TURKISH REPUBLIC OF NORTH CYPRUS
NEAR EAST UNIVERSITY
HEALTH SCIENCES INSTITUTE

THE METHYLDETECT METHOD OPTIMIZATION FOR IDENTIFY METHYLATION IN BREAST CANCER SAMPLES

SILA GANİM

MASTER THESIS

MEDICAL BIOLOGY AND GENETIC PROGRAMME

Supervisor:
Prof. Dr. Nedime SERAKINCI

Northern Cyprus, Nicosia
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NEAR EAST UNIVERSITY

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ACKNOWLEDGMENTS

I am pleased to record my grateful thanks to many individuals and organizations without whose help it would have been hard to produce such a worthy project. I want to thank especially to my supervisor, Prof. Dr. Nedime SERAKINCI, for her dedicated work and endless support.

I am also grateful to Near East University Hospital Medical Pathology Laboratory, Assist. Prof. Hanife ÖZKAYALAR for providing samples for the project.

MethylDetect is acknowledged for providing the materials for the presented work in particular to Prof. Dr. Lisa Lotte HANSEN.

I am grateful to Serhat DEMİRKOL from Intron Sağlık Ltd. and Metin CEYHAN from of Roch for their contribution to the study.

Special thanks go to Near East University Hospital Medical Genetic Laboratory colleagues and in particular Ayşegül BOSTANCI for their support, encouraging me and keeping my motivation at high levels during my thes studies.

Finally, I am pleased to record my sincere gratitude to my family, especially my husband, Seçkin, my mum, Züler, my dad, Caner, and my brother, Sıtkı, and friends for keeping my motivation high all the time during my studies, without whose help it would have been hard to work on this project.

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SYMBOLS AND ABBREVIATIONS

APC	Adenomotous Polyposis Coli Gene
Asef1	APC Stimulated Guanine Nucleotide Exchange Factor
ATM	Ataxia Telangiectasia Mutated
BER	Base Excision Repair
BRCA1	Breast Cancer Gene 1
BRCA2	Breast Cancer Gene 2
BRCT	BRCA1 C-Terminal Domain
CBF-β	Core-Binding Factor-B
CDH1	E Cadherin 1 Gene
CGIs	CpG Islands
CHEK2	Checkpoint Kinase 2
CpG	Cytosine-Phosphate-Guanine
DBD	DNA Binding Domain
DNMT	DNA Methyltransferase
DSB	Double-Strand Breaks
DSS1	Deleted In Split Hand/Split Foot Protein
ER	Estrogen Receptor
GEF	Guanine-Nucleotide-Exchange Factor
HER2	Human Epidermal Growth Factor Receptor 2
HR	Homologous Recombination
HHR	Homologous Recombination Repair

MBD	Methyl Binding Domain
MCR	The Mutation Cluster Region
MSP/USP PCR	Methylation-Specific PCR
MSREs	Methylation-Sensitive Restriction Enzymes
MCR	Mutation Cluster Region
NGS	Next-Generation Sequencing
NHEJ	Non-Homologous End-Joining
NLS	Nuclear Localization Sequences
OB	Oligonucleotide Binding
OB2	Oligonucleotide Binding 2
PALB2	Partner And Localizer Of The BRCA2 Gene
PARP	Poly (ADP-Ribose) Polymerase
PR	Progesterone
PTEN	Phosphatase And Tensin Homolog
RNA	Ribonucleic Acid
RUNX-3	Runt-Related Transcription Factor 3
SCD	Serine Containing Domain
ssDNA	Single-Stranded DNA
TD	Tower Domain
TP53	Tumor Protein P53

The Methyldetect Method Optimization for Identify Methylation in Breast Cancer Samples

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ABSTRACT

Ganim, S. The MethylDetect Method Optimization For Identify Methylation In Breast Cancer Samples. Near East University Institute of Health Sciences, M.Sc. Thesis in Medical Biology and Genetic Programme, Nicosia, 2020.

DNA methylation is a major epigenetic mechanism which is catalyzed by a family of DNA methyltransferases (Dntms) that containing the transfer of a methyl group to the C-5 position of the cytosine ring of DNA. This study is aimed, to optimize the methyldetect method for identfy methylation profile of the following genes BRCA1, BRCA2, APC, RUNX3 and CDH1 thus overall goal is to optimize methyldetect assay for rutine clinical applications for evaluation of the methylation profile between the samples normal, invasive ductal carcinoma (IDC), ductal carcinoma in situ (DCIS). Also, this study conducted with methyldetect company was aimed to be adapted to routine working life by working with methylation PCR kits produced compatible with LightCycler 480 instrument. In this study, to be able to optimized the method paraffin-embedded tumor tissues from patients with a history of breast cancer were used a total of 17 paraffin-embedded tissues were collected from the Near East Hospital Pathology Laboratory (10 samples IDC, 5 samples DCIS and 2 normal). First, nucleic acid were isolated from Formalin-Fixed Paraffin-Embedded Tissue Sections by using the Roche High Pure PCR Template Preparation kit and PCRs were run according to the specified criteria. However, our results indicate that quality of calibration sample, primer design and as well as stability

is very curicial for optimal evaluation. Despite the facts methods seemed to worked of most of the samples but since the calibration samples did not we failed to obtain significant results on patient materials which could be explained by due to the failure to primer stability as well as quality of calibration samples.

Keywords: Methylation, Breast Cancer, PCR, Gene

Supported by Near East University

Meme Kanseri Örneklerinde Metilasyonu Tanımlamak İçin Metildetect Yöntemi Optimizasyonu

Öğrencinin adı: Sıla Ganim

Danışman: Prof. Dr. Nedime SERAKINCI

Bölüm: Medical Biology and Genetic

ÖZ

Ganim, S. Meme Kanseri Örneklerinde Metilasyonu Tanımlamak İçin MethylDetect Yöntemi Optimizasyonu. Yakın Doğu Üniversitesi Sağlık Bilimleri Enstitüsü, Tıbbi Biyoloji ve Genetik Programı, Yüksek Lisans Tezi, Lefkoşa, 2020.DNA metilasyonu, bir metil grubunun DNA'nın sitozin halkasının C-5 pozisyonuna transferini içeren bir DNA metiltransferaz (Dntms) ailesi tarafından katalize edilen majör bir epigenetik mekanizmadır.

Bu çalışma, BRCA1, BRCA2, APC, RUNX3 ve CDH1 genlerinin metilasyon profilini tanımlaması amacı ile methyldetect yönteminin optimizasyonu ve rutin klinik uygulamada normal, invaziv duktal karsinom (IDC), yerinde duktal karsinom (DCIS) örnekleri arasındaki metilasyon profilinin değerlendirilebilmesi. Dolayısı ile bu çalışmada Methyldetect firması ile yapılan bu çalışmanın, lightcycler 480 cihazı ile uyumlu üretilen metilasyon pzr kitleri ile çalışılarak rutin çalışma hayatına adapte edilmesi amaçlanmıştır. Bu çalışmada, meme kanseri öyküsü olan hastalardan alınan parafine gömülü tümör dokularının yöntemini optimize edebilmek için Yakın Doğu Üniversitesi Hastanesi Pataloji Laboratuvarından toplam 17 parafine gömülmüş tümör dokusu toplanmıştır (10 örnek IDC, 5 örnek DCIS ve 2 örnek normal). Önce, nükleik asit Formalinle Sabitlenmiş Parafin Gömülü Doku Bölümlerinden Roche High Pure PCR Template Preparation kiti kullanılarak izole edildi ve PZR'ler, belirtilen kriterlere göre çalıştırıldı. Bununla birlikte, sonuçlarımız kalibrasyon numunesinin kalitesinin, primer tasarımının ve stabilitenin de optimum değerlendirme için çok önemli olduğunu göstermektedir. Gerçeklere rağmen, yöntemlerin çoğu numunede işe yaradığı görüldü,

ancak kalibrasyon numuneleri işe yaramadığından hasta materyalleri üzerinde, primer stabilitesinin yanı sıra kalibrasyon kalitesindeki başarısızlıkla açıklanabilecek önemli sonuçlar elde edemedik.

Anahtar Kelimeler: Metilasyon, Meme Kanseri, PZR, Gen

Destekleyen kurum: Yakın Doğu Üniversitesi

1. INTRODUCTION

Breast cancer is the most common malignancy and the second most common cancer in women. The lifetime risk of developing breast cancer in women is %12 or 1 in 8 women in the United States (Momenimovahed and Salehiniya, 2019). In 2018, it was an estimated 523.000 new cases and 138,000 death in Europe (Ferlay et al., 2018). Studies have shown that the risk of breast cancer is resulting from a combination of several factors. These are including, dietary factors, lifestyle and environmental factors, and hereditary (Nindrea et al., 2018).

Most inherited cases of breast cancer are associated with mutations in two genes Breast Cancer Gene 1 (BRCA1) and Breast Cancer Gene 2 (BRCA2). On the other hand, many other genes, that are related to the risk of breast cancer development, are Ataxia telangiectasia mutated (ATM), Phosphatase and tensin homolog (PTEN), Tumor Protein p53 (TP53), Partner and localizer of the BRCA2 gene (PALB2), E Cadherin 1 (CDH1), Checkpoint Kinase 2 (CHEK2), and more (Feng et al., 2018). Breast cancer is a multiplex disease. It encompasses a heterogeneous for the molecular and clinical level and classified into two groups as molecular and histological. The breast cancer developmental pathway is generally encompassing to complex genetic and epigenetic alterations (Kanwal and Gupta, 2010; Lorinez, 2014). The activation of cellular oncogenes or/and silencing tumor suppressor genes are critical for aberrant patterns of gene expression in sporadic and familial breast cancer (Fucito et al., 2008).

However, epigenetic alterations, related to gene expression that can promote the development of several cancer types, such as breast cancer. Epigenetic alterations generally can be divided into three interacting processes: DNA methylation, histone modification, and chromatin remodeling. Deoxyribonucleic Acid (DNA) methylation is the most studied epigenetic mechanism. It does not affect genomic DNA itself. However, this mechanism involves the covalent binding of a methyl group (-CH3) to the 5 positions of the pyrimidine ring of a cysteine (C) nucleotide within CpG dinucleotides. This reaction can be regulated by the DNA methyltransferase (DNMT) enzyme family. This chemical modification impacts gene expression through two main mechanisms.

Firstly, DNA methylation can directly affect the binding of transcription factors. Secondly, the methyl binding domain (MBD) protein family recognizes methylated cytosines (Delpu et al., 2013). DNA methylation has several methods for the detection including, methylation-sensitive restriction enzymes (MSREs), methylation-specific PCR (MSP/USP PCR), pyrosequencing and methylation-specific next-generation sequencing (NGS) (Delpu et al., 2013; Krygier et al., 2016). Principally, this project uses the MSP/USP PCR method for the detection of promoter methylation levels in these genes BRCA1, BRCA2, CDH1, Adenomatous Polyposis Coli Gene (APC), and Runt-Related Transcription Factor 3 (RUNX3). This method uses sodium bisulfite for the identify methylated or unmethylated DNA. The sodium bisulfite provides the conversion of all unmethylated cytosines to uracil while leaving methylated cytosines unchanged. The silencing of functionally significant genes plays an essential role in cancer development (Sharma et al., 2010). These genes, known as tumor suppressors, appear to play a significant role in breast cancer development. Family history is an important risk factor in breast cancer.

BRCA1 has several pathways to involved in maintaining genome integrity includes DNA repair, the control of cell cycle checkpoints and apoptosis (Li et al., 2015). Mutations in BRCA1/2 genes are related to 20%-40% of familial breast cancer. Studies demonstrated that lifetime possibility of developing breast cancer with a BRCA1 mutation is 57%-65% while BRCA2 is 45%-49% (Kwong et al., 2016). BRCA1 gene mutations usually lead to a more aggressive phenotype, while BRCA2 gene mutations are similar to sporadic breast cancer (Dziadkowieck et al., 2016). In women with the BRCA1/2 mutations have a risk of up to %87 of developing breast cancer in a lifetime. Mainly, the analysis of these two genes has become a critical factor in the World, not only for the prevention of healthy women carrying a mutation but also for the development of new and personal treatment (Ossa and Torres, 2016).

In general, the APC gene is responsible for both hereditary and sporadic colorectal cancer. However, the mutation in the APC gene is rare in lung and breast cancer, whereas somatic mutations have demonstrated %18 in breast cancers (Virmani et al., 2001). Many studies have reported that APC methylation is highly specific for breast cancer and can

be used as a biomarker in the diagnosis of breast cancer (Van der Auwera et al., 2008; Dumitrescu, 2012). Jin et al. found a significant association between APC methylation and breast cancer pathogenesis.

CDH1 gene, a subtype of the classical cadherin family, plays a critical role in Ca2+ dependent homophilic cell to cell adhesion interactions. In the mammary gland, it provides a tight junction between epithelial cells and interacts with components by adhesion junctions and plays a significant role in suppressing invasion and metastasis of breast cancer cells (Andrews, Kim, and Hens, 2012). Studies showed that; in many human carcinoma E-cadherin expression is decreased or absent. In breast cancer, reduced E-cadherin expression has reported %50 of invasive ductal carcinomas. In contrast, invasive lobular carcinomas showed a complete loss of E-cadherin expression in %84 (De Leeuw et al., 1997).

The RUNX3 gene, a member of the RUNT family, plays a significant role in the proliferation, growth, and apoptosis of cells. The RUNX3 expression is associated with the development of various tumors and their metastasis and prognosis (G. Li., 2019). It is related to different cancer types such as liver, larynx, lung, breast, prostate and endometrial cancers (Kim et al., 2004). The downregulation of the Runx is controlled by several mechanisms such as promoter region hypermethylation, loss of heterozygosity, hemizygous deletion and mutation. These mechanisms have shown to be related to the carcinogenesis of solid human tumors (Hwang et al., 2007). Studies showed that the RUNX3 methylation occurs in a primary role in human gastric carcinoma and aberrant methylation of promoter RUNX3 relationship with loss of RUNX3 expression (Li et al., 2002).

In the light of these, this research was conducted to follow examining the methylation profiles of five different genes (BRCA1, BRCA2, RUNX3, APC, CDH1) that associated with breast cancer using the MethylDetect DNA methylation kit. Our main goal was investigating the usability of breast cancer in personalized treatment planning along with the identification of its possible use as a genetic biomarker in treatment selection.

2. GENERAL INFORMATION

2.1 Cancer

Cancer is a major health problem worldwide. In 2018, Global Cancer Statistics reported that 18,1 million new cases (17 million excluding Non Melanoma Skin Cancer) and 9.6 million deaths with cancer.

Cancer is a group of diseases characterized by abnormal cell growth**.** The cancerous cells have specific properties which classified into ten different “hallmarks” (Figure 2.1). These are acquired capabilities, which are necessary for neoplastic development. These hallmarks of cancer includes Genomic instability and mutation, tumor-promoting inflammation, sustaining proliferative signaling, evading growth suppressors, enabling replicative immortality, avoiding immune destruction, activating invasion and metastasis, inducing angiogenesis, resisting cell death and deregulating cellular energetics (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Cancer development is a process of the combination of events in the body. Such as, mutations occur within cells when they divide. The tumor suppressor genes and oncogenes are affected genes to regulate cell growth and differentiation. An oncogene, that promotes cell growth and reproduction and tumor suppressor genes that inhibit cell division and survivor. Malignant transformation may occur when inactivated tumor suppressor gene or hyper-activated proto-oncogenes (Fymat, 2017).

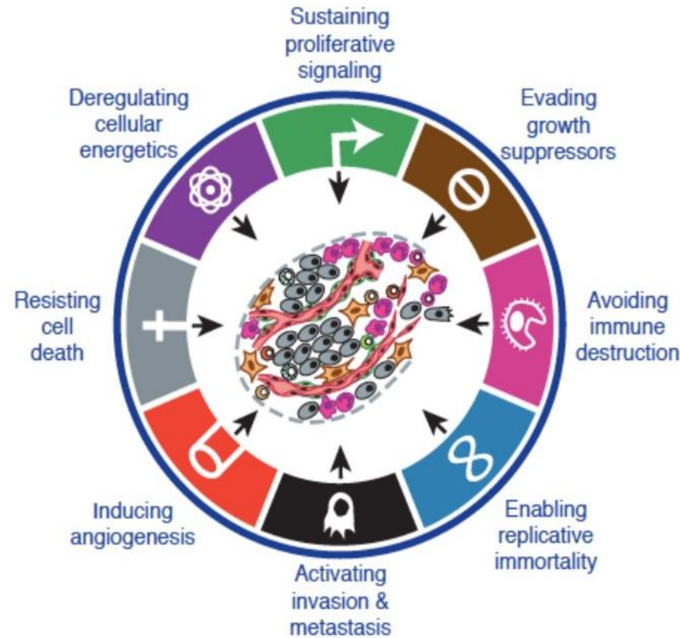

Figure 2.1: The hallmarks of cancer (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

2.2 Breast Cancer

Breast cancer is the most commonly diagnosed cancer type in women, with over 2.1 million newly diagnosed female breast cancer cases in 2018 all over the world (Bray et al., 2018). Statistics showed that half of the breast cancer incidences and 60 % of concerned deaths are occurring in economically developing countries (Hasan et al., 2013). Breast cancer has multiple risk factors: dietary factors, lifestyle and environmental factors, and hereditary (Nindrea et al., 2018). These are related to menstruation (early age at menarche, later age at menopause), reproduction (late age at first birth, and fewer children), exogenous hormone intake (oral contraceptive use and hormone replacement therapy), nutrition (alcohol), and anthropometry (The greater weight, weight gain during adulthood, and body fat distribution) and physical activity (Bray et al., 2018).

The development of breast cancer is generally specified to be a result of complex genetic and epigenetic alternations (Kanwal and Gupta, 2010; Lorinez, 2014). DNA methylation is a mechanism of epigenetic alteration concerned in gene expression programming that can promote the development of several cancers such as breast cancer. It occurs most commonly with an addition of a methyl group in the fifth position of the pyrimidine ring of cytosine on CpG sites within the genome. DNA methylation as a biomarker for early detection of cancer. (Pouliot et al., 2015). A PubMed search for the keywords 'epigenetic' and 'breast cancer' reveals that the first publication was in 1983 (Huang et al., 2011).

2.2.1 Breast Cancer Classification

Breast cancer is classified into two groups as molecular or intrinsic and histological. Histologically it can be categorized into 'In Situ Carcinoma and Invasive (Infiltrating) Carcinoma'. Breast cancer is not a single disease. It encompasses a heterogeneous for the molecular and clinical level. Molecular subtypes of breast cancer are classified according to gene expression profiles. These are divided into five subgroups according to the hormonal receptor state; Luminal A, Luminal B, Human Epidermal Growth Factor Receptor 2 (HER2) over-expression, Basal-like, and Normal-like (Figure 2.2). Luminal cancers express hormone receptors (estrogen receptor (ER)/ progesterone receptor (PR)) and are of low grade; HER2 subtypes overexpress HER2 gene products and are of high grades. Luminal B tumors have the worst prognosis than Luminal A tumors. The basal-like tumors have a triple-negative receptor status (ER, PR, HER2) and have the worst prognosis like Luminal B (Polyak, K. 2007; Allison, K.H., 2012; Feng et al., 2018).

	Molecular Subtype			
	Luminal A	**Luminal B**	**HER2/neu**	**Basal like[a]**
Gene expression pattern	Expression of luminal (low molecular weight) cytokeratins, high expression of hormone receptors and related genes	Expression of luminal (low molecular weight) cytokeratins, moderate-low expression of hormone receptors and related genes	High expression of HER2/*neu*, low expression of ER and related genes	High expression of basal epithelial genes and basal cytokeratins, low expression of ER and related genes, low expression of HER2/*neu*
Clinical and biologic properties	50% of invasive bresat cancer, ER/PR positive, HER2/neu negative	20% of invasive breast cancer, ER/PR positive, HER2/neu expression variable, higher proliferation than Luminal A, higher histologic grade than Luminal A	15% of invasive breast cancer, ER/PR negative, HER2/neu positive, high proliferation, diffuse TP53 mutation, high histologic grade and nodal positivity	~15% of invasive breast cancer, most ER/PR/HER2/*neu* negative (triple negative), high proliferation, diffuse TP53 mutation, BRCA1 dysfunction (germline, sporadic)
Histologic correlation	Tubular carcinoma, Cribriform carcinoma, Low grade invasive ductal carcinoma, NOS, Classic lobular carcinoma[b]	Invasive ductal carcinoma, NOS Micropapillary carcinoma	High grade invasive ductal carcinoma, NOS	High grade invasive ductal carcinoma, NOS Metaplastic carcinoma, Medullary carcinoma
Response to treatment and prognosis	Response to endocrine therapy	Response to endocrine therapy (tamoxifene and aromatase inhibitors) not as good as Luminal A	Response to trastuzumab (Herceptin)	No response to endocrine therapy or trastuzumab
	Variable response to chemotherapy	Variable response to chemotherapy (better than Luminal A)	Response to chemotherapy with antracyclins	Sensitive to platinum group chemotherapy and PARP inhibitors
	Good prognosis	Prognosis not as good as Luminal A	Usually unfavorable prognosis	Not all, but usually worse prognosis

PARP poly-adenosinediphosphate ribose polymerase

[a] Basal like tumor group includes a low-grade group with low proliferation but expression of basal type (high molecular weight) cytokeratin and triple negative phenotype (like adenoid cystic carcinoma, secretuar carcinoma).

Figure 2.2. Major Molecular/intrinsic subtypes of Breast Cancers (Eliyatkin, N. and et al. (2015).

2.2.2. Factors Contributing To Breast Cancer Development

Cancer development is induced by multiple factors. Breast cancer is affected by both environmental and genetic risk factors. Environmental factors are associated with such as diet/obesity, lack of exercise, alcohol consumption, using oral hormonal contraceptives, radiation (Stewart, 2014; Kaminska et al., 2015).

On the other hand, aging is one of the risk factors that increase the risk of most breast cancer between women age 55 and older. Beyond the risk of gender and aging concerning breast cancer, it is well documented that if a woman who has a first-degree relative (mother, sister or daughter) with a history of the disease are at increased risk. A small fraction (5-10%) of all breast cancers (Feng et al., 2018), are linked to inherited gene mutations. The most common gene mutations which cause the hereditary breast and ovarian cancers are BRCA1 and BRCA2. In many other genes in which germline mutations are associated with the risk of breast cancer development, are ATM, TP53, CHEK2, CDH1, PTEN, and more (Polyak, 2007; Davies et al., 2017; Feng et al.,2018).

2.2.3 Breast Cancer Treatment

Surgery, radiotherapy, endocrine (hormonal) therapy, chemotherapy, targeted therapy can be used for breast cancer treatment. Besides, immunotherapy was also used as a therapeutic option. The use of these options, depending on the type and location of cancer, such as tumor stage, hormonal receptor status, age, HER2 status, lymph node. Most breast cancers are treated with radiation therapy in two ways: primary or adjuvant. Adjuvant radiation therapy uses radiation with surgery but primary radiation therapy to treat by alone. Chemotherapy is a combination of drugs treatment and it can be given before or after surgery. The use of this treatment can be possible to stop the metastasis of cancer cells. Chemotherapy is also used before surgery, for large tumors (neo-adjuvant treatment) (Senkus et al., 2013, Peart, O., 2015; Curigliano et al., 2016). Doxorubicin is the most common chemotherapeutic drug used in breast cancer as well as many other cancer forms. Doxorubicin is an anthracycline with a variety of molecular mechanisms, involving topoisomerase II inhibition, DNA intercalation, free radicals production (Chen et al., 2017).

Humanized monoclonal antibody trastuzumab (Herceptin) drug can be used in the treatment of HER2 (+) positive or HER2 over-expressing breast cancer patients (Curigliano et al., 2016). Poly (ADP-ribose) polymerase (PARP) inhibitors, is a new cancer therapy that is given to patients with BRCA1/2 mutations (Rafii et al., 2017). PARPs create a large family of 18 proteins involved in single-stranded DNA breaks

through base excision repair (BER). The inhibition of PARP causes single-strand breaks that will eventually turn into double-strand breaks. In normal cells, DNA double-strand breaks (DSB) are repaired by mechanisms including BRCA1/2 dependent homologous recombination repair (HHR). PARP inhibitors are used as a therapeutic strategy for homologous recombination repair dysfunction. PARP inhibition leads to cell death in HRR-deficient cells. The PARP inhibitors also are known as 'synthetic lethality', are related to the tumor suppressor and DNA repair genes of BRCA1 and BRCA2. Synthetic lethality is the most effective when it causes dysfunctioning of both BER and homologous recombination (HR) repair mechanisms, whereas, individual disruption is not enough for causing tumor cell death (Nijman, 2011; Burgess and Puhalla, 2014). Olaparib is the most known PARP-inhibitor drug using for treatment of the breast and ovarian cancers (Miller and Ledermann, 2016; Murata et al., 2016). However, early detection of cancer still provides the best treatment options and the highest survival rate.

2.3 Epigenetics and DNA Methylation

The term "epigenetics" refers to the heritable alterations of genes without any changes in the sequence of the DNA (Weinhold, B., 2006). Besides, disordered epigenetic gene regulation causes important human diseases such as cancer. Epigenetic mechanisms include a variety of gene regulatory events such as DNA methylation, histone modifications, and noncoding ribonucleic acids (RNAs). The most studied epigenetic mechanism is DNA methylation. It involves the covalent binding of a methyl group (-CH3) to the 5 positions of the pyrimidine ring of a cysteine (C) nucleotide within CpG dinucleotides (the prefers to the phosphodiester bond between the nucleotides). This reaction is regulated by the DNA methyltransferase (DNMT) enzyme family (Handy et al., 2011; Jin et al., 2011; Kanwal and Gupta, 2012; Sawalha, 2017).

The term CpG islands (CGIs) are short regions containing a high-density CG sequence that controls the expression of human genes (known as promoters) (Illingworth et al., 2010). CpG islands are estimated to amount to 1-2% of the mammalian genome. In a genome, a lack of methylation of CpG is conditional on the persistence of CpG dinucleotides (Hisano et al., 2003). In carcinogenesis, hypomethylation and

hypermethylation play a participative role such as transcriptional silencing. In a normal transcribed gene generally have unmethylated promoter regions (Petr et al., 2008). Several molecular mechanisms can lead to the silencing of genes by DNA methylation. One of the mechanisms that involve, directly blocks the binding sites of the transcription factors by methylation. A second mechanism proposes that methylation attracts proteins that specifically bind hence, block the access of other factors required for gene expression (Curradi et al., 2002).

DNA methylation is known to be essential for several processes like regulating tissue-specific gene expression, genomic imprinting, inactivation of the X chromosome (Brenet et al., 2011; Moore et al., 2012).

2.3.1 The Role of DNA Methylation in Cancer

Aberrant DNA methylation, like hypo-hyper methylation, is widely related to cancer development. The activation of protooncogenes or the inactivation of tumor suppressor genes are the genetic models of cancer that may lead to malignant status (Wajed et al., 2001).

Aberrant hypermethylation in a specific promoter region is one of the most common ways of silencing tumor suppressor gene expression, open the way for the progression of cancer. Aberrant methylation is found regularly in breast cancers with more than 40 genes, including CDH1, BRCA1, RASSF1A, APC, CyclinD2 are frequently methylated (Cho et al., 2010). The focus in this project is on methylation in the promoters of BRCA1, BRCA2, CDH1, APC and RUNX3.

2.3.2 Detection of Methylation

The detection of DNA methylation has numerous methodological approaches, including methylation-sensitive restriction enzyme digestion (MSREs), methylation specific (MSP/USP) PCR, pyrosequencing and methylation-specific NGS (Delpu et al., 2013; Krygier et al., 2016). MSREs is a method that is used to analyze the methylation status of cytosine residues in CpG sequences. They separate among methylated and unmethylated alleles. When unmethylated DNA is digested by MSREs, the methylated DNA is one and only amplification products that are detected. The MSP/USP PCR is the first and most common susceptible method to apply the detection of methylation in tissues and body fluids (Cottrell et al., 2014). This method uses primer sequences designed to identifying methylated or unmethylated DNA sequences via sodium bisulfite. (Sant and Goodrich, 2018). The sodium bisulfite is used to the conversion of all unmethylated cytosines to uracil while leaving methylated cytosines unchanged (Cottrell et al., 2004). A drawback of this method is that the amplification of unconverted bisulfite DNA could give false-positive results (Murgatroyd, 2014). The methylation-specific PCR (MSP) is the qualitative analysis and it is generally used for the detection of DNA methylation but it is unsuited for the clinical setting. Despite this, pyrosequencing is a quantitative MSP analysis and it might be more suitable for clinical detection (Hu and Liu., 2017). Also, pyrosequencing uses bisulfite converted DNA. This technique is rapid, cost-effective, highly sensitive and easily standardized (Delpu et al., 2013).

Nowadays, the technique that greatly increases sensitivity and resolution in preclinical and clinical epigenetic studies, called as NGS (Hu and Liu., 2017). This technique, which is rapidly expanding to the clinical environment in oncology, can provide great benefit to many patients for diagnosis and/or treatment selection (Petrackova et al., 2019). It allows a large analysis of the methylation status of almost any CpG site and the creation of genomic maps of DNA methylation in a single basic resolution (Barros-Silva et al., 2018). These approaches which have been developed and continue to be developed today will open the way for studies that quantify DNA methylation patterns and differences in DNA methylation (Masser et al., 2015).

2.4 Breast Cancer Gene 1 (BRCA1)

BRCA1 was first identified in 1990 and isolated in 1994 (Hall et al., 1990, Xu et al., 1997; Clark et al., 2012). The gene is a tumor suppressor gene located on chromosome 17q12-21 (Figure 2.3), contains 22 exons and encodes a large protein of 220 kDa, consisting of 1863 amino acids (Ashworth, 2001; Godet and Gilks, 2017).

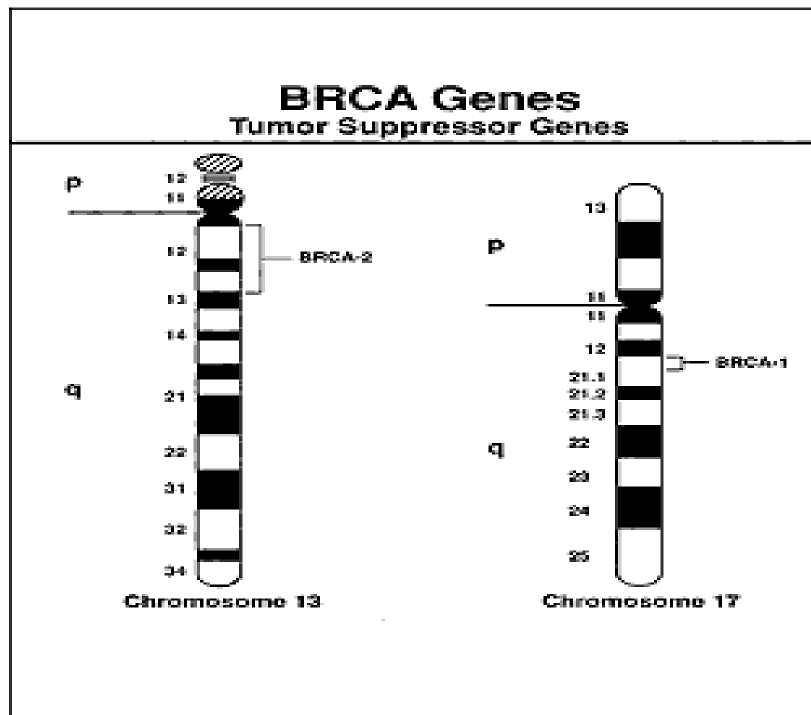

Figure 2.3: The structure of two different genes BRCA1 and BRCA2. They are both play a role as tumor suppressor genes. (Fleming, R.M. and et al. (2017)).

2.4.1 BRCA1 Protein Domains and Function

The BRCA1, a versatile protein, is often mutated in three functional domains that include, the N-terminal RING domain involved in heterodimerization of BRCA1/BARD1, allowing that E3 ubiquitin ligase activity, exons 11-13 is encoded the central part of BRCA1 and these regions include two nuclear localization sequences (NLS), one coiled-coil domain which is essential for interaction with BRCA2 through partner and localizer of BRCA2 (PALB2) along with a serine containing domain (SCD) that is the region for

phosphorylated by ATM (Figure 2.4). BRCA1 C-terminal domain (BRCT) is divided into two categories that responsible for phospho-protein binding (Figure 2.4) (Rohini et al., 2011; Clark et al., 2012, Gorodetska et al., 2019). BRCA1 phosphorylation takes place as a part of the subcellular localization of the protein (Scully et al., 1997; Scully and Livingston, 2000; Brodie and Henderson, 2010). Through its ability to interact with a large range of different protein complexes which participates in several cellular processes including, cell cycle regulation, protein ubiquitination, chromatin remodeling, transcriptional regulation, mRNA splicing, apoptosis, maintenance of genome integrity, DNA damage signaling, DNA repair through homologous recombination (HR) (Savage and Harkin., 2015).

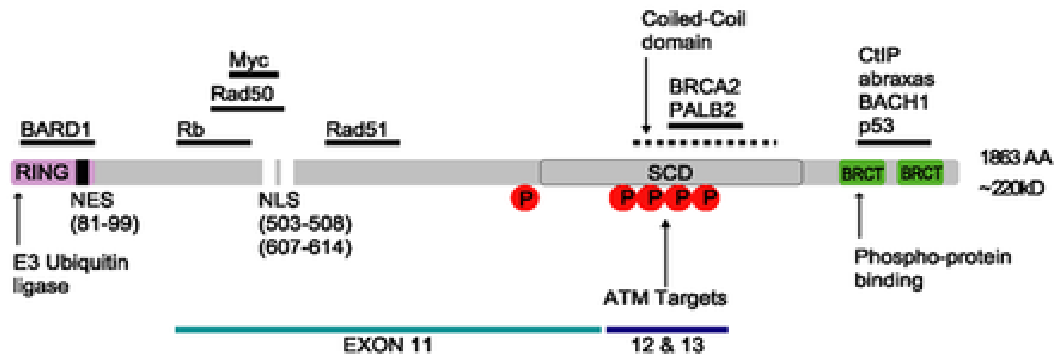

Figure 2.4: Functional domains of BRCA1 protein (Alwosaibi, K. (2016).

DSB is mediated to be one of the perilous types of DNA damage and can be repaired by two major pathways called homologous recombination (HR) and non-homologous end-joining (NHEJ). It is approved that BRCA1 plays an essential role in HR as shown in figure 2.3 (Rohini et al., 2011).

DSB has sensors, effectors and mediators, that have different functions include detecting broken ends, executing repair and facilitating interactions between sensors and effectors, respectively. BRCA 1 plays a primer role as a mediator in the DSB repair mechanism and it binds to several proteins and allows the involvement of molecules related to HR. Phosphorylation of histone H2AX is the first response to DSB which initiates a series of

functions leading to binding of complex abraxas, RAP80 and BRCA1. BRCA1 is important for strand resection by interactions with CtIP and MRN complex (MRE11-RAD50-NBS1). Moreover, BRCA1 interacts with the PALB2-BRCA2 complex which causes RAD51 mediated HR by the invasion of a single strand. The BRCA1-BRIP1-TOPBP1 complex is associated with DNA repair but the exact mechanisms are unknown (Rohini et al., 2011; Savage and Harkin, 2015) (Figure 2.5).

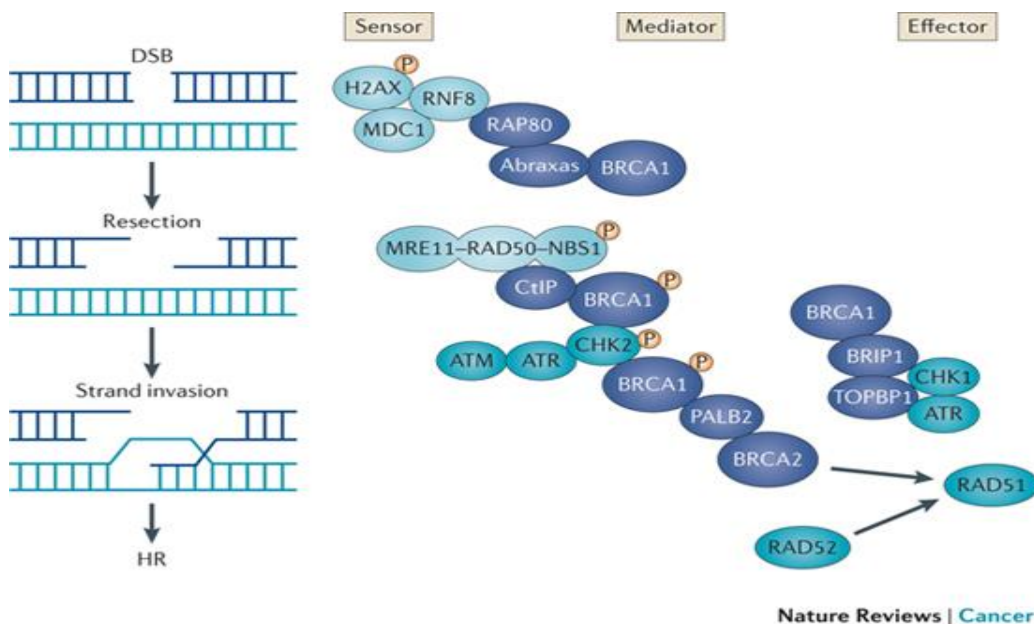

Figure 2.5: The role of BRCA1 in HR. BRCA1 is involved in DSB repair. DSB are detected by sensors (light blue) which lead to a cascade of reactions and the recruitment of BRCA1, which is considered a mediator (dark blue). BRCA1 is involved in recruiting molecules, involved in HR repair. BRCA1 bound to relevant complexes, is involved in both resection and strand invasion by binding to various effectors (turquoise) (Roy, R. and et al. (2016)).

2.5 Breast Cancer Gene 2 (BRCA2)

BRCA2 was discovered in 1995 (Filippini and Vega, 2013). This gene, which is a tumor suppressor gene like, BRCA1 gene. It is mapping on chromosome 13q12-q13, contains 27 exons and encodes 384 kDa of protein, containing 3418 amino acids (Bieche et al., 1999, Guenard and Durocher, 2010).

2.5.1 BRCA2 Protein Domains and Function

BRCA2 has the primary function in HR. BRCA2 is a central mediator of DSB by HR. BRCA2 includes a DNA binding domain and eight BRC repeats. The DNA binding domain (DBD) is a site for binds single-stranded DNA (ssDNA) and double-stranded DNA and the eight BRC repeats site that binds RAD51. BRCA2 plays an essential role in controlling the function and localization of RAD51, during HR (Moynahan and Jasin, 2010; Roy et al., 2012). The DBD contains five items: a 190 amino-acid α-helical domain, three oligonucleotide binding (OB) folds and a tower domain (TD). These oligonucleotide binding (OB) folds are ssDNA binding modules, however, TD that bulges form oligonucleotide binding 2 (OB2) and binds dsDNA (Yang et al., 2002; Roy et al., 2012) (Figure 2.6). The helical domain, OB1 and OB2 interact with the deleted in split hand/split foot protein (DSS1). However, the mechanism by which DSS1 participates in the BRCA2 function remains unclear (Li et al., 2006).

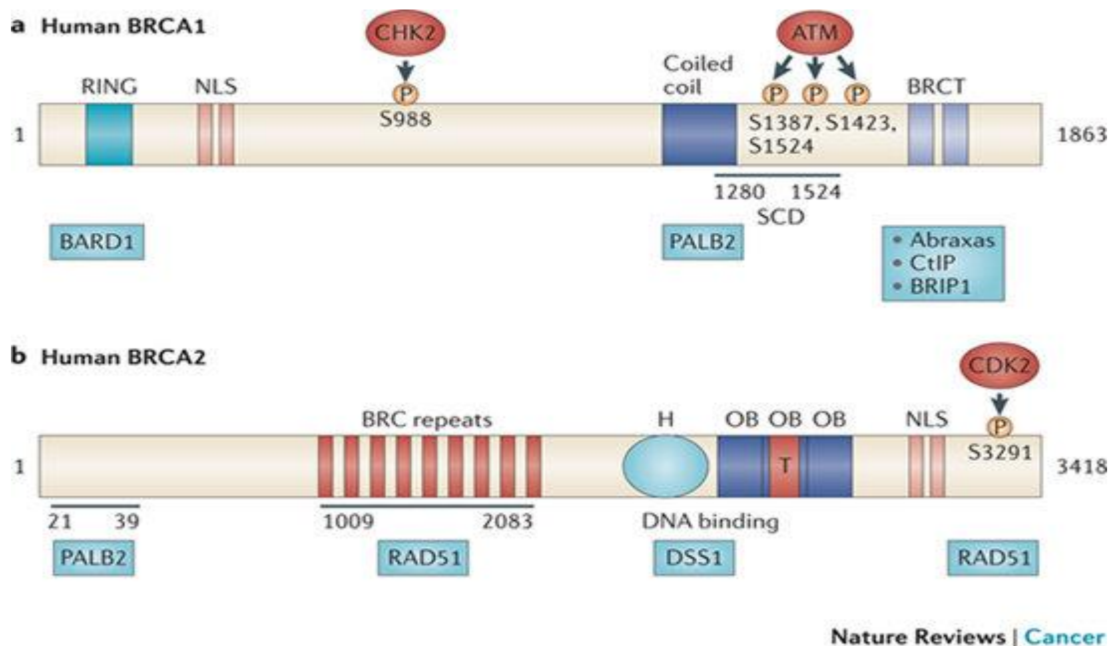

Figure 2.6: Functional Domains of BRCA1 and BRCA2.(Roy, R. And et al. (2016)

2.6 The Adenomotous Polyposis Coli Gene (APC)

The APC gene is a tumor suppressor gene. Mutations in this gene are responsible for both hereditary (familial adenomatous polyposis-FAP) and sporadic colorectal cancers but have also been described in several epithelial cancers such as breast cancer (Dihlmann et al., 1999, Lesko et al., 2014). The gene is located on chromosome 5q21-q22 and contains 8535 nucleotides and 21 exons (Groden et al.,1991, Nıshısho et al.,1991). The APC gene encodes a 310 kDa protein (Zhang and Shay, 2017). This multifunctional protein consists of 2843 amino acids and multiple binding domains (Lesko et al., 2014). APC gene is expressed in most tissues such as the lung, liver, kidney and mammary gland (Furuuchi et al., 2000).

2.6.1 APC Protein Domains and Function

APC is a multi-domain protein which has multiple functions and contains binding partners for numerous protein. These domains of APC include: an oligomerization domain, an armadillo repeat-domain, a 15- or 20-residue repeat domain, SAMP repeats domain, a

basic domain and C-terminal domains, from the N terminus to the C terminus (Figure 2.7) (Aoki and Taketo, 2007; Zhang and Shay, 2017). The proteins which it contains as follows: microtubules, the Wnt/Wg pathway components -catenin and axin, the cytoskeletal regulators EB1 and IQGAP1, and the Rac guanine-nucleotide-exchange factor (GEF) Asef1 (APC stimulated guanine nucleotide exchange factor) (Aoki and Taketo, 2007). The functions of the APC protein areas include the Regulation of β-catenin, cytoskeleton organization, putative neuronal role, putative involvement in apoptosis and cell-cycle control and classical FAB (Sieber et al., 2000). The best-known function of APC is interaction with β-catenin in the cytoplasm. Levels of β-catenin are modulated with APC functions, in the Wnt signaling pathway (Watanabe et al., 2004). Most APC mutations occur in the site of the mutation cluster region (MCR) and resulting in the C-terminal truncation of the protein. These truncations induce loss of the domains which is essential for binding to β-catenin. The APC –β catenin interaction is required for its tumor suppressor activity (Aoki and Taketo, 2007). In breast cancer, mutations of the APC that regulate Wnt signaling pathway and cause in the accumulation of cytosolic and nuclear β-catenin. Mutations in the MCR, leading to allelic loss are often found in colorectal cancers. Interestingly, most APC mutations have also been identified with sporadic breast cancer occur outside the MCR and its function independently of the Wnt pathway to lead cancer progression (Lesko et al., 2014). Promoter methylation is the most common method of APC inactivation in breast cancer.

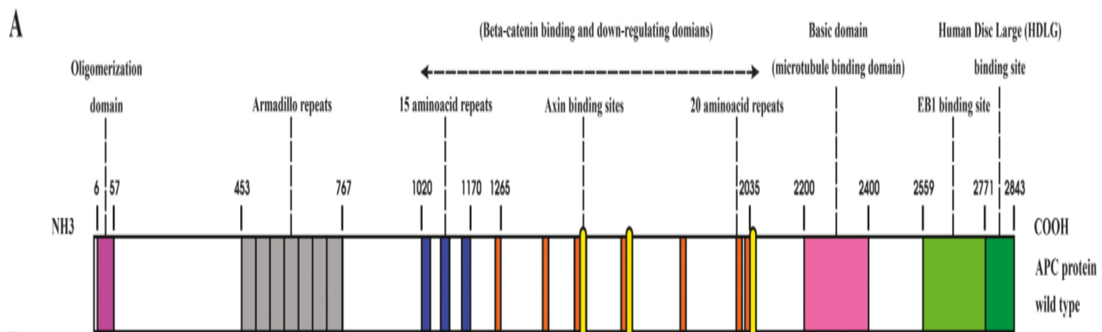

Figure 2.7: The APC protein structure with functional domains (Pouya, et al. (2018))

2.7 E Cadherin Gene 1 (Epithelial Cadherin-CDH1)

Cadherins include a large family of cell surface glycoproteins that mediate calcium ions ($Ca2+$) dependent homophilic cell to cell adhesion interactions. The cadherin family consists of five major subfamilies: classical cadherins of type I, closely related cadherins of type II, desmosome cadherins (desmocollins and desmogleins), protocadherins, and a variety of cadherin-related molecules (Halbleib and Nelson, 2006; Van Roy and Berx, 2008). E-cadherin, a type 1 classical cadherins, is a tumor suppressor gene that encodes a 120 kDa glycoprotein with a large extracellular domain. It is located on chromosome 16q22.1. E-cadherin is modified in many cancers, such as breast cancer. However, E-cadherin is expressed in all of the mammary epithelial cells (Andrews et al., 2011; Liu et al., 2016).

2.7.1 CDH1 Protein Domains and Function

Classical cadherins were first identified cadherin family which is divided into two different subtypes (Type I and Type II). Type I classical cadherins are segregated by embryonic germ layer or tissue type and they include epithelial (E), neuronal (N), placental (P) and retinal (R) cadherins are expressed in the mammary gland (Patel et al., 2006; Andrews, Kim and Hens, 2012). E-cadherin is a member of type I classical cadherins, thus its contain three major domain: a large extracellular domain, described as extracellular cadherin repeats 1-5 (EC1-EC5) (beginning with the N-terminus of the protein), a single transmembrane and a short cytoplasmic domain. (Pecina-Slaus., 2003). The E-cadherin protein is a calcium-dependent cell-cell adhesion molecule expressed in adherents junctions between epithelial cells (Figure 2.8). The cytoplasmic domain is associated with the cytoplasmic protein catenins (alpha, beta and gamma catenins), which are intermediate linkers between the cadherins and actin filaments. The cadherin-catenin complex that is essential for providing normal cell-cell adhesion. The function of the cytoplasmic domain is bound to the actin cytoskeleton via these intracellular linkers protein, the catenins. Extracellular domain structure of classical E-cadherin includes five tandem repeats and these repeats contain the sites with the adhesive activity of the biggest part of N-terminal. The extracellular cadherin domains have a

function of homophilic recognition and binding in itself (Ivanov et al., 2001; Pecina-Slaus., 2003).

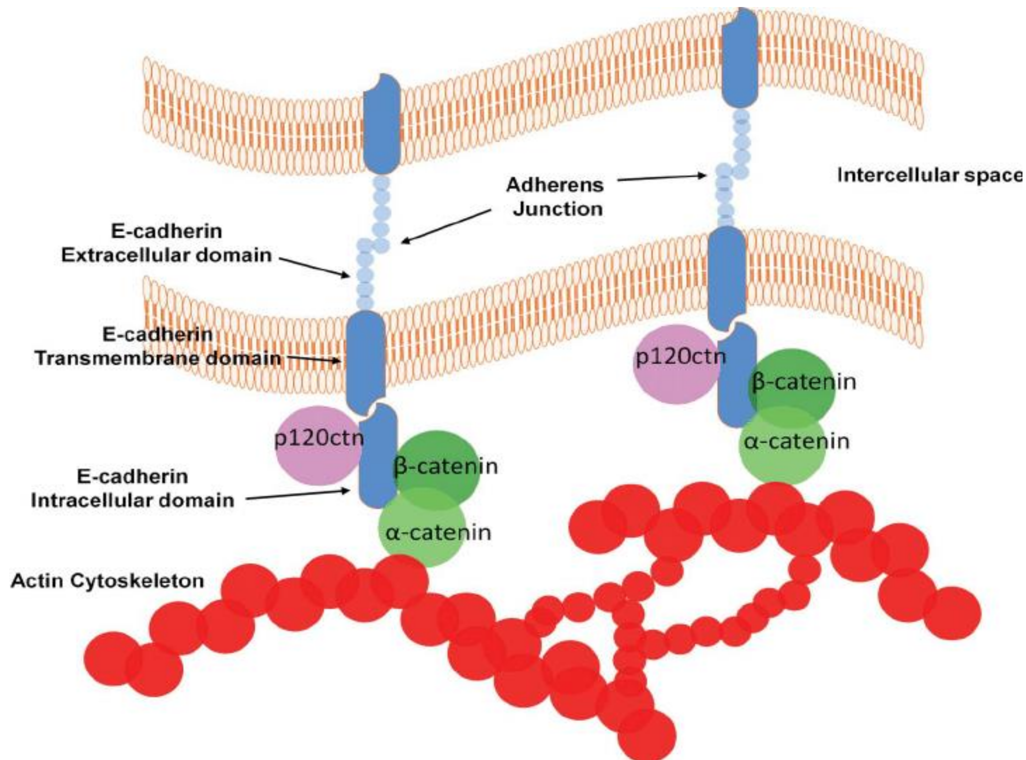

Figure 2.8: The interface of the E-cadherin mediating cell-cell adhesion. (Gall, T.M.H. and Frampton, A.E.F (2013)).

2.8 The Runt Related Transcription Factor 3 Gene (RUNX3)

The RUNX gene has a large family, including RUNX1, RUNX2, RUNX3. The RUNX3 gene was first identified as a tumor suppressor gene in gastric cancer. It is located at the short arm of chromosome 1 at 1p36. The gene that plays an important regulatory effect on the proliferation, growth and apoptosis of cells. However, genomic loss in this region leads to different human cancers, such as stomach, breast and lung (Hwang et al., 2007, Liu et al., 2018). The downregulation of the Runx3 is being controlled by several mechanisms such as promoter region hypermethylation, loss of heterozygosity, hemizygous deletion and mutation. These mechanisms are shown to be related to the carcinogenesis of human solid tumors (Hwang et al., 2007).

2.8.1. RUNX3 Protein Domains and Function

The RUNX family members demonstrate homology in a 128-amino-acid region called as the RD. This region directs connecting to RUNX proteins to DNA and mediates their interaction with the protein core-binding factor-β (CBF-β). The RD involves that contains three different RUNX proteins, RUNX1 (also called PEBP2aB/CBFA2/AML1), RUNX2 (PEBP2aA/CBFA1/AML3) and RUNX3 (PEBP2aC/CBFA3/AML2), which are transcriptional regulators. All of the three RUNX proteins play an important role in cancer development (Ito., 2004; Kim et al., 2004; Duncan et al., 2008). The CBFs are heterodimeric transcription factors containing a DNA-binding α-subunit and a non-DNA-binding β-subunit. These DNA-binding α-subunit encoded by one of three members of the RUNX family (Warren, 2000). The RD in which related to RUNX3 comprises the N-terminal part of the molecule and has an S-type immunoglobulin fold (Bangsow et al., 2001).

3. MATERIAL AND METHODS

3.1 Collection of Samples and Ethical Approval

In this study, paraffin-embedded tumor tissues who have had a history of breast cancer were chosen and included. A total of 17 paraffin-embedded tissues were collected from the Near East Hospital Pathology Laboratory that the existence of tumor tissue was confirmed by Roche High Pure PCR Template Preparation kit (Ref: 11796828001). After used for nucleic acid isolation. Following the nucleic acid isolation, Epitech Bisulfite (Qiagen) kit was used for bisulfite conversions and Methyl Detect was used for DNA investigation of methylation status of BRCA1, BRCA2, APC, CDH1, RUNX3. Lastly, LightCycler 480 High-Resolution Melting Master kit (Ref: 04909631001) was used as a master mix for PCR. Following the bisulfite conversions, the concentration of the isolated DNAs' was measured with ATC Gene UVS-99 Nano-Drop. LightCycler 480 instrument was used for PCR.

Ethical approval for the study was obtained from the Near East University Scientific Research Assessment Ethics Committee (Project no: YDU/2019/71-861).

3.2 Nucleic Acid Isolation

Isolation of Nucleic acids from Formalin-Fixed Paraffin-Embedded Tissue Sections was done by using the Roche High Pure PCR Template Preparation kit (Ref: 11796828001) according to the manufacturer's instructions.

3.3 Bisulfide DNA Conversion

DNA was isolated from the paraffin-embedded tissues for methylation analysis as bisulfite conversion has been done as following the Bisulfide Mix. The sample was prepared by adding 800 µl RNase-free water to each aliquot then vortexed 5 seconds until the Bisulfide Mix was completely dissolved. Sodium Bisulfide Conversion of Unmethylated Cytosines in DNA from Low-Concentration Solution was carried out according to the manufacturer's instructions (Table 3.1). Then, it mixed the bisulfide reactions and stored

at room temperature (15-25°C) and used a thermal cycler for performed the bisulfite DNA conversion (Table 3.2).

Table 3.1. Bisulfide reaction components

Component	**Volume per reaction(µl)**
DNA solution (1-500 ng)	Variable* (Maximum 40 µl)
RNase-free water	Variable*
Bisulfite Mix(dissolved)	85
DNA Protect Buffer	15
Total Volume	**140**

(* The combined volume of DNA solution and RNase-free water must total 40 µl)

Table 3.2. Bisulfide conversion thermal cycler conditions

Step	**Time**	**Temperature**
Denaturation	5 min	95 °C
Incubation	25 min	60 °C
Denaturation	5 min	95 °C
Incubation	85 min(1 h 25 min)	60 °C
Denaturation	5 min	95 °C
Incubation	175 min (2 h 55 min	60 °C
Hold	Indefinite*	20 °C

(* Converted DNA can be left the thermal cycler overnight without any loss of performance).

3.4 Polymerase Chain Reaction (PCR) and HRM PCR

The MethylDetect DNA Methylation Assay Kit was used for analysis to investigate the methylation status of BRCA1, BRCA2, RUNX3, CDH1, APC. Amplification reactions carried out by using the LightCycler 480 system. Amplification reactions were performed in a 20 µl volume containing 10 µl of HRM Master 2x conc, 1.0 µl of Primer mix, 2.4 µl of MgCl2 (25mM), 0.6 µl of H2O (PCR grade) and 6 µl of the bisulfide treated DNA (50-100 ng) (Table 3.3). The HRM program is suitable for the LightCycler 480 System (Table 3.4)

Table 3.3. The List of PCR Materials

COMPONENT	VOLUME
HRM Master 2 x conc.	10 µl
Primer Mix	1.0 µl
$MgCl_2$ (25mM)	2.4 µl
H_2O (PCR grade)	0.6 µl
Bisufide Treated DNA	6.0 µl
Positive Control	6.0 µl
Calibration Control	6.0 µl
Negative Control	6.0 µl

Table 3.4. The HRM thermal cycler conditions

Program	Cycles	Temperature (°C)	Hold (sec)	Ramp Rate (°C/sec)	Acquisitions (per °C)
Pre-Incubation	1	95	600		
Amplification	50	95 58* 72	15 10 15	4.4 2.2 4.4	None Single None
High Resolution Melting		95 60 95	15 60 Continous	4.4 2.2 0.01	None None 50

* The optimal annealing temperature for each MethylDetect DNA Methylation Assay can be different.

4. RESULTS

This study conducted from February 2019 until March 2020 for the optimize the methyldetect method for identify methylation profile of the following genes BRCA1, BRCA2, APC, RUNX3, CDH1 in breast cancer samples.

A total of 17 paraffin-embedded tissues were collected from the Near East University Hospital Pathology Laboratory who have had a history of breast cancer. Among 17 tissues samples, 10 samples were studied as invasive ductal carcinoma (IDC), 5 samples ductal carcinoma in situ (DCIS) and 2 samples normal (non-cancerous tissues).

4.1 Evalution of BRCA1, BRCA2, APC, CDH1 and RUNX3 gene methylation status:

Optimization and analysis of this study was done with LightCycler 480 system, high resolution melting (HRM) based gene scanning. For HRM-based gene scanning, the optimal temperature and suitable primer concentration were selected to obtain PCR products with effiecient amplification and a satisfactory melting profile. LightCycler 480 Gene Scanning 1.5 Software was used to analyze to the normalized and difference melting curves for the methylation status of patients.

When using PCR for methylation studies, all DNA samples have to be bisulfite so that the methylated cytosines in the template are preserved. Following the bisulfite treatment, unmethylated cytosines are converted to uracil while the DNA sequence and methylated cytosines remain unchanged. After PCR, products have different melting profile after HRM will allow us to distinguish between methylated and unmethylated (Figure 4.1).

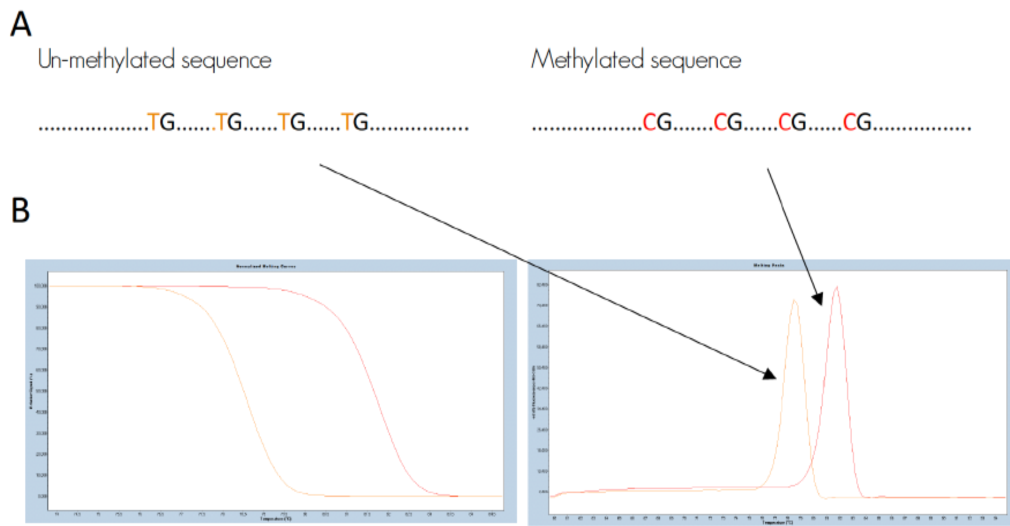

Figure 4.1: The principle of the behind HRM analysis. A) the difference between methylated genomic region and unmethylated genomic region after bisulfite conversion. B) the difference in melting properties of the PCR products. The methylated template (red) and the unmethylated template (orange). (Copyright from MethylDetect DNA methylation assays).

The melting temperatures of the control genes that has been used for optimization in these study should be as following respectively, for negative control 80 $C^{0\,\pm}$, for calibration control 82 $C^{0\,\pm}$, for positive control 83 $C^{0\pm}$ (Figure 4.2) and normalized melting curves in expected results are shown in the figure 4.3.

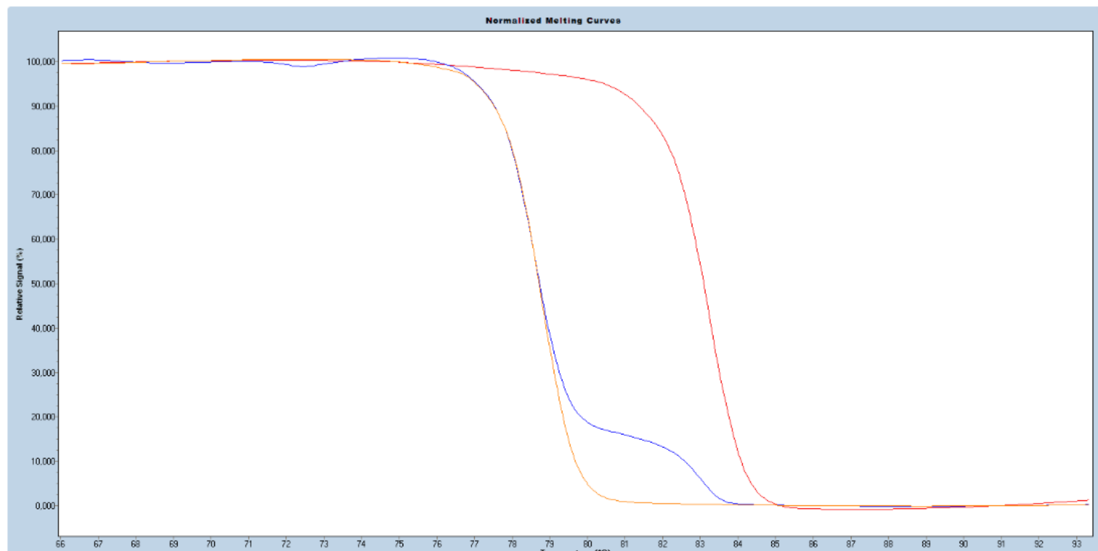

Figure 4.2: Normalized melting curves for all genes (BRCA1, BRCA2, RUNX3, APC and CDH1) controls. Positive control (Red), the assay calibration control (blue) and negative control (orange). (Copyright from MethylDetect DNA methylation assays).

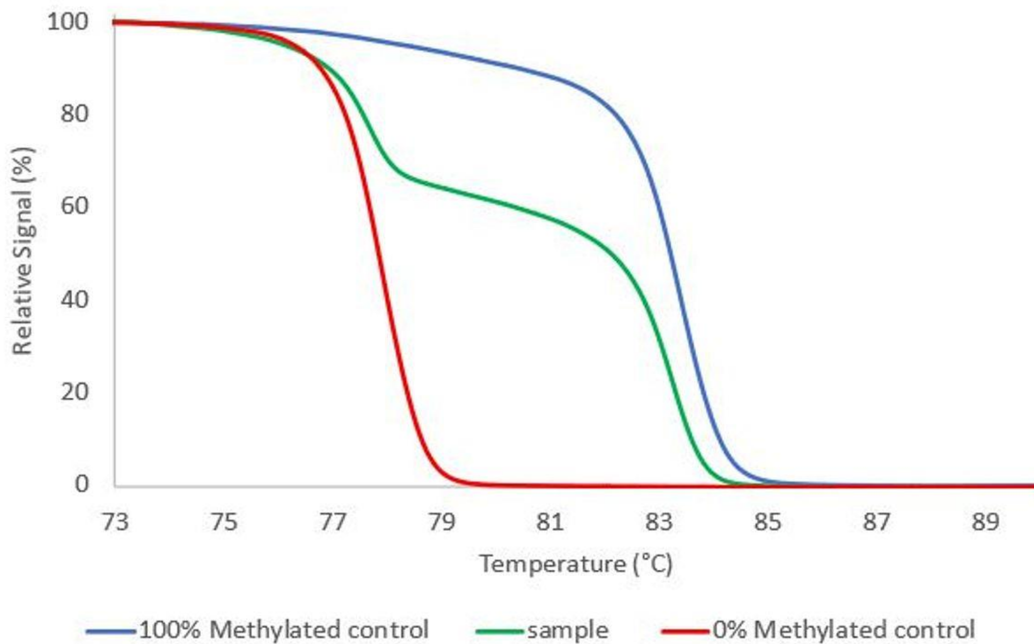

Figure 4.3: Normalized melting curves, illustrating unmethylated (red), sample (green), methylated (blue) (Copyright from MethylDetect DNA methylation assays).

The results obtained are as follows,

Based on the above mentioned standards first we investigated BRCA1 gene, it has been observed that, all negative, positive and calibration controls give a melting curve at the same temperatures. Optimally, the negative control is expected to give a melting curve before the positive control. These results suggested that the controls did not operate at the expected temperature (Figure 4.3) needs further optimization.

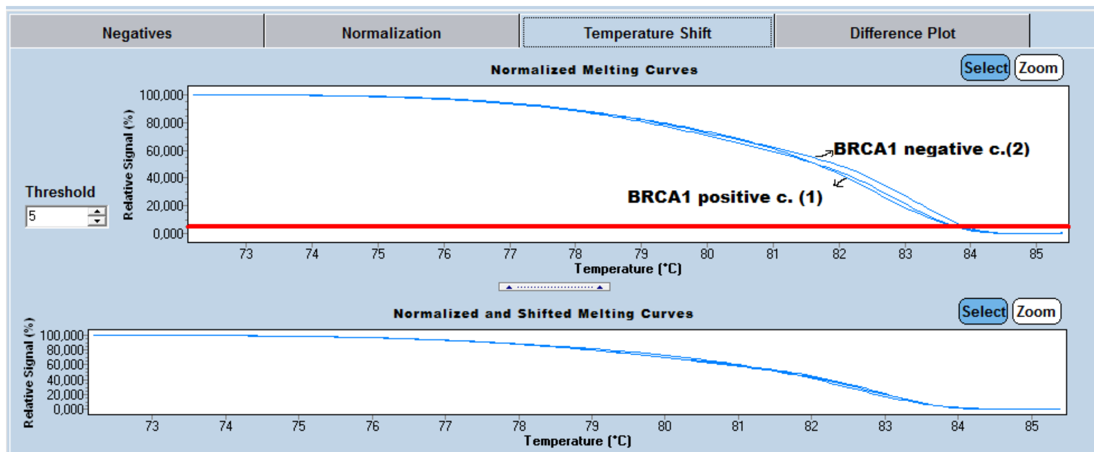

Figure 4.4: Controls of BRCA1 (Positive, Calibration, Negative).

(1) Show the Positive Control of BRCA1

(2) Show the Negative Control of BRCA1

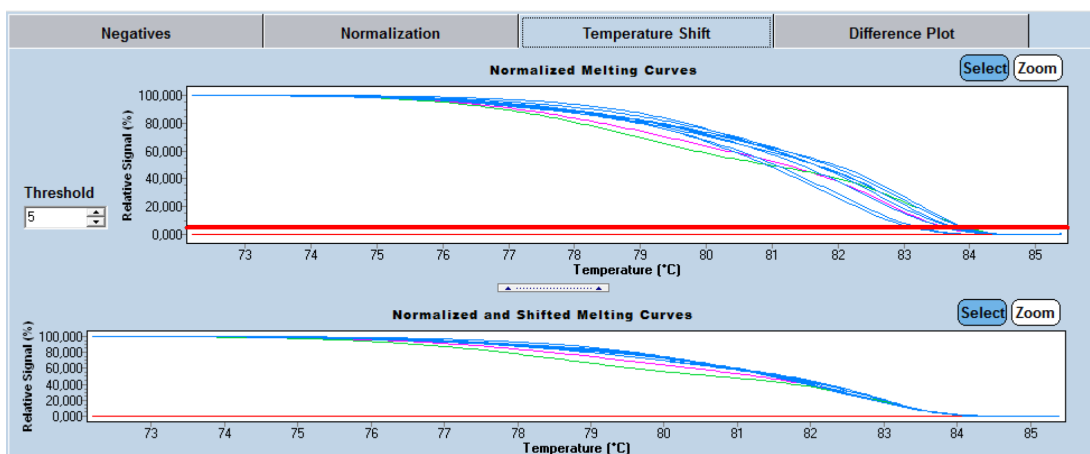

Figure 4.5: Both of BRCA1 samples and controls

Simillarly BRCA2, look the same as for BRCA1. Perhaps simly due to the optimal annealing temperature is the same for gene both. Annealing temperature, which is the optimal binding temperature of the primers, is different for each PCR. When we evaluate the annealing temperature of a MethylDetect DNA Methylation Assay Kit protocol for BRCA1 and BRCA2 is 55-57 C^0 (Table 3.4) given results suggest that the system has not been optimally worked this could be explained either this system has not been fully optimized to the given PCR machine or the kit and/or primers are not effective (Figure 4.3).

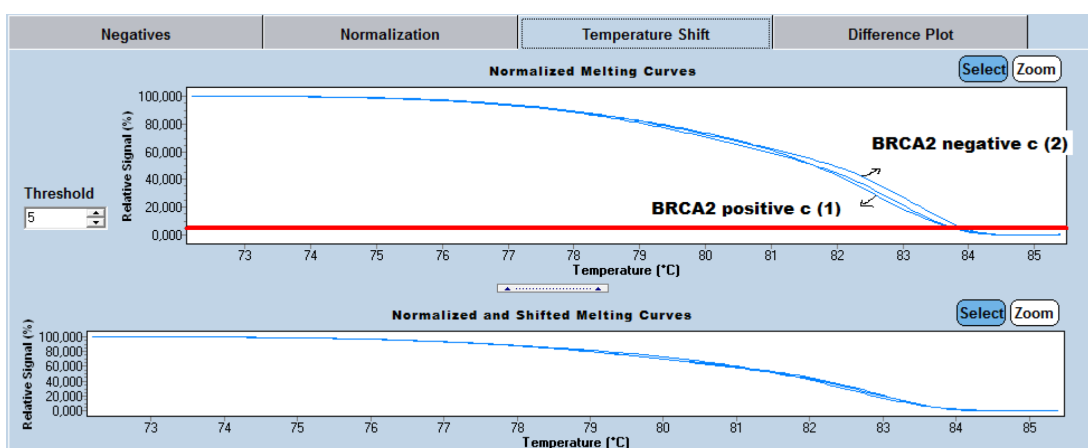

Figure 4.6: Controls of BRCA2 (Positive, Calibration, Negative)

(1) Show the Positive Control of BRCA2

(2) Show the Negative Control of BRCA2

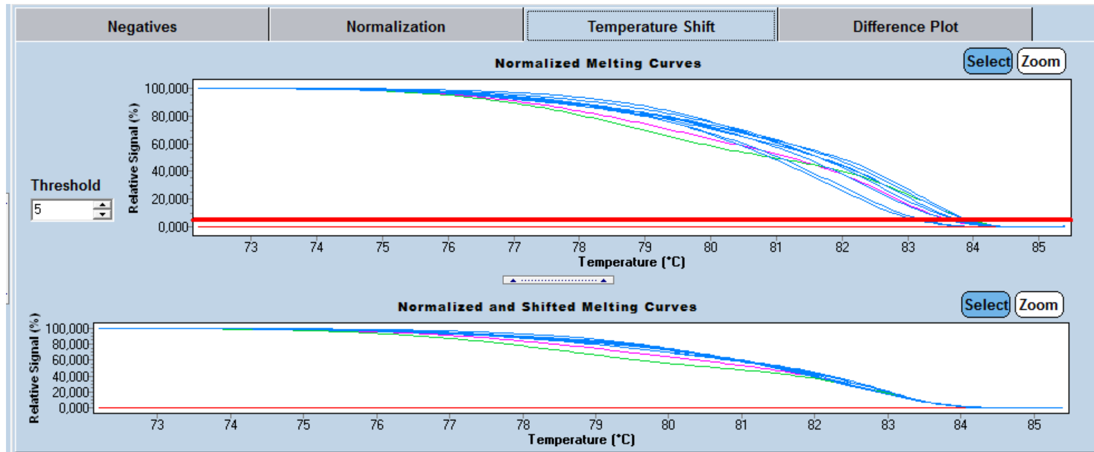

Figure 4.7: Both of BRCA2 samples and controls

In APC gene, again control samples did not run at the temperature they should worked. The negative control is expected to give a melting curve before the calibration control and positive control and the calibration control must also be between positive and negative controls(Figure 4.11). Calibration control give a melting curve before the negative and positive control (Figure 4.5). The optimal annealing temperature of a MethylDetect DNA Methylation Assay Kit protocol for APC is 64-66 C^0 (Table 3.4). This difference simply could be explained by either the calibration and positive controls quality were not correct simply due to the during transport conditions or wrong chosen but unfortunately we could not check.

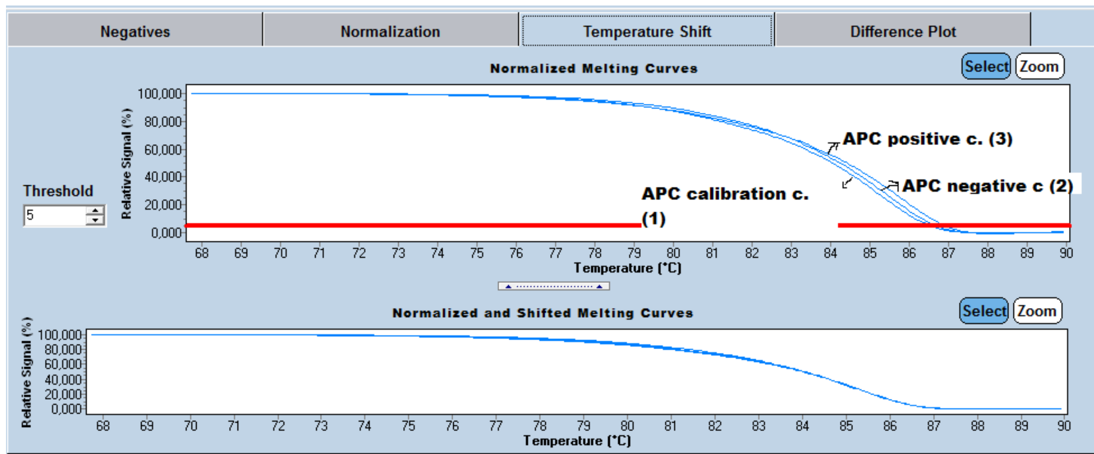

Figure 4.8: Controls of APC (Positive, Calibration, Negative)

(1) Show the Calibration Control of APC

(2) Show the Negative Control of APC

(3) Show the Positive Control of APC

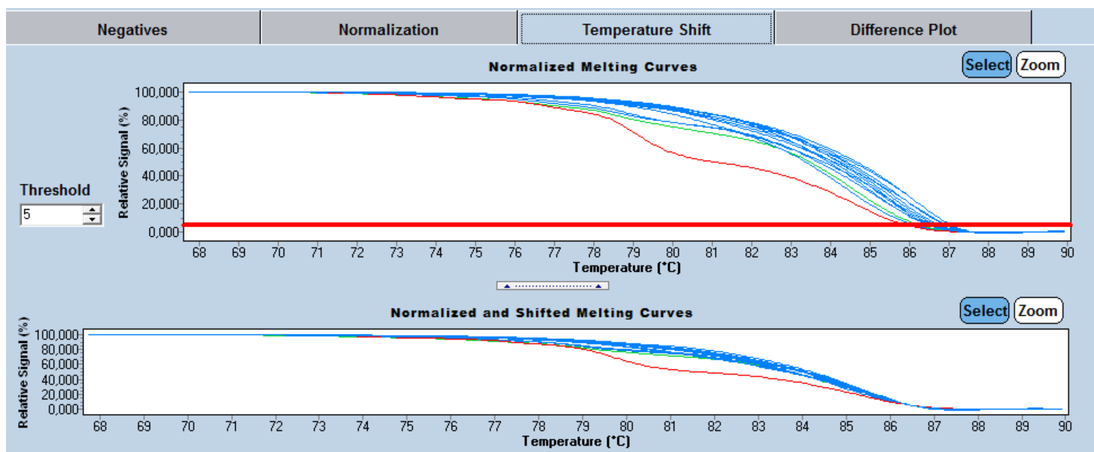

Figure 4.9: Both of APC samples and controls

In CDH1 gene, the results showed that, the melting temperatures of controls appear to lower than they shold be and it is seen that the melting curves of calibration control and positive control are opposite to each other (Figure 4.7). The optimal annealing temperature

of a MethylDetect DNA Methylation Assay Kit protocol for CDH1 is 61-63 C^0 (Table 3.4).

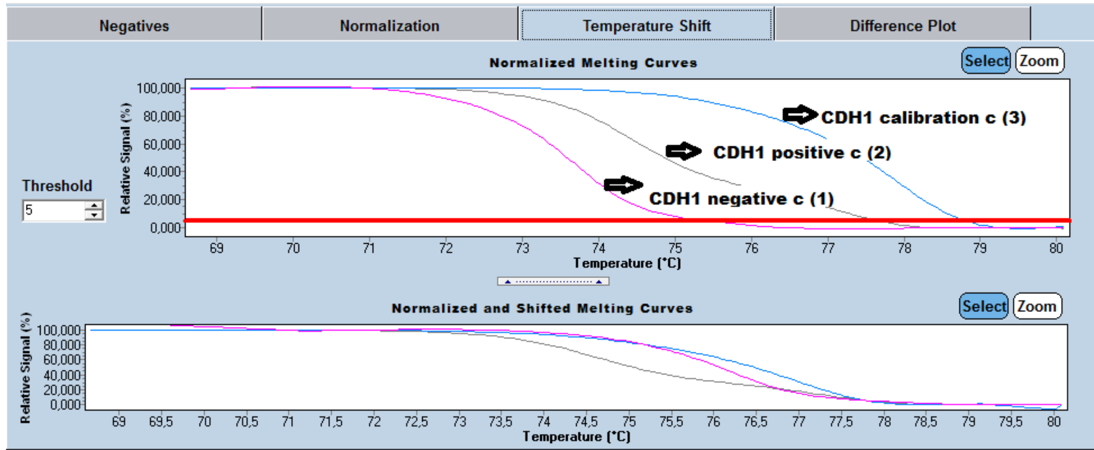

Figure 4.10: Controls of CDH1 (Positive, Calibration, Negative)

(1) Show the Negative Control of CDH1

(2) Show the Positive Control of CDH1

(3) Show the Calibration Control of CDH1

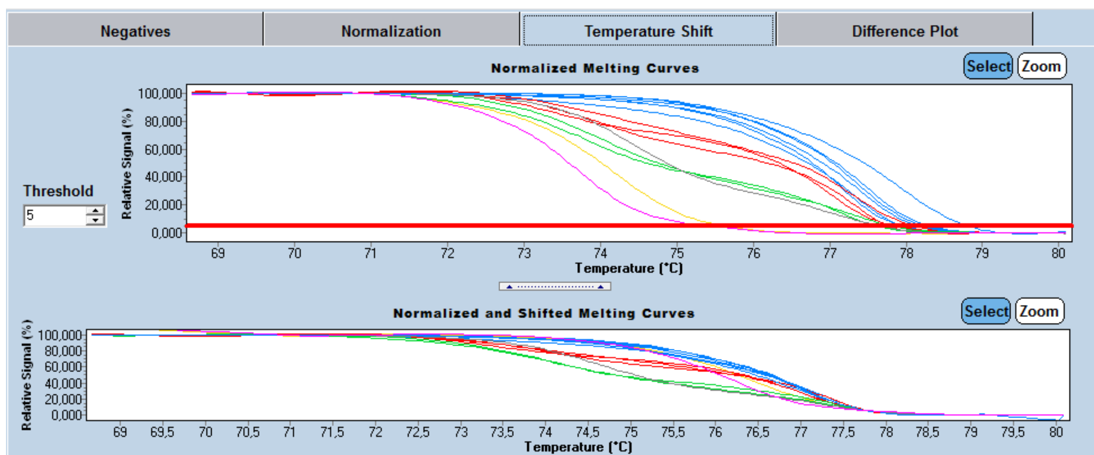

Figure 4.11: Both of CDH1 samples and controls

Results in RUNX3 gene indicate that the controls did not work. (Figure 4.9). The optimal annealing temperature of a MethylDetect DNA Methylation Assay Kit protocol for RUNX 3 is 54-56 C^0 (Table 3.4). These results could suggest that the primers either has dising problem or the optimal temperature has not been calculate correctly.

All patient samples that has been used as experimental samples nucleic acids were isolated under the same conditions and all PCRs were set up same conditions. Thus our results suggest that the samples that we studies in principle worked for BRCA1, BRCA2, APC, CDH1 but there is low amplification due to DNA isolation. The situation for RUNX3 is interpreted differently because the positive and calibration controls did not work and only two of the patient/experimental samples worked (Figure 4.9, Figure 4.10). Thus, a meaningful analysis could not be done.

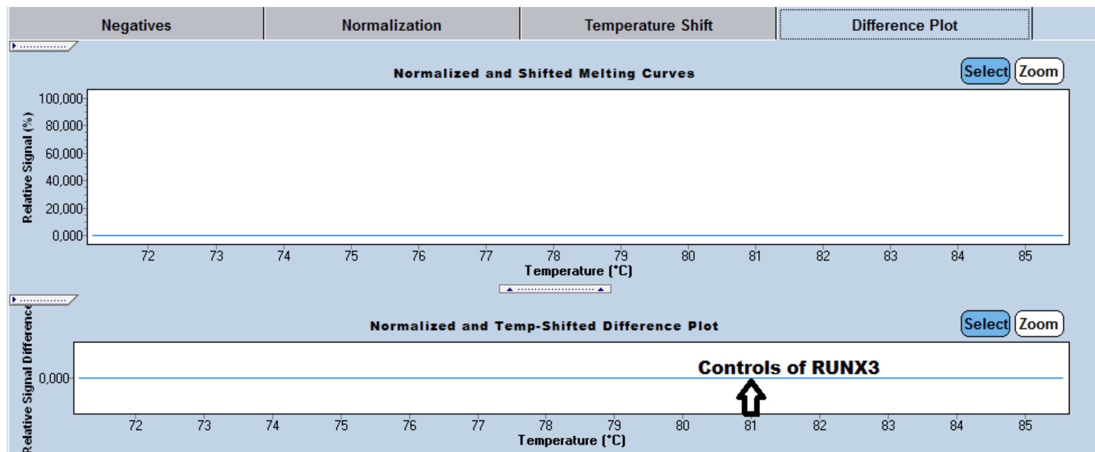

Figure 4.12: Controls of RUNX3 (Positive, Calibration, Negative)

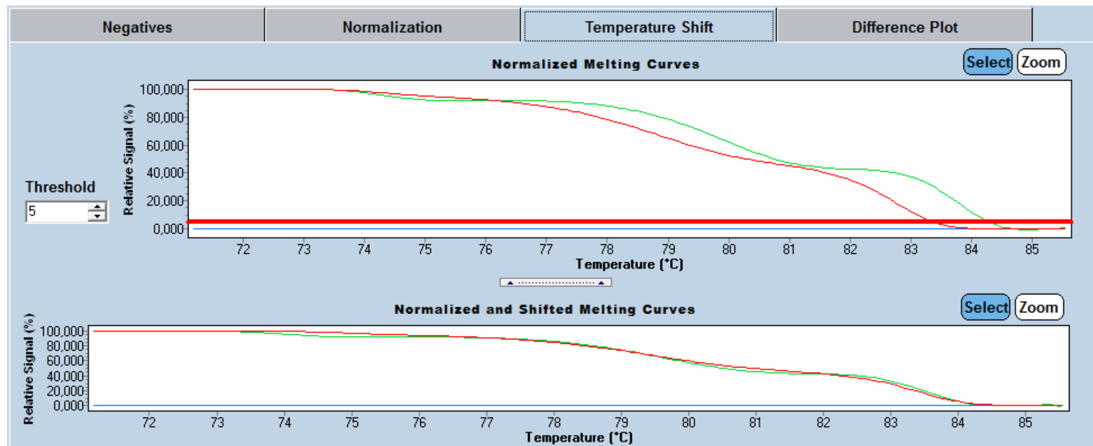

Figure 4.13: Both of RUNX3 samples and controls

In light of these PCR results, positive, negative, and calibration controls seemingly did not worked as expected at the required temperature at the BRCA1, BRCA2, APC, CDH1 and RUNX3 genes.

This could be perhaps explained by;

The optimal range of annealing temperatures are different for each instrument. This temperature which is special for each primer, affects the connection of the primers and thus working of the PCR. This may affected primer optimization.

The primers may have expired especially if they have been exposed to repetitive freezings and thawings.

A problem with amplification

It can be said that no meaningful results were obtained when these comparisons are made.

5. DISCUSSION

In women worldwide, the breast cancer has the highest incidence for in all the cancer types. It is estimated that 627,000 women died from breast cancer, in 2018. That is roughly 15% of all cancer deaths among women (Feng et al., 2018,). Breast cancer, has various risk factors that can be divided in to two groups. The first group, which include inherent factors such as age, sex, race, genetic make up and the second group are environmental factors such as lifestyle, diet or long-term medical intervention (Sun, et al., 2017).

Breast cancer is classified into two groups as histological and molecular or/and intrinsic. 'In Situ Carcinoma and Insvasive (Infiltrating) Carcinoma' are in histological group and molecular subtypes of breast cancer are divided into five subgroups, include; Luminal A, Luminal B, Her2 over-expression, Basal-like, and Normal-like (Table 2.1).

Methylation of DNA is an important epigenetic alteration which is promote gene silencing. In breast cancer, silencing of functionally important genes play an essential roles in cancer development (Sharma et al., 2010). The familial breast cancer gene 1 (BRCA1) have several pathyways to involved in maintaining genome integrity including DNA repair, the control of cell cycle check points and apoptosis (Li et al., 2015). Several studies report that methylation of BRCA1 gene has been associated with decreased gene expression in sporadic breast cancer. (Birgisdottir et al., 2006, BenGacem et al., 2012). Sharma et al. (2010) observed that reduced expression level in %85 with methylation. However, several studies propose that, methylation is not the only mechanism to loss or reduce expression of BRCA1 protein. Therefore, some mechanisms such as, mutations, loss of heterozygosity and deletion which may also inactivate BRCA1 expression in sporadic breast tumors (Birgisdottir et al., 2006, Sharma et al., 2010, Li et al., 2015). Many studies exploring association between BRCA1 methylation status and clinico-pathological parameters (histological types, clinical stages) have reported divergent results. BenGacem et al. (2012) found a significant correlation between BRCA1 methylation and high histological tumor grade and Wei et al. found as the same results but did not found a correlation with tumour size or lymph node metastasis. In addition to this, Feng et al. (2009) showed a significant correlation between BRCA1 promoter methylation and lymph

node metastasis. In contrast, BRCA2, like BRCA1, is not frequently mutated in sporadic breast cancer (Miki et al., 1996). Previous studies have shown that BRCA2 promoter methylation has not been observed in breast cancers while it has been reported in ovarian cancer. In breast tumors the methylation status of BRCA2 has been investigated and most studies established that BRCA2 promoter methylation is rarely (BenGacem et al., 2012). In an earlier study, Collins et al. (1997) have explored BRCA 2 methylation in 64 cases of sporadic breast cancer, in different types of cancer cell lines and in normal human tissues, but they reported negative result using HpaII/MspI digestion PCR assays. However, BenGacem et al. (2012) analyzed the impact of BRCA1 and BRCA2 promoter methylation on patients' survival. They found that patients with BRCA1 and/or BRCA2 methylated tumors have better survival than those with unmethylated tumors.

Many studies demonstrated that several different tumor suppressor and other genes have been found to be hypermethylated in breast cancer such as adenomatous polyposis coli (APC), E cadherin gene 1 (CDH 1), Runt related transcription factor 3 gene (RUNX 3) (Dulaimi et al., 2004).

Studies have reported that, CDH1 promoter methylation occurs in ˜%30 of in situ ductal carcinomas in breast cancer. Furthermore, this gene has been shown to be one of the most commonly inactivated by methylation in sporadic breast cancer (Caldiera et al., 2006).

Liu et al. (2018) demonstrated that, the RUNX3 protein was expressed in %42 breast cancers and hypermethylation of RUNX3 was found in %58 breast cancers.

APC is a tumor suppressor gene which is an important of the Wnt signaling pathway. Inactivaion of the gene promotes development of the familial adenomatous polyposis and most sporadic colorectal tumors (Li et al., 2017, Virmani et al., 2001). In many studies have reported that APC methylation is highly specific for breast cancer and can be used as a biomarker in the diagnosis of breast cancer (Dumitrescue, 2012, Van der Auwera et al., 2008). Jin et al. (2001) found significant association between APC methylation and breast cancer pathogenesis. They demonstrate that the rate of hypermethylation of the APC promoter CpG islands was detected in 18 of 50 (36%) breast cancer.

In this study, we aimed that to optimize the methyldetect method for identfy methylation profile of the following genes BRCA1, BRCA2, APC, RUNX3 and CDH1 thus overall goal is to optimize methyldetect assay for rutine clinical applications for evaluation of the methylation profile between the samples normal (non-cancerous tissues), invasive ductal carcinoma (IDC), ductal carcinoma in situ (DCIS). The detection of DNA methylation has numerous methodological approaches. The method used in this study is MSP/USP PCR. This method is the most common susceptible method to apply the detection of methylation in tissues which use primer sequences designed for identifying methylated or unmethylated DNA sequences via sodium bisulfite (Cottrell et al., 2014, Sant and Goodrich, 2018). After bisulfite conversion, the DNA is subjected to PCR where a saturating fluorescent dye is present. This dye makes it possible to follow the amplification process during PCR when bound to double-stranded DNA.

The software generates melting curves showing the relationship between temperature and measured fluorescence. End of amplification until all amplicons are completely separated exist as single strands of DNA. MS-HRM is based on controlled amplification of all templates disregarding methylation status. The PCR bias brought about by the difference in base composition between methylated and unmethylated templates after bisulfite conversion is overcome by a special primer design, facilitating AT-rich amplification compared to CG rich templates (Hansen et al., 2008, Hussmann and Hansen, 2018). The basis is to design primers are to be complementary when selecting methylated templates and temperature both primers methylated and unmethylated similar efficiency templates. MS-HRM can be detected the methylation status of a single CpG and in preference, a limited number of CpG dinucleotides should be included in the amplicon to avoid additional melting areas that could interfere with the melting profile and consequently interfere with the interpretation of results (Wang et al., 2014). The valuation of the methylation level is semi-quantitative and is performed by comparing the melting profile of the test sample to a standard dilution range of totally methylated DNA in unmethylated DNA.

The specific primer design supporting the amplification of the methylated allele resulted in high sensitivity in MS-HRM tests and the ability to detect methylation levels between 0.1% and 1% (Wojdacz et al., 2009).

Optimization of the PCR is depens on both the quality and quantity of elements, besides the reaction to establish reaction temperatures and laboratory infrastructure. Multiple factors can affect the amplification of PCR, thus interfering with the results of various molecular biology procedures.

High Resolution Melting (HRM) is a novel technique that enables researchers to discover genetic variations quickly and efficiently (e.g., SNPs, mutations, methylation). HRM supply exceptional specifity and sensitivity with high sample throughput. Data from HRM must be analyzed in a complex way to generate meaningful results. This type of analysis requires that all experimental parameters be meticulously controlled and highly reproducible from sample to sample. Thus, the first step to achieving optimum results is to choose a real-time PCR instrument that can achieve this repeatability. The results of all HRM expreiments are highly depend on the quality of the individual PCR product. Therefore, the second step to achieving optimum HRM results is to properly set up the PCR.

There are some general guidelines for optimizing: reaction mixture and the PCR and melting programs. Recommendations for optimization of the reaction mixture: using LightCycler 480 High Resolution Melting Master, saltsa re important for the concentration of buffer Mg^2 so the concentration of the salts should be as homogeneous as possible in all samples, always determine the optimum $MgCl_2$ concentration for each experimental system to ensure both the specificity and robustness of the PCR. The recommended $MgCl_2$ concentration in this study is between 1.5 and 3.5 mM. Recommendation for optimization of the PCR and melting programs: using a touchdown PCR protocol covering a range of annealing temperatures between 65 C^0 and 55 C^0 (LightCycler 480 Real-Time PCR System Technical Note 1).

In this study, the optimization of the methyldetect method could not be achieved and the expected result could not be obtained. The reasons for not getting the result are thought to

be as follows; firstly, the optimal range of annealing temperatures are different for each instrument. This temperature, which is special for each primer, affects the connection of the primers and thus the working of the PCR. This may have affected primer optimization. Secondly, the primers may have expired especially if they have been exposed to repetitive freezings and thawings. Third, it may be associated with low amplification of DNA losses that may occur during isolation.

As a result of assessments, it is planned to repeat our work by considering all the factors required for optimization.

6. CONCLUSION

Studies have contributed to increasing the understanding of a significant number of people worldwide affected by breast cancer and the major impact of epigenetics on the diseases. This study aimed to optimize the methyldetect method for identfy methylation profile of the following genes BRCA1, BRCA2, APC, RUNX3 and CDH1 thus overall goal is to optimize methyldetect assay for rutine clinical applications for evaluation of the methylation profile between the samples normal (non-cancerous tissues), invasive ductal carcinoma (IDC), ductal carcinoma in situ (DCIS).

No meaningful results were obtained, in our study. This situation can be explained by the system not working optimally and not being fully optimized to the PCR machine.

Since calibration, positive and negative controls did not work at the expected value, it is aimed to rework the controls and primers with new ones and to ensure optimization.

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