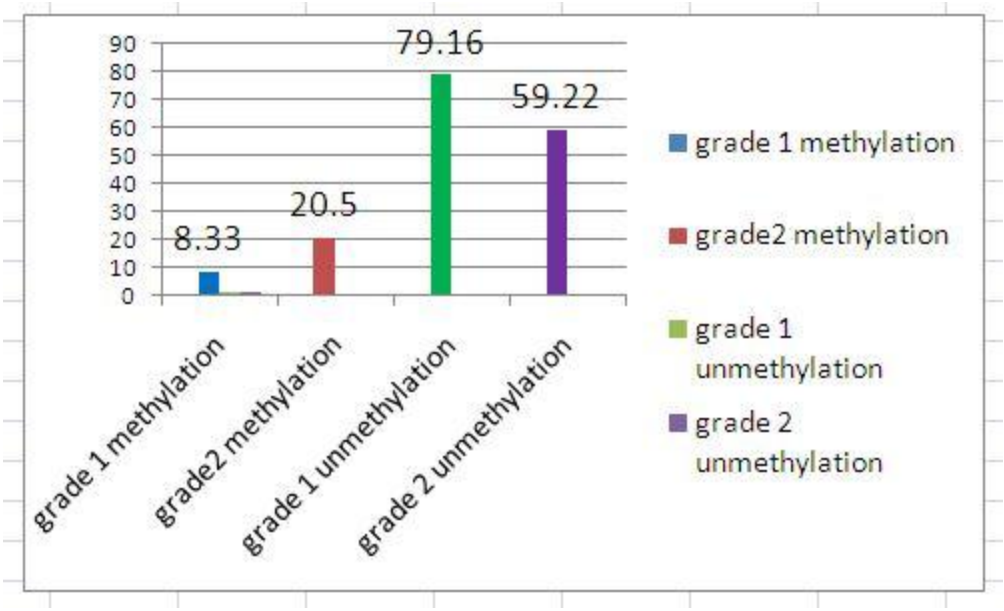


Figure 4.17: Total methylation and unmethylation between grade 1 and grade 2 of breast cancer samples of ESR2 gene.

An Iranian study shows that DNA methylation increased during breast cancer. In tumor tissue samples, 13/34 (38.2%) of ER promoter was methylated. However, in 100% (34/34) of samples the unmethylated allele was detected.Furthermore, ER2 promoter methylation was detected in 13(32.5%) tissue samples and 4(10.0%) peripheral blood specimens. Of these ER2-methylated cases, the Co-occurrent methylation of ER2 promoter in peripheral blood and tissue samples was evident in 1(7.7%) patient [133].

On the other hand, one study has demonstrated a frequent occurrence of ESR2 promoter methylation in ERβ of breast cancer in Chinese women. ESR2 methylation was significantly higher in high-grade of breast cancer (45%) than in starting neoplasia and strongly correlated with ESR1 methylation.They Suggest common epigenetic mechanisms of regulation [208].

Table 4.10 Differennt in DNA methylation, based on the single CPG of ESR2

NO	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	N	M	U	M	U	U	U	N	N	U	U	U	U	U	U	U	U	U	U	U	U	U	N	M
2	M	M	N	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	M	N
3	N	N	U	M	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	M
4	U	M	N	N	U	U	U	U	N	U	U	U	U	U	U	U	U	U	U	U	U	U	N	M
5	M	M	U	M	U	U	U	N	N	U	U	U	U	U	U	U	U	U	U	U	U	U	N	N
6	M	M	M	M	U	U	N	U	U	U	U	U	U	U	U	N	N	M	M	N	N			
7	N	U	U	N	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	M	U
8	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	M	U
9	U	N	U	N	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	N	N

M= methylattion ,U= Unmethylation , N = not present

Among many genes involved in human breast cancer, those related to estrogen actions are excellent candidates for investigation because estrogen exerts as a tumor promoter, through receptors [102]. Estrogen is vital for growth and development of the mammary glands and has been associated with the promotion and growth of breast cancer [172].

Studies have demonstrated that ESR2 gene promoter is subject to regulation by DNA-methylation. The discovery of ESR2 gene inactivate through promoter increased DNA methylation mainly in tumors with an unfavorable prognosis may

indicate a role for the ESR2 gene. In human breast cancer development or growth, may be used as a prognostic molecular biomarker. Expression of ESR2 also seems to be regulated by histone acetylation. Hypermethylation of the ESR2 promoter associated with a marked decreased in ESR2 mRNA expression in breast tumors and breast cancer cell lines compare to normal epithelial cells. The inhibition of DNA-methyltransferases reactivates ESR2 expression in these cell lines [158,209].

The bisulfite-specific sequence is a sensitive technique designed to analyze methylation of CpG dinucleotides within islands and has been used to detect abnormal methylation of the genes involved in human cancer. This method can also be used to decide the methylation status of individual CpG dinucleotides [20].

However, Next-Generation Sequencing techniques provide a single-nucleotide sequence resolution; thus, open new possibilities in this field. Bisulfite sequencing (BS-Seq) is the gold-standard technology to analyze methylation patterns at a single-base resolution. It makes use of the different characteristics of methylated and unmethylated Cs under bisulfite treatment. When single-stranded DNA gets treated with bisulfite, unmethylated Cs deaminate to the nucleotide uracil (U), whereas methylated Cs remain unaffected. In the subsequent sequencing process, these Us previously unmethylatedCs are sequenced as Ts. That implies the encoding of the individual methylation states in the read sequence and enables a later decoding [112].

Studies revealed that these sequencing errors in high-throughput sequencing techniques such as Illumina are not equally distributed over the whole set of possible error types [39]. Different analyzes showed significant biases in sequencing errors dependent on the genomic and erroneous base type [121].

Overall, twelve dissimilar substitution error are possible, but analyzes of a eukaryotic dataset released a mistake frequency of only 2% for C>G substitutions, whereas the substitution error T>C occurs in 15% of the cases. C>T substitution errors again occur low-frequently with only 4%. A similar strong bias holds for indel errors, where insertion, as well as deletion errors of A and T nucleotides, occur up to 6 times more often than indel errors of the nucleotides C and G. Due to C>T conversions, bisulfite sequencing data contains a higher fraction of T [39, 121].

Pairwise Sequence Alignment used to identify regions of similarity that may indicate functional, structural and evolutionary relationships among two biological sequences (protein or nucleic acid). The program has the type of Alignment and choice for determining the two sequences can be aligned globally using different algorithms. The Needleman-Wunsch algorithm is one of the best algorithms for global alignment, which can be performed using the by the biQ analyzers.

4.6.2. Quantitative Identification of CPG methylation of OPN gene

To calculate the OPN gene promoter region, DNA methylation among breast cancer patients (14) samples subjected to DNA sequencing, (11) of blood samples were from breast cancer patients (7) grade1 and (4) grade2) and the remaining were healthy.

The results showed that the percentage of DNA methylation is different between healthy (0 %) and cancer samples (57.57 %). DNA methylation is different between grade1and grade2 samples (**Table 4.13**).

As appeared from the results, DNA methylation increased (hypermethylation) in breast cancer samples if compared to healthy samples as well as between grade1 (61.89 %) and grade2 (49.99%).

Table 4.11: Cytosine conversion rates among OPN gene

Sample number	**conversion rates in the final pileup**	**Percent of conversion %**
14	50 out of 51) forward strand	98
15	(48 out of 52) forward strand	92
16	(50 out of 50) forward strand	100
17	(49 out of 49) forward strand	100
18	(51 out of 51) forward strand	100
19	(36 out of 40) forward strand	90
20	(46 out of 51) forward strand	90
21	(45 out of 51) forward strand	88
22	(48 out of 51) forward strand	94
23	(13 out of 35) forward strand	37
24	(51 out of 51) forward strand	100
25	(41 out of 47) forward strand	87
26	(47 out of 49) reverse strand	96
27	(44 out of 44) reverse strand	100
Positive Control	(40 out of 49)	82

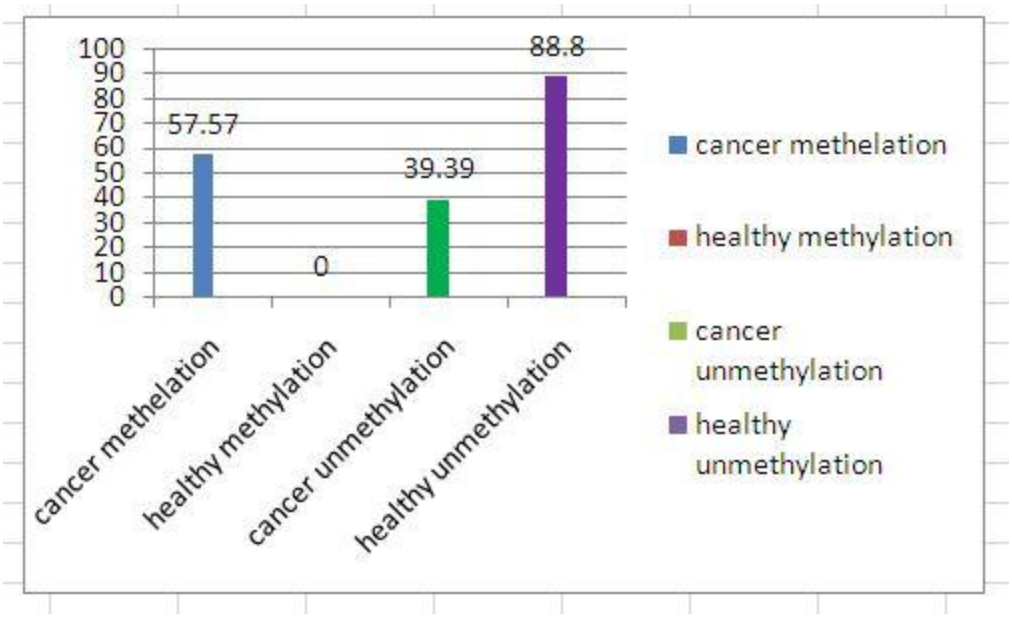


Figure 4.18: Total methylation and unmethylation between cancer and healthy samples of OPN gene

To better understand more about OPN gene DNA methylation status, CpG methylation was calculated. Totally (0%) CpGs from the healthy sample was methylated while (57.57%) from cancer sample was methylated.

Total unmethylated CPG from the healthy sample (88.8%) while from cancer samples (39.39%).Total CpGs, not present from cancer samples (3.03%) while from healthy specimens (11.11%) (**Table 4.12)**

Table 4.12: Percent of DNA methylation in OPN gene

Sample number	Unmethylated CpGs %	Methylated CpGs %	CpGs, not present %
14	(2 cases) 66.66%	(1 cases) 33.33%	(0 cases) 0%
15	(1 cases) 33.33%	(2cases) 66.66%	(0 cases) 0%
16	(1cases) 33.33%	(2 cases) 66.66%	(0cases) 0%
17	(1cases) 33.33%	(2cases) 66.66%	(0cases) 0%
18	(2cases) 66.66%	(1 cases) 33.33%	(0cases) 0%
19	(2cases) 66.66%	(0cases) 0%	(1cases) 33.33%
20	(1cases) 33.33%	(2cases) 66.66%	(0cases) 0%
21	(1cases) 33.33%	(2cases) 66.66%	(0cases) 0%
22	(1cases) 33.33%	(2 cases) 66.66%	(0cases) 0%
23	(0cases) 0%	(2cases) 66.66%	(1cases) 33.33%
24	(2cases) 66.66%	(1cases) 33.33%	(0cases) 0%
25	(1cases) 33.33%	(2cases) 66.66%	(0cases) 0%
26	(2cases) 100%	(0cases) 0%	(0cases) 0%
27	(2cases) 100%	(0cases) 0%	(0cases) 0%

Table 4.13: Percent of DNA methylation in OPN gene of grades 1 and 2 in breast cancer samples

Samples	Grades	Percent methylation	Percent unmethylation	Total methylation	Total unmethylation
14	1	33.33	66.66	61.89	38.09
15	1	66.66	33.33		
16	1	66.66	33.33		
17	1	66.66	33.33		
20	1	66.66	33.33		
21	1	66.66	33.33		
22	1	66.66	33.33		
18	2	33.33	66.66	49.99	41.66
23	2	66.66	0		
24	2	33.33	66.66		
25	2	66.66	33.33		

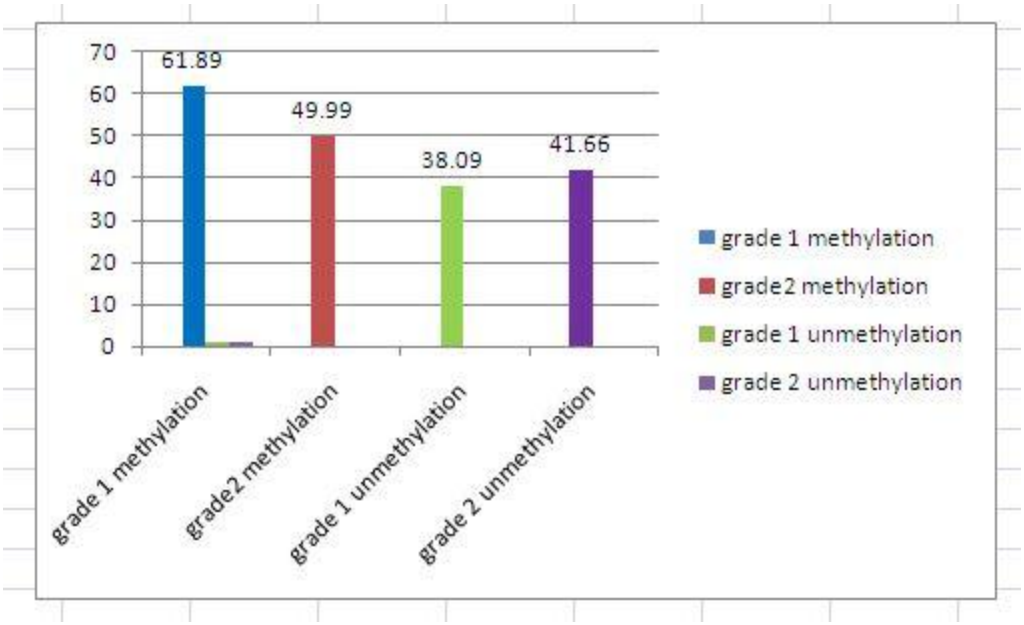


Figure 4.19: Total methylation and unmethylation between grad1 and grade 2 breast cancer samples of OPN gene.

Table 4.14: Different in DNA methylation, based on the single CPG of OPN gene

NO	1	2	3
14	U	U	M
15	U	M	M
16	M	U	M
17	M	U	M
18	U	U	M
19	N	U	U
20	M	U	M
21	M	U	M

22	**M**	**U**	**M**
23	**M**	**M**	**N**
24	**U**	**U**	**M**
25	**M**	**U**	**M**
26	**U**	**U**	
27	**U**	**U**	

M= methylattion ,U= Unmethylation , N = not present

OPN belongs to a group of proteins that is possibly referred to as Intrinsically Disordered Proteins (IDPs) that composition a biologically active proteins lacking defined the secondary and tertiary structure [100].

The one nucleotide resolution of this approach has secured bisulfite analyzes as the gold standard for 5mC studies. Bisulfite conversion reaction causes the deamination of unmethylated cytosine nucleotides to uracil even as part methylated cytosine intact. This property lets the discrimination of 5-methylcytosine (5mC) for cytosines [57]. The recently produced uracils pair with adenosines, which consequently pair by way of thymidines as PCR steps forward, efficiently altering unmethylated cytosine to thymidines. After the last PCR product is sequenced and compare to the original cytosine that remain cytosine were methylated, while cytosine converted to thymidines were unmethylated [29].

The mainly general gene-specific approach is bisulfite-sequencing. In bisulfite, specific PCR is performed on the bisulfite converted DNA amplifying a small (<500bp) region of interest. Pyrosequencing is the most excellent option for this since it provide a proportion methylation at each one cytosine [30].

If sequencing is not required, several bisulfite PCR approaches are possible. These rely on PCR primers that are designed detect the only specific possible outcome of bisulfite conversion. These techniques let the high-throughput analysis of many methylation sites, but at the reduced sensitivity compared to sequencing.

Bisulfite analysis can interrogate as well methylation at the genome-wide point [110]. Via coupling bisulfite conversion to next-gen DNA sequencing platforms, the percent methylation of any given C in the genome can be detected. Approaches such as whole-genome shotgun bisulfite sequencing and reduced representation bisulfite sequencing utilize next-gen platforms in this way.

There are numerous reports on methylation profiles of breast cancer patients in various populations. Moreover, CpG methylation of OPN gene is commonly found in breast cancer, including early stages of tumor development. Also numerous reports documented CpG methylation of SPP1 in several common human malignancies, with an especially high prevalence of the breast cancer [111].

OPN handled instituting the promotes tumor progression in breast cancer. It reported that OPN has been in down expressed status in breast cancer and these finding associated with hypermethylation status of SPP1 gene promoter, which plays a significant role in the progression of breast cancer.

The result more evaluation of SPP1 gene promoter methylation in blood and tissue, for example, carcinoma in situ can provide the foundation for its development as a biomarker for early detection [167].

4.6.3. Quantitative Identification of CPG methylation of BRCA1 gene

Out of (11) blood samples selected to determine DNA methylation of BRCA1 gene promoter region (4) samples were selected for DNA sequencing, from which (2) were grade1, (1) grade2 and (1) healthy.

The percentage of DNA methylation is different between healthy (36.36%) and cancer (63.3%) samples, cancer samples considered as hypermethylated BRCA1. Among breast cancer samples, grade1 DNA methylation was (49.99%), while grade 2 DNA methylation ratio was (36.36%) (**Table 4.16**) (**Figure 4.20 and 4.21**).

Table 4.15: Cytosine conversion rates of BRCA1 gene

Sample number	**conversion rates in the final pileup**	**Percent of conversion**
28	(19 out of 38)	50
29	(37 out of 48)	77
30	(36 out of 58)	62
31	(30 out of 49)	61

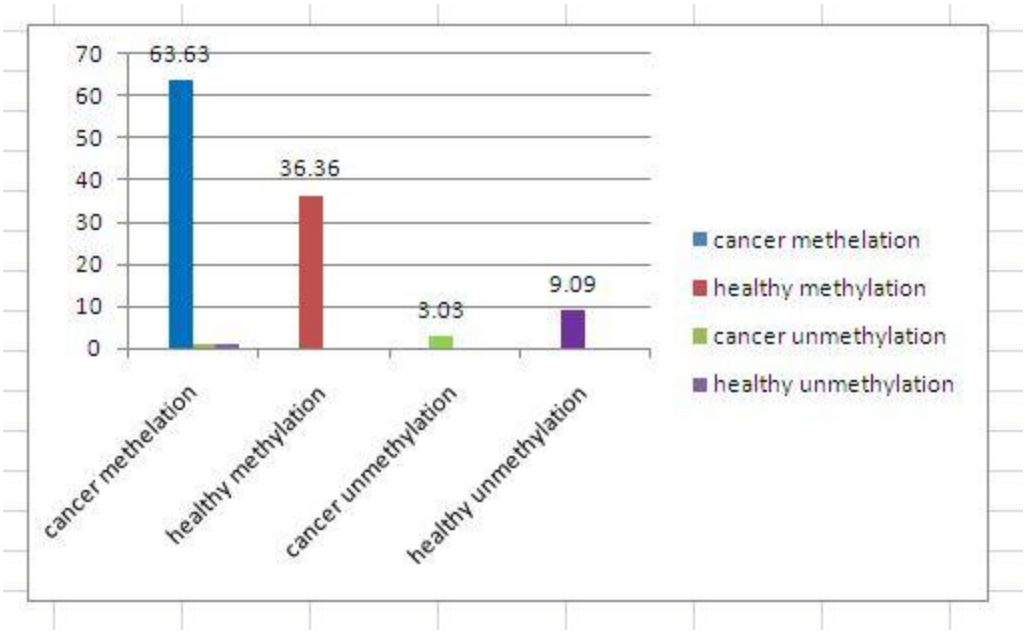


Figure 4.20: Total methylation and unmethylation between cancer and healthy samples of BRCA1 gene.

Total unmethylated CPG from the healthy sample was (9.09%) while from cancer samples it was (3.03%).Total CpGs, not present from cancer samples was (33.33%) while from healthy samples it was (54.54%) (**Table 4.16**).

Table 4.16: CPG methylation in BRCA1 gene

Sample number	Unmethylated CpGs %	Methylated CpGs%	CpGs, not present%
28	(0 cases) 0%	(7cases)63.63%	(4 cases)36.36%
29	(1 cases)9.09%	(4cases)36.36%	(6cases)54.54%
30	(0 cases) 0%	(10 cases)90.9%	(1cases) 9.09%
31	(1 cases)9.09%	(4cases) 36.36%	(6cases)54.54%

Table 4.17: Percent of DNA methylation in BRCA1 gene of grades 1 and 2 in breast cancer samples

Samples	Grades	Percent methylation	Percent unmethylation	Total mehylation	Total unmethylation
28	1	63.63	0	49.9	4.5
29	1	36.36	9.09		
30	2	90.9	0	90.9	0

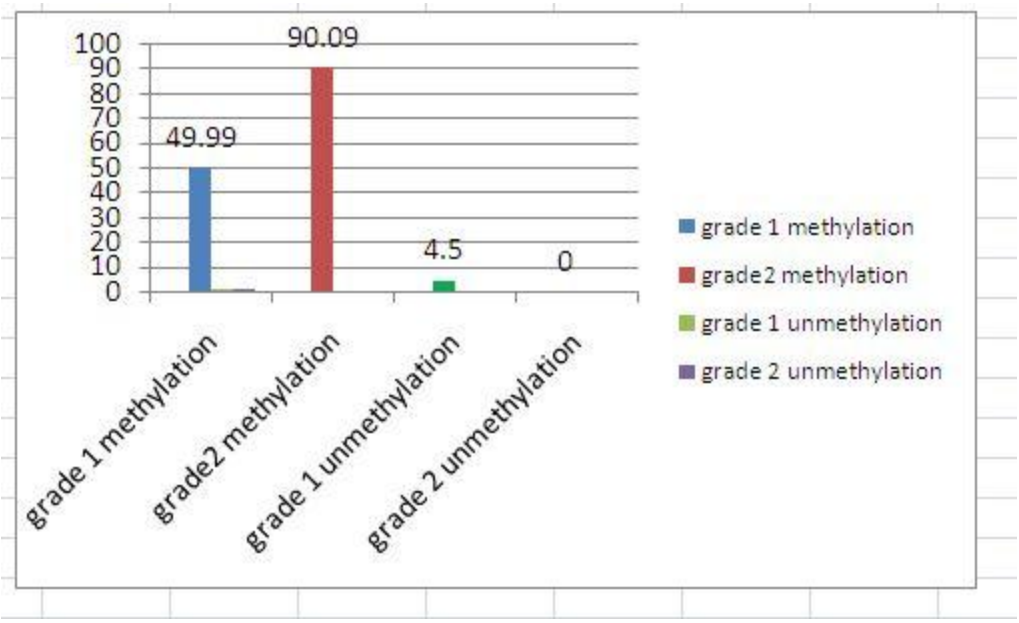


Figure 4.21: Total methylation and unmethylation between grad1 and grade 2 breast cancer samples of BRCA1 gene.

Bisulfite conversion reactions are predictable to deaminate cytosine to uracil and to leave 5-methylcytosine unchanged [57].

When bisulfite treated PCR amplified DNA, 5-methylcytosine on the strand template pairs with guanine on the recently synthesized strand converted cytosine, which is uracil pairs with adenine. The methylation model of individual DNA molecules, for that reason, be capable of being inferred from the sequences of subcloned PCR products A cytosine site interpreted as having been unmethylated if thymine occupies it and as having been methylated if cytosine occupies it .

DNA methylation plays at least two important and specific roles in human cancers. First, DNA methylation is a significant contributor of point mutations at CpG dinucleotides in a variety of growth regulatory genes. Second, DNA methylation can play a dramatic role in suppressing gene expression through aberrant methylation of cytosine and guanine-rich gene regulatory regions called CpG islands. Both regional and locus-specific changes in DNA methylation can result in a selective growth advantage for a cell. These changes appear to be particularly relevant to tumor-suppressor genes such as BRCA1 [117].

BRCA has two alternative first exons, Ia, and lb. each with its promoter region. Exon la-containing transcripts are the forms predominantly expressed in the mammary epithelium [203].The findings that the BRCA1 promoter aberrantly methylated in sporadic breast cancer could have clinical as well as biological importance. Analysis of the BRCA1 promoter region for the presence of 5-methylcytosine could serve as a diagnostic or prog- notice indicator for sporadic breast cancer [153].

Alteration of DNA methylation kinds is a very frequent event in cancer cells, and it has shown that hypermethylation is related to the down-regulation of some

suppressor genes in human cancers [31]. In addition the CCGG and GCGC sites located around the exon 1a of BRCA1 found to be hypermethylated in some breast cancers samples and abnormal CpG methylation detected in a small percentage (16% and 21% at CpG sites of DNA molecules from two out six breast cancer samples analysed using the bisulfite genomic sequencing method [114].

Catteauet in 1999 have shown that the promoter region of the BRCA1 gene is hypermethylated in the samples of breast tumors 11%.

Niwa in 2000 reported highest frequency (31%) BRCA1 methylation in sporadic breast cancer by using molecular biological techniques dissimilar from Southern blot hybridization. Also Esteller, 2000 reported that hypermethylation of the BRCA1 gene promoter found in a considerable proportion of primary sporadic breast carcinomas, which was 9.1 [44].

The most reliable analysis of data from bisulfite-treated DNA is those that account for two types of conversion error occurs and cause the failed conversion and inappropriate conversion [60].

The better studied realize these mistakes failed conversion was said to happen at what time an unmethylated cytosine fails to be deaminated and consequently appears in resulting data as if it had been methylated. For the reason that five methylcytosine in somatic cells of mammals occurs completely or almost absolutely at CpG cytosines [15].

The failed conversion prevalence for bisulfite action of mammalian DNA indicates the portion of nonCpG cytosines that appear as cytosines in sequence information. When not explicitly integrated as a parameter in data analysis, failed conversion can inflate estimates of methylation densities, and can undermine labors to determine the sequence design preference of DNA methyltransferases.

The failed conversion frequency can typically be abridged by increasing the period of bisulfite cure [69]. By increasing the quantity of thermal denaturation steps used during conversion [103].

The second kind of inappropriate error conversion was supposed to take place once a methylated cytosine was deaminated, yielding thymine. Similar to uracils that result from deamination of cytosines, thymine that happen throughout inappropriate conversion of 5-methylcytosine will pair with adenine during PCR. As a consequence, 5-methylcytosines that undergo inappropriate conversion will be present misinterpreted as unmethylated. When an inappropriate conversion occurred and ignored in data analysis, it will lead to underestimates of genomic methylation densities. In contrast, when the inappropriate conversion occurs, and its frequency is known, it can be there include as a parameter in the data analysis. Information on failed and inappropriate-conversion frequencies is, therefore, essential for the inference from specific DNA methylation patterns [69].

Two previous studies have explicitly investigated failed and inappropriate-conversion frequencies under the conventional bisulfite conversion protocol, which uses 5.5M bisulfite and 58C. term these conditions LowMT (low molarity/temperature). Grunau et al. 2011, treated enzymatically methylated DNA under LowMT conditions and report unsuitable conversion frequencies that may be as elevated as 6%.

Shiraishi and Hayatsu reported a comparable inappropriate conversion rate under similar circumstances intended for conversion of DNA from a tightly methylated tumor cell line. Neither analysis was capable explicitly of excluding alternate explanations for these events[168] (**Table 4.18**)

Table 4.18: Different in DNA methylation based on the single CPG of BRCA1

NO	1	2	3	4	5	6	7	8	9	10	11
1	M	N	M	N	M	N	M	N	M	M	M
2	N	N	N	M	U	N	N	M	N	M	M
3	N	M	M	M	M	M	M	M	M	M	M
4	M	N	M	U	M	N	N	M	N	N	M

M= methylattion ,U= Unmethylation , N = not present

4.7. Results of Statistical Analysis:

Fisher exact test used to compare methylation and unmethylation between healthy and cancer samples and also between grade 1 and grade 2 of each gene. For ESR2 genes, the value was (**0.033**) between healthy and breast samples that indicate significant result (**p < 0.05**). The result of statistical analysis between grade 1 and grade 2 of ESR2 samples was (**0.007**) which also showed significantly (**p < 0.05**).

For OPN gene, neither statistical analysis between healthy and breast cancer samples (**0.0**) nor between grad1 and grade2 (**0.376**) at (**p < 0.05**) was significant.

Regarding BRCA1 gene, the Fisher exact test statistic value was (**0.013**) between healthy and breast cancer samples (**p < 0.05**) and the value was (**0.017**) between grade 1 and grade 2 of breast cancer. The two values were significant.

Chapter five

Conclusions and Recommendations

Conclusions and Recommendations

5.1. Conclusions

From this study the following are concluded:

1- DNA quantity decrease after bisulfite conversion reaction.

2- Bisulfate conversion rates are different from the samples among genes (ESR2, OPN and BRCA1).

3- ESR2 are more converted than OPN and OPN more converted than BRCA1.

4- Total CPG DNA methylation is different between all three genes.

5- The percentage of DNA methylation in ESR2 (12.39%) for breast cancer and healthy samples (4.16%), in grade 1 (8.33%) and grad 2 (20.5%) of breast cancer samples.

6- The percentage of DNA methylation in OPN (57.57 %) for breast cancer and healthy samples (0 %).in garde 1 (61.89%) and in grade 2 (49.99%) of breast cancer samples.

7- The percentage of DNA methylation in BRCA1 (63.63%) for breast cancer and healthy samples (36.36%). In grade 1 (49.9%) and grade 2(90.9.36%) of breast cancer samples.

8- Total CPG unmethylated different among to three genes.

10-The percentage of unmethylated CPG of ESR2 is (72.5%) for breast cancer but healthy samples(87.48%). in grade 1 of patient (79.16%) and (59.22%) in grade2.

11-The percentage of unmethylated CPG of OPN is(39.3 %)but for healthy samples(88.8%).in grade 1(38.09%) of patients and grade 2(41.66%).

12-The percentage of unmethylated CPG of BRCA1is (3.03%) but for healthy samples it is (9.09%). in grade 1 of patients (4.5%) and grade 2(%0)

5.2. Recommendations

The followings are recommended for future research

1-Using other techniques to determine DNA methylation statuses such as CoBRA (combined bisulphite restriction analysis), Methylation-specific PCR (MSP), Pyrosequencing and MethyLight™.

2- Evaluation of the relation between DNA methylation on the gene expression.

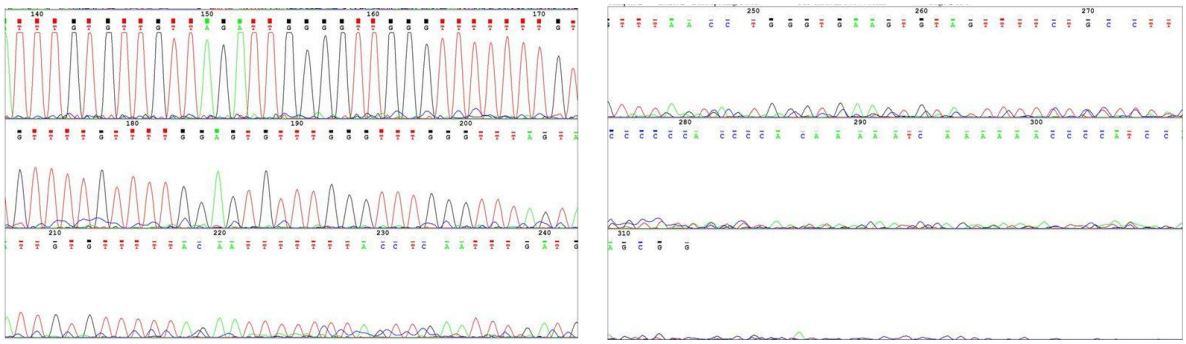
3- DNA methylation profiling of different breast cancer genes.

4- Comparison of DNA methylation between different stages of breast cancer.

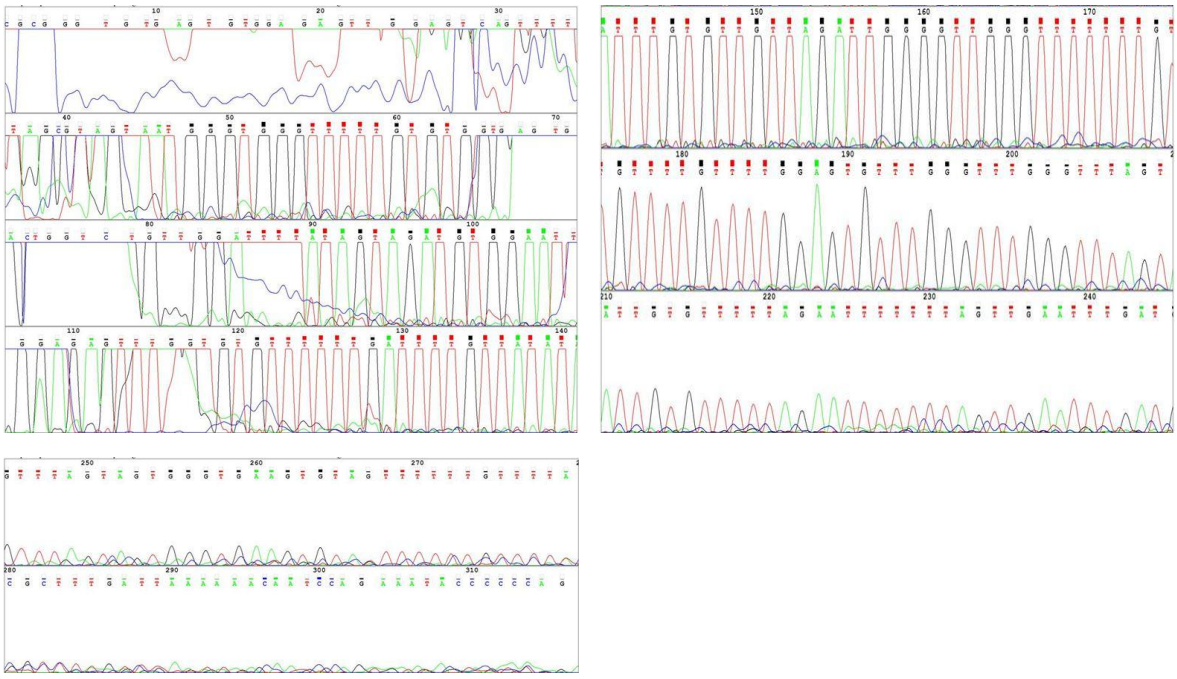
Appendix

Appendix A Electropherograms and sequences of ESR2

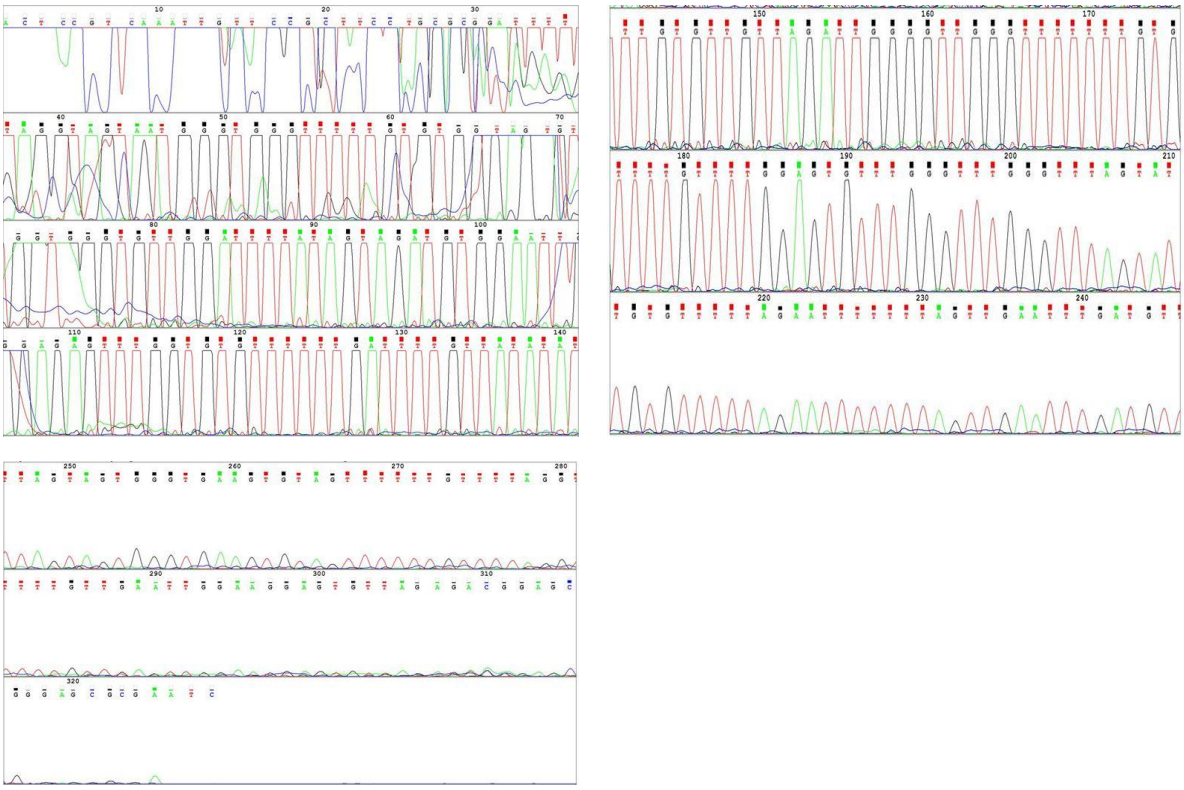
Sample 1



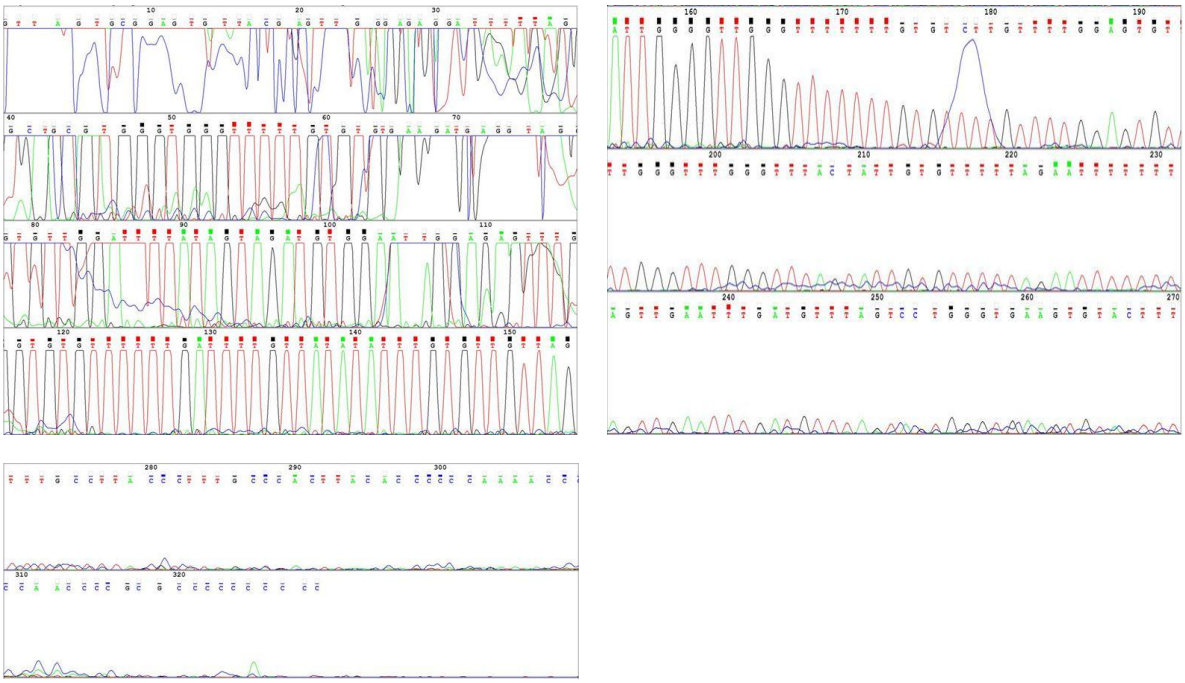
Sample 2



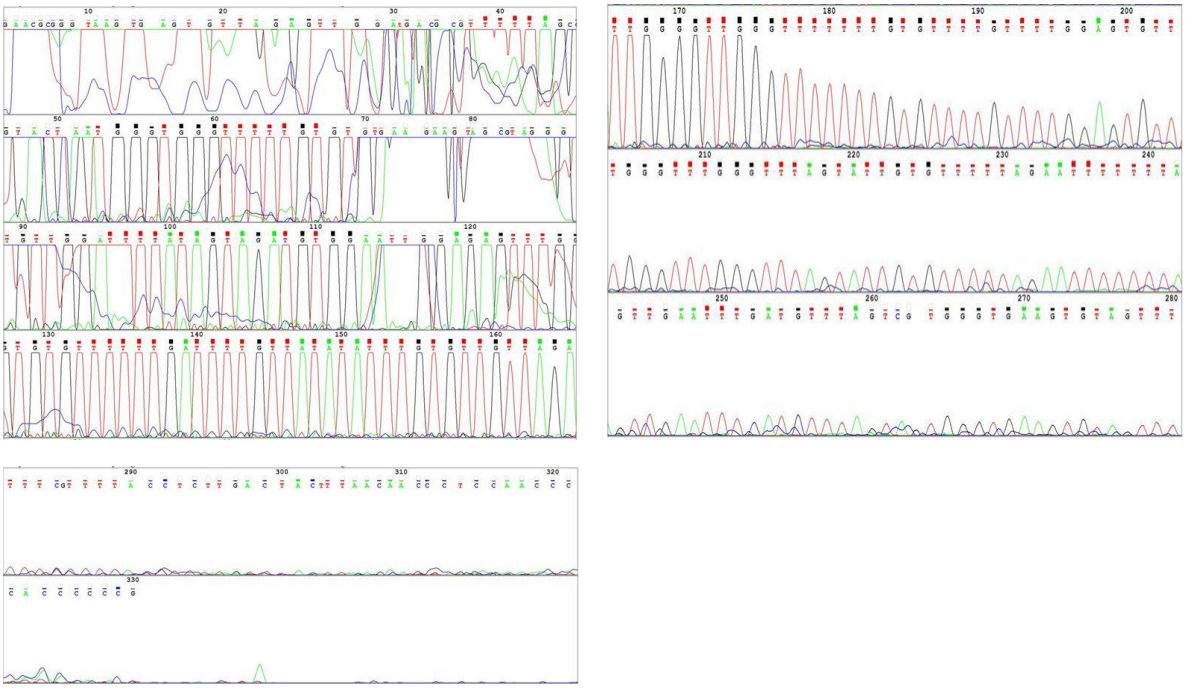
Sample 3



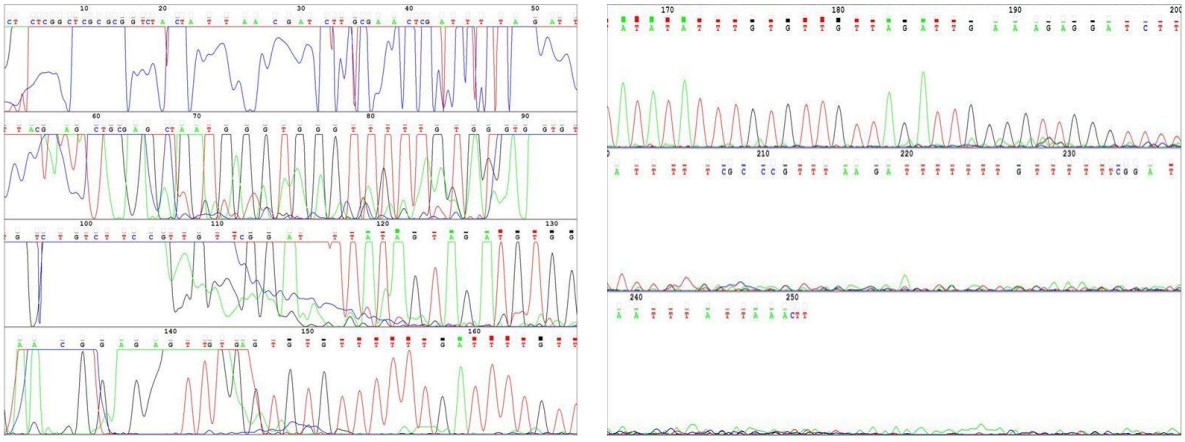
Sample 4



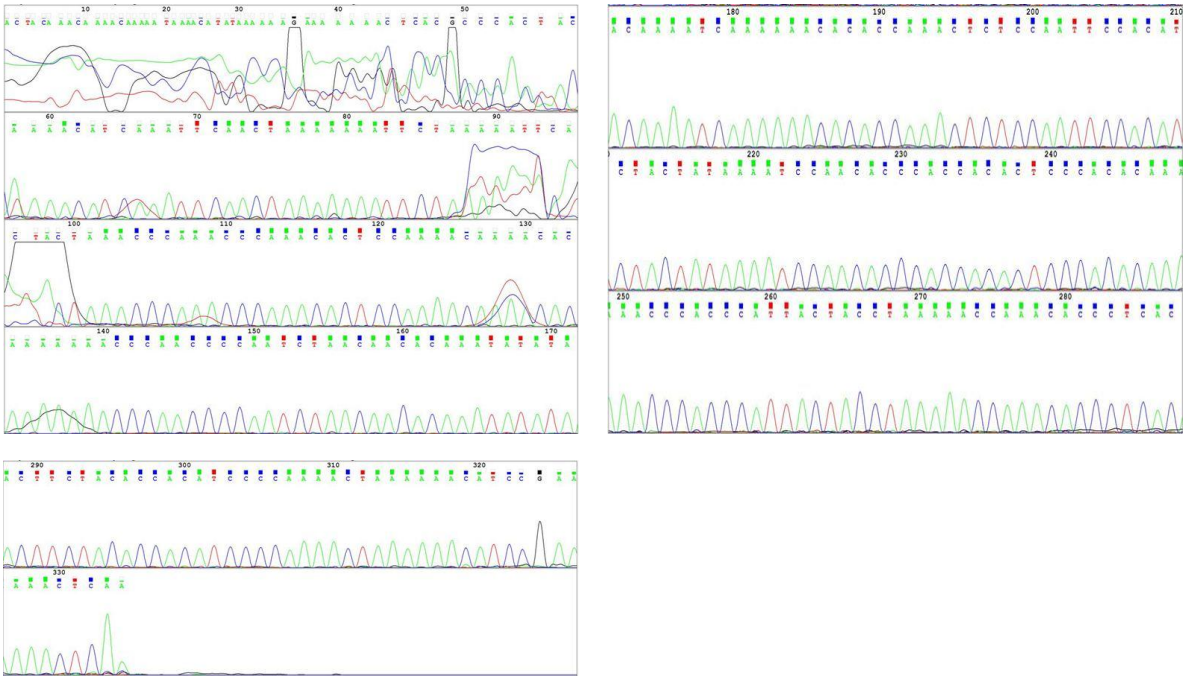
Sample 5



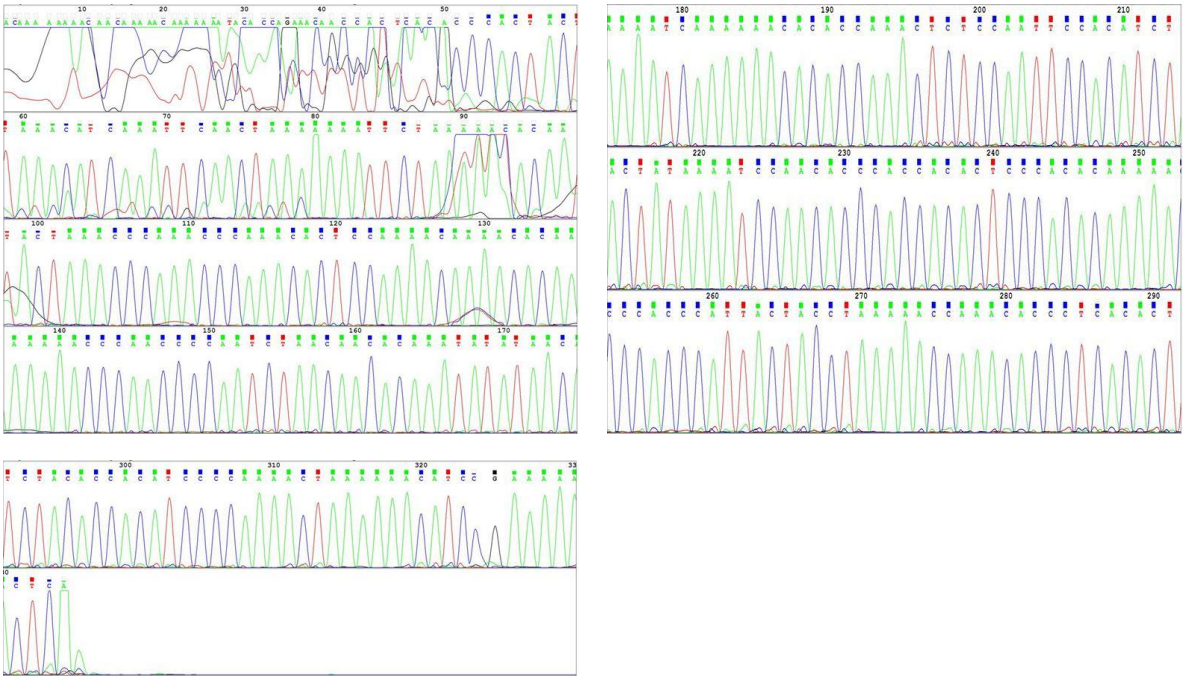
Sample 6



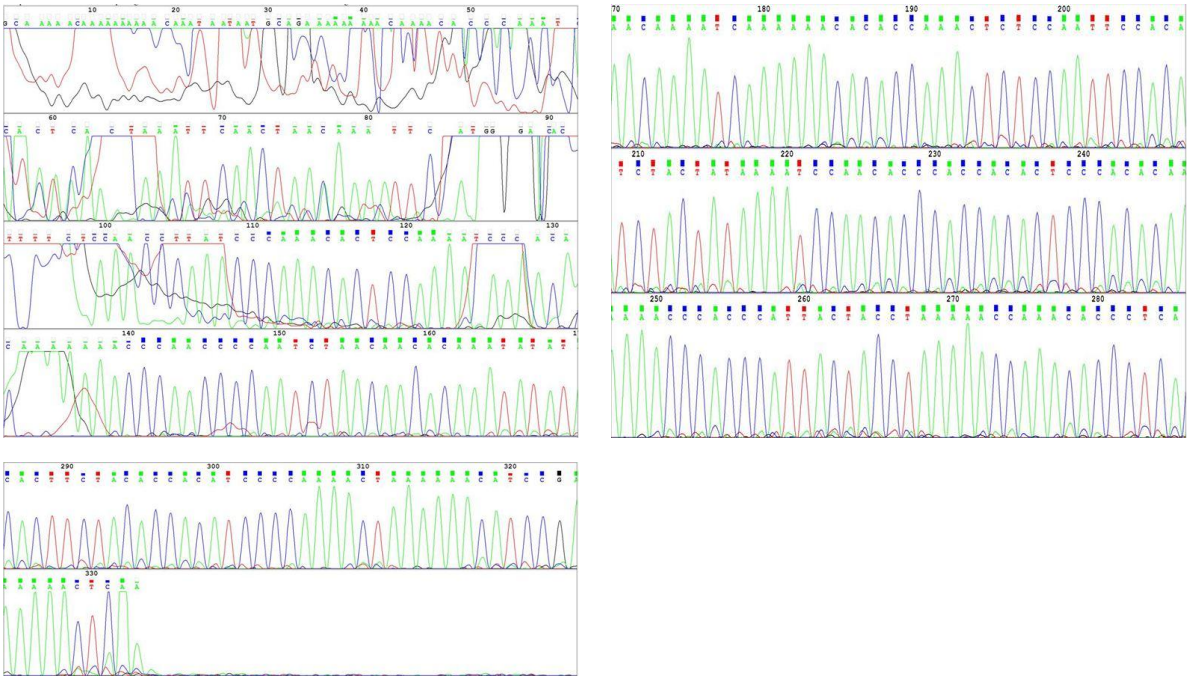
Sample 7



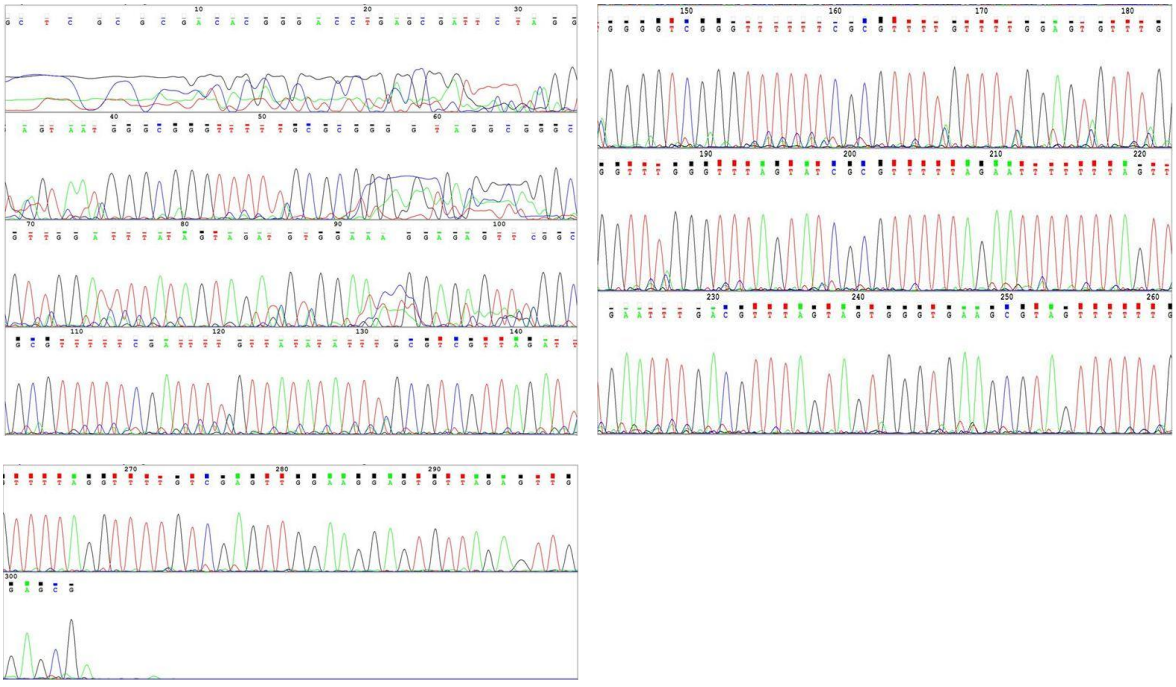
Sample 8



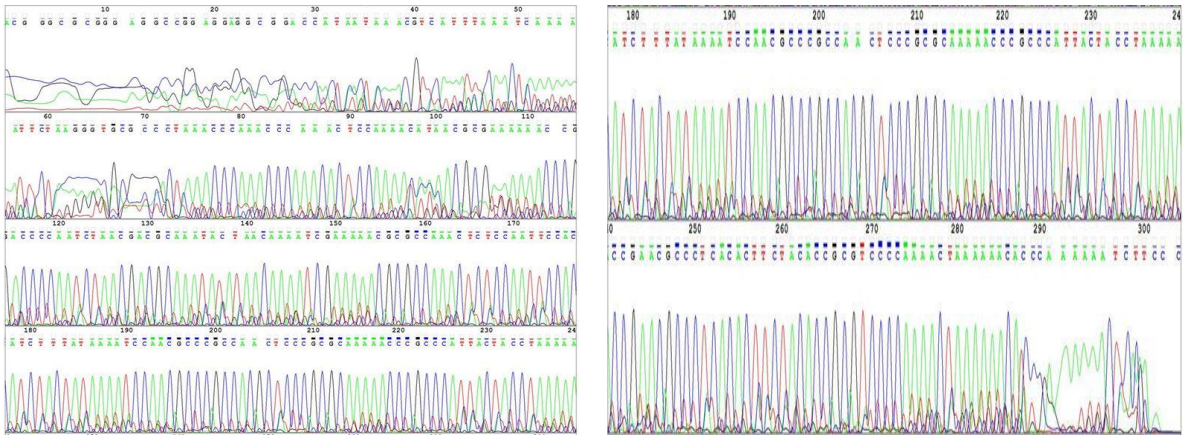
Sample 9



ESR2 converted control forward

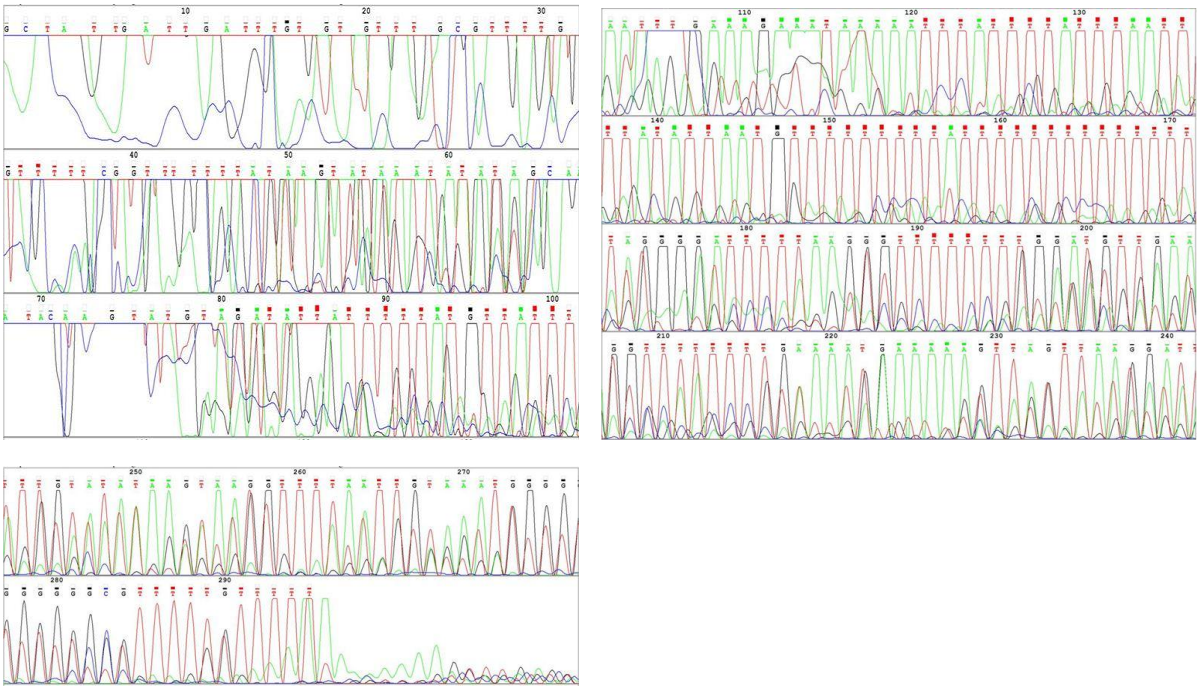


ESR2 converted control reverse

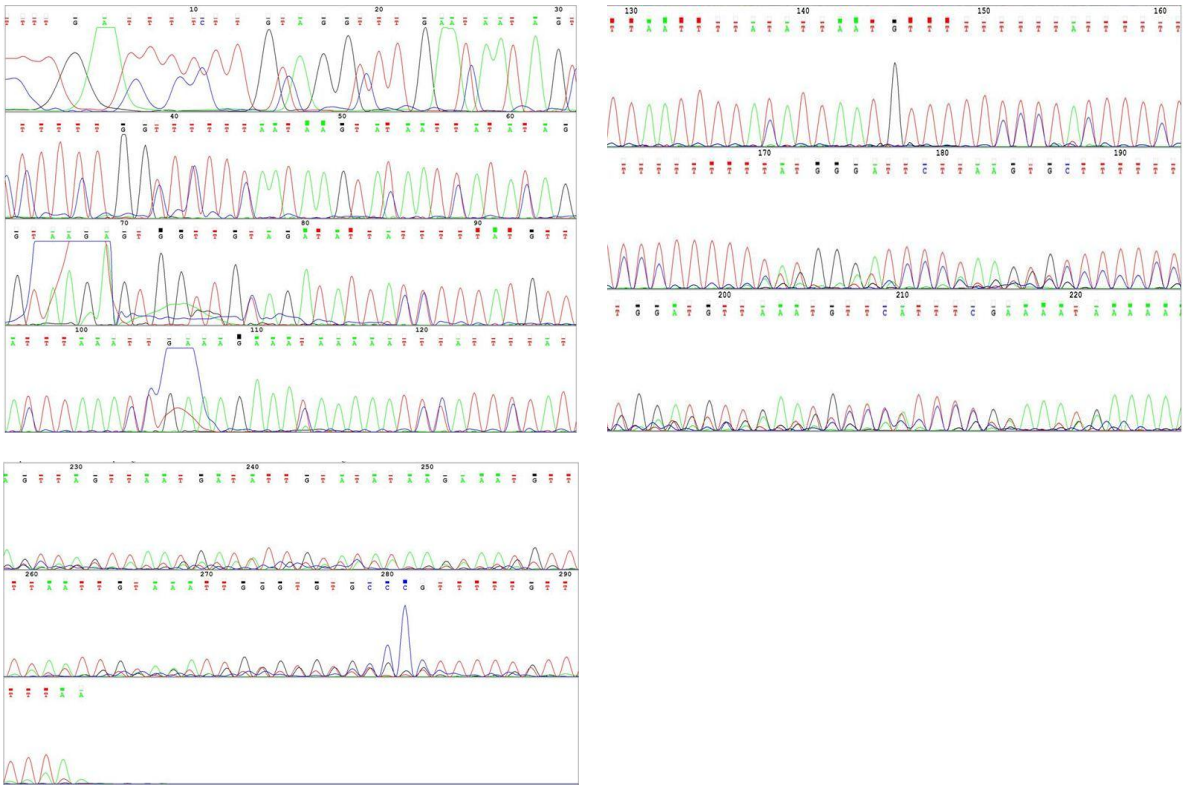


appnedix B Electropherograms and sequences of OPN

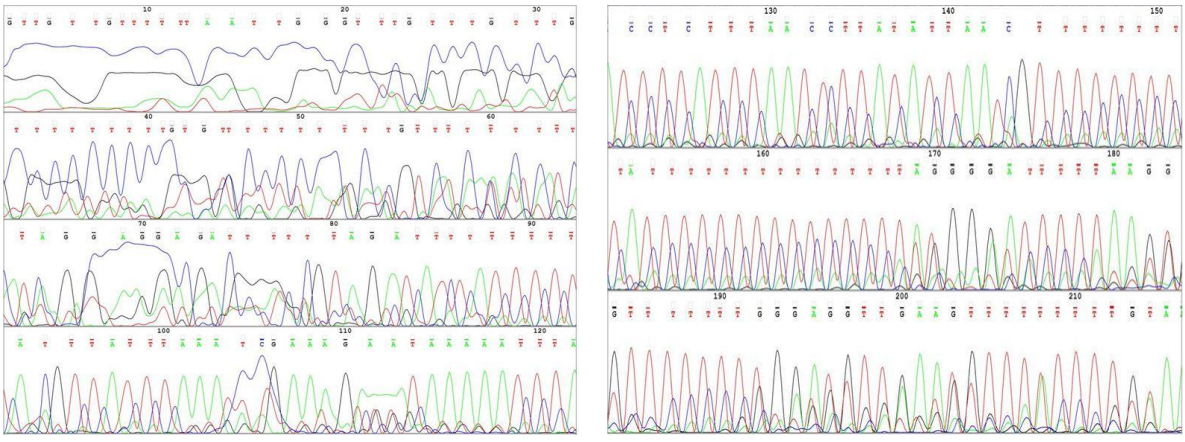
Sample 14

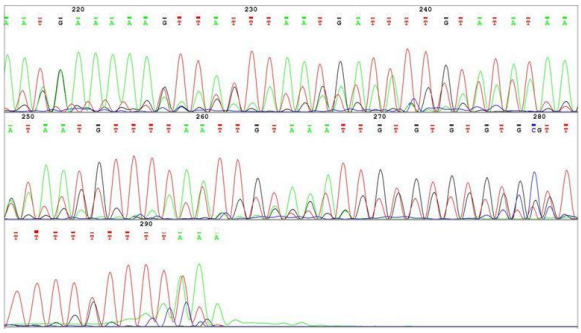


Sample 15

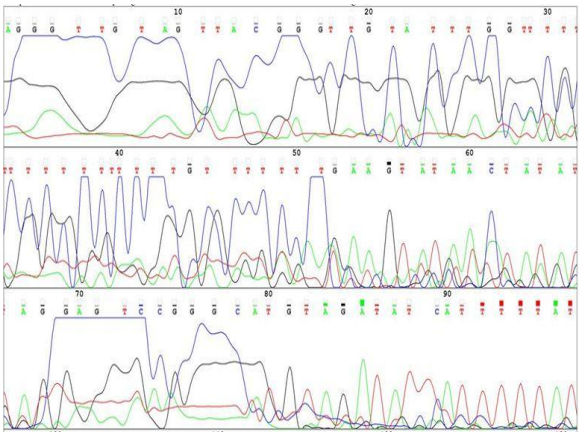


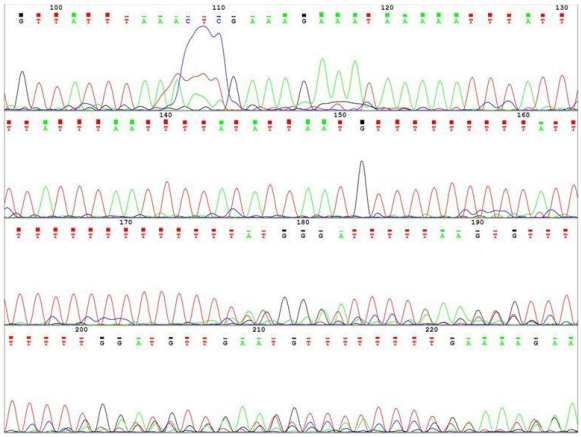
Sample 16

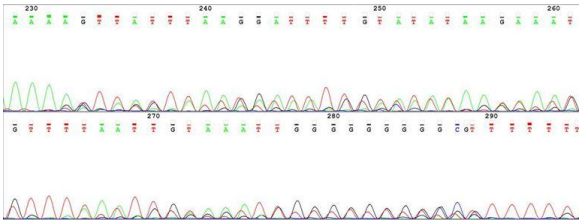




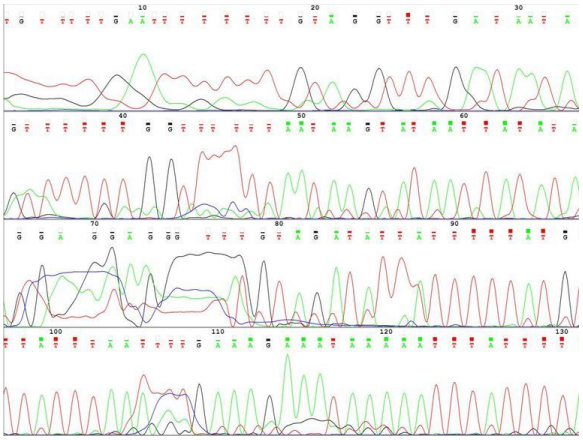
Sample 17

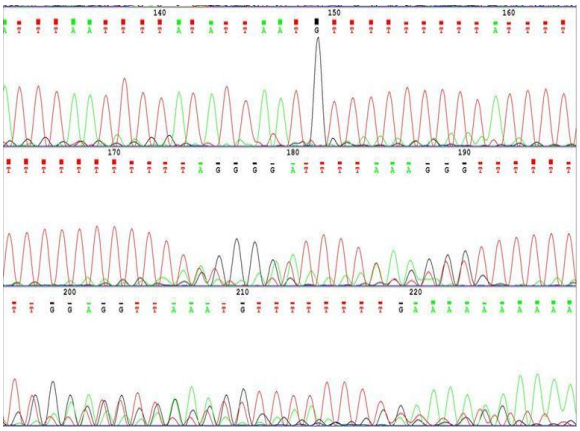


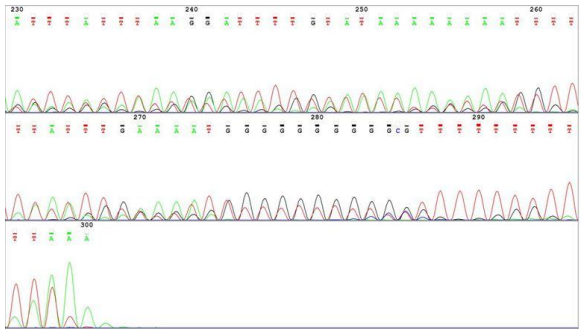




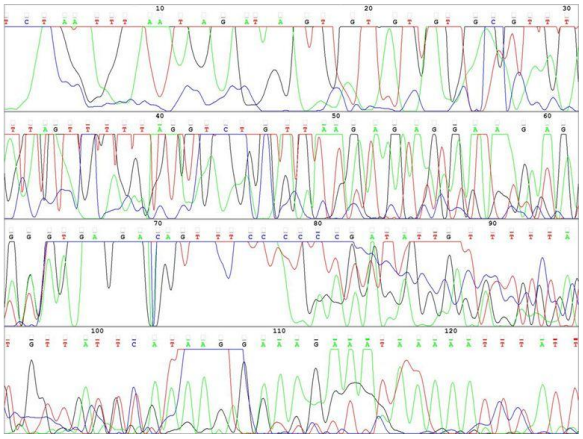
Sample 18

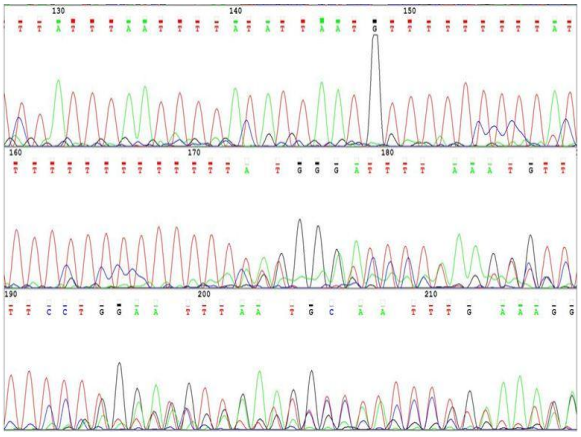


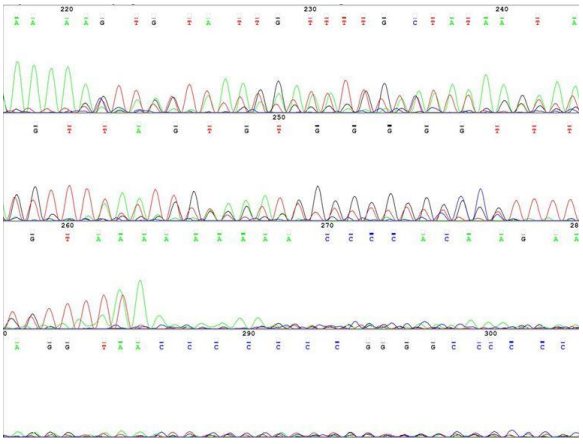




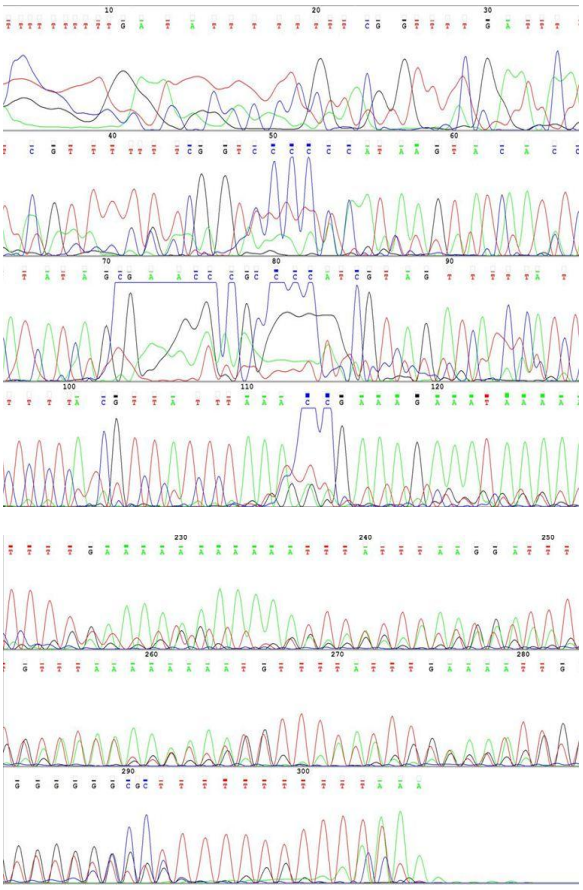
Sample 19



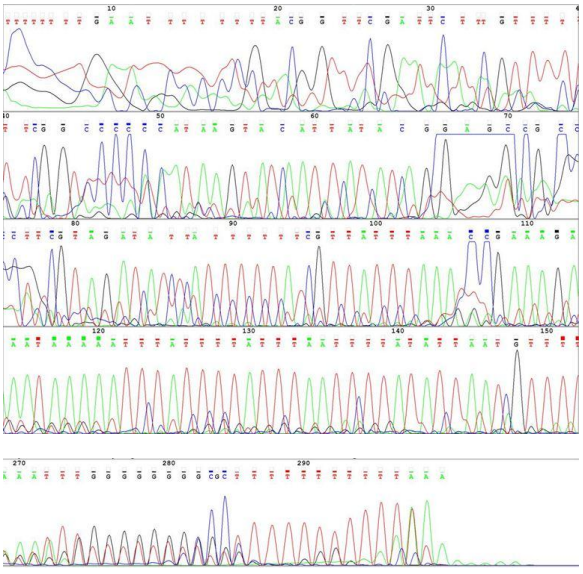




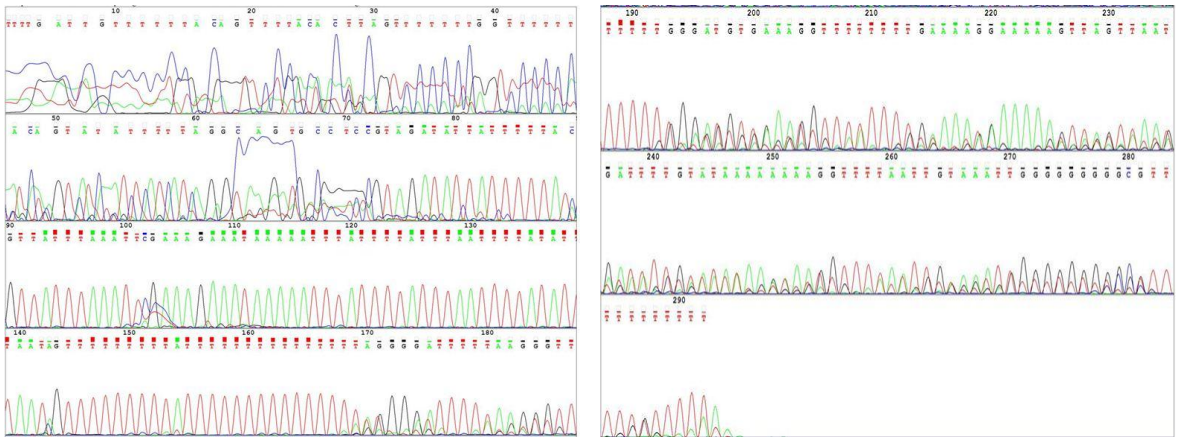
Sample 20



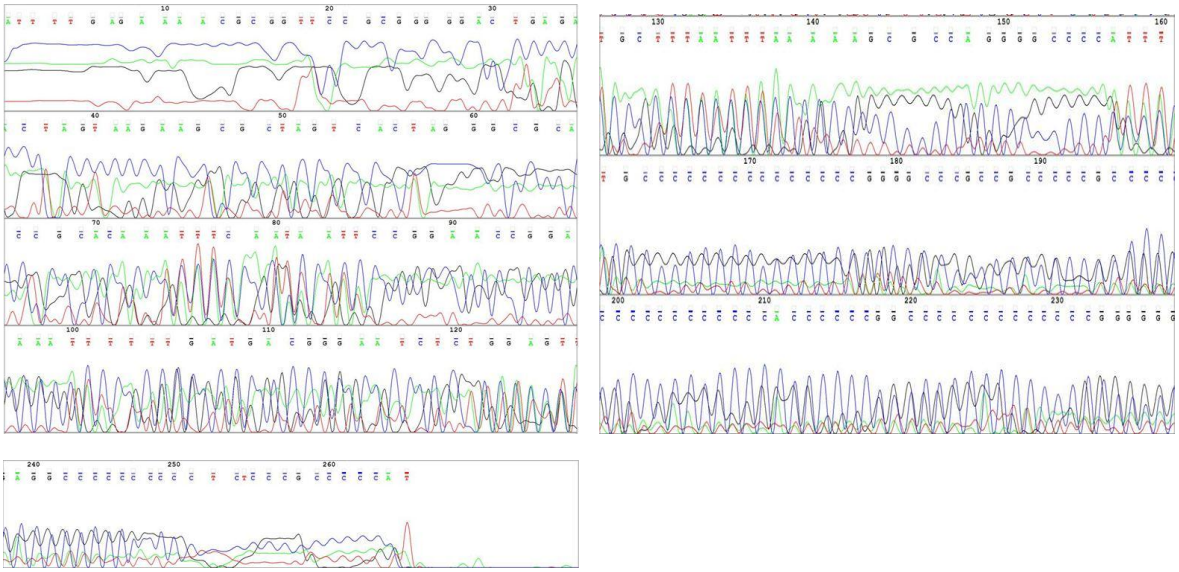
Sample 21



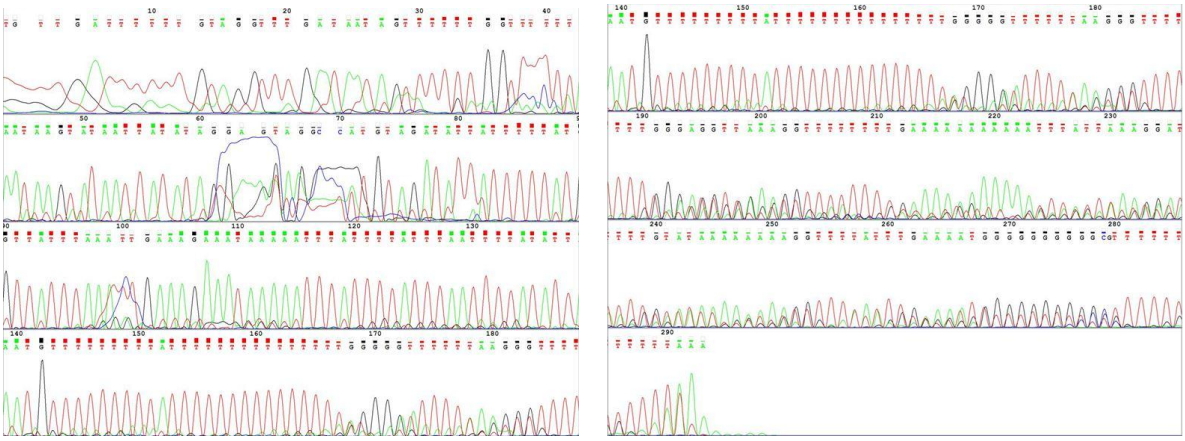
Sample 22



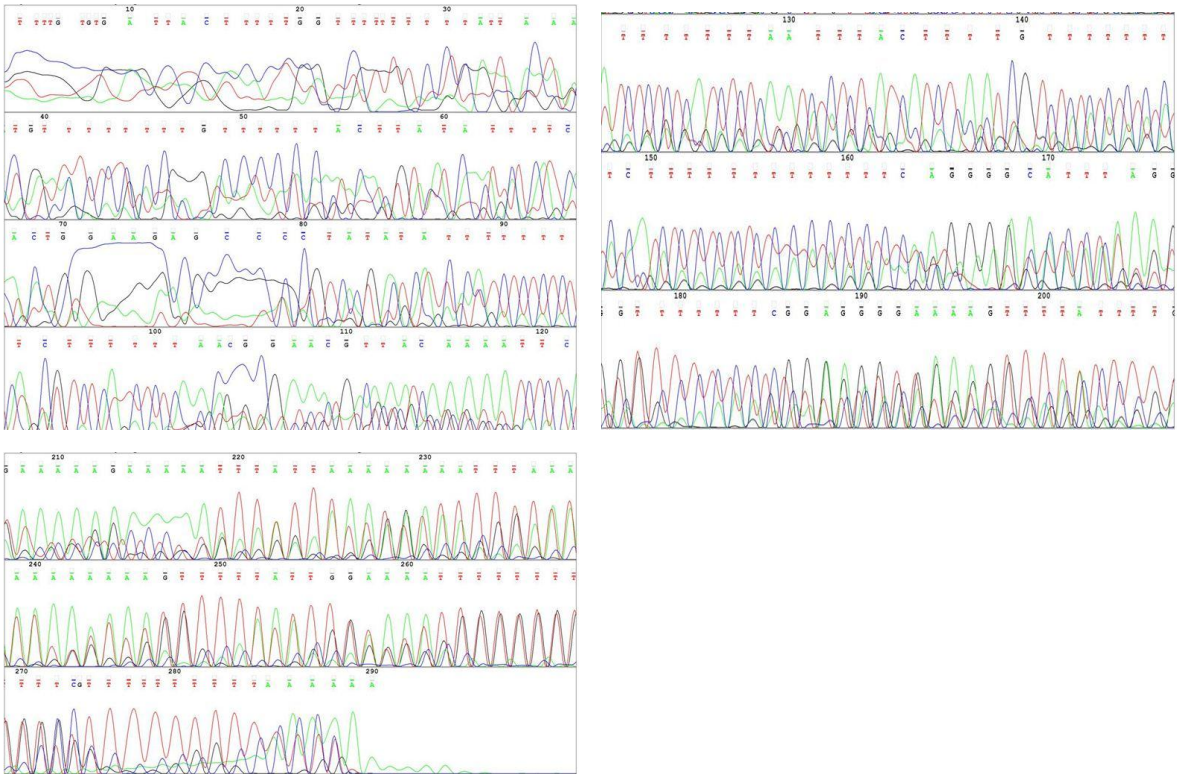
Sample 23



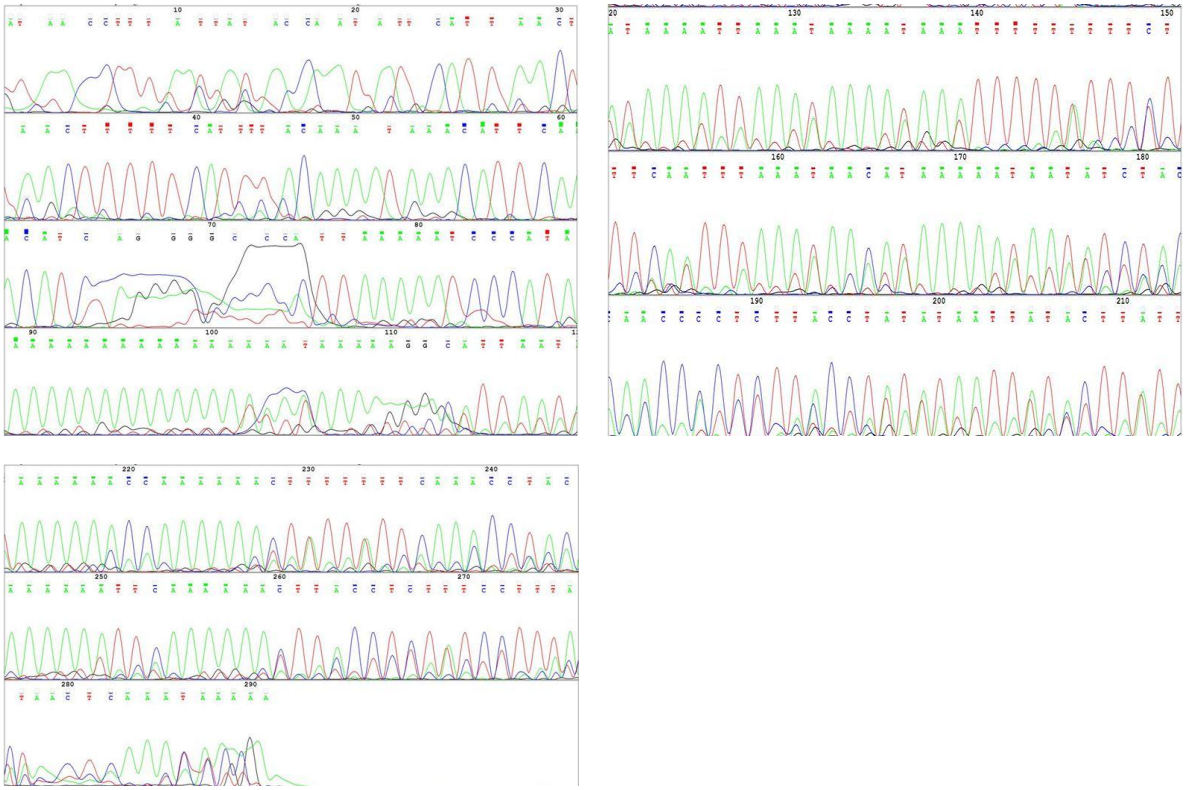
Sample24



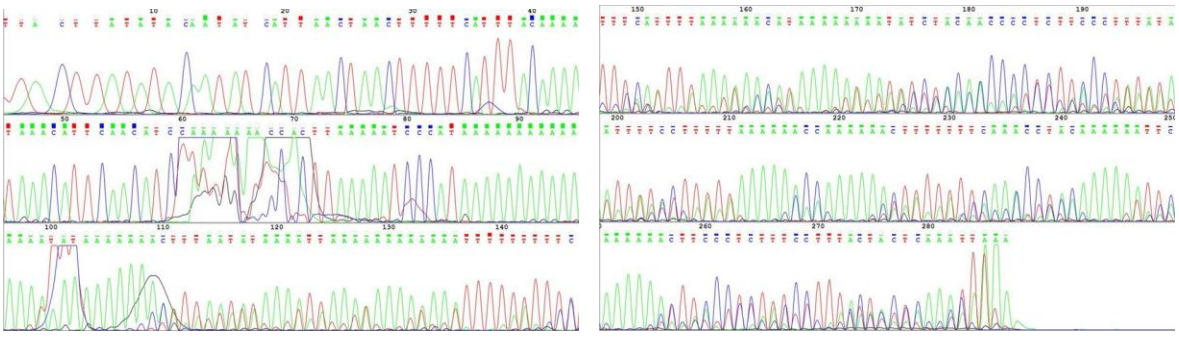
Sample 25



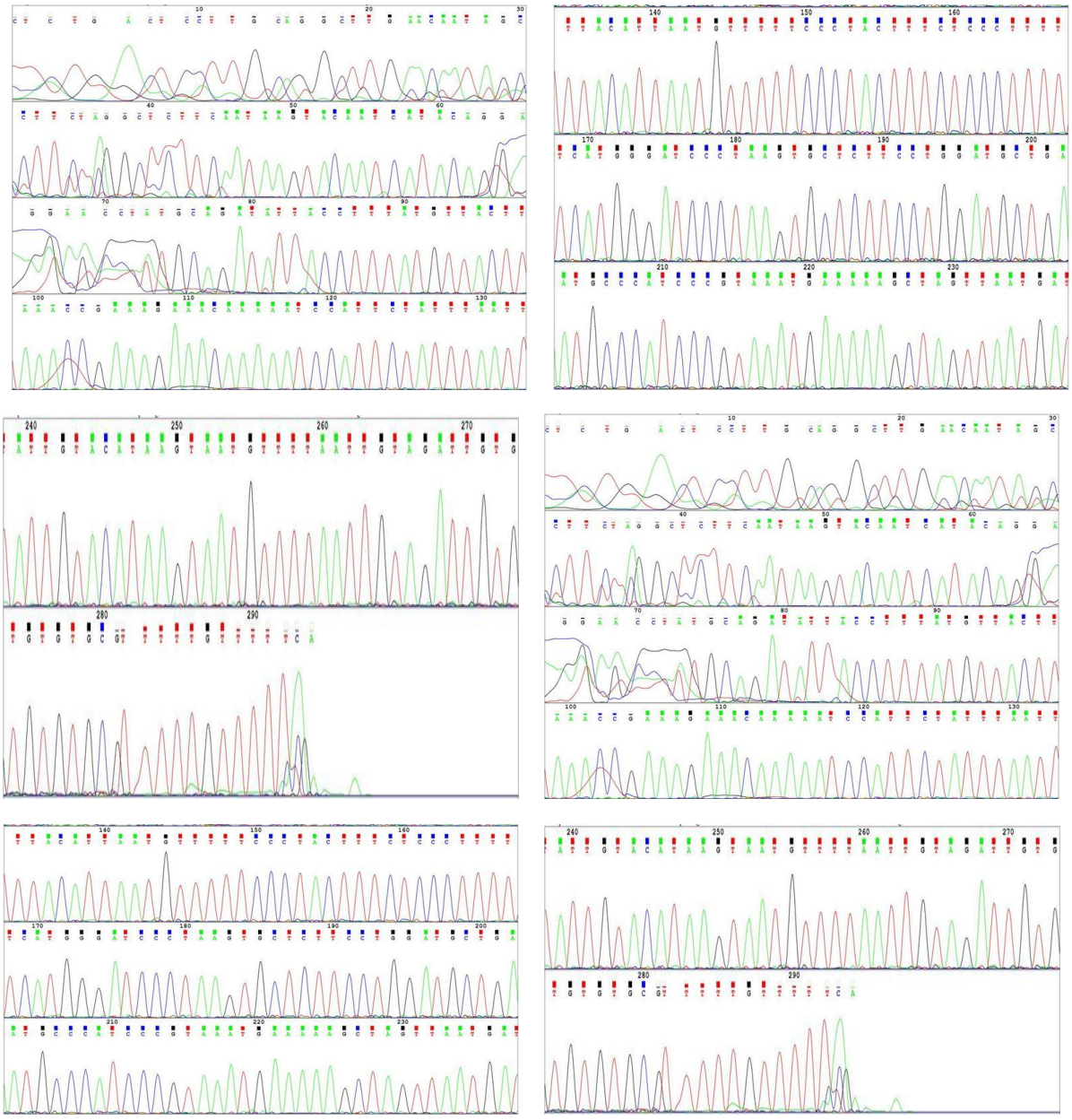
Sample 26



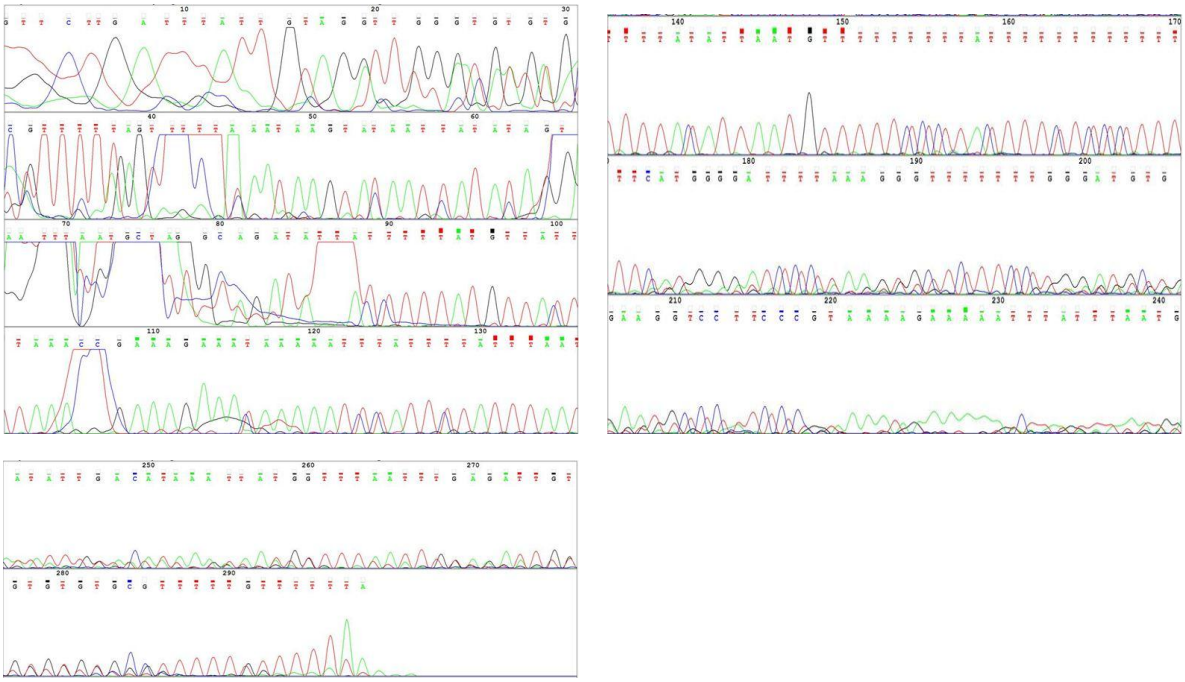
Sample 27



OPN methylated control forward

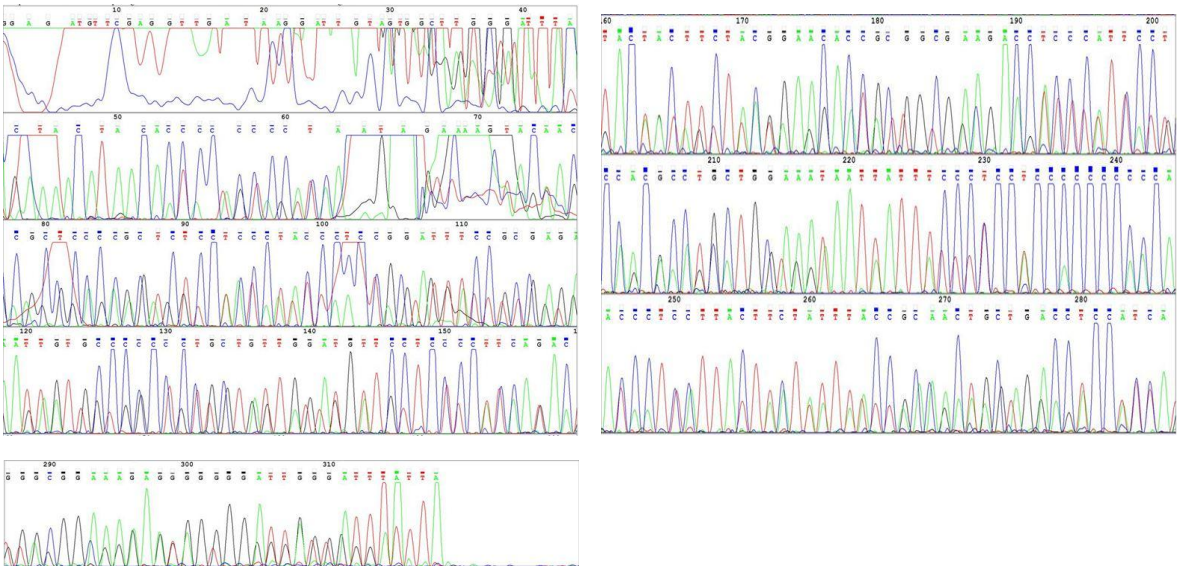


OPN converted control forward

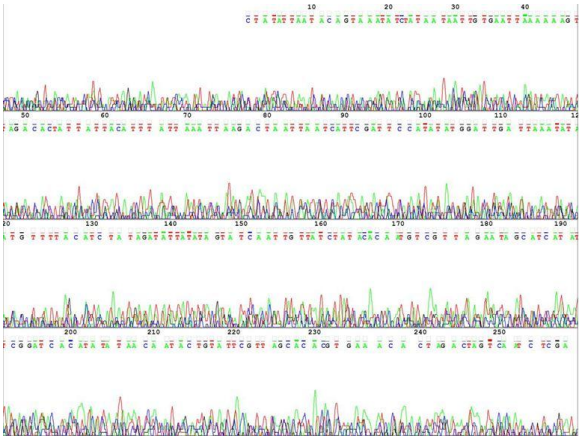


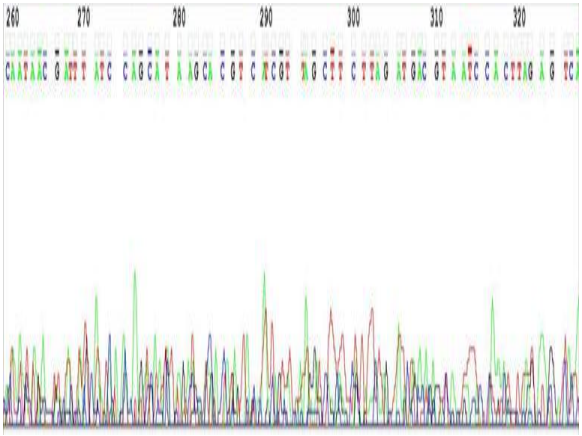
Appendix C Electropherograms and sequences of BRCA1

Sample 28

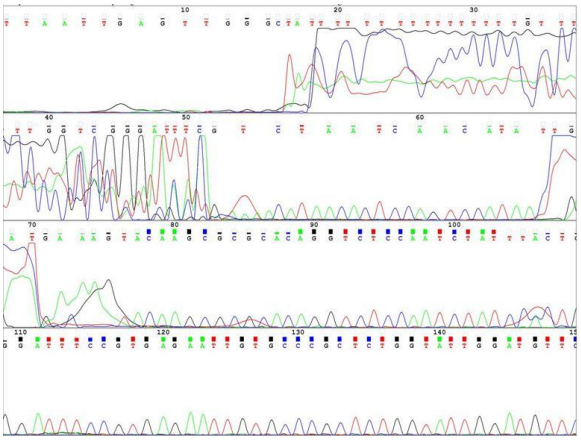


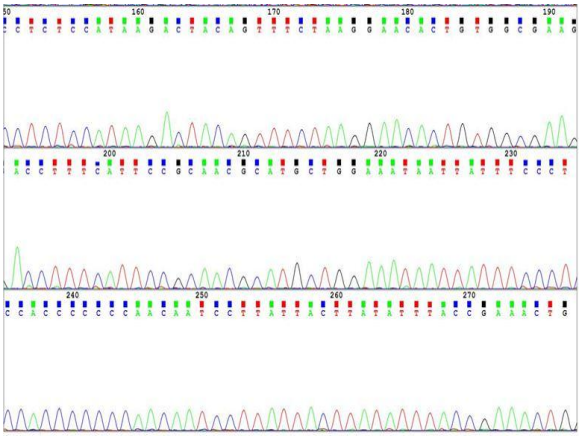
Sample 29

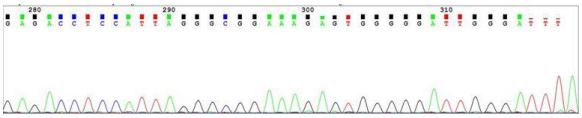




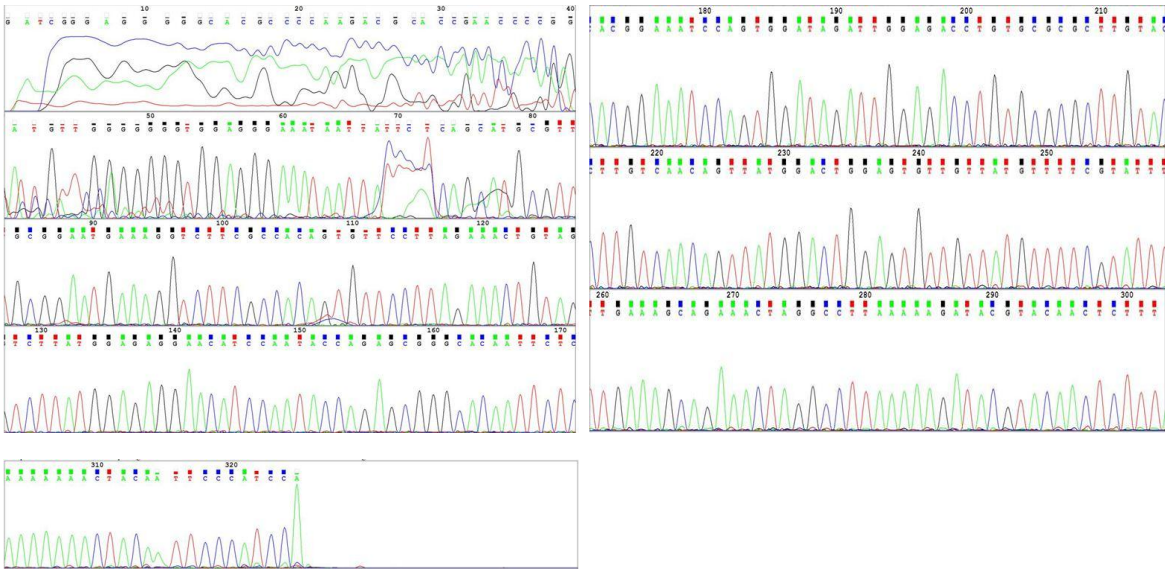
Sample 30







Sample 31



Appendix D Needleman-Wunsch pairwise alignment of ESR2

Sample 1

```
Genomic_Sequence    ACGCGGT-GT-AGAAGTGT-GAGGGCGT-TCGG-TTTTTAG-GTAGTAATGGGCGGGTTT
[2]1.seq            A-GCGGTGGTGAG-AGT-TAGA-GTTGGAGCGAGTTTTTAGCGC-CT-ATGGGTGGGTTT

Genomic_Sequence    TTGCGCGG-GAGCGTGGCGGGCGTTGGATTTTATAGTAGATGTGGAATTGGAGAGTTTGG
[2]1.seq            TTGTGTGTTTATTAACACTAGTGTTGGATTTTATAGTAGATGTGGAATTGGAGAGTTTGG

Genomic_Sequence    CGCGTTTTTCGATTTTGTTATATATTTGCGTCGTTAGATTGGGGTCGGGTTTTTTCGCGT
[2]1.seq            TGTGTTTTTTGATTTTGTTATATATTTGTGTTGTTAGATTGGGGTTGGGTTTTTTTGTGT

Genomic_Sequence    TTTGTTTTGGAGTGTTTGGGTTTGGGTTTAGTATCGCGTTTTTAGAATTTTTTTAGTTGA
[2]1.seq            TTTGTTTTGGAGTGTTTGGGTTTGGGTTTAGTATTGTGTTTTTACAATTTTTTTACCTCA

Genomic_Sequence    ATTTGACGTTTAGTAGTGGGTGAAGCGTAGTTTTTTGTTTTAGGTTTTGTCGAGTTGGAA
[2]1.seq            ATTTGATGTTTAAC-CTGGGTGAAGTGTAGTTTTCTGCCTTCCCCCCACCCCA-CAAAAA

Genomic_Sequence    --GGAGTGTTAGAGTTGGAGCG-
[2]1.seq            TCAAAAAACCCCA-TC-CAGCGG
```

Sample 2

```
Genomic_Sequence    ACGCGG-TGTAGAAGTGT-GAG-GGCGT--TCGGTTTTTAG-GTAGTAATGGGCGGGTTT
[2]2.seq            -CGCGGGTGT-G-AGTGTGGAGAGTTGGAGTCAGTTTTTAGCGTAGTAATGGGTGGGTTT

Genomic_Sequence    TTGCGCGG-GAGCGTGGCGG-GCGTTGGATTTTATAGTAGATGTGGAATTGGAGAGTTTG
[2]2.seq            TTGTGTGGTGAGTG-ACTGGTCTGTTGGATTTTATAGTAGATGTGGAATTGGAGAGTTTG

Genomic_Sequence    GCGCGTTTTTCGATTTTGTTATATATTTGCGTCGTTAGATTGGGGTCGGGTTTTTTCGCG
[2]2.seq            GTGTGTTTTTTGATTTTGTTATATATTTGTGTTGTTAGATTGGGGTTGGGTTTTTTTGTG

Genomic_Sequence    TTTTGTTTTGGAGTGTTTGGGTTTGGGTTTAGTATCGCGTTTTTAGAATTTTTTTAGTTG
[2]2.seq            TTTTGTTTTGGAGTGTTTGGGTTTGGGTTTAGTATTGTGTTTTTAGAATTTTTTTAGTTG

Genomic_Sequence    AATTTGACGTTTAGTAGTGGGTGAAGCGTAGTTTTTTGTTTTAGGTTTTGTCGAGTT-GG
[2]2.seq            AATTTGATGTTTAGTAGTGGGTGAAGTGTAGTTTTTTGTTTTACG--CT-TTGA-TTAAA

Genomic_Sequence    AAGGAGTGTTAGAGTTGGAGCG-----
[2]2.seq            AAACAAT-CCAGAAAT--ACCCCCCAG
```

Sample 3

```
Genomic_Sequence    ACGCGGTGTAGAAGTG-T--G-AGGGCGTTCGG-TTTTTAGGTAGTAATGGGCGGGTTTT
[2]3.seq            ACTCCGT-CA-AATTGTTCCGCTTCCTGCGCGGATTTTTAGGTAGTAATGGGTGGGTTTT

Genomic_Sequence    TGCGCGGGAGCGTGGCGGGCGTTGGATTTTATAGTAGATGTGGAATTGGAGAGTTTGGCG
[2]3.seq            TGTGTGGTAGTGTGGTGGGTGTTGGATTTTATAGTAGATGTGGAATTGGAGAGTTTGGTG

Genomic_Sequence    CGTTTTTCGATTTTGTTATATATTTGCGTCGTTAGATTGGGGTCGGGTTTTTTCGCGTTT
[2]3.seq            TGTTTTTTGATTTTGTTATATATTTGTGTTGTTAGATTGGGGTTGGGTTTTTTTGTGTTT

Genomic_Sequence    TGTTTTGGAGTGTTTGGGTTTGGGTTTAGTATCGCGTTTTTAGAATTTTTTTAGTTGAAT
[2]3.seq            TGTTTTGGAGTGTTTGGGTTTGGGTTTAGTATTGTGTTTTTAGAATTTTTTTAGTTGAAT

Genomic_Sequence    TTGACGTTTAGTAGTGGGTGAAGCGTAGTTTTTTGTTTTAGGTTTTGTCGAGTTGGAAGG
[2]3.seq            TTGATGTTTAGTAGTGGGTGAAGTGTAGTTTTTTGTTTTAGGTTTTGTTGAATTGGAAGG

Genomic_Sequence    AGTGTTAGAGTTGGAGCG----------
[2]3.seq            AGTGTTAGAGACGGAGCGGGAGCGCGAATC
```

Sample 4

```
Genomic_Sequence    ---A-CGCGG--TG-TA-GAAGTGTGAGGGCGTTCGG-TTTTTAGGTAGTAATGGGCGGG
[2]4.seq            GTTAGTGCGGAGTGTTACG-AGT-TG-GGA-G-A-GGATTTTTTAGGCTGC-GTGGGTGGG

Genomic_Sequence    TTTTTGCGCGGG-AG-CGTGGCGGGCGTTGGATTTTATAGTAGATGTGGAATTGGAGAGT
[2]4.seq            TTTTTGTGTGTGAAGATGAGGTAGGTGTTGGATTTTATAGTAGATGTGGAATTGGAGAGT

Genomic_Sequence    TTGGCGCGTTTTTCGATTTTGTTATATATTTGCGTCGTTAGATTGGGGTCGGGTTTTTTC
[2]4.seq            TTGGTGTGTTTTTTGATTTTGTTATATATTTGTGTTGTTAGATTGGGGTTGGGTTTTTTT

Genomic_Sequence    GCGTTTTGTTTTTGGAGTGTTTGGGTTTGGGTTTAGTATCGCGTTTTTAGAATTTTTTTAG
[2]4.seq            GTGTCTTGTTTTGGAGTGTTTGGGTTTGGGTTTACTATTGTGTTTTTAGAATTTTTTTAG

Genomic_Sequence    TTGAATTTGACGTTTAGTAGTGGGTGAAGCGTAGTTTTTTGTTTTAGGTTTTGTCGAGTT
[2]4.seq            TTGAATTTGATGTTTAGTCCTGGGTGAAGTGTACTTTTTTGCCTTACCCTTTGCCCACTT

Genomic_Sequence    GGA-AGGAGTGTTAGAGTTGGA--GCG----------
[2]4.seq            --ACA-CCCCCCAAAACCCCAACCCCGCGCCCCCCCCC
```

Appendix

Sample 5

Sample 6

Sample 7

Sample 8

Sample 9

Control converted methylated forward strand

Control converted methylated reverse strand

Appendix E Needleman-Wunsch pairwise alignment of OPN

Sample 14

```
Genomic_Sequence    TTTTTTGAATT-TTTTGTAG-GTTTGAATAATAGTTTTTTGGTTTTTTAATAAGTATAAT
[2]1.seq            GCTATTG-ATTGATTTGT-GTGTTTGCGTTTTGGTTTTTCGGTTTTTTTATAAGTATAAA

Genomic_Sequence    TATATAGGTAAGAGTGGT-TGTAGATATTATTTTTATGTTATTTAAATCGAAAGAAATAA
[2]1.seq            TATATA-GCAATACAAGTATGTAGATATTATTTTTATGTTATTTAATTTGAAAGAAATAA

Genomic_Sequence    AAATTTATTTTATTTAATTTTATATTAATGTTTTTTTTTATTTTTTTTTTTTTTATGGG
[2]1.seq            AAATTTATTTTATTTAATTTTATATTAATGTTTTTTTTTATTTTTTTTTTTTTTAGGG

Genomic_Sequence    ATTTTTAAGTGTTTTTTTTGGATGTTGAATGTTTATTTCGTAAATGAAAAAGTTAGTTAA
[2]1.seq            ATTTTTAAGGGTTTTTTTTGGATGTTGAAGGTTTTTTTGAAAATGAAAAAGTTAGTTAA

Genomic_Sequence    TGATATTGTATATAAGTAATGTTTTAATTGTAGATTGTGTGTGTGCGTTTTTGTTTTT
[2]1.seq            GGATTTTGTATATAAGTAAGGTTTAATTGTAAATGGGGGGGGGGCGTTTTTGTTTTT
```

Sample 15

```
Genomic_Sequence    TTTTTTGAATTTTTTGTAGGTTTGAATAATAGTTTTTTGGTTTTTTAATAAGTATAATTA
[2]2.txt            --TTTTGATTTTCTTGTAGGTTTGAATAATAGTTTTTTGGTTTTTTAATAAGTATAATTA

Genomic_Sequence    TATAGGTAAGAGTGGTTGTAGATATTATTTTTATGTTATTTAAATCGAAAGAAATAAAAA
[2]2.txt            TATAGGTAAGAGTGGTTGTAGATATTATTTTTATGTTATTTAAATTGAAAGAAATAAAAA

Genomic_Sequence    TTTATTTTATTTAATTTTATATTAATGTTTTTTTTTATTTTTTTTTTTTTTTATGGGATT
[2]2.txt            TTTATTTTATTTAATTTTATATTAATGTTTTTTTTTATTTTTTTTTTTTTTTATGGGATT

Genomic_Sequence    TTTAAGTGTTTTTTTTGGATGTTGAATGTTTATTTCGTAAATGAAAAAGTTAGTTAATGA
[2]2.txt            CTTAAGTGCTTTTTTTGGATGTTAAATGTTCATTTCGAAAATAAAAAAGTTAGTTAATGA

Genomic_Sequence    TATTGTATATAAGTAATGTTTTAATTGTAGATTGTGTGTGTGCGTTTTTGTTTTT--
[2]2.txt            TATTGTATATAAGAAATGTTTTAATTGTAAATTGGGTGTGCCCGTTTTTGTTTTTAA
```

Sample 16

```
Genomic_Sequence    -------TTTTTTGAATT--TTTTGTAGGTTTGAATAATAGTTTTTTG-GTTTTTTAATA
[2]3.txt            GTTGTTGTTTTTTT-AATTGGGTTTGTTTGTTTG--T--T-TTTTTTTGTGTTTTTT-TTT

Genomic_Sequence    AGTATAATTATATAGGTAAGAG-TGGTTGTAGATATTATTTTTATGTTATTTAAATCGAA
[2]3.txt            TGT-TTTTTTTTTAGG-AGGAGAT-TTTTTAGAT-TTTTTTTTAT-TTATTTAAATCGAA

Genomic_Sequence    AGAAATAAAAATTTATTTTATTTAATTTTATATTAATGTTTTTTTTTATTTTTTTTTTT
[2]3.txt            AG-AATAAAAATTTACCTC-TTTAACCTTATATTAAC-TTTTTTTTTATTTTTTTTTTT

Genomic_Sequence    TTTATGGGATTTTTAAGTGTTTTTTTTGGATGTTGAA-TGTTTATTTCGTAAATGAAAAA
[2]3.txt            TTTAGGGGATTTTTAAGGGTTTTTTTGGGAGGTTGAAGT-TTTTTTTGTAAATGAAAAA

Genomic_Sequence    GTTAGTTAATGATATTGTATATAAGTAATGTTTTAATTGTAGATTGTGTGTGTGCGTTTT
[2]3.txt            GTTATTTAATGATTTTGTATATAAATAATGTTTTAATTGTAAATTGTGTGTGTGCGTTTT

Genomic_Sequence    TGTTTTT---
[2]3.txt            TTTTTTTAAA
```

Sample 17

```
Genomic_Sequence    -------T-TTTTTGAATT-TTTTGTAGGTTTGAATAATAGTTTTTTGGTTTTTTAAT-A
[2]4.seq            AGGGTTGTAGTTACGGGTTGTATT-T-GGTTT---TTTTTTTTTTTTGTTTTTT--TGA

Genomic_Sequence    AGTATAATTATATAGGTAAGAGT-GGT-TGTAGATATTATTTTTATGTTATTTAAA-TCG
[2]4.seq            AGTATAACTATATAGG--AGTCCGGGCATGTAGATATCATTTTTATGTTATTTAAACTCG

Genomic_Sequence    AAAGAAATAAAAATTTATTTTATTTAATTTTATATTAATGTTTTTTTTTATTTTTTTTT
[2]4.seq            AAAGAAATAAAAATTTATTTTATTTAATTTTATATTAATGTTTTTTTTTATTTTTTTTT

Genomic_Sequence    TTTTTATGGGATTTTTAAGTGTTTTTTTTGGATGTTGAATGTTTATTTCGTAAATG-AAA
[2]4.seq            TTTTTATGGGATTTTTAAGTGTTTTTTTTGGATGTTGAATGTTTTTTTTG-AAAAGAAAA

Genomic_Sequence    AAGTTAGTTAATGATATTGTATATAAGTAATGTTTTAATTGTAGATTGTGTGTGTGCGTT
[2]4.seq            AAGTTATTTAAGGATTTTGTATATAAGAAATGTTTTAATTGTAAATTGGGGGGGGGCG--

Genomic_Sequence    TTTGTTTTT
[2]4.seq            -TT-TTTTT
```

Sample 18

```
Genomic_Sequence    T-TTTTTGAA--TTTTTTGTAGGTTTGAATAATAGTTTTTTGGTTTTTTAATAAGTATAA
[2]5.seq            TGTTTTTGAATTTTTTTTGTAGGTTTG-ATAATAGTTTTTTGGTTTTTTAATAAGTATAA

Genomic_Sequence    TTATATAGGTAAGAGTGG-TTGTAGATATTATTTTTATGTTATTTAA-ATCGAAAGAAAT
[2]5.seq            TTATATAGG-AGGAG-GGTTTGTAGATATTATTTTTATGTTATTTAATTTTGAAAGAAAT

Genomic_Sequence    AAAAAATTTATTTTATTTAATTTTATATTAATGTTTTTTTTTATTTTTTTTTTTTTTATG
[2]5.seq            AAAAATTTATTTTATTTAATTTTATATTAATGTTTTTTTTTATTTTTTTTTTTTTTAGG

Genomic_Sequence    GGATTTTTAAGTGTTTTTTTTGGATGTTGAATGTTTATTTCGTAAATGAAAAAGTTAGTT
[2]5.seq            GGATTTTAAAGGGTTTTTTTTGGAGGTTAAATGTTTTTTTTGAAAAAAAAAAATTTATTT

Genomic_Sequence    AATGATATTGTATATAAGTAATGTTTTAATTGTAGATTGTGTGTGTGCGTTTTTGTTTTT
[2]5.seq            AAGGATTTTGTATAAAAAAAAATTTTTTATTTGAAAATGGGGGGGGGGCGTTTTTTTTTT

Genomic_Sequence    ---
[2]5.seq            AAA
```

Sample 19

```
Genomic_Sequence    -----TTT-TTTGA-ATTTTTTGTAG-GTTTGAATAATAGTTTTTTGGTTTTTTAATAAG
[2]6.seq            TCTAATTTAATAGATAGTGTGTGT-GCGTTT---T--TAGTTTTTAGGTCTGTTAA-GAG

Genomic_Sequence    TATAATTATATAGGT-A-AGAG-TGGTTGTAGATATTATTTTTATGTTATTTA-AATCGA
[2]6.seq            -AGGA--AGAGGGGTGAGACAGTTTCCCCCCGATATTGTTTTTATGTTATTCATAAG-GA

Genomic_Sequence    AAGAAATAAAAATTTATTTTATTTAATTTTATATTAATGTTTTTTTTTATTTTTTTTTT
[2]6.seq            AAGAAATAAAAATTTATTTTATTTAATTTTATATTAATGTTTTTTTTTA-TTTTTTTTT

Genomic_Sequence    TTTTATGGGATTTTTAAGTGTTTTTTTTGG-ATGTTGAATGTTTATTTCGTAAATGAAAA
[2]6.seq            TTTTATGGGA-TTTTAAATG-TTTTCCTGGAAT-TT-AATG--CAATTTG-AAA-GGAAA

Genomic_Sequence    AGTTAGTTAATGATATTGTATATAAGTAATGTTTTAATTGTAGATTGTGTGTGTGCGTTT
[2]6.seq            AG-T-G-TATTG-TTTTGC-TATAA-T-A-G--TT-A--GT-G--TG-G-GGGTTTGTAA

Genomic_Sequence    TTGTTTTT----------
[2]6.seq            AAAAAAACCCCACAAGAAAGGTAACCCCCCCGG
```

Sample 20

```
Genomic_Sequence    ----TTTTTTGA-ATTTTTTGTAGG-TTTGAATAATAG-TTTTTT-GGTTTTTTAATAAG
[2]7.txt            TTTTTTTTTTGATATTTTTTTTCGGTTTTG-ATTTTCGTTTTTTTCGGTCCCCCCATAAG

Genomic_Sequence    TATAATTATATAG-GTAA---GAGTGGTTGTAGATATTATTTTTATGTTATTTAAATCGA
[2]7.txt            TAC-ACC-TATAGCG-AACCCGCCCCATCGTAGTTTTTATTTTTACGTTATTTAAACCGA

Genomic_Sequence    AAGAAATAAAAATTTATTTTATTTAATTTTATATTAATGTTTTTTTTTATTTTTTTTTT
[2]7.txt            AAGAAATAAAAATTTATTTTATTTAATTTTATATTAATGTTTTTTTTTATTTTTTTTTT

Genomic_Sequence    TTTTATGGGATTTTTAAGTGTTTTTTTTGGATGTTGAATGTTTATTTCGTAAATGAAAAA
[2]7.txt            TTTTAGGGGTTTTTTAAGGGTTTTTTTTGGATTTTGAATTTTTTTTTTGAAAAAAAAAAA

Genomic_Sequence    GTTAGTTAATGATATTGTATATAAGTAATGTTTTAATTGTAGATTGTGTGTGTGCGTTTT
[2]7.txt            TTTATTTAAGGATTTTGTTTAAAAAAAATGTTTTATTTGAAAATTG-GGGGGGGCGCTTTT

Genomic_Sequence    TGTTTTT----
[2]7.txt            TTTTTTTTAAA
```

Sample 21

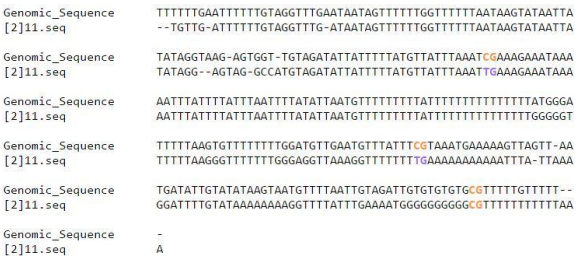
```
Genomic_Sequence    --TTTTTTGAATTTTTTGTA-GGTTTGAATAATAGTTTTTT-GGTTTTTTAATAAGTATA
[2]8.seq            TTTTTTTTGAATTTTTTTTACGGTTCGATTCTTTGTTTTTTCGGCCCCCC-ATAAGTAC-

Genomic_Sequence    ATTATATAGGTAAG-AGTGGTT-GTAGATATTATTTTTTATGTTATTTAAATCGAAAGAAA
[2]8.seq            ATTATAC-GG--AGCCGCCCTTCGTAGATATTATTTTTTTCGTTATTTAAACCGAAAGAAA

Genomic_Sequence    TAAAAATTTATTTTATTTAATTTTATATTAATGTTTTTTTTTATTTTTTTTTTTTTTTAT
[2]8.seq            TAAAAATTTATTTTATTTAATTTTATATTAATGTTTTTTTTTATTTTTTTTTTTTTTTAG

Genomic_Sequence    GGGATTTTTAAGTGTTTTTTTTGGATGTTGAATGTTTATTTCGTAAATGAAAAAGTTAGT
[2]8.seq            GGGTTTTTTAAGGGTTTTTTTTGGATGTTAAAGGTTTTTTTTGAAAAAAAAAATTTATT

Genomic_Sequence    TAATG-ATATTGTATATAAGTAA-TGTTTTAATTGTAGA-TTGTGTGTGTGCGTTTTTGT
[2]8.seq            TAA-GAATTTTTTTTATAAAAAAGT-TTTTATTTG-AAATTTG-GGGGGGGCGCTTTTTT

Genomic_Sequence    TTTT----
[2]8.seq            TTTTTAAA
```

Appendix

Sample 22

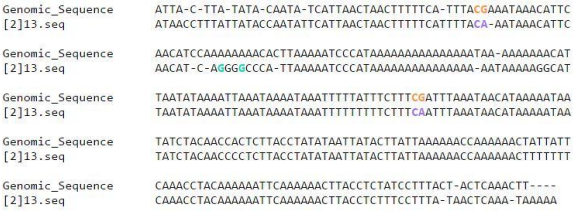
Sample 23

Sample 24

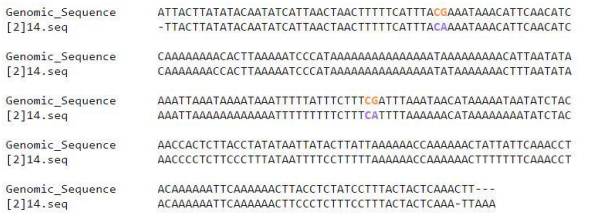


Sample 25

Sample 26



Sample 27



Converted methylated control forward

methylated control forward

Appendix F Needleman-Wunsch pairwise alignment of BRA1

Sample 28

```
Genomic_Sequence    -------TACGTATTTTTTTAAGGTTTAGTTTTTGTTTTTAAAATACGAAAATATAATAT
[2]1.seq            GGAGATGTTCG-AGGTTGATAAGGATT-GTAGTGGCTTGGGATTTAC-----TACTACAC

Genomic_Sequence    TTTAGTTTATAATTGTTGATAAGTATAAGCGCGTATAGGTTTTTAATTTATTTATTGGAT
[2]1.seq            CCC--CCCCTAA---TAGAAAAGTACAACCGCTCCCCGCTCCTCCCTACCCTCCGGAT

Genomic_Sequence    TTTCGTGAGAATTGTGTTCGTTTTGGTATTGGATGTTTTTTTTTATAAGATTA-TAGTTT
[2]1.seq            TTCCGCGAGAATTGTGCCCCCCCTGCTGTTGGATGTTCCTCCCCTTCAGACTACTA-CTT

Genomic_Sequence    TTAAGGAATATTGTGGCGAAGATTTTTTATTTCGTAACGTATGTTGGAAATAATTATTTT
[2]1.seq            CTACGGAACACCGCGGCGAAGACCTCCCATTCCTCCACGCCTGCTGGAAATAATTATTTC

Genomic_Sequence    TTTTTATTTTTTTAATAATTTTTATTATTTATATTTATCGAAATTGGAGATTTTTATTAG
[2]1.seq            CCTCCTCCCCCCCCCCAACCCTCCTTACTTCTATTTACCGCAACTGCTGACCTCCATCAG

Genomic_Sequence    GGCGGAAAGAGTGGGGGATTGGGATTT----
[2]1.seq            GGCGGAAAGAGGGGGGGATTGGGATTTATTA
```

Sample 29

```
Genomic_Sequence    ---TACGT-ATTTTTTTAAGGT-T-TAGTTTTTGT-TTTTAAAATACGAAAATA-TAATA
[2]2.txt            CTATAT-TAATACAGTAAATATCTATAATAATTGTGAATTAAAAAA-GTAGACACTATTA

Genomic_Sequence    TTTTAGTTTA-T-AATTGTTGA-TAAGT-ATAAGCGCGTATAGGTTTTTA-ATTTATTTA
[2]2.txt            TTACA-TTTATTAAATT-AAGACTAATTAATCATT-CG-AT---TCCATATATGGATTGA

Genomic_Sequence    TTGGATTTTCGTGAGA-AT-TGT-G-T-TCGTTTTGGTATTGGA-TGTT-TTTTTTTATA
[2]2.txt            TTAAATATATGTTTTACATCTATAGATATT-ATATAGTATC-AATTGTTATCTATACACA

Genomic_Sequence    A-G--ATT---ATAGT-TTTTA-AGGA--ATAT-TGTGGCGAAGATTTTTTATTTCGT-A
[2]2.txt            ATGTCGTTAGAATAGCATCATATCGGATCACATATATAAC--A-ATACTGTA-TTCGTTA

Genomic_Sequence    ACGTATGTTGGAAATAATTATTTTTTTTTATTTTTTTAATAAT-TTTTAT-TATTTATAT
[2]2.txt            GCACACG-T-GAAAC-ACTAGACTAGTCAATCTCGACAATAACGATTTATCCA-GCATAA

Genomic_Sequence    TTATCGAAATTG-GAGATTTTTATTAGGGCGGAAAGAGTGGGGGATTGGGATTT--
[2]2.txt            GCA-CGTCATCGTTAGCTTCTTA-GATGACG--TA-A-T---CCACTTAGAGTCAG
```

Sample 30

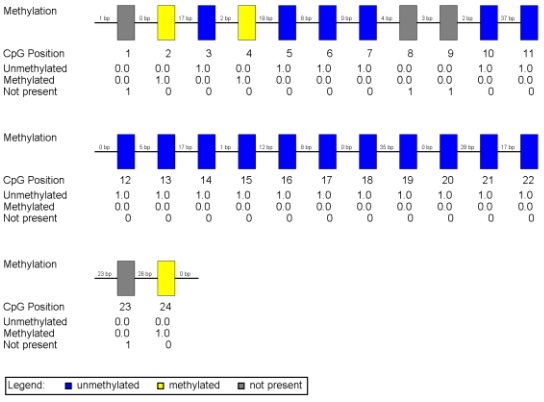
```
Genomic_Sequence    ----------TACGTATTTTTTTAAGGTTTAGTTTTTGTTTTTAAAATACGAAAATAT
[2]3.seq            TTAATTGAGTTGGGC-TATTTTTTT----TTT-TTTTTTGTTTTT--GGT-CG-GGAT-T

Genomic_Sequence    AATATTTTAGTTTATA-ATTGTTGATAAGTATAAGCGCGTATAGGTTTTTAATTTATTTA
[2]3.seq            --TCGTCTAATCAACATATTGATGA-AAGTACAAGCGCGCACAGGTCTCCAATCTATTTA

Genomic_Sequence    TTGGATTTTCGTGAGAATTGTGTTCGTTTTGGTATTGGATGTTTTTTTTTATAAGATTAT
[2]3.seq            CTGGATTTCCGTGAGAATTGTGCCCGCTCTGGTATTGGATGTTCCTCTCCATAAGACTAC

Genomic_Sequence    AGTTTTTAAGGAATATTGTGGCGAAGATTTTTTATTTCGTAACGTATGTTGGAAATAATT
[2]3.seq            AGTTTCTAAGGAACACTGTGGCGAAGACCTTTCATTCCGCAACGCATGCTGGAAATAATT

Genomic_Sequence    ATTTTTTTTTTATTTTTTTAATAATTTTTATTATTTATATTTATCGAAATTGGAGATTTTT
[2]3.seq            ATTTCCCTCCACCCCCCCAACAATCCTTATTACTTATATTTACCGAAACTGGAGACCTCC

Genomic_Sequence    ATTAGGGCGGAAAGAGTGGGGGATTGGGATTT
[2]3.seq            ATTAGGGCGGAAAGAGTGGGGGATTGGGATTT
```

Sample 31

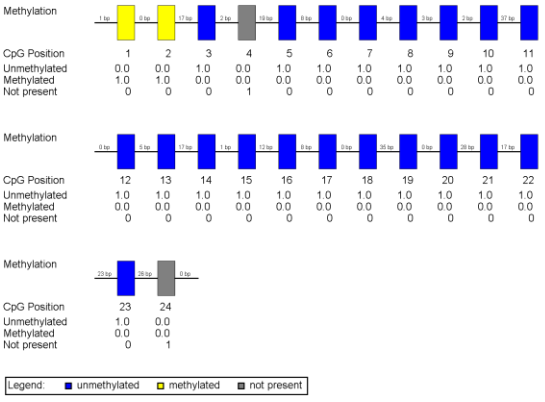
```
Genomic_Sequence    ------------------CCCTCCACCCCCCCCAACAATCC--TTATTACTTATATTTACC
[2]4.seq            GATCGGGAGGGGGCACGCCCCAAGACGCACCGAAC--CCCGGATGTTGGGGGGGTGGAGG

Genomic_Sequence    GAAACTAA-AAACCTCCATTAAAACGA-AAAAAATAAAAAA--TT-AAAAC-CTCTT-CT
[2]4.seq            GAAA-TAATTATTCT-CA-GCATGCGTTGCGGAATGAAAGGTCTTCGCCACAGTGTTCCT

Genomic_Sequence    TACGACTACTTTAAAC-AAT--A-AATAACGATTCTA-ACC--TTCGTACAACAATTACT
[2]4.seq            TA-GA-AACTGTAGTCTTATGGAGAGGAACA-TCCAATACCAGAGCGGGC-ACAATT-C-

Genomic_Sequence    ATAATACAATAAACCGCAACTAAA-AAAATAAAAA-CTAAAAAACAAACACTTTATAACA
[2]4.seq            -T--CACGG-AAATC-CAG-TGGATAGATTGGAGACCT---GTGC--GCGC-TTGT-ACT

Genomic_Sequence    AACTCAAATAAAATTCTTCCTCTTCCGTCTCT-TTCCT-TTTACGTCA-TCCGAAAACAA
[2]4.seq            TG-TC--A-ACAGTT-ATGGACTGGAGTGT-TGTT-ATGTTTTCGT-ATTTTGAAAGCAG

Genomic_Sequence    ACTAAATA-ACCAATCCAAAACCCCGAAAAACGCT-TAACTCTTT-CTATCCCTCCCA-T
[2]4.seq            A--AACTAGGCC--T--TAAA----AAGATACG-TACAACTCTTTAAAAAAACTACAATT

Genomic_Sequence    CCTCTAATT--
[2]4.seq            CC-C--ATCCA
```

Appendix G Aggregated Representation of Methylation Data of ESR2 gene

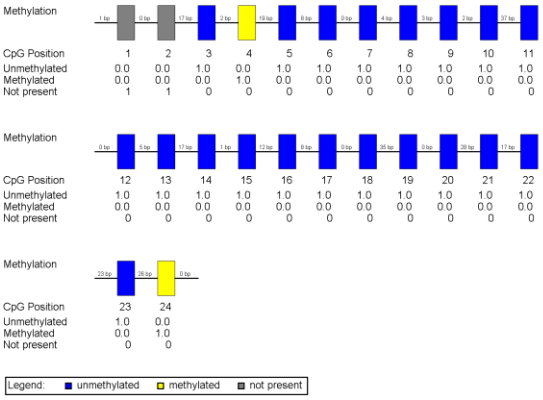
Sample 1



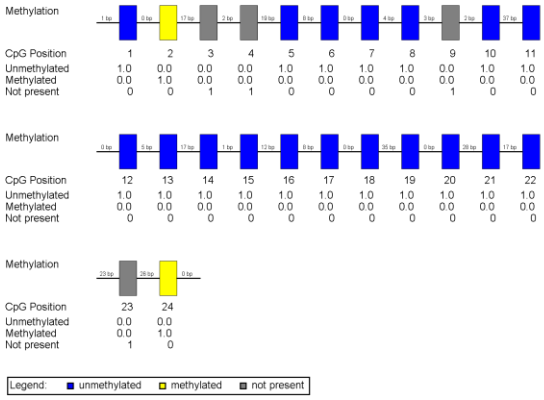
Sample 2



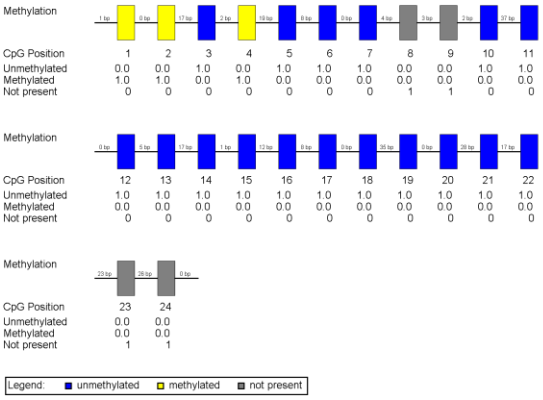
Sample 3



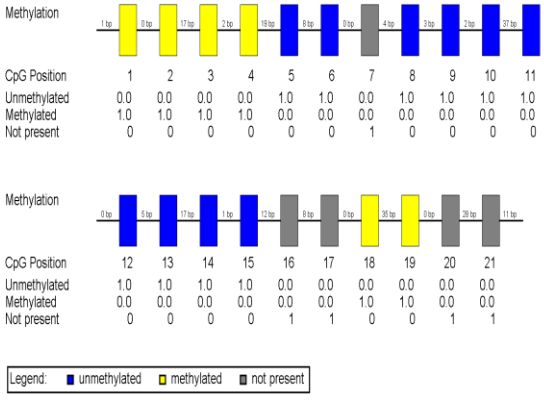
Sample 4



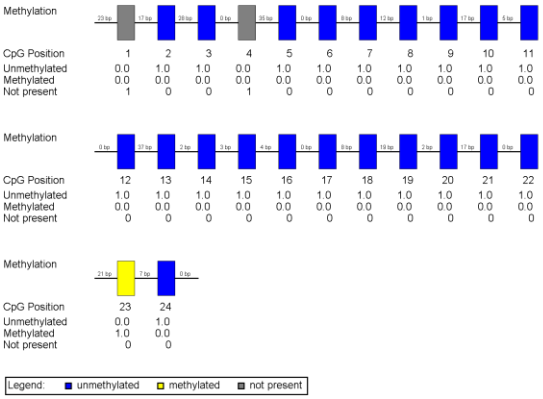
Sample 5



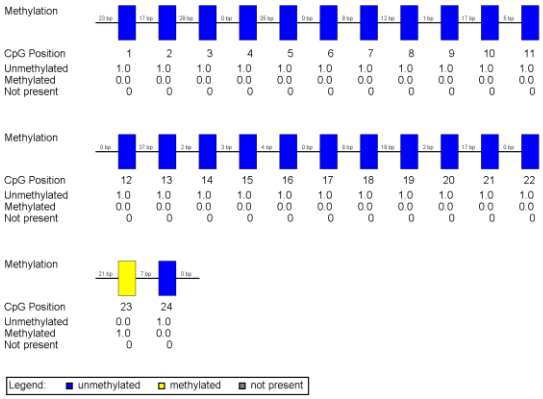
Sample 6



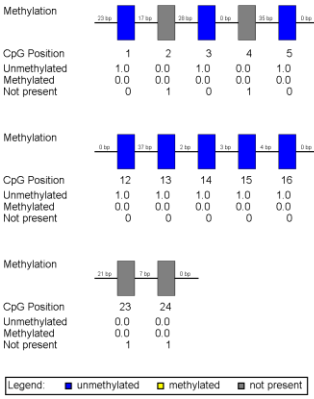
Sample 7



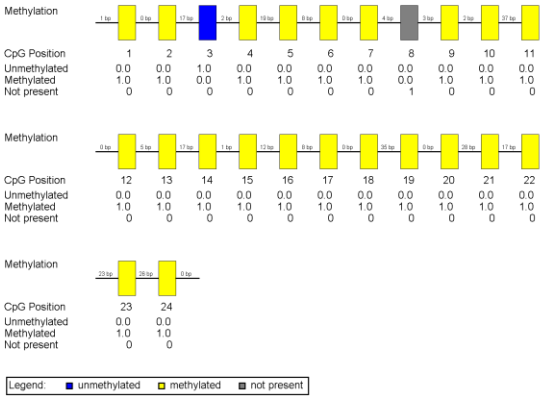
Sample 8



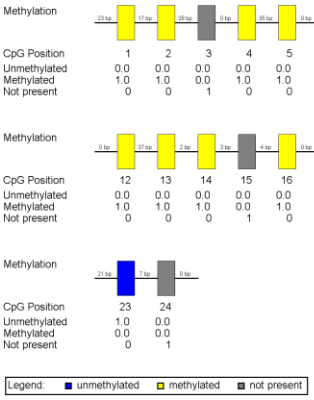
Sample 9



Converted methylated control forward strand

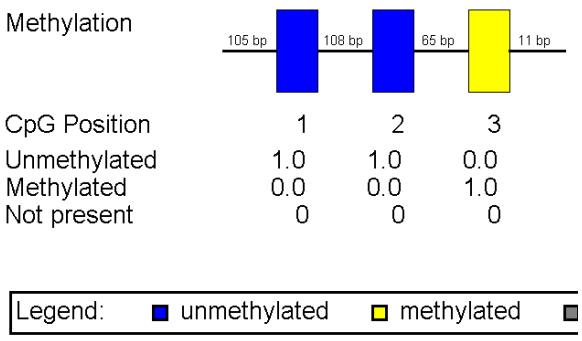


Converted methylated control reverse strand

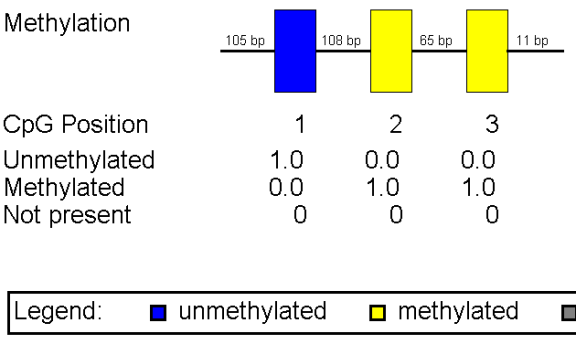


Appendix H Aggregated Representation of Methylation Data of OPN gene

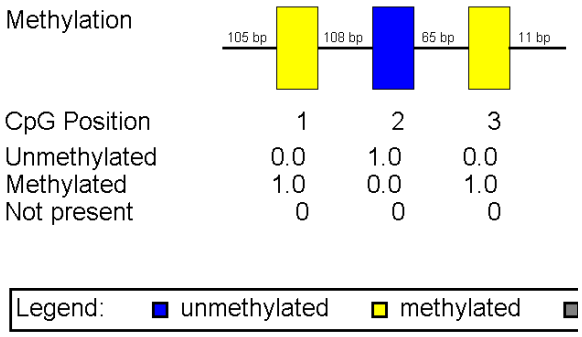
Sample 14



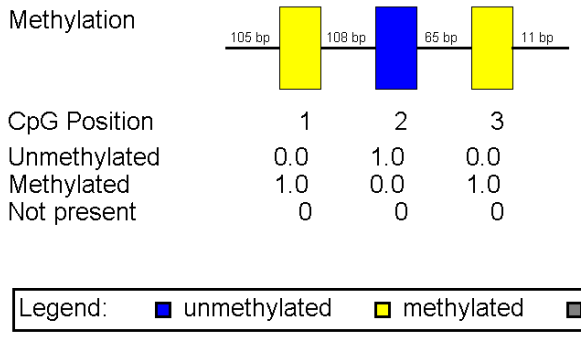
Sample 15



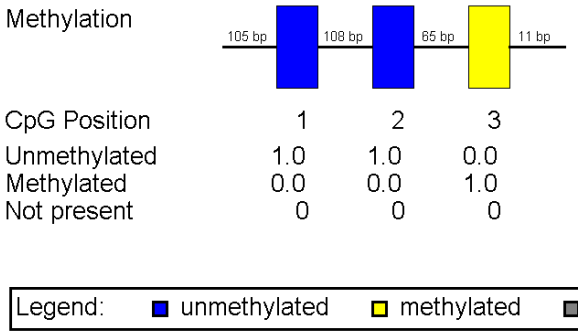
Sample 16



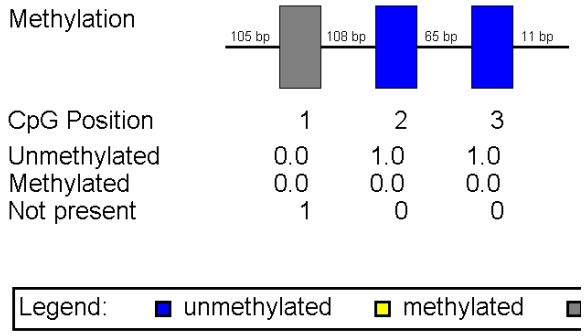
Sample 17



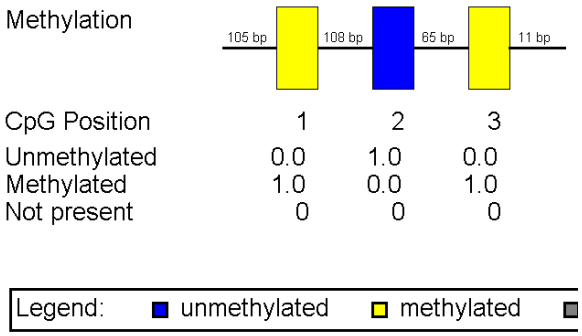
Sample 18



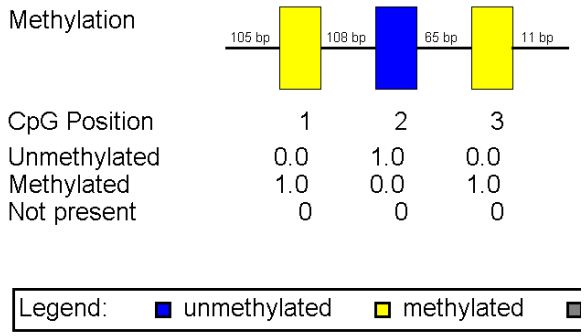
Sample 19



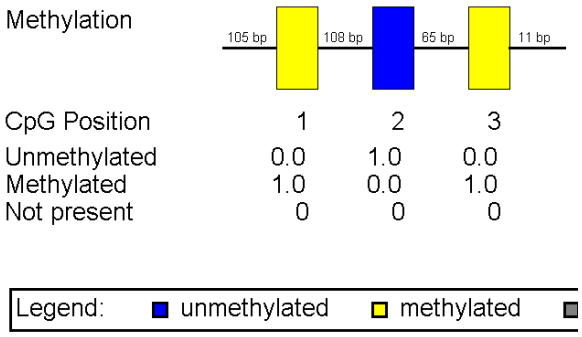
Sample 20



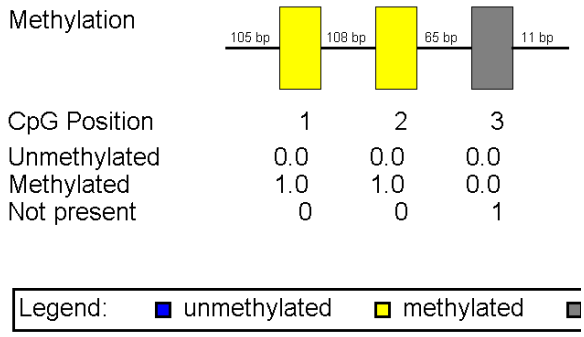
Sample 21



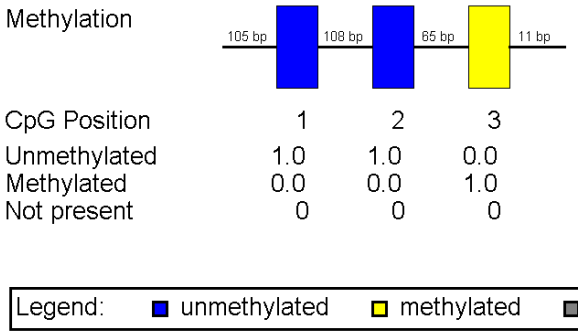
Sample 22



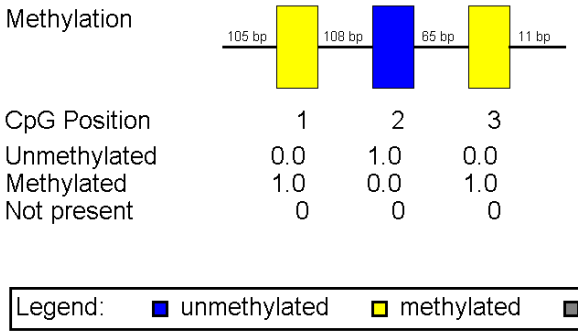
Sample 23



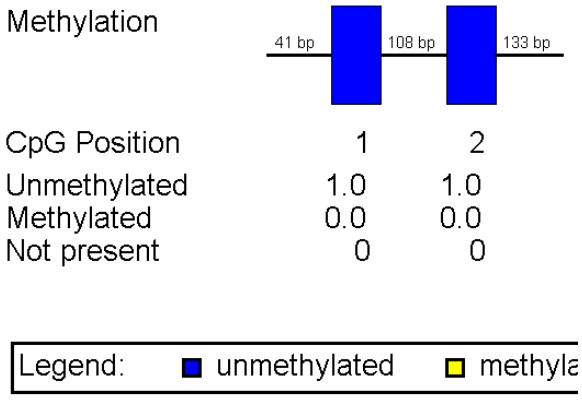
Sample1 24



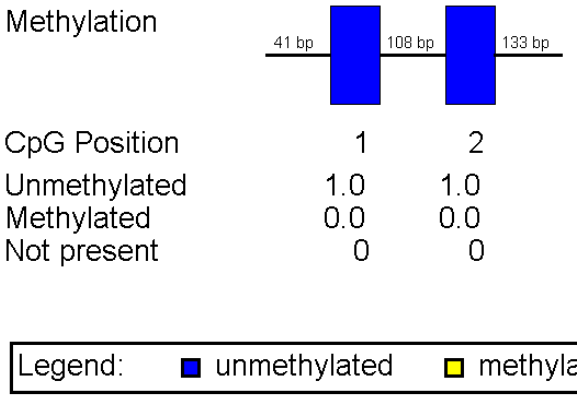
Sample 25



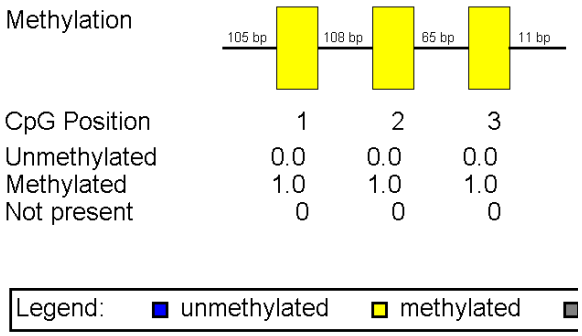
Sample 26



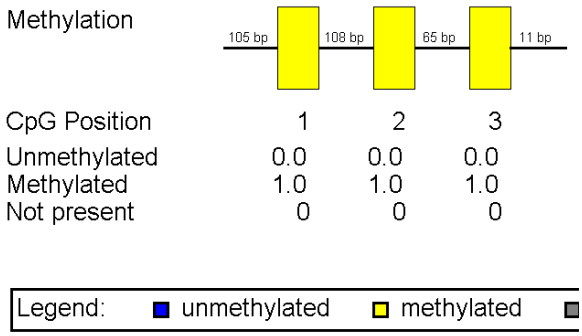
Sample 27



Converted control methylated forward

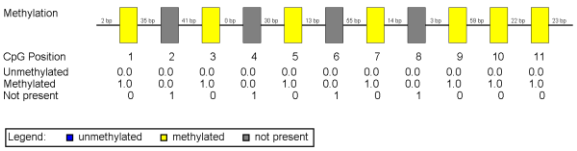


Control methylated

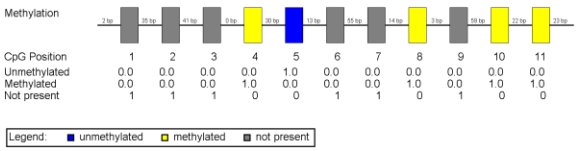


Appendix IAggregated Representation of Methylation Data of BRCA1 gene

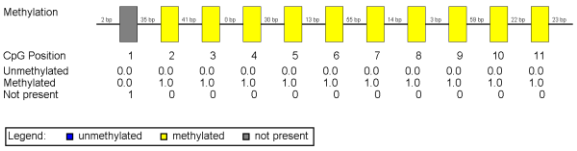
Sample 28



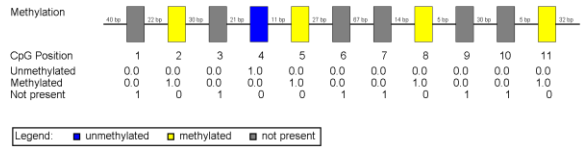
Sample 29



Sample 30



Sample 31



References

[1] Agrawal, A., Murphy, R. and Agrawal, D. (2007). DNA methylation in breast and colorectal cancers. *Mod Pathol*, 20(7), pp.711-721.

[2] American Cancer Society. Cancer Facts & Figures 2014. Atlanta: American Cancer Society; 2014.

[3] American Cancer Society. Global Cancer Facts & Figures 2nd Edition. Atlanta: American Cancer Society; 2011.

[4] B., K. (2013). Bisulfite Conversion and DNA Methylation Analysis. EpiBeat. Retrieved 4 July 2015, from http://www.epibeat.com/tools-technology/the-basics-of-bisulfite-conversion-for-dna-methylation-analysis/721/

[5] Balic, M., Schwarzenbacher, D., Stanzer, S., Heitzer, E., Auer, M., Geigl, J., Cote, R., Datar, R. and Dandachi, N. (2013). Genetic and epigenetic analysis of putative breast cancer stem cell models. *BMC Cancer*, 13(1), p.358.

[6] Barekati, Z., Radpour, R., Lu, Q., Bitzer, J., Zheng, H., Toniolo, P., Lenner, P. and Zhong, X. (2012). Methylation signature of lymph node metastases in breast cancer patients. *BMC Cancer*, 12(1), p.244.

[7] Baxter, E.(2011).Investigating the association between BRAFV600E and methylation in sporadic colon cancer. Ph.D. Thesis.The University of Edinburgh.

[8] Baylin, S. (2001). Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. *Human Molecular Genetics*, 10(7), 687-692.

[9] Bediaga, N., Acha, A., Guerra, I., Viguri, A., Albaina, M., Ruiz, I., Rezola, R., Alberdi, M., Dopazo, J., Montaner, D., Renobales, M., Fernandez, A., John, F., Fraga, M., Liloglou, T. and M. de Pancorbo, M. (2010). DNA methylation

epigenotypes in breast cancer molecular subtypes. *Breast Cancer Research*, 12(5), p.R77.

[10] Ben Gacem, R., Hachana, M., Ziadi, S., Ben Abdelkarim, S., Hidar, S., and Trimeche, M. (2012). Clinicopathologic significance of DNA methyltransferase 1, 3a, and 3b overexpression in Tunisian breast cancers. *Human Pathology*, 43(10), pp.1731-1738.

[11] Benton J, (2010). laminin-1 mediates epigenetic control of the epithelial cadherin for breast cancer cells in three-dimensional culture.Ph.D. thesis.Graduate School of Arts and Sciences of Georgetown University.Doctor of Philosophy in Biochemistry and Molecular Biology.

[12] Bestor TH (1992) Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain. EMBO Journal 11: 2611-2617.

[13] Bianco, T. 2000. 'Tumour-Specific Distribution Of BRCA1 Promoter Region Methylation Supports A Pathogenetic Role In Breast And Ovarian Cancer.' *Carcinogenesis* 21 (2): 147-151.

[14] Bird A (2002) DNA methylation patterns and epigenetic memory. *Genes and Development* 16(1):6-21.

[15] Bird, A. (1992). The essentials of DNA methylation. *Cell*, 70(1), 5-8.

[16] Birgisdottir V., Stefansson A., Bodvarsdottir K., Hilmarsdottir H., Jonasson G., and Eyfjord E.,(2006). Epigenetic silencing and deletion of the BRCA1 gene in sporadic breast cancer. *Breast Cancer Research*.Vol 8 No 4.

[17] Blay, P., Santamaría, I., Pitiot, A., Luque, M., Alvarado, M., Lastra, A., Fernández, Y., Paredes, Á., Freije, J. and Balbín, M. (2013). Mutational analysis of BRCA1 and BRCA2 in hereditary breast and ovarian cancer families from Asturias (Northern Spain). *BMC Cancer*, 13(1), p.243.

[18] Bock, C., S. Reither, T. Mikeska, M. Paulsen, J. Walter and T. Lengauer (2005). "BiQ Analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing." *Bioinformatics* 21(21): 4067-8

[19] Bonadonna, G., Hortobagyi, G. and Valagussa, P. (2006). Textbook of breast cancer. London: Taylor & Francis.

[20] Boyd, V., Moody, K., Karger, A., Livak, K., Zon, G., & Burns, J. (2006). Methylation-dependent fragment separation: Direct detection of DNA methylation by capillary electrophoresis of PCR products from bisulfite-converted genomic DNA. *Analytical Biochemistry*, 354(2), 266-273.

[21] Brooks, J., Cairns, P. and Zeleniuch-Jacquotte, A. (2009). Promoter methylation and the detection of breast cancer. *Cancer Causes Control*, 20(9), pp.1539-1550.

[22] Bryant, C., Rawlinson, R. and Massey, A. (2014). Chk1 Inhibition as a novel therapeutic strategy for treating triple-negative breast and ovarian cancers. *BMC Cancer*, 14(1), p.570.

[23] Butcher, D., Mancini-DiNardo, D., Archer, T. and Rodenhiser, D. (2004). DNA binding sites for putative methylation boundaries in the unmethylated region of theBRCA1 promoter. *International Journal of Cancer*, 111(5), pp.669-678.

[24] Cassidy, J. (2010). Oxford Handbook of oncology. Oxford: Oxford University Press.

[25] Catteau, A., Harris, W., Xu, C., & Solomon, E. (1999). Methylation of the BRCA1 promoter region in sporadic breast and ovarian cancer: correlation with disease characteristics. *Oncogene*, 18(11), 1957-1965.

[26] Chander, H., Brien, C., Truesdell, P., Watt, K., Meens, J., Schick, C., Germain, D. and Craig, A. (2014). Toca-1 is suppressed by p53 to limit breast cancer cell invasion and tumor metastasis. *Breast Cancer Research*, 16(6).

[27] Chen, A., Beetham, H., Black, M., Priya, R., Telford, B., Guest, J., Wiggins, G., Godwin, T., Yap, A. and Guilford, P. (2014). E-cadherin loss alters cytoskeletal organization and adhesion in non-malignant breast cells but is insufficient to induce an epithelial-mesenchymal transition. *BMC Cancer*, 14(1), p.552.

[28] Cho, H., Kim, S., Kim, K., Kang, W., Kim, J., Oh, S., Kim, J. and An, C. (2009). The combination effect of sodium butyrate and 5-Aza-2'-deoxycytidine on radiosensitivity in RKO colorectal cancer and MCF-7 breast cancer cell lines. *World Journal of Surgical Oncology*, 7(1), p.49.

[29] Clark, S., Harrison, J., Paul, C., and Frommer, M. (1994). High-Sensitivity Mapping of Methylated Cytosines. *Nucleic Acids Res.* 22, 2990-2997.

[30] Colella, S., Shen, L., Baggerly, K., Issa, J., and Krahe, R. (2003). Sensitive and quantitative universal Pyrosequencing (TM) methylation analysis of CpG sites. *BioTechniques* 35, 146-+.

[31] Counts, J., & Goodman, J. (1995). Hypomethylation of DNA: a nongenotoxic mechanism involved in tumor promotion. *Toxicology Letters*, 82-83, 663-672.

[32] Craig, A.M., Smith, J.H., Denhardt, D.T., 1989. Osteopontin, a transformation-associated cell adhesion phosphoprotein, is induced by 12-O-tetradecanoylphorbol 13-acetate in mouse epidermis. J. *Biol. Chem*. 264, 9682–9689.

[33] David, C. (2013). The International Agency for Research on Cancer (IARC).Lyon/Geneva: WHO, p.1.

[34] De la Hoya, M. (2003). Pre-test prediction models of BRCA1 or BRCA2 mutation in breast/ovarian families attending familial cancer clinics. *Journal of Medical Genetics*, 40(7), pp.503-510.

[35] Deb, S., Do, H., Byrne, D., Jene, N., Dobrovic, A. and Fox, S. (2013). PIK3CA mutations are frequently observed in BRCAX but not BRCA2 - associated male breast cancer. *Breast Cancer Research*, 15(4), p.R69.

[36] Dedeurwaerder, S., Desmedt, C., Calonne, E., Singhal, S., Haibe-Kains, B., Defrance, M., Michiels, S., Volkmar, M., Deplus, R., Luciani, J., Lallemand, F., Larsimont, D., Toussaint, J., Haussy, S., Rothé, F., Rouas, G., Metzger, O., Majjaj, S., Saini, K., Putmans, P., Hames, G., van Baren, N., Coulie, P., Piccart, M., Sotiriou, C. and Fuks, F. (2011). DNA methylation profiling reveals a predominant immune component in breast cancers. *EMBO Mol Med*, 3(12), pp.726-741.

[37] Delpu, Y., Cordelier, P., Cho, W. and Torrisani, J. (2013). *DNA Methylation and Cancer Diagnosis.* IJMS, 14(7), pp.15029-15058.

[38] Desjardins, P., & Conklin, D. (2010). NanoDrop Microvolume Quantitation of Nucleic Acids. *Journal of Visualized Experiments : JoVE*, (45), 2565. Doi:10.3791/2565.

[39] Dohm, J., Lottaz, C., Borodina, T., & Himmelbauer, H. (2008). Substantial biases in ultra-short read data sets from high-throughput DNA sequencing. *Nucleic Acids Research*, 36(16), e105-e105.

[40] Dorairaj, J., Salzman, D., Wall, D., Rounds, T., Preskill, C., Sullivan, C., Lindner, R., Curran, C., Lezon-Geyda, K., McVeigh, T., Harris, L., Newell, J.,

Kerin, M., Wood, M., Miller, N. and Weidhaas, J. (2014). A germline mutation in the BRCA1 3'UTR predicts Stage IV breast cancer. *BMC Cancer*, 14(1), p.421.

[41] Ellis M. J. and Perou C. M. (2013) The genomic landscape of breast cancer as a therapeutic roadmap. *Cancer Discov* 3: 27-34.

[42] Ellis M. J., Ding L., Shen D., Luo J., and Suman V. J., (2012) Whole-genome analysis informs breast cancer response to aromatase inhibition. *Nature* 486: 353-360.

[43] Elmore, S. (2007). Apoptosis: A Review of Programmed Cell Death. *Toxicologic Pathology*, 35(4), 495–516.

[44] Esteller, M. (2000). Promoter Hypermethylation and BRCA1 Inactivation in Sporadic Breast and Ovarian Tumors. *Journal of the National Cancer Institute*, 92(7), 564-569.

[45] Esteller, M. (2002). CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene*, 21(35), pp.5427-5440.

[46] Fabbri M, Garzon R, Cimmino A, Liu Z, Zanesi N, Callegari E, Liu S, Alder H, Costinean S, Fernandez-Cymering C, Volinia S, Guler G, Morrison CD, Chan KK, Marcucci G, Calin GA, Huebner K, Croce CM (2007) MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *PNAS* 104(40):15805-10.

[47] Fackler, M. (2004). Quantitative Multiplex Methylation-Specific PCR Assay for the Detection of Promoter Hypermethylation in Multiple Genes in Breast Cancer. *Cancer Research*, 64(13), pp.4442-4452.

[48] Fackler, M., Umbricht, C., Williams, D., Argani, P., Cruz, L., Merino, V., Teo, W., Zhang, Z., Huang, P., Visvananthan, K., Marks, J., Ethier, S., Gray, J.,

Wolff, A., Cope, L. and Sukumar, S. (2011). Genome-wide Methylation Analysis Identifies Genes Specific to Breast Cancer Hormone Receptor Status and Risk of Recurrence. *Cancer Research*, 71(19), pp.6195-6207.

[49] Falahi, F., van Kruchten, M., Martinet, N., Hospers, G. and Rots, M. (2014). Current and upcoming approaches to exploit the reversibility of epigenetic mutations in breast cancer. *Breast Cancer Research*, 16(4).

[50] Feinberg, A. (2001). Cancer epigenetics takes center stage. Proceedings of the National Academy of Sciences, 98(2), 392-394.

[51] Feng, W., Orlandi, R., Zhao, N., Carcangiu, M., Tagliabue, E., Xu, J., Bast, R. and Yu, Y. (2010). Tumor suppressor genes are frequently methylated in lymph node metastases of breast cancers. *BMC Cancer*, 10(1), p.378.

[52] Feng, W., Shen, L., Wen, S., Rosen, D., Jelinek, J., Hu, X., Huan, S., Huang, M., Liu, J., Sahin, A., Hunt, K., Bast, R., Shen, Y., Issa, J. and Yu, Y. (2007). Correlation between CpG methylation profiles and hormone receptor status in breast cancers. *Breast Cancer Research*, 9(4), p.R57.

[53] Filkowski j., (2010).the role of epigenetic changes in chemoresistant breast cancer cells.msc Thesis.Department of Biological Sciences the University of Lethbridge.

[54] Ford, D., Easton, D., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D., Weber, B., Lenoir, G., Chang-Claude, J., Sobol, H., Teare, M., Struewing, J., Arason, A., Scherneck, S., Peto, J., Rebbeck, T., Tonin, P., Neuhausen, S., Barkardottir, R., Eyfjord, J., Lynch, H., Ponder, B., Gayther, S., Birch, J., Lindblom, A., Stoppa-Lyonnet, D., Bignon, Y., Borg, A., Hamann, U., Haites, N., Scott, R., Maugard, C., Vasen, H., Seitz, S., Cannon-Albright, L., Schofield, A. and Zelada-Hedman, M. (1998). Genetic Heterogeneity and

Penetrance Analysis of the BRCA1 and BRCA2 Genes in Breast Cancer Families. *The American Journal of Human Genetics*, 62(3), pp.676-689.

[55] Franklin, Tamara B., and Isabelle M. Mansuy. "Epigenetic Inheritance in Mammals: Evidence For The Impact Of Adverse Environmental Effects". *Neurobiology of Disease* 39.1 (2010): 61-65. Web.

[56] Franzen, A., Heinegard, D., 1985. Isolation and characterization of two sialoproteins present only in bone calcified matrix. *Biochem. J.* 232, 715–724.

[57] Frommer, M., McDonald, L., Millar, D., Collis, C., Watt, F., & Grigg, G. et al. (1992). A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proceedings of the National Academy of Sciences, 89(5), 1827-1831. doi:10.1073/pnas.89.5.1827

[58] Gardiner-Garden M, Frommer M (1987) CpG islands in vertebrate genomes. *Journal of Molecular Biology* 196(2):261-282.

[59] Gawish H., Hagras A., and Abdel Bary H (2012). RASSF1A Gene Hypermethylation in Tissue and Serum Together with Tissue Protein Expression in Breast Cancer Patients. *Life Science Journal*. 9(3).

[60] Genereux, D., Johnson, W., Burden, A., Stoger, R., & Laird, C. (2008). Errors in the bisulfite conversion of DNA: modulating inappropriate- and failed-conversion frequencies. *Nucleic Acids Research*, 36(22), e150-e150.

[61] Gheibi A, Kazemi M, Baradaran A, Akbari M, Salehi M.(2012). Study of promoter methylation pattern of 14-3-3 sigma gene in normal and cancerous tissue of breast: A potential biomarker for detection of breast cancer in patients. *Adv Biomed Res* 2012; 1:80.

[62] Gibbon, S. (2007). Breast cancer genes and the gendering of knowledge. Houndmills, Basingstoke, Hampshire: Palgrave MacMillan.

[63] Giles RH, Dauwerse JG, Higgins C, Petrij F, Wessels JW, Beverstock GC, Döhner H, Jotterand-Bellomo M, Falkenburg JH, Slater RM, van Ommen GJ, Hagemeijer A, van der Reijden BA, Breuning MH (1997) Detection of CBP rearrangements in acute myelogenous leukemia with t(8;16). *Leukaemia* 11(12):2087-2096.

[64] Giordano, A. and Normanno, N. (2009). Breast cancer in the post-genomic era. Totowa, N.J.: Humana.

[65] Gkazepis A. (2011).Tissue kallikrein-related peptidases as novel biomarkers in cancer: Cellular analysis of expression, epigenetic regulation and relation to the course of the disease.Ph.D. thesis.Universität München.

[66] Goll MG, Kirpekar F, Maggert KA, Yoder JA, Hsieh CL, Zhang X, Golic KG, Jacobsen SE, Bestor TH (2006) Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. *Science* 311(5759):395-398.

[67] Goto T., and Monk M.(1998).Regulation of X-chromosome inactivation in development in mice and humans.*Microbiol Mol Biol* Rev. 1998 Jun;62(2):362-78.

[68] Greenman, C., Stephens, P., Smith, R., Dalgliesh, G. L., Hunter, C., Bignell, G., Stratton, M. R. (2007). Patterns of somatic mutation in human cancer genomes. *Nature*, 446(7132), 153–158. Doi:10.1038/nature05610.

[69] Grunau, C. (2001). Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. *Nucleic Acids Research*, 29(13), 65e-65.

[70] Haanpää, M., Pylkäs, K., Moilanen, J. and Winqvist, R. (2013). Evaluation of the need for routine clinical testing of PALB2 c.1592delT mutation in BRCA negative Northern Finnish breast cancer families. *BMC Med Genet*, 14(1), p.82.

[71] Hanahan D and Weinberg RA (2000) The Hallmarks of Cancer Cell 100:57-70.

[72] Hayashi A, Horiuchi A, Kikuchi N, Hayashi T, Fuseya C, Suzuki A, Konishi I, Shiozawa T (2010) Type-specific roles of histone deacetylase (HDAC) overexpression in ovarian carcinoma: HDAC1 enhances cell proliferation and HDAC3 stimulates cell migration with downregulation of E-cadherin. *International Journal of Cancer* 127(6):1332-1346.

[73] 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. Series B, Physical and Biological Sciences*, 84(8), 321–330. http://doi.org/10.2183/pjab/84.321.

[74] Hayatsu, H., Wataya, Y., & Kai, K. (1970). Addition of sodium bisulfite to uracil and to cytosine. J. Am. *Chem. Soc.*, 92(3), 724-726. doi:10.1021/ja00706a062

[75] Hervouet, E., Cartron, P., Jouvenot, M., & Delage-Mourroux, R. (2013). Epigenetic regulation of estrogen signaling in breast cancer. *Epigenetics*, 8(3), 237-245.

[76] Hill k., identification of dna methylation changes in sporadic breast and other cancers.(2011). ph.d. thesis.University of Birmingham.

[77] Hill, V., Hesson, L., Dansranjavin, T., Dallol, A., Bieche, I., Vacher, S., Tommasi, S., Dobbins, T., Gentle, D., Euhus, D., Lewis, C., Dammann, R., Ward, R., Minna, J., Maher, E., Pfeifer, G. and Latif, F. (2010). Identification of 5 novel genes methylated in breast and other epithelial cancers. *Molecular Cancer*, 9(1), p.51.

[78] Hinshelwood, R.A., Clark, S.J., 2008. Breast cancer epigenetics: normal human mammary epithelial cells as a model system. *J.Mol. Med*. 86, 1315e1328.

[79] Holm, K., Hegardt, C., Staaf, J., Vallon-Christersson, J., Jönsson, G., Olsson, H., Borg, Å. and Ringnér, M. (2010). Molecular subtypes of breast cancer are associated with characteristic DNA methylation patterns. Breast *Cancer Research*, 12(3), p.R36.

[80] Hon, G., Hawkins, R., Caballero, O., Lo, C., Lister, R., Pelizzola, M., Valsesia, A., Ye, Z., Kuan, S., Edsall, L., Camargo, A., Stevenson, B., Ecker, J., Bafna, V., Strausberg, R., Simpson, A. and Ren, B. (2011). Global DNA hypomethylation coupled to repressive chromatin domain formation and gene silencing in breast cancer. *Genome Research*, 22(2), pp.246-258.

[81] Honrado, E. (2005). Immunohistochemical Expression of DNA Repair Proteins in Familial Breast Cancer Differentiate BRCA2-Associated Tumors. *Journal of Clinical Oncology*, 23(30), pp.7503-7511.

[82] Hsieh A. C., Liu Y., Edlind M. P., Ingolia N. T., and Janes M. R., (2012) The translational landscape of mTORsignalling steers cancer initiation and metastasis. *Nature* 485: 55-61

[83] Huang, T. (1999). Methylation profiling of CpG islands in human breast cancer cells. *Human Molecular Genetics*, 8(3), pp.459-470.

[84] Ida K, Kitabayashi I, Taki T, Taniwaki M, Noro K, Yamamoto M, Ohki M, Hayashi Y (1997) Adenoviral E1A-associated protein p300 is involved in acute myeloid leukemia with t(11;22)(q23;q13). Blood 90(12):4699-4704.

[85] Imyanitov, E. and Byrski, T. (2013). Systemic treatment for hereditary cancers: a 2012 update. *Hered Cancer Clin Pract*, 11(1), p.2.

[86] Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, Cui H, Gabo K, Rongione M, Webster M, Ji H, Potash JB, Abunciyan S, Feinberg AP (2009) The human colon cancer methylation shows similar hypo- and hypermethylation at conserved tissuespecific CpG island shores. *Nature Genetics* 41(2):178-186.

[87] Izadi, P., Noruzinia, M., Fereidooni, F. and Nateghi, M. (2012). *Asian Pacific Journal of Cancer Prevention*, 13(8), pp.4113-4117.

[88] Janiszewska, H., Haus, O., Lauda-Świeciak, A., Pasińska, M., Laskowski, R., Szymański, W., Górski, B. and Lubiński, J. (2003). Frequency of three BRCA1 gene founder mutations in breast/ovarian cancer families from the Pomerania-Kujawy region of Poland. *Clinical Genetics*, 64(6), pp.502-508.

[89] Jin KL, Pak JH, Park JY, Choi WH, Lee JY, Kim JH, Nam JH (2008) Expression profile of histone deacetylases 1, 2 and 3 in ovarian cancer tissues. *Journal of Gynecological* Oncology 19(3):185-190.

[90] Johnson, J. (1999). molecular mechanism of nucleotide excision repair deficiency in novel breast cancer cell lines. ph.d. thesis. University of Pittsburgh School of Medicine.

[91] Kaplan W., (2013).Priority Medicines for Europe and the World "A Public Health Approach to Innovation".Update on 2004 Background Paper.

[92] Kim C., Thorat A., Lee R., Cho H., Vasiljevi´c N., Scibior-Bentkowska D., Wu K., Ahmad S., Duffy S., Cuzick M., and Lorincz T.,(2012). Quantitative DNA methylation and recurrence of breast cancer: A study of 30 candidate genes. *Cancer Biomarkers11*. 75–88.

[93] Klajic, J., Fleischer, T., Dejeux, E., Edvardsen, H., Warnberg, F., Bukholm, I., Lønning, P., Solvang, H., Børresen-Dale, A., Tost, J. and Kristensen, V. (2013). Quantitative DNA methylation analyzes reveal stage dependent DNA methylation and association to clinicopathological factors in breast tumors. *BMC Cancer*, 13(1), p.456.

[94] Kloten, V., Becker, B., Winner, K., Schrauder, M., Fasching, P., Anzeneder, T., Veeck, J., Hartmann, A., Knüchel, R. and 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(1), p.R4.

[95] Ko, E., Park, S., Cho, E., Kim, Y., Hwang, J., Lee, Y., Nam, S., Bang, S., Park, J. and Kim, D. (2010). Cystatin M loss is associated with the losses of estrogen receptor, progesterone receptor, and HER4 in invasive breast cancer. *Breast Cancer Research*, 12(6), p.R100.

[96] Kornegoor, R., Moelans, C., Verschuur-Maes, A., Hogenes, M., de Bruin, P., Oudejans, J. and van Diest, P. (2012). Promoter hypermethylation in male breast cancer: analysis by multiplex ligation-dependent probe amplification. *Breast Cancer Research*, 14(4), p.R101.

[97] Koshiishi N, Chong JM, Fukasawa T, Ikeno R, Hayashi Y, Funata N, Nagai H, Miyaki M, Matsumoto Y, Fukayama M (2004) p300 gene alterations in intestinal and diffuse types of gastric carcinoma. Gastric Cancer 7(2):85-90.

[98] Krans, B. (2012). Benign Tumors. [online] Healthline. Available at: http://www.healthline.com/health/benign#Overview1 [Accessed 13 Mar. 2015].

[99] Kuiper, G. G. and Gustafsson, J. A. (1997) the novel estrogen receptor-β subtypes: potential role in the cell- and promoter-specific actions of estrogens and anti-estrogens FEBS Lett 410, 87-90.

[100] Kurzbach, D., Platzer, G., Schwarz, T.C., Henen, M.A., Konrat, R., Hinderberger, D., 2013. Cooperative unfolding of compact conformations of the intrinsically disordered protein osteopontin. *Biochemistry*.

[101] Lacroix, J. (1994). DNA fragment size determination on agarose gel by using the application GEL. *Bioinformatics*, 10(2), 185-187.

[102] Laidlaw, I. (1995). The proliferation of normal human breast tissue implanted into athymic nude mice is stimulated by estrogen but not progesterone. *Endocrinology*, 136(1), 164-171.

[103] Laird, C., Pleasant, N., Clark, A., Sneeden, J., Hassan, K., & Manley, N. et al. (2003). Hairpin-bisulfite PCR: Assessing epigenetic methylation patterns on complementary strands of individual DNA molecules. *Proceedings Of The National Academy Of Sciences*, 101(1), 204-209.

[104] Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, Harris K, Heaford A, Howland J, Kann L, Lehoczky J, LeVine R, McEwan P, McKernan K, Meldrim J, Mesirov JP, Miranda C.International Human Genome Sequencing Consortium (2001) Initial sequencing and analysis of the human genome. *Nature* 15(409):860-921.

[105] Larsen F, Gundersen G, Lopez R, Prydz H (1992) CpG islands as gene markers in the human genome. *Genomics* 13:1095–1107.

[106] Larsen, M., Thomassen, M., Tan, Q., Lænkholm, A., Bak, M., Sørensen, K., Andersen, M., Kruse, T. and Gerdes, A. (2014). RNA profiling reveals familial

aggregation of molecular subtypes in non-BRCA1/2 breast cancer families. *BMC Medical Genomics*, 7(1), p.9.

[107] Lehmann, U., Hasemeier, B., Lilischkis, R. and Kreipe, H. (2001). Quantitative Analysis of Promoter Hypermethylation in Laser-Microdissected Archival Specimens. *Lab Invest*, 81(4), pp.635-638.

[108] Lewis M., Cler R., Bu W., Mu¨ ller Z., Milchgrub S., Naftalis Z., Leitch M., Minna D., Euhus M., and Minna D.,(2005). Promoter Hypermethylation in Benign Breast Epithelium in Relation to Predicted Breast Cancer Risk. *Clin Cancer Res*. 11:166-172.

[109] Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, Kandoth C, Payton JE, Baty J, Welch J, Harris CC, Lichti CF, Townsend RR, Fulton RS, Dooling DJ, Koboldt DC, Schmidt H, Zhang Q, Osborne JR, Lin L, O'Laughlin M, McMichael JF, DelehauntyKD, McGrath SD, Fulton LA, Magrini VJ, Vickery TL, Hundal J, Cook LL, Conyers JJ,

[110] Li, N., Ye, M., Li, Y., Yan, Z., Butcher, L.M., Sun, J., Han, X., Chen, Q., Zhang, X., and Wang, J. (2010). Whole genome DNA methylation analysis based on high throughput sequencing technology. Methods 52, 203-212.

[111] Lin, I., Chen, D., Chang, Y., Lee, Y., Su, C., & Cheng, C. et al. (2015). Hierarchical Clustering of Breast Cancer Methylomes Revealed Differentially Methylated and Expressed Breast Cancer Genes. PLoS ONE, 10(2).

[112] Lister, R., & Ecker, J. (2009). Finding the fifth base: Genome-wide sequencing of cytosine methylation. *Genome Research*, 19(6), 959-966.

[113] Loss, L., Sadanandam, A., Durinck, S., Nautiyal, S., Flaucher, D., Carlton, V., Moorhead, M., Lu, Y., Gray, J., Faham, M., Spellman, P. and Parvin, B.

(2010). Prediction of epigenetically regulated genes in breast cancer cell lines. *BMC Bioinformatics*, 11(1), p.305.

[114] Magdinier, F., Ribieras, S., Lenoir, G., Frappart, L., & Dante, R. (1998). Down-regulation of BRCA1 in human sporadic breast cancer; analysis of DNA methylation patterns of the putative promoter region. *Oncogene*, 17(24), 3169-3176.

[115] Magistri, Marco (2011). Promoter-associated noncoding RNAs constitute an epigenetic switch controlling gene transcription in human cells. Thèse de doctorat: Univ. Genève, 2011, no. Sc. 4293.

[116] Majid, R., Mohammed, H., Saeed, H., Safar, B., Rashid, R., & Hughson, M. (2009). Breast cancer in Kurdish women of northern Iraq: incidence, clinical stage, and case-control analysis of parity and family risk. *BMC Women's Health*, 9(1), 33.

[117] Mancini, D., Rodenhiser, D., Ainsworth, P., O'Malley, F., Singh, S., Xing, W., & Archer, T. (1998). CpG methylation within the 5′ regulatory region of the BRCA1 gene is tumor specific and includes a putative CREB binding site. *Oncogene*, 16(9), 1161-1169.

[118] Masson, A., Talseth-Palmer, B., Evans, T., Grice, D., Hannan, G. and Scott, R. (2014). Expanding the genetic basis of copy number variation in familial breast cancer. *Hered Cancer Clin Pract*, 12(1), p.15.

[119] MDEldridge, L. (2015). What's the Difference Between Benign and Malignant Tumors?. [online] About.com Health. Available at http://lungcancer.about.com/od/*Biology-of-Cancer*/a/Benign-Vs-Malignant.htm [Accessed 13 Mar. 2015].

[120] Melnikov, A. A., Gartenhaus, R. B., Levenson, A. S., Motchoulskaia, N. A., & Levenson (Chernokhvostov), V. V. (2005). MSRE-PCR for analysis of gene-specific DNA methylation. *Nucleic Acids Research*, 33(10), e93. http://doi.org/10.1093/nar/gni092

[121] Minoche, A., Dohm, J., & Himmelbauer, H. (2011). Evaluation of genomic high-throughput sequencing data generated on Illumina HiSeq and Genome Analyzer systems. *Genome Biol*, 12(11), R112.

[122] Misra, S., Sharma, S., Agarwal, A., Khedkar, S., Tripathi, M., Mittal, M. and Chaudhuri, G. (2010). Cell cycle-dependent regulation of the bi-directional overlapping promoter of human BRCA2/ZAR2 genes in breast cancer cells. *Molecular Cancer*, 9(1), p.50.

[123] Mondal K. and Dutta S.,(2014). Review on apoptosis and its role in cancer.*World Journal of Pharmacy and Pharmaceutical Sciences.*3 (3).714.

[124] Moreno DA, Scrideli CA, Cortez MA, de Paula Queiroz R, Valera ET, da Silva Silveira V, Yunes JA, Brandalise SR, Tone LG (2010) Differential expression of HDAC3, HDAC7 and HDAC9 is associated with prognosis and survival in childhood acute lymphoblastic leukaemia. *British Journal of Haematology* 150(6):665-673.

[125] Mouse Genome Sequencing ConsortiumConsortium, Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, Agarwala R, Ainscough R, Alexandersson M, An P.(2002) Initial sequencing and comparative analysis of the mouse genome. *Nature* 420(6915):520-562.

[126] Muhittin Yılmaz, Cem Ozic and İlhami Gok (2012). Principles of Nucleic Acid Separation by Agarose GelElectrophoresis, Gel Electrophoresis -

Principles and Basics, Dr. Sameh Magdeldin (Ed.), ISBN: 978-953-51-0458-2, InTech, Available from: http://www.intechopen.com/books/gel-electrophoresis-principles-andbasics/principles-of-nucleic-acid-separation-by-agarose-gel-electrophoresis.

[127] Nalwoga H.,(2010).Molecular markers in breast carcinoma A study with focus on molecular phenotypes, angiogenesis and stem cells in an African population.Dissertation for the degree of philosophiae Doctor (Ph.D.) The Gade Institute, Section for Pathology University of Bergen Norway.

[128] Nass, S., Henderson, I. and Lashof, J. (2001). Mammography and beyond. Washington, DC: National Academy Press.

[129] NIH Cancer Trends Report, 2010.

[130] Nikkilä , j.(2013).palb2 and rap80 genes in hereditary breast cancer predisposition. ph.d. thesis. university of oulu.

[131] Niwa, Y., Oyama, T., & Nakajima, T. (2000). BRCA1 Expression Status in Relation to DNA Methylation of the BRCA1 Promoter Region in Sporadic Breast Cancers. *Japanese Journal Of Cancer Research*, 91(5), 519-526.

[132] nne Marie Helmenstine, P. (2015). Get the Recipe for 0.5M EDTA Solution. About.com Education. Retrieved 21 July 2015, from http://chemistry.about.com/od/labrecipes/a/Edta-Solution.htm

[133] Noruzinia M., Loghmani H., Abdul-Tehrani H., Taghizadeh M. and Hamid Karbassian M (2014).*Molecular and Biochemical diagnosis* (MBD).21-33.

[134] Ola R,(2012).DNA methylation and the control of genes involved in malignant processes. Ph.D. thesis. babes-bolyai university – cluj napoca faculty of biology and geology Department of Molecular Genetics.

[135] Ottaviano L., Issa P, Pan F., Smith S., Baylin B., and Davidson E...(1994).Methylation of the Estrogen Receptor Gene CpG Island Marks Loss of Estrogen Receptor Expression in Human Breast Cancer Cells. *Cancer Res*. 54:2552-2555.

[136] Palacios J., Honrado H., and Osorio A.,(2003).Immunohistochemical Characteristics Defined by Tissue Microarray of Hereditary Breast Cancer Not Attributable to BRCA1 or BRCA2 Mutations: Differences from Breast Carcinomas Arising in BRCA1 and BRCA2 Mutation Carriers.*Clinical Cancer Research*.3606.pp:9, 3606–3614.

[137] panigrahi k.(2011). Regulatory circuit of p300 and dna methylation in cancer.department of life science national institute of technology rourkela-769008, odisha.

[138] Park, S., Kwon, H., Choi, Y., Lee, H., Kim, S., Kim, J., Kim, I., Jung, N., Cho, N. and Kang, G. (2011). Distinct patterns of promoter CpG island methylation of breast cancer subtypes are associated with stem cell phenotypes. *Mod Pathol.*

[139] Parrella, P. (2004). Nonrandom Distribution of Aberrant Promoter Methylation of Cancer-Related Genes in Sporadic Breast Tumors. *Clinical Cancer Research*, 10(16), pp.5349-5354.

[140] Patra, Samir Kumar, et al. 'Demethylation of (Cytosine-5-C-Methyl) DNA and Regulation Of Transcription In The Epigenetic Pathways Of Cancer Development'. *Cancer Metastasis* Rev 27.2 (2008): 315-334.

[141] Pattabiraman V. (2011).Characterization of BIX-01294 and its analogs E67 and E11 on growth and reactivation of epigenetically silenced tumor suppressor genes in human breast cancer cells. MSc thesis. In the Graduate Division of

Biological and Biomedical Sciences Genetics and Molecular Biology.Bharathiar University.

[142] Phillips, T. (2015). 3 Ways to Prepare a TBE Buffer. About.com Money. Retrieved 21 July 2015, from http://biotech.about.com/od/buffersandmedia/ht/MakeTBE.htm

[143] Platzer, G., Schedlbauer, A., Chemelli, A., Ozdowy, P., Coudevylle, N., Auer, R., Kontaxis, G., Hartl, M., Miles, A.J., Wallace, B.A., Glatter, O., Bister, K., Konrat, R., 2011. The metastasis-associated extracellular matrix protein osteopontin forms a transient structure in ligand interaction sites. *Biochemistry* 50, 6113–6124.

[144] purdon j.(2010).epigenetic methylation and its implication in cancer and neurodegeneration.msc degree.The Graduate School of Biomedical Sciences University of Medicine and Dentistry of New Jersey.

[145] Qiu, P. and Zhang, L. (2012). Identification of markers associated with global changes in DNA methylation regulation in cancers. *BMC Bioinformatics*, 13(Suppl 13), p.S7.

[146] Quina AS, Buschbeck M, Di Croce L (2006) Chromatin structure and epigenetics. *Biochemical Pharmacology* 72(11):1563-1569.

[147] Ramos, E., Camargo, A., Braun, K., Slowik, R., Cavalli, I., Ribeiro, E., Pedrosa, F., Souza, E., Costa, F. and Klassen, G. (2010). Simultaneous CXCL12 and ESR1 CpG island hypermethylation correlates with poor prognosis in sporadic breast cancer. *BMC Cancer*, 10(1), p.23.

[148] Rankin, T., & Mansuy, I. (2010). Epigenetic inheritance in mammals: Evidence for the impact of adverse environmental effects. *Neurobiology of Disease*, 39(1), 61-65.

[149] Rebbeck, T., Kantoff, P., Krithivas, K., Neuhausen, S., Blackwood, M., Godwin, A., Daly, M., Narod, S., Garber, J., Lynch, H., Weber, B., and Brown, M. (1999). Modification of BRCA1-Associated Breast Cancer Risk by the Polymorphic Androgen-Receptor CAG Repeat. *The American Journal of Human Genetics,* 64(5), pp.1371-1377.

[150] Rezano, A., Kuwahara, K., Yamamoto-Ibusuki, M., Kitabatake, M., Moolthiya, P., Phimsen, S., Suda, T., Tone, S., Yamamoto, Y., Iwase, H. and Sakaguchi, N. (2013). Breast cancers with high DSS1 expression that potentially maintains BRCA2 stability have poor prognosis in the relapse-free survival. *BMC Cancer*, 13(1), p.562.

[151] Rhee I, Bachman KE, Park BH, Jair K-W, Yen R-W C, Schuebel KE, Cui H, Feinberg AP, Lengauer C, Kinzler KW, Baylin SB, Volgelstein B (2002) DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature* 416:552-556.

[152] Riazalhosseini, Y. (2010). Identification of a DNA methylation signature and distinct microRNA variants in breast cancer. Ph.D. thesis. Combined Faculties for the Natural Sciences and for Mathematics of the Ruperto-Carola University of Heidelberg, Germany.

[153] Rice, J., Massey-Brown, K., & Futscher, B. (1998). Aberrant methylation of the BRCA1 CpG island promoter is associated with decreased BRCA1 mRNA in sporadic breast cancer cells. *Oncogene*, 17(14), 1807-1812.

[154] Ripamonti, C., Colombo, M., Mondini, P., Siranoush, M., Peissel, B., Bernard, L., Radice, P. and Carcangiu, M. (2013). First description of an acinic

cell carcinoma of the breast in a BRCA1 mutation carrier: a case report. *BMC Cancer*, 13(1), p.46.

[155] Rivenbark, A., Jones, W., Risher, J. and Coleman, W. (2006). DNA Methylation-Dependent Epigenetic Regulation of Gene Expression in MCF-7 Breast Cancer Cells. *Epigenetics*, 1(1), pp.33-45.

[156] Robert MF, Morin S, Beaulieu N, Gauthier F, Chute IC, Barsalou A, MacLeod AR (2003) DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. *Nature Genetics* 33(1):61-65.

[157] rodic, n. (2005). Identification and initial characterization of genes controlled by dna methylation in embryonic stem cells. Ph.D. thesis. University of florida.

[158] Rody, A., Holtrich, U., Solbach, C., Kourtis, K., von Minckwitz, G., Engels, K., Kissler, S., Gatje, R., Karn, T., Kaufmann, M., 2005. Methylation of estrogen receptor beta promoter correlates with loss of ER-beta expression in mammary carcinoma and is an early indication marker in premalignant lesions. *Endocr. Relat. Cancer* 12, 903–916.

[159] S.A.W. Fuqua (ed.)(,2009.Hormone Receptors in Breast Cancer, Cancer Treatment and Research 312, DOI 10.1007/978-0-387-09463-2_1, ˇ Springer *Scienceþ Business Media*, LLC.

[160] Sadikovic B., Haines R., Butcher T., and Rodenhiser I.,(2004). Chemically induced DNA hypomethylation in breast carcinoma cells detected by the amplification of inter methylated sites. . *Breast Cancer Research*. 6 No 4.

[161] Sahoo, m. (2012). Cloning of dna methyltransferase1. MSc. National institute of technology rourkela-769008, odisha.

[162] Sang Y., Cheng C., Tang XF., Zhang MF and Lv XB., (2015).Hypermethylation of TET1 Promoter Is a New Diagnostic Marker for Breast Cancer Metastasis.*Asian Pac J Cancer Prev*, 16 (3), 1197-1200.

[163] Sassi, A., Popielarski, M., Synowiec, E., Morawiec, Z. and Wozniak, K. (2013). BLM and RAD51 Genes Polymorphism and Susceptibility to Breast Cancer. *Pathology & Oncology Research*, 19(3), pp.451-459.

[164] Sawan C and Herceg Z (2010) Histone Modifications and Cancer. *Advances in Genetics* 70:57-85.

[165] Shapiro, R., Servis, R., & Welcher, M. (1970). Reactions of Uracil and Cytosine Derivatives with Sodium Bisulfite. *J. Am. Chem. Soc.*, 92(2), 422-424. doi:10.1021/ja00705a626

[166] Sharma, D., Blum, J., Yang, X., Beaulieu, N., Macleod, A. and Davidson, N. (2005). Release of Methyl CpG Binding Proteins and Histone Deacetylase 1 from the Estrogen Receptor α (ER) Promoter upon Reactivation in ER-Negative Human Breast Cancer Cells. *Molecular Endocrinology*, 19(7), pp.1740-1751.

[167] Shen, C., Tsou, Y., Chen, H., Huang, H., Wu, S., & Cheng, W. et al. (2014). Osteopontin Promoter Controlled by DNA Methylation: Aberrant Methylation in Cloned Porcine Genome. *Biomed Research International*, 2014, 1-16.

[168] Shiraishi, M. (2004). High-Speed Conversion of Cytosine to Uracil in Bisulfite Genomic Sequencing Analysis of DNA Methylation. *DNA Research*, 11(6), 409-415.

[169] Singh, T.(2014).Comprehensive Analysis of Differentially Expressed mRNAs and miRNAs in Breast Cancer.MSc thesis. Department Of Life Science National Institute of Technology Rourkela.

[170] Sinilnikova, O., Antoniou, A., Spurdle, A., Healey, S., Schmutzler, R., Neuhausen, S., Couch, F., Stoppa-Lyonnet, D., Chenevix-Trench, G. and Easton, D. (2008). Common breast cancer-predisposition alleles are associated with breast cancer risk in BRCA1 and BRCA2 mutation carriers. *European Journal of Cancer Supplements*, 6(9), pp.207-208.

[171] Snell, C., Krypuy, M., Wong, E., Loughrey, M. and Dobrovic, A. (2008). BRCA1 promoter methylation in peripheral blood DNA of mutation negative familial breast cancer patients with a BRCA1 tumor phenotype. *Breast Cancer Research*, 10(1), p.R12.

[172] Speirs, V., Skliris, G.P., Burdall, S.E., Carder, P.J., 2002. Distinct expression patterns of ER alpha and ER beta in normal human mammary gland. J. *Clin. Pathol*. 55, 371– 374.

[173] SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.

[174] Suganuma T, Kawabata M, Ohshima T, Ikeda MA (2002) Growth suppression of human carcinoma cells by reintroduction of the p300 coactivator. PNAS 99(20):13073-13078.

[175] Sunami, E., Shinozaki, M., Sim, M., Nguyen, S., Vu, A., Giuliano, A. and Hoon, D. (2008). Estrogen receptor and HER2/neu status affect epigenetic differences of tumor-related genes in primary breast tumors. *Breast Cancer Research*, 10(3), p.R46.

[176] Suzuki, M., & Bird, A. (2008). DNA methylation landscapes: provocative insights from epigenomics. *Nat Rev Genet*, 9(6), 465-476.

[177] Swift GW, Reed JP, Alldredge PA, Wylie T, Walker J, Kalicki J, Watson MA, Heath S, Shannon WD, Varghese N, Nagarajan R, Westervelt P,

Tomasson MH, Link DC, Graubert TA, DiPersio JF, Mardis ER, Wilson RK (2010) DNMT3A mutations in acute myeloid leukemia. *New England Journal of Medicine* 363:2424-2433.

[178] Tan, A., Jimeno, A., Lin, S., Wheelhouse, J., Chan, F., Solomon, A., Rajeshkumar, N., Rubio-Viqueira, B. and Hidalgo, M. (2009). Characterizing DNA methylation patterns in pancreatic cancer genome. *Molecular Oncology*, 3(5-6), pp.425-438.

[179] Tapia, T., Smalley, S., Kohen, P., Muñoz, A., Solis, L., Corvalan, A., Faundez, P., Devoto, L., Camus, M., Alvarez, M. and Carvallo, P. (2008). Promoter hypermethylation of BRCA1 correlates with absence of expression in hereditary breast cancer tumors. *Epigenetics*, 3(3), pp.157-163.

[180] Tillinghast GW, Partee J, Albert P, Kelley JM, Burtow KH, Kelly K (2003) Analysis of genetic stability at the EP300 and CREBBP loci in a panel of cancer cell lines. *Genes Chromosomes and Cancer* 37(2):121-131.

[181] Today, M. (2013). What Is Cancer? What Causes Cancer?. [online] Medicalnewstoday.com.Available at: tttp://www.medicalnewstoday.com/info/cancer-oncology/ [Accessed 13 Mar. 2015].

[182] Tollefsbol Trygve O. (ed.), Epigenetics Protocols: Second Edition, Methods in Molecular Biology, vol. 791, DOI 10.1007/978-1-61779-316-5_11, © Springer Science+Business Media, LLC 2011.

[183] Tripathy , t.(2013).modulation of notch signaling components in presence of epigenetic modulators in breast cancer.msc thesis.department of life science national institute of technology rourkela.

[184] Tung, N., Miron, A., Schnitt, S., Gautam, S., Fetten, K., Kaplan, J., Yassin, Y., Buraimoh, A., Kim, J., Szasz, A., Tian, R., Wang, Z., Collins, L., Brock, J., Krag, K., Legare, R., Sgroi, D., Ryan, P., Silver, D., Garber, J. and Richardson, A. (2010). Prevalence and predictors of loss of wild type BRCA1 in estrogen receptor positive and negative BRCA1-associated breast cancers. *Breast Cancer Research*, 12(6), p.R95..

[185] usan, J., Harrison, J., Paul, C., & Frommer, M. (1994). High sensitivity mapping of methylated cytosines. *Nucl Acids Res*, 22(15), 2990-2997.

[186] Valente, A., Rummel, S., Shriver, C. and Ellsworth, R. (2014). Sequence-based detection of mutations in cadherin 1 to determine the prevalence of germline mutations in patients with invasive lobular carcinoma of the breast. *Hered Cancer Clin Pract*, 12(1), p.17.

[187] Visvanathan, K., Sukumar, S., and Davidson, N. (2006). Epigenetic Biomarkers and Breast Cancer: Cause for Optimism. *Clinical Cancer Research*, 12(22), pp.6591-6593.

[188] W. Doerfler (2006).DNAMethylation: BasicMechanisms.Springer-Verlag Berlin Heidelberg.ISBN-10 3-540-29114-8.

[189] Wajed, S., Laird, P. and DeMeester, T. (2001). DNA Methylation: An Alternative Pathway to Cancer. *Annals of Surgery*, 234(1), pp.10-20.

[190] Walsh CP1., and Xu GL (2006).Cytosine methylation and DNA repair.*Curr Top Microbiol Immunol*. 2006; 301:283-315.

[191] Walter MJ, Ding L, Shen D, Shao J, Grillot M, McLellan M, Fulton R, Schmidt H, Kalicki-Veizer J, O'Laughlin M, Kandoth C, Baty J, Westervelt P, Dipersio JF, Mardis ER,Wilson RK, Ley TJ, Graubert TA (2011) Recurrent DNMT3A mutations in patients with myelodysplastic syndromes.

[192] Wang WW, Spurdle AB, Kolachana P, Bove B, Modan B, Ebbers SM, Suthers G, Tucker MA, Kaufman DJ, Doody MM, Tarone RE, Daly M, Levavi H, Pierce H, Chetrit A, Yechezkel GH, Chenevix-Trench G, Offit K, Godwin AK, Struewing JP (2001) A single nucleotide polymorphism in the 5' untranslated region of RAD51 and risk of cancer among BRCA1/2 mutation carriers. *Cancer epidemiology, biomarkers and prevention* 10(9):955- 960.

[193] Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schübeler D (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nature Genetics* 37(8):853-62.

[194] Weinberg RA (2007) The Biology of Cancer. Garland Scince Publishing.

[195] WHO (2008) "Cancer."

[196] Widschwendter, M. (2004). Association of Breast Cancer DNA Methylation Profiles with Hormone Receptor Status and Response to Tamoxifen. *Cancer Research*, 64(11), pp.3807-3813.

[197] Wild, C. (2014). The International Agency for Research on Cancer (IARC). Yon/London: WHO, p.1.

[198] William W. Wilfinger, Karol Mackey, and Piotr Chomczynski. (1997. Effect of pH and Ionic Strength on the Spectrophotometric Assessment of Nucleic Acid Purity: *BioTechniques* 22:474-481.

[199] Wojdacz, T., Thestrup, B., Overgaard, J. and Hansen, L. (2011). Methylation of cancer-related genes in tumor and peripheral blood DNA from the same breast cancer patient as two independent events. *Diagnostic Pathology*, 6(1), p.116.

[200] Wong M. (2010).Examination of Promotor Hypermethylation Patterns in Magnetically Enriched Exfoliated Breast Milk Epithelial Cells. MSc thesis. University of Massachusetts. Department of Veterinary & Animal Sciences Program in Animal Biotechnology & Biomedical Sciences.

[201] Xiang, T., Li, L., Fan, Y., Jiang, Y., Ying, Y., Putti, T., Tao, Q. and Ren, G. (2010). PLCD1 is a functional tumor suppressor inducing G 2 /M arrest and frequently methylated in breast cancer. *Cancer Biology & Therapy*, 10(5), pp.520-527.

[202] Xiao, F., Kim, Y., Snyder, C., Wen, H., Chen, P., Luo, J., Becirovic, D., Downs, B., Cowan, K., Lynch, H. and Wang, S. (2014). Genome instability in blood cells of a BRCA1+ breast cancer family. *BMC Cancer*, 14(1), p.342.

[203] Xu, C., Brown, M., Chambers, J., Griffiths, B., Nicolai, H., & Solomon, E. (1995). Distinct transcription start sites generate two forms of BRCA1 mRNA. *Hum Mol Genet*, 4(12), 2259-2264.

[204] Xu, J., Shetty, P., Feng, W., Chenault, C., Bast, R., Issa, J., Hilsenbeck, S. and Yu, Y. (2012). Methylation of HIN-1, RASSF1A, RIL and CDH13 in breast cancer is associated with clinical characteristics, but only RASSF1A methylation is associated with outcome. *BMC Cancer*, 12(1), p.243.

[205] Yan XJ, Xu J, Gu ZH, Pan CM, Lu G, Shen Y, Shi JY, Zhu YM, Tang L, Zhang XW, Liang WX, Mi JQ, Song HD, Li KQ, Chen Z, Chen SJ (2011) Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nature Genetics.*

[206] Yang, X. (2001). DNA methylation in breast cancer. *Endocrine-Related Cancer*, 8(2), pp.115-127.

[207] Zangana, A., & Garota, S. (2012). Risk factors of breast cancer in a sample of Kurdish women of Kurdistan Region - Iraq: A comparative study between pre-menopausal and post-menopausal women. *Zanco Journal of Medical Sciences*, 16(3), 262-267.

[208] zhao et al ., (2012).clinical implication of ERBmethylation on sporadic breast cancer in chinese women.*med oncol*.vol.29.issue 3 . 1569-1575

[209] Zhao, C., Lam, E.W., Sunters, A., Enmark, E., De Bella, M.T., Coombes, R.C., Gustafsson, J.A., Dahlman-Wright, K., 2003. Expression of estrogen receptor beta isoforms in normal breast epithelial cells and breast cancer: regulation by methylation. *Oncogene* 22, 7600–7606.

[210] Zurita, M., Lara, P., del Moral, R., Torres, B., Linares-Fernández, J., Arrabal, S., Martínez-Galán, J., Oliver, F. and Ruiz de Almodóvar, J. (2010). Hypermethylated 14-3-3-σ and ESR1 gene promoters in serum as candidate biomarkers for the diagnosis and treatment efficacy of breast cancer metastasis. *BMC Cancer*, 10(1), p.217.

دیاریکردنی ئاست و چەندیەتی گۆڕانکاری مەثیلەیشنی ترشی ناوەکی لە خوێنی شێرپەنجەی مەمک

نامەیەکە

پیشکەش کراوە بە ئەنجومەنی فاکەلتی زانست و پەروەردەی زانستەکان

سکولّی زانست لە زانکۆی سلێمانی

وەک بەشێک لە پێداویستیەکانی بە دەستهێنانی بروانامەی ماستەر لە زانستی زیندەوەرزانی

(گەردی زیندەوەرزانی)

لەلایەن

هەڕێم عثمان سمایل

بەکالۆریۆس- زیندەوەزانی (٢٠١١)، زانکۆی کۆیە

بە سەرپەرشتی

د. دلّنیا ئەسعەد مەحمود

پرۆفیسۆری یاریدەدەر

٢٠١٦ شوبات ٢٧١٥ ڕێبەندان

پوختە

لەم سالانەی دوای دا ، گۆران کاری لە مەتیلەیشنی ی ترشی ناوەکی وەکو گۆران کاریەکی سەرەکی ئیپیجێنەتیکی دەست نیشان کراوە لە شێرپەنجەی مەمک لە مرۆڤ وە وەکوو داهاتوویەکی گەش وایە بۆ دەست نیشان کردنی شێرپەنجەی مەمک لە قۆناغی سەرەتایدا .ئەم لێکۆڵینەوەیە تایبەت بوو لە دەست نیشان کردنی گۆران کاری لە مەتیلەیشنی ترشی ناوەکی بۆ ناوچەی promoter ی ترشی ناوەکی بۆ سێ جینی جیاواز(ESR2, OPN, and BRCA1) لە شێرپەنجەی مەمک .لە دیاری کردنی شێرپەنجەی مەمک زۆر جین دەست نیشان کراوە کە رێژەی مەتیلەیشنی تێدا زۆر دەبێت ، بەلام لێکۆڵینەوەی کەم کراوە لە بەکارهێنانی ئەم جینانە لە شێرپەنجەی مەمک .

بە تێکرای ٣٠ حالەتی شێرپەنجەی مەمک لەگەڵ ٨ حالەتی ئاسایی لە خوێن دا لێکۆڵینەوەی لە سەر کرا.ترشی ناوەکی لە خوێن دەرهێندرا، وە کارلێکی گۆرینی bisulfate بە کار هێنرا ، umethylated cytosine دەاتوانیت بگۆرێت بۆ یراسیل بەلام methylated cytosine ناگۆری بۆ یوراسیل .جۆری Promoter methylation بریاری لێدارا بە هۆی بەکارهێنانی bisulfite sequencing. بەرنامەی BiQ Analyzer software بەکارهێنرا بۆ شیکردنەوەی ئەنجامەکان ، وە بۆ بریار دان لە ڕێژەی گۆرینی bisulfate ، رێژەی سەدی مەتیلەیشنی، رئژەی سەدیunmethylated ، رێژەی هەڵە لە sequencing وەهەروەها alignment ی ریزبوونی جینەکان.

پشت بەستن بە sequence کە شی کراوەتەوە بە بەرنامەی BiQ Analyzer software ئەنجامەکانی بۆهێلی ESR2 بەم شیوەیە 4.16% CPG لە حاڵەتی ئاسایی methylated بوو بەلام methylated CPG (12.39%) بوو . وە هەروەها ریژەی unmethylated CPG ی جیاوازە لە نیوان حالەتی ئاسایی لەگەل شیرپەنجەی مەمک ، .(87.5%) unmethylated بوو لە حالەتی ئاسایی بەلام لە شێرپەنجەی مەمک (72.5%) بوو ، وە تێکرای رێژەی سەدی CPG ی کە دەرنەکەوتوو لە کاتی sequencing بۆ حالەتی ئاسایی .(8.31%) وە بۆ شیرپەنجەی مەمک (14.99%) بوو.تێکرای مەتیلەیشنی CPG لە بۆجینی OPN لە حالەتی ئاسایی (0%) بوو بەلام بۆ شێرپەنجەی مەمک (57.57%) بوو، وە هەروەها ریژەی unmethylated CPG لە حالەتی ئاسایی (88.8%) بوو بەلام لە شێرپەنجە (39.3%) بوو . کەمترین رێژەی CPG کە دەرنەکەوتووە لە sequencing بۆ حالەتی ئاسایی .(11.11%) بوو بەلام بۆ شێرپەنجە (3.03%) بوو. رێژەی CPG مەتیلەیشنی بۆ جینی BRCA1 لە شێرپەنجە (63.3%) بوو بەلام بۆ حالەتی ئاسایی (9.09%) بوو.رێژەی unmethylated from CPG

بۆ شێرپەنجەی مەمک (3.3%) بەلام بۆ حاڵەتی ئاسایی (36.36%) بوو وە رێژەی CPG کە دەرنەکەوتوو لە sequencing بۆ حالەتی ئاسایی (54.54%) بەلام بۆ شێرپەنجە (33.6%) بوو .

ئەم لێکۆڵینەویە دەسەلمێنی کە مەثیلەیشنی ترشی ناوەکی دەگۆردرێت لە شێرپەنجەی مەمک بۆ سێ جینی جیاواز (ESR2 ,OPN and BRCA1) وە دەتوانرێت وەکو potential biomarker لە دیاری کردنی شێرپەنجەی مەمک.وە لە رووی سەرژمیریەوە جیاوازی هەیەلە نیوان هەرسێ جینەکە بۆ حاڵەتی ئاسایی و نەخۆشەکان، وە هەروەها جیاوازی هەیە(مەثیلەیشنی) لە نێوان قۆناغی یەک و دوو شیرپەنجەی مەمک بۆ (BRCA1،ESR2) بەلام بۆ OPN نیە لە روو سەرژمێریەوە.

النوعي والكمي تحديد التغييرات الحامض النووي في الدم لمرضى سرطان الثدي

رسالة

مقدمة الى مجلس فاكلتي العلوم و تربية العلوم

سكول العلوم في جامعة السليمانية

كجزء من متطلبات نيل شهادة

ماجستير في علوم الحياة

(البيولوجيا الجزيئية)

من قبل

هەريم عثمان سمايل

بكالوريوس في علوم الحياة (٢٠١١) ، جامعة كويسنجق

باشراف

دكتور دلّينا اسعد محمود

استاذ المساعد

شباط ٢٠١٦ ربيع الثانى ١٤٣٧

الخلاصة

في الآونة الأخيرة، الحامض النووي هو تعديل جينية الرئيسي في سرطان الثدي البشرية التي يتم اكتشافها والعلامات البيولوجية واعدة في الكشف المبكر عن سرطان الثدي. وقد أجريت الدراسة لتحديد التغيرات الحامض النووي في منطقة المروج من ثلاثة جينات مختلفةESR2) ، OPN، و (BRCA1في سرطان الثدي. وقد تم الإبلاغ عن العديد من الجينات لتكون hypermethylation في النساء بسرطان الثدي في تشخيص سرطان الثدي، ولكن لم يكن هناك دراسة شاملة قليلا على فائدة سريرية من هذه الجينات في سرطان الثدي.

في المجموع، تم دراسة 30 حالة من النساء في سرطان الثدي و 8 حالات الدم بصحة جيدة. تم استخدام الحمض النووي المستخرج من عينات الدم، وردود الفعل تحويل ثنائي سلفات لالسيتوزين تحويل unmethylatedلالأورالية وخال من الرصاص السيتوزين لم تتأثر. تم تحديد نمط المروج مثيلة باستخدام التسلسل بيسلفيت. تم استخدام برنامج BIQ محلل لتحليل البيانات لتحديد معدل بيسلفيت التحويل، نسبة الحامض، نسبةunmethylated ، أخطاء أثناء تسلسل ومحاذاة تسلسل الجينات.

استنادا إلى sequencing بواسطة برنامج BIQ محلل نتائج ESR2 هي (4.16٪) من الدليل السياسي الشامل من كان ميثليته العينة صحية بينما كان ميثليته (12.39٪ CPG (من عينة السرطان، وكانت أيضا unmethylated CPGالفرق بين عينة صحية والسرطان . إجمالي unmethylated CPG من العينة صحية (87.5٪). في حين من عينات السرطان (72.5٪) إجماليCpGs ، وليس تقديم ليس من عينات سرطان (14.99٪)، في حين من العينات الصحية (8.31٪ OPN .(إجمالي مثيلة CPG من عينة السرطان (57.57٪)، في حين من عينة صحية (0٪) ولكن unmethylated CPG من عينة السرطان (39.39٪)، في حين شكل صحية العينة كان (88.8٪). الحد الأدنى من الشعبية المركزية لا يعرض من عينة السرطان (3.03٪)، في حين زاد من العينات السليمة إلى (11.11٪). كان مثيلة CPGpercentage ل BRCA1 في عينة السرطان (63.63٪)، ولكن في عينات سليمة كان (36.36٪). الحد الأدنى unmethylated من CPGكان (3.03٪) الحالي من عينة السرطان لكن في عينات صحية (٪ 9.09) موجود Maximun. الدليل السياسي الشامل لم تكن موجودة من كلا صحية وسرطان عينات كانت (33.6٪)، ولكن كان لعينات صحية (54.54٪)

وتوضح هذه الدراسة أن التغيرات الحامض النووي في سرطان الثدي لمدة ثلاثة جينات مختلفة (ESR2، OPN، وBRCA1) ويمكن أن يكون استخدام مثل العلامات البيولوجية المحتملة للكشف عن سرطان الثدي وذات دلالة إحصائية بين السرطان وعينات صحية لجميع الجينات الثلاثة في P - القيم <The.0.05 الصف 1 والصف 2 عينات من ESR2 وBRCA1، هم كبير ولكن ليس لOPN في القيم P- <0.05 إحصائيا.