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INVESTIGATION OF GSTP1 PROMOTOR REGION METHYLATION IN PERIPHERAL BLOODS OF PROSTATE CANCER PATIENTS

DOCTOR OF PHILOSOPHY THESIS

SEMA AKYÜREK, MSc

İSTANBUL – 2019

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T.C.

THESIS APPROVAL FORM

Institute	: Yeditepe University Institute of Health Sciences
Programme	: Molecular Medicine
Title of the Thesis	: Investigation of GSTP1 promotor regıon methylatıon ın perıpheral bloods of prostate cancer patıent
Owner of the Thesis	: Sema AKYÜREK
Examination Date	: 18.07.2019

This study have approved as a Doctorate Thesis in regard to content and quality by the Jury.

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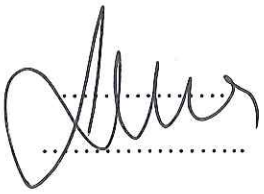
APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated 31.07.2019... and numbered 2019/13-13

Prof. Dr. Bayram YILMAZ
Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.



Sema AKYÜREK

I dedicate this thesis to my lovely daughter Defne AKYÜREK

and

my better half Mustafa AKYÜREK

ACKNOWLEDGEMENTS

First, I would like to thank to my thesis advisor, Professor Doctor Turgay İSBİR for his support. He made me become a better and patient scientist.

I also would like to thank to Associate Professor Doctor Müslüm AKGÖZ, the head of Bioanalysis Laboratory of TÜBİTAK UME for his suggestions and support during my studies in the laboratory.

Finally, I gratefully thank to my daughter, Defne AKYÜREK and my better half, Mustafa AKYÜREK for their endless patience, support and encouragement during this study.

This study was supported by The Scientific and Technological Research Council of Turkey (TUBITAK) project number 214S321.

July 2019 Sema AKYÜREK

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LIST of SYMBOLS and ABBREVIATIONS

bp	: Base Pair
cfDNA	: Cell Free DNA
CpG	: Cytosine-phosphate-Guanine
ctDNA	: Circulating Tumour DNA
ddPCR	: Droplet Digital Polymerase Chain Reaction
DNA	: Deoxyribonucleic Acid
DNMT	: DNA methyltransferase
DRE	: Digital Rectal Examination
gDNA	: Genomic DNA
GSTP1	: Glutatyon S-transferaz P1
HRM	: High Resolution Melting
MI	: Methylation Independent
MS	: Methylation Specific
MS-HRM	: Methylation Sensitive High Resolution Melting
MSP	: Methylation Specific Polymerase Chain Reaction
NCBI	: National Center for Biotechnology Information
PC	: Prostate Cancer
PCR	: Polymerase Chain Reaction
PSA	: Prostate Specific Antigen
qPCR	: Quantitative Polymerase Chain Reaction
TET	: Ten-Eleven Translocation methyl-cytosine dioxygenases enzymes

ABSTRACT

Akyürek, S. Investigation of GSTP1 Promotor Region Methylation in Peripheral Bloods of Prostate Cancer Patients. Yeditepe University Health Sciences Institute, Department of Molecular Medicine. Doctorate Thesis. İstanbul, 2019.

In this project, it was investigated whether it is possible to analyse the promoter region methylation of GSTP1 gene in peripheral blood. For this purpose, DNA was isolated from peripheral blood of prostate cancer patients and the promoter region methylation of GSTP1 gene was examined by using the obtained isolate. After bisulfite modification of the isolated DNAs, Methylation Sensitive High-Resolution Melting (MS-HRM) was performed using primer pairs designed for methylated and unmethylated DNA sequences. As a result, when MS-HRM method applied to the peripheral blood DNA isolates, GSTP1 promoter methylation could not detected due to due to the presence of excessive amount of unmethylated leukocyte gDNA. The same DNA isolates were examined by ddPCR, which is known to be one of the most accurate and reliable tools due to its high sensitivity and specificity and 2 of 65 samples were detected as GSTP1 methylated. In this study, the third method used to detect GSTP1 promoter methylation was Nested MS-PCR method which consists of Nested PCR and MS-HRM. The purpose of this method is to increase the number of copies of methylated GSTP1 fragments released from the tumour cells which has very low concentration compared to unmethylated GSTP1 fragments of leukocyte gDNA and to increase the sensitivity and specificity of the MS-HRM to be performed to detect methylation. In the first step, a 124bp region determined around the transcription start site of the GSTP1 gene was amplified by nested PCR using methylation independent (MI) primers. The resulting PCR products were diluted and MS-HRM was performed using methylation specific (MS) primers. With this method, methylation was detected in the promoter region of GSTP1 gene in 9 of 65 samples. Since it is not possible to quantify with qPCR without using control material, ddPCR performed with nested PCR products to quantify the methylated GSTP1 fragments. As a result of this study, although the sample could not be collected in accordance with ctDNA studies, it was shown that it is possible to detect promoter region methylation using appropriate methods.

Key words: Methylation, GSTP1, Nested-MSP, ddPCR, Peripheral Blood

ÖZET

Akyürek, S. Prostat Kanserli Hastaların Periferal Kanlarında GSTP1 Promotor Bölge Metilasyonunun Araştırılması. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Moleküler Tıp Anabilim Dalı. Doktora Tezi. İstanbul, 2019.

Bu projede, periferal kanda GSTP1 genine ait promotör bölge metilasyon analizinin mümkün olup olamayacağı araştırılmıştır. Bu amaçla, prostat kanserli hastalardan alınan periferal kanlardan DNA izolasyonu yapılmış ve elde edilen izolat kullanılarak GSTP1 genine ait promotor bölge metilasyonu incelenmiştir. İzole edilen DNA'ların bisülfit çevirim işleminden sonra metile ve metile olmayan DNA dizileri için tasarlanan primer çiftleri ve metilasyona özgü polimeraz zincir tepkimesi (MS-HRM) gerçekleştirilmiştir. Sonuç olarak bu metot ile periferik kandan elde edilen DNA izolatı incelendiğinde, lökosit gDNA'sının içerdiği yoğun miktarda metile olmayan DNA varlığı nedeniyle, GSTP1 genine ait promotor bölge metilasyonunun tespit edilemediği gözlenmiştir. Aynı DNA izolatları, yüksek hassasiyeti ve özgünlüğü sayesinde en doğru ve güvenilir araçlardan biri olarak bilinen ddPCR ile incelenmiş ve 65 adet örnekten 2 tanesinde GSTP1 geni promotor bölgesi metile olarak saptanabilmiştir. Bu çalışmada, GSTP1 promotor metilasyonunu saptayabilmek için kullanılan üçüncü metot ise nested PZT ve ardından MS-HRM yöntemlerini içeren kantitatif Nested-MS-HRM metodudur. Bu metodun kullanılmasındaki amaç lökosit gDNA'sına ait metile olmayan GSTP1 fragmentleri ile kıyaslandığında konsantrasyonu çok düşük olan tümörlü hücrelere ait metile GSTP1 fragmentlerinin kopya sayısının arttırılması ve metilasyonu saptamak için gerçekleştirilecek MS-HRM' in hassasiyetinin ve özgünlüğünün arttırılmasıdır. İlk basamakta nested PZT ile GSTP1 geninin transkripsiyon başlangıç noktası civarında belirlenen, 124bp büyüklüğünde bir bölge metilasyondan bağımsız primerler kullanılarak çoğaltılmıştır. Elde edilen PZT ürünleri seyreltilmiş ve metilasyona özgü primerler kullanılarak MS-HRM gerçekleştirilmiştir. Bu yöntem ile toplamda 65 örnekten 9 tanesinde GSTP1 geni promotor bölgesinde metilasyon varlığı saptanmıştır. qPZT ile kontrol materyali kullanılmadan miktar tayini yapmak mümkün olamayacağı için nested PZT ile çoğaltılan örnekler ddPZT ile çalışılmış ve miktar tayinleri yapılmıştır. Bu çalışmanın sonucunda ctDNA çalışmalarına uygun olarak örnek toplanamamış olsa da uygun yöntemler kullanılarak promotor bölge metilasyonunun saptanabileceği gösterilmiştir.

Anahtar Kelimeler: Metilasyon, GSTP1, Nested-MSP, ddPCR, Periferal Kan,

1. INTRODUCTION and PURPOSE

The genome, which makes each individual unique, contains the information necessary for an organism to form and to survive. In humans, a copy of the entire genome consisting of 3 million base pairs is located in the nucleus of the cells. The genome carries the nucleotide sequences that carry the information to form the organism and the chemical changes controlling the flow of this information [1].

The epigenetic word "on genetics" refers to the mechanisms involved in the regulation of gene expression by controlling the flow of information carried in nucleotides, without causing changes in the DNA sequence [2]. DNA methylation, histone modifications, chromatin modifying complexes and microRNAs are epigenetic mechanisms involved in the regulation of gene expression [3]. DNA methylation is the most researched epigenetic mechanism [4]. Methylation refers to the binding of a methyl group covalently to the 5-carboxyl end of the Cytosine base in the Cytosine-phosphate-Guanine (CpG) binary-nucleotides on the DNA sequence [5,6].

In the formation process of each individual, all the methylation patterns in the gametes are deleted and then the individual pattern is regenerated by means of enzymes. The new methylation pattern formed during the embryo period is carried in each cell with a core [1,7].

The methylation pattern in healthy cells can be transferred to the newly formed cell with high accuracy and it is known that these patterns are impaired in aging and disease processes. Studies have shown that DNA methylation patterns have changed in heart diseases, diabetes and neurological diseases, particularly cancer [6,8].

When tumour tissues were examined, hypomethylation on genome basis and hypermethylation on gene basis were observed [5,6]. Hypomethylation and hypermethylation are methylation mechanisms that cause changes in the methylation pattern of cells. Hypomethylation occurs on the genome, while hypermethylation is gene-specific in the promoter regions of the gene. Both mechanisms play a role in the formation of cancerous cells. As a result of hypomethylation, the formation of proto-oncogenes leads to tumour formation. The genes involved in the cell cycle and DNA repair mechanisms and the inactivation of tumour suppressor genes by hypermethylation also cause tumour formation [2].

These changes in DNA methylation enable them to be used as, non-invasive biomarkers because they can be detected in blood and other body fluids, because they can be used to diagnose diseases and to monitor the effectiveness of the treatment [2,9].

When cancer is diagnosed in early period, it is possible to treat with surgical intervention and in advanced cases there is no response to surgical intervention. Therefore, early diagnosis of cancer is of great importance for prolonging life. It is unfortunately not possible to detect cancer at an early stage with tests such as colonoscopy, mammography and cervical cytology, which are approved tests in cancer diagnosis. However, studies have shown that early detection of cancer is possible by analysing ctDNAs (circulating tumour DNA) released from tumour tissues to body fluids by means of apoptosis and necrosis [10]. But the detection of the ctDNA is challenging due to the amount of ctDNA (~ 0.01 to 5 ng ctDNA per mL plasma, ctDNA relative to cfDNA (cell free DNA) ranges from < 0.1% to 10%.) and high fragmentation [11]. Methylation studies on ctDNA has been studied in many of the cancer types and it is a promising alternative method on the diagnosis and prognosis of the patients [12]. In order to perform methylation studies on ctDNAs, firstly, ctDNAs must be isolated from blood. The most important point to be considered at this stage is to prevent the lysis of leukocytes and to prevent contamination of ctDNAs with large amount of leukocyte DNA which can lead to false negative results. To avoid lysis of the leukocytes commercial tubes designed to prevent cell lysis can be used or peripheral blood can be centrifuged at 1600 x g for 10 min at 4°C [13].

In clinical trials, sample collection in accordance with the research is the most important step of the clinical studies. Therefore, clinical samples which are commonly difficult to access should be evaluated in the best way. In this study, in case of compulsory situations, even if the samples were not prepared in accordance with ctDNA analysis, it was aimed to answer the questions whether this samples are suitable for methylation analysis and can methylation be detected by using different methods and whether information can be obtained about tumour size by quantifying the methylated GSTP1? For this purpose, peripheral bloods of prostate cancer patients were collected.

Prostate cancer is the most common type of cancer diagnosed in middle-aged men, living in developed and developing countries. It is known that the probability of developing prostate cancer increases with advancing age. While this probability is 0.6% in the 35-44 age range, it is 9.7%, 32.7%, 36.3% and 16,8%, in the 45-54, 55-64, 65-74,

75-84 age ranges, respectively. The probability of cancer development in prostate tissue, which is clinically important during the lifetime of a man, is 17.8% [14].

Prostate cancer, which ranks second among cancer-related deaths, is a disease that can be effectively treated by retropubic prostatectomy if it has not spread to other organs. Early diagnosis is important because prostate cancer is curable. Today, "PSA in serum" (prostate-specific antigen) is used as a biomarker in the clinic for early diagnosis and follow-up of the treatment process. However, since there is no definite cut-off value for serum PSA value, it is not clear whether a person has prostate cancer or not by looking at the PSA value. PSA is not a cancer-specific biomarker. Serum PSA levels are also high in patients with benign prostatic hyperplasia. These data indicate that PSA is a biomarker with low selectivity and specificity [15].

As a result of the studies, it has been shown that methylation observed in the promoter regions of related genes is a potential biomarker that can be used in early diagnosis of prostate cancer and follow-up of the treatment process [16]. Methylation observed in the GSTP1 gene, which is responsible for detoxification and protecting cells from DNA damage, is considered as a potential biomarker that can be used in the diagnosis and treatment of prostate cancer [15]. Therefore, in this study we aimed to find answers to our questions using peripheral bloods of prostate cancer patients and investigating promotor methylation status of GSTP1 gene.

2. GENERAL INFORMATION

The human genome consists of Adenine, Cytosine, Guanine and Thymine nucleotides, and the sequence of the genome is the same in almost all cells of the individual. It makes each individual unique, contains the information necessary for an organism to form and to survive. In humans, nucleus of the cells contains a copy of the entire genome consisting of 3 million base pairs. The genome carries the nucleotide sequences that carry the information to form the organism and the chemical changes controlling the flow of this information [1,17].

While the genome is the same in almost all cells of an individual, the epigenome shows significant changes in different cells depending on time and environmental factors. Epigenetic mechanisms create the Epigenome through a number of chemical changes without causing any change in the genome. These chemical changes on the genome, alter the flow of information in the nucleotides by regulating gene expression [17,18].

The epigenetic mechanisms that make up the epigenome are chemical changes that occur either on the nucleotides that make up the genome or on the histone proteins that allow the genome to be packed and fit into the cell [17]. DNA methylation, histone modifications, chromatin modifying complexes and microRNAs are epigenetic mechanisms involved in the regulation of gene expression [3]. DNA methylation is the most researched epigenetic mechanism [4].

2.1 DNA Methylation

DNA methylation which is essential for regulating tissue specific gene expression, X chromosome inactivation and silencing retroviral elements [19], refers to the binding of a methyl group covalently to the 5-carboxyl end of the Cytosine base in the Cytosine-phosphate-Guanine (CpG) dinucleotides on the DNA sequence [5,6].

Methylation of cytosine is a covalent modification that occurs after the DNA synthesis by transfer of a methyl group to the carbon-5 position of the cytosine via enzymes from the methyl donor S-adenosylmethionine. Those enzymes are called as DNA methyltransferases (DNMTs) family enzymes. There are two different subfamilies of DNMTs: DNMT3 and DNMT1[17,18,20–22]. DNMT3 subfamily consist of DNMT3a and DNMT3b enzymes and those enzymes are responsible for de novo methylation patterns in early development. After establishment of the de novo

methylation on one strand of the double helix, DNMT1 copy methylation patterns to the opposite strand during DNA replication. In some cases, like a failure on copying the methylation pattern to the other strand, cytosine methylation needs to be erased. This is called as demethylation and it occurs during the early development of embryo by passive or active demethylation. Passive demethylation occurs during the mitosis via reducing the two daughter cells by half which has failure in the copied methylation patterns. In active demethylation there are two hypotheses: Base excision repair and enzymatic demethylation. According to the base excision repair cytosines carrying methyl tag are excised from the genome and replaced with cytosines. Enzymatic demethylation mechanism involves "ten-eleven translocation methyl-cytosine dioxygenases enzymes" (TET), which convert methyl cytosine to hydroxymethyl cytosine and initiate a series of reactions in which we obtain unmodified cytosine [17,20] .

Figure 1 and Figure 2 shows the enzymes used in the methylation and demethylation processes.

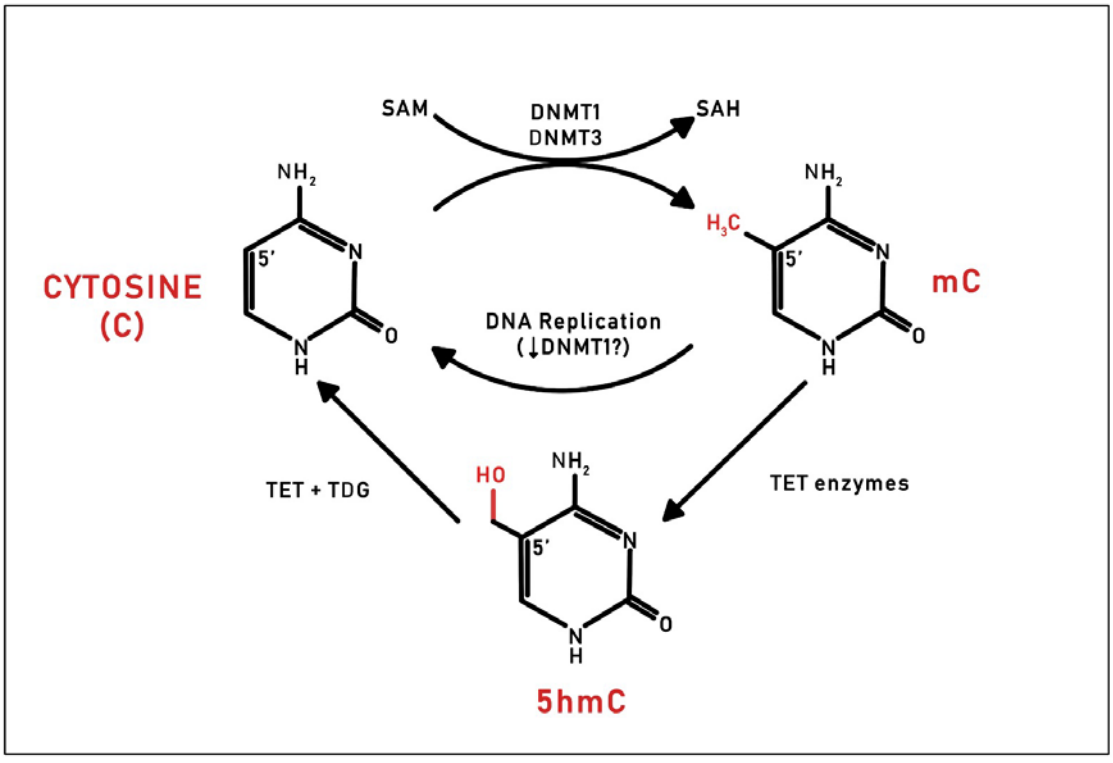


Figure 1.Methylation and demethylation of cytosine

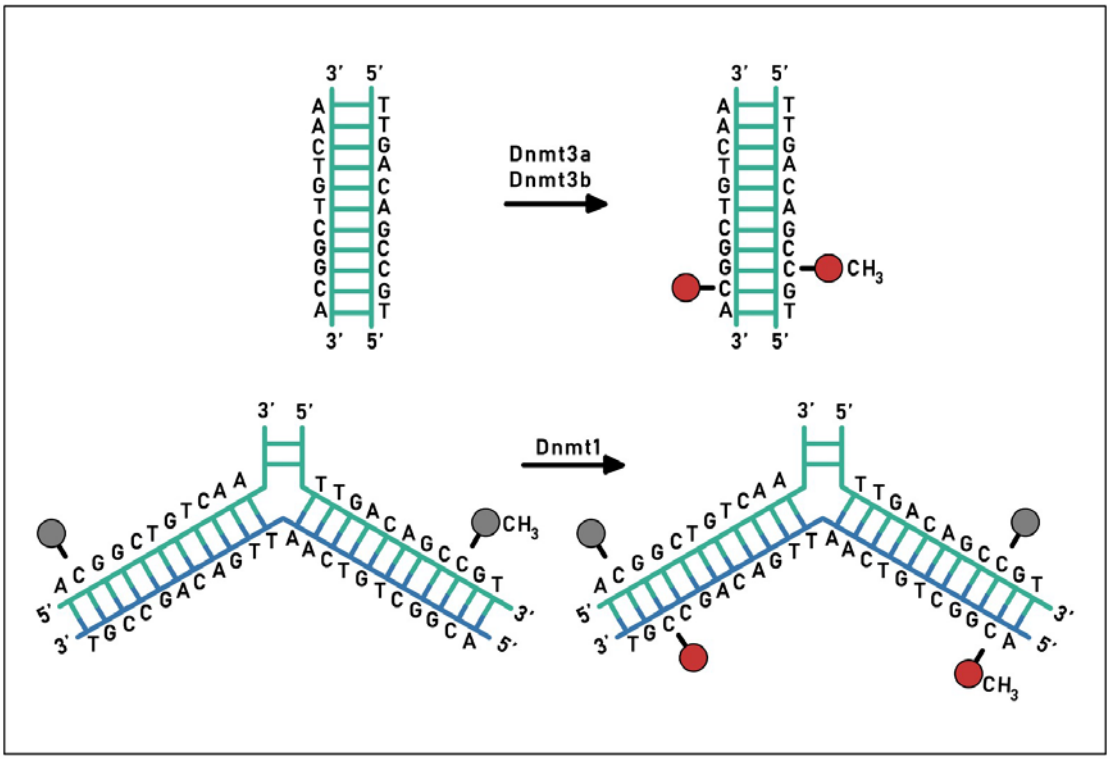


Figure 2. DNA methylation pathways

When tumour tissues were examined, hypomethylation and hypermethylation which cause changes in the methylation pattern of cells observed. While hypomethylation occurs on genome basis and hypermethylation occurs on gene basis [5,6]. Both mechanisms play a role in the formation of cancerous cells. As a result of hypomethylation, the formation of proto-oncogenes leads to tumour formation. The genes involved in the cell cycle and DNA repair mechanisms and the inactivation of tumour suppressor genes by hypermethylation also cause tumour formation [2].

Hypermethylation in the human genome is most commonly observed in regions called CpG islands which presents in the promoter regions. CpG islands are DNA sequences which are more than 200bp length and have more than 50% CG content [20]. CpG islands of genes are unmethylated with the exception of imprinted genes and genes on the inactive X chromosome [6]. DNA methylation controls gene expression in eukaryotes. If a promotor region get methylated it means that there will be an inhibition on expression of this gene [23]. Promotor methylation of CpG islands regulates the differentiation and development [19]

In the whole genome high resolution DNA methylation analysis with primary human fibroblast cell line, 4.25% of total cytosines found in genomic DNA, 67.7% of CpG dinucleotides were found methylated and 99.98% of DNA methylation was found to be CpG dinucleotides. In a similar study with human embryonic stem cells, 5.83% of all cytosines and 82.7% of CpG dinucleotides were found to be methylated and 25% of all methylations were observed in regions other than CpG [24]. In light of this information, it is possible to speak of a methylation pattern of each cell. It is known that the methylation pattern in cells, the frequency of methylation of cytosines on a gene, is transferred to the new cell with high accuracy during mitosis while the transfer of the pattern is impaired in aging and disease processes [6]. Abnormal gene methylation patterns seen in many complex diseases such as cancer, diabetes and neurological disorders are biomarkers that can be used in the diagnosis, course and follow-up of the treatment process. Therefore, accurate determination of gene methylation patterns is of great importance for the diagnosis of diseases and the follow-up of the response to treatment [6]. Figure 3 shows the relation between methylation and cancer.

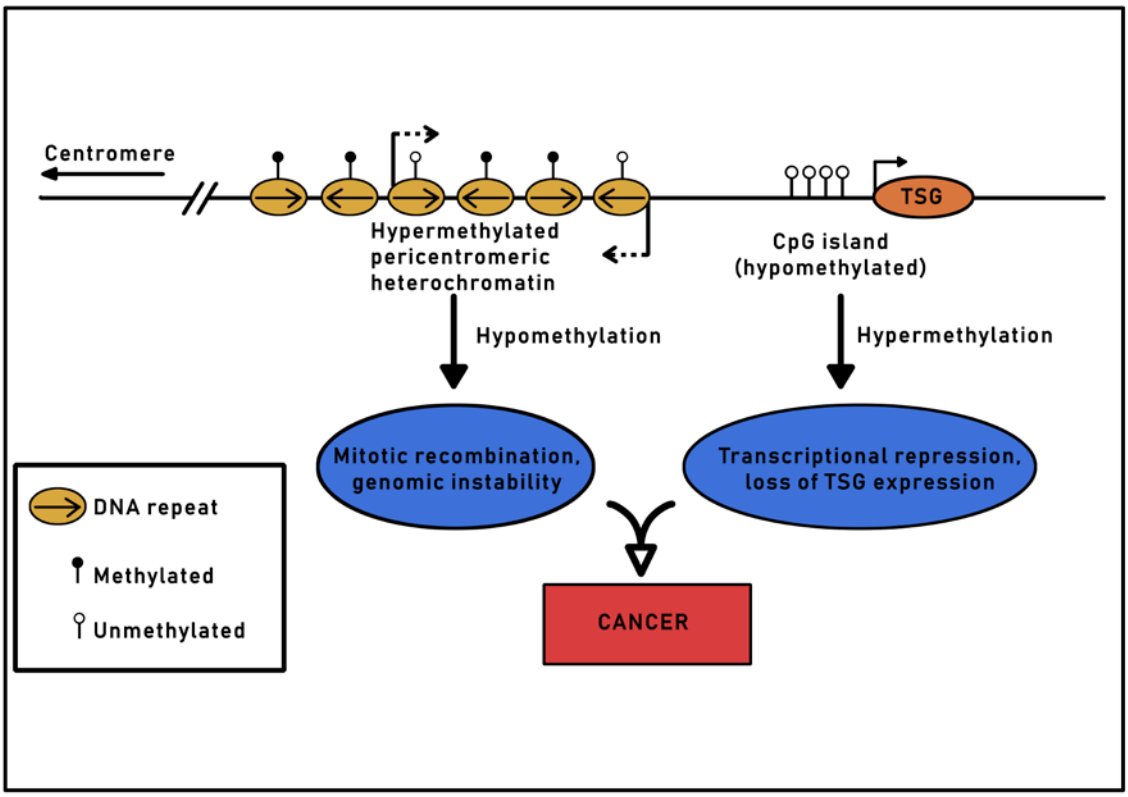


Figure 3. DNA methylation and cancer

2.2 ctDNA

Changes in DNA methylation can be detected using liquid biopsies such as urine, blood, saliva etc. Cell free DNA (cfDNA) that are shed from normal cells and circulating tumour DNA (ctDNA) shed from tumour cells are non-invasive blood-based biomarkers that have potential for detection, diagnosis and to monitor the cancer [25]. The difference to be expected to observe between normal cells and tumour cells is that normal cells have unmethylated and tumour cells have methylated promotor region [2,9]. While the concentration of cfDNAs found in the bloodstream of healthy individuals is very low, there is a significant increase in the concentrations of cfDNAs due to the release of ctDNA from the necrotic and apoptotic cells of cancer tissue into the bloodstream during the tumour formation [9,26].

There are many reasons why liquid biopsies are potentially replacing the standard biopsy. The first reason is biopsy is a surgical procedure and risky than taking the blood sample. It is not always possible to get sample to monitor the diseases during the treatment, for some types of tumours it is difficult even sometimes it is not possible to take the samples. Tumour tissues have intra tumoral heterogeneity so it may not give same information in each case [25].

Studies have shown that early detection of cancer is possible by analysing ctDNAs (circulating tumour DNA) released from tumour tissues to body fluids by means of apoptosis and necrosis [10]. But the detection of the ctDNA is challenging due to the amount of ctDNA (~ 0.01 to 5 ng ctDNA per mL plasma, ctDNA relative to cfDNA (cell free DNA) ranges from < 0.1% to 10%.) and high fragmentation [11]. Methylation studies on ctDNA has been studied in many of the cancer types and it is a promising alternative method on the diagnosis and prognosis of the patients [12]. In order to perform methylation studies on ctDNAs, firstly, ctDNAs must be isolated from blood. The most important point to be considered at this stage is to prevent the lysis of leukocytes and to prevent contamination of ctDNAs with large amount of leukocyte DNA which can lead to false negative results. To avoid lysis of the leukocytes commercial tubes can be used designed to prevent cell lysis or as soon as peripheral blood is drawn, blood can be centrifuged at 1600 x g for 10 min at 4°C [13].

2.3 Prostate Cancer

The prostate is a gland about the size of a ping-pong ball and is part of the reproductive system in men. The prostate gland surrounds the urethra and takes place below the bladder, in front of the rectum. The prostate produces the fluid called as

seminal fluid that protects and support the sperm and helps transport of the sperm. Figure 4 shows the structure of reproductive system in men [27].

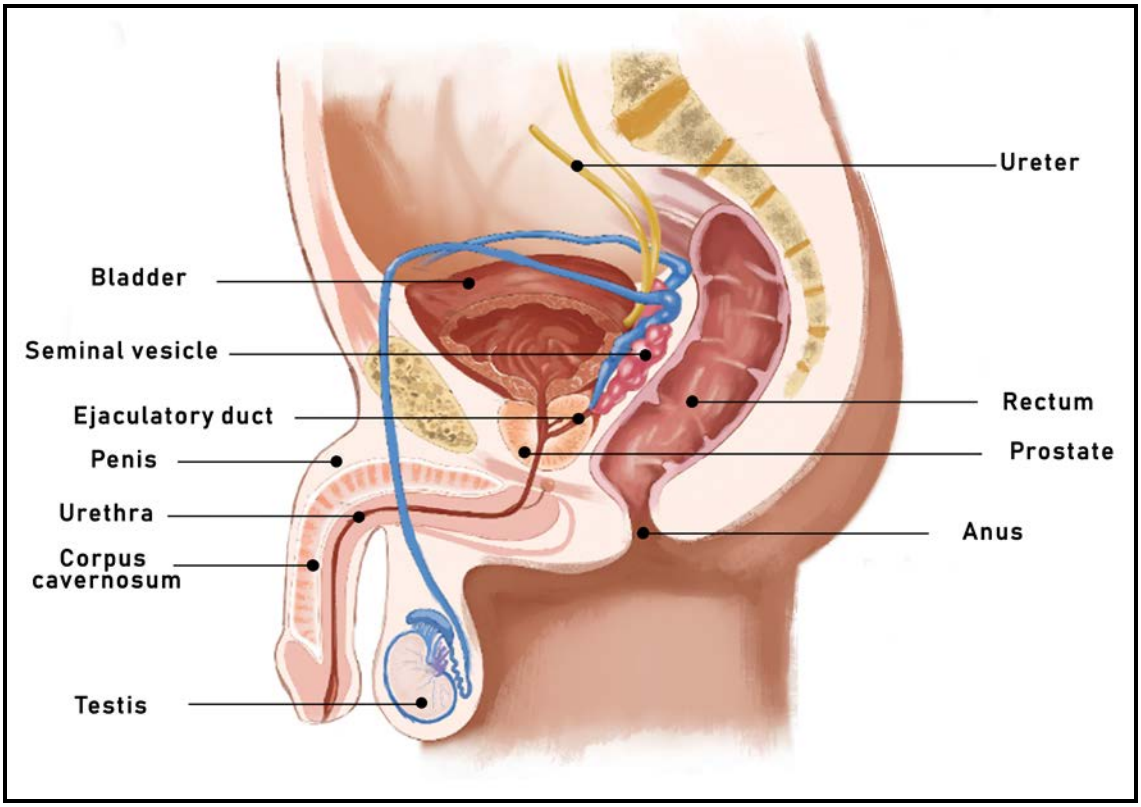


Figure 4. Prostate gland [(27)]

Prostate cancer is the second most common cancer type among men and a major cause of 1–2% of cancer deaths in men. It occurs in the developed world more frequently as a result of lifestyle and environmental risk factors [28,29].

Prostate cancer is thought to be multifactorial disease that is affected by genetic and environmental conditions. The probability of developing prostate cancer increases with age. Those with a history of cancer among first-degree relatives, are 3.5 times more likely to develop prostate cancer [30]. Smoking cigarette, being overweight are also risk factors to develop prostate cancer [29].

Prostate cancer generally has no symptoms in early phases, but it can be detected by screening. While prostate cancer can remain latent in the body without any harm, in advanced cases, individuals may experience weak or interrupted urine flow. The other signs of the advanced cases are inability to urinate, starting or stopping urination

difficultly, frequently urination need, blood in the urine; or pain or burning during urination. On the other hand those symptoms can also be seen with benign conditions, that's why they are not specific to prostate cancer[29].

In the Classification of the PC, Gleason score is the recommended methodology. Gleason score is a grading system to get information about aggressiveness of prostate cancer. Treatment options can be chosen according to this score. Most of the prostate cancer has a Grade of 3 or higher. If the grade is higher it means that cancer can grow and spread quickly. Tumours generally have different grade of tumour cells. In this case Gleason score can be written as 3 +4=7, it means that the highest amount of tumour cells is grade 3 and the next one is grade 4, and the total Gleason score for this biopsy is 7. If the Gleason score is less than 3, it means that the type of the cancer is slowly growing cancer and cells look like normal cells. If it is 7, intermediate risk for aggressive prostate cancer. Scores 8 and higher means that the cancer is high grade and it can spread more rapidly. Gleason grading scale is given in Figure 5 and new grading system explanation is given in Table 1. The Gleason score is very important to understand the behaviour of PC. But other factors are also used to determine the stage of prostate cancer like PSA level, rectal examination. Physician can understand if cancer is found on both sides of the prostate or not and has spread outside or not. Screening for prostate cancer involves digital rectal examination (DRE) and prostate specific antigen (PSA) blood test. As a result of DRE, physician can determine if the prostate gland is enlarged, has lumps, if there are any hard areas or not. PSA is a substance, produced by both normal and cancerous prostate cells. In case of prostate cancer and prostate disease, the concentration of the PSA in blood often increases. The normal concentration of PSA is likely to be 0-1,5ng/mL. Higher levels of PSA can be a sign of prostate cancer but also benign prostatic hyperplasia (BPH) and prostatitis. That's why it is important to track PSA levels to see if it is rising in time or not. With its low sensitivity and specificity around 75% [31], PSA cannot be used as a biomarker for PC. It can only give information about prostate cancer possibility [15,27].

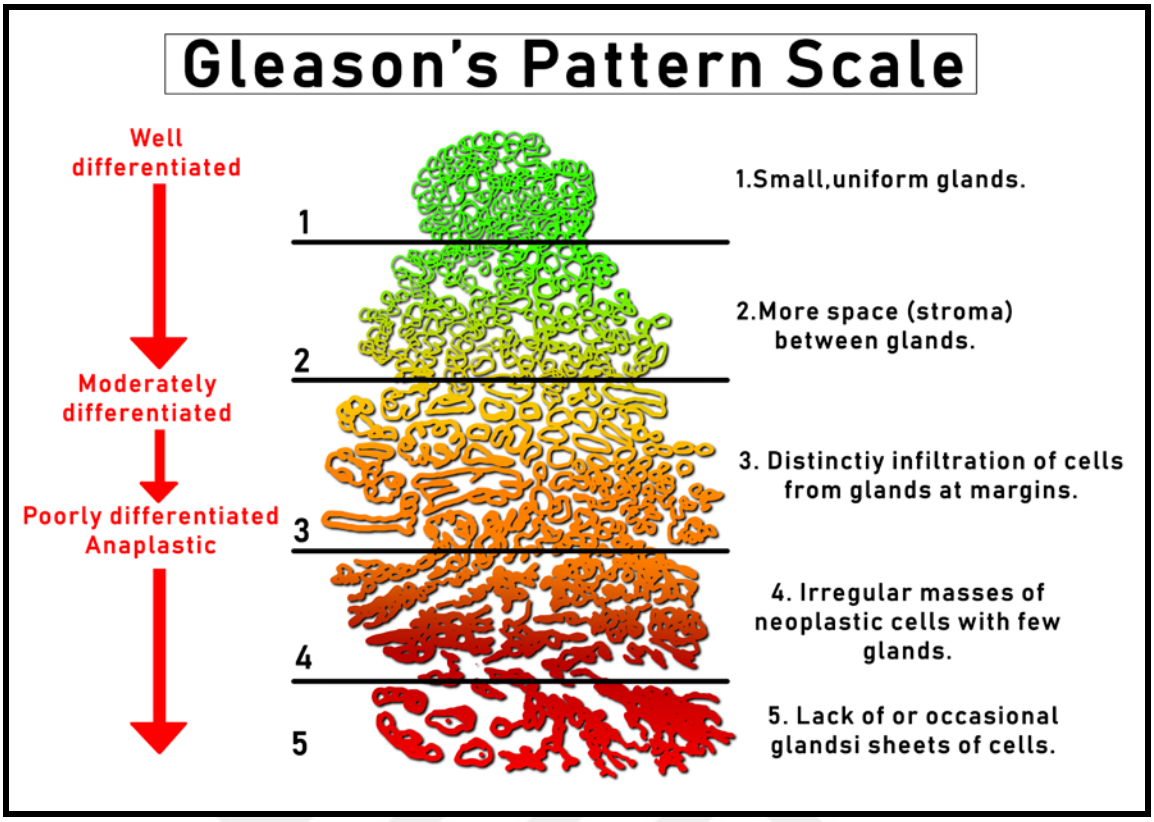


Figure 5. Gleason grading scale [27]

Table 1. New prostate cancer grading system

Traditional Gleason Score	New Grading System
Gleason 3+3 = 6	GRADE 1
Gleason 3+4 = 7	GRADE 2
Gleason 4+3= 7	GRADE 3
Gleason 4+4= 8	GRADE 4
Gleason 9-10	GRADE 5

2.4 DNA Methylation Based Biomarkers in Prostate Cancer

Like other cancer types prostate cancer development also involves genetic alterations that cause normal cells to transform abnormal cells. Detection and identification of those genetic alterations can be used in early diagnosis of PC. DNA-based biomarkers, such as DNA methylation biomarkers, are very advantageous because of their high stability, accessibility and easy analysis. [32].

In early tumorigenesis promotor methylation occurs and it effects key regulatory genes. It is known that it is possible to detect one or more promotor methylation in

tumour tissues. Hence promotor methylation might have clinically important information on detection and follow-up the prostate cancer [32].

The most studied DNA methylation-based biomarkers that found to be methylated and their functions are given in Table 2.

Table 2. DNA methylation based prostate cancer biomarkers

Gene Altered	Function	Significance in Prostate Cancer	Biomarker	References
GSTP1	Detoxification and protection of the cell from DNA Damage	Methylated in all stages of the of disease	Early detection, diagnosis, prognosis	[33,34]
APC	Tumour suppressor gene (cell migration and adhesion)	Methylated in all stages of the of disease	Early detection, diagnosis, prognosis	[33,34]
RASSF1A	Tumour suppressor gene (cell cycle and apoptosis)	Methylated in early stages of disease	Early detection	[33]
RARβ2	Hormone receptor (cell growth and differentiation)	Methylated in all stages of the of disease	Early detection, diagnosis, prognosis	[34,35]
PTGS2	Prostaglandin biosynthesis	Methylated in all stages of the of disease	Early detection, diagnosis, prognosis	[34]
CD44	Cell to cell interaction, cell migration and adhesion	Methylated in all stages of the of disease	Early detection, diagnosis, prognosis	[36]

Glutathione-S-transferase P1 (GSTP1) is the most frequently methylated gene, in prostate cancer, out of large number of methylated genes [34]. GSTP1 comprise a family of enzymes involved in protection cells from electrophilic metabolites of carcinogens and detoxification of cells from oxidants [15,32]. It is shown that GSTP1 promotor methylation accurately discriminates normal or hyperplastic prostate tissue and prostatic adenocarcinoma [33].

Reasons for being a potential DNA-methylation based biomarker for prostate cancer are [15] :

- With its high specificity for prostate cancer >90% GSTP1 is a better biomarker than PSA (~20%)

- GSTP1 promotor methylation levels can differentiate prostate cancer from other prostatic diseases
- Quantification of the GSTP1 promotor region gives information about different stages of the prostate cancer
- It is possible to use serum, plasma or urine to measure the promotor methylation (non-invasive)

Although GSTP1 has higher specificity than PSA, it is known that promotor methylation can also occur in other types of cancers. As a result, we cannot say that GSTP1 is 100% specific biomarker to PC. Using other methylation biomarkers alongside GSTP1 is a better way to improve the specificity and sensitivity of the measurement. In previous studies it is showed that using 4-gene panel APC, RASSF1A, RAR β2 and GSTP1 it was possible to discriminate cancer cells from normal cells at 86% sensitivity and 89% specificity [15].

In methylation analysis sample type, the timing of sample collection (before or after treatment) and methods that will be used to measure the methylation are also very important. When different sample types, whole blood, serum/plasma, used to measure GSTP1 methylation, it is observed that sensitivity of the GSTP1 decreased in whole blood [15].

2.5 Promotor Methylation Detection Methods

Promotor methylation can be detected using non- bisulfite and bisulfite methods. Non-bisulfite methods use methylation sensitive restriction endonucleases and Southern blot analyses or PCR detection. This method generally has limitation because of the cleavage sites. Bisulfite methods can be a solution for this limitation. Methods involves bisulfite modification allow quantitative determination of promotor methylation [37].

2.5.1 Bisulfite modification

DNA methylation information is lost after the PCR amplification. To keep the methylation information during PCR, DNA must be modified with bisulfite [38]. Bisulfite modification occurs on the single strand of the DNA. That's why the first step of the modification is denaturation of the DNA into single strands. After denaturation the following reactions are, reaction of bisulfite with cytosine to give cytosine bisulphite derivative, hydrolytic deamination to get uracil sulphonate derivative, removal of sulphonate and to get uracil and the last reaction is PCR. As a result of modification of cytosines to uracils non-complementary strands occur. Resulted DNA's can be

amplified either using different sets of primers that are specific to differentiated DNA strands or primers designed for high resolution melting analyses. After PCR, uracils amplified as thymines, but 5mC are amplified as cytosine [39]. Figure 6 shows bisulfite conversion of DNA.

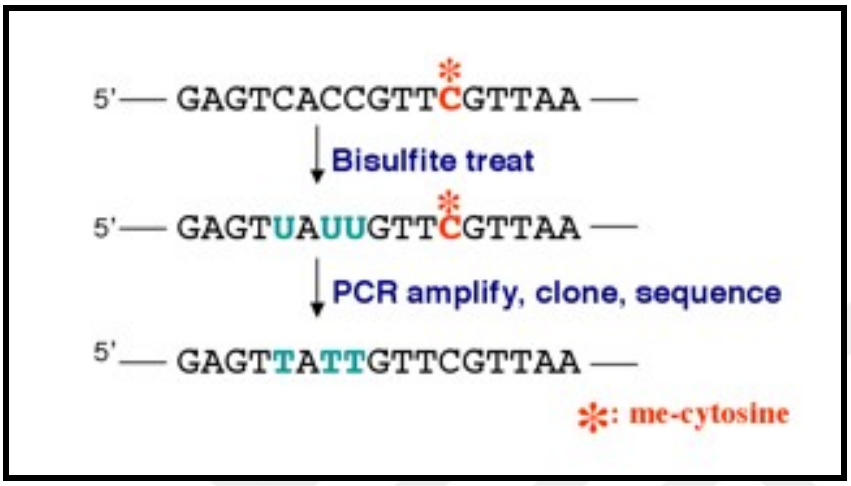


Figure 6. Bisulfite modification of DNA

There are many PCR-Based methods for the detection of promotor methylation. Sanger sequencing and pyrosequencing, methylation specific PCR, quantitative methylation specific PCR, methylation sensitive high-resolution melting (MS-HRM).

2.5.2 Methylation sensitive PCR and HRM analyses

MS-PCR is a widely used method that has sensitivities as low as 0.1-1 % methylation sequences. Because its high sensitivity this method is a useful method for mosaic methylation analyses and detection of ctDNAs. When using this method, it is very important to have short amplicons to get information from fragmented DNA. The working principle of MS-PCR is to distinguish between methylated and unmethylated DNA sequences by making use of sequence differences resulting from bisulfite modification. In MS-PCR analysis, two pairs of primers are used to specifically amplify the methylated and unmethylated DNA sequences, respectively. In MS-PCR studies primers designed for both methylated and unmethylated sequences. After the PCR has been completed, the amplicons analysed by high resolution melting analyses [38].

High resolution melting (HRM) is a post-PCR technique that is widely used in promotor methylation analysis [40,41]. Promotor region is amplified with methylation

specific primers in the presence of a saturating fluorescent dye. When this fluorescent dye binds to the double stranded DNA, dye fluoresces. After the MS-PCR in the presence of saturating fluorescent dye, temperature is slowly increased as a result DNA starts to melt. When melting curves analysed methylated and unmethylated DNA sequences gave characteristic shapes. Usually methylated DNA sequence has melting temperature (Tm) higher than the unmethylated DNA sequence. Using control materials specific to interested gene will give exact information about the expected methylated and unmethylated curves and Tm of the amplicons [42].

2.5.3 ddPCR

Low level of mutations in ctDNA can be detected by droplet digital PCR, which is a droplet based, microfluidic platform. PCR samples are separated by water oil emulsion and nanolitre-sized droplets are formed. A droplet is totally same as a well in a plate that PCR occurs. PCR occurs in droplets and droplets are read by the microfluidic system to determine positive and negative droplets. Afterwards, data are analysed using microfluidic droplet reader [43]. Workflow of the ddPCR is given in the Figure 7.

dPCR enable to make detection of rare variants in the presence of intense wild type sequence concentration. In terms of diagnosis and staging of the cancer, detection and quantification of the rare variants are very important. By performing the PCR in the generated nanoliter volume droplet, it is possible to detect the rare variant in the droplet independently of the signal generated by the wild type DNA sequence. Studies have shown that variants as low as 1 per 100000 can be measured [44].

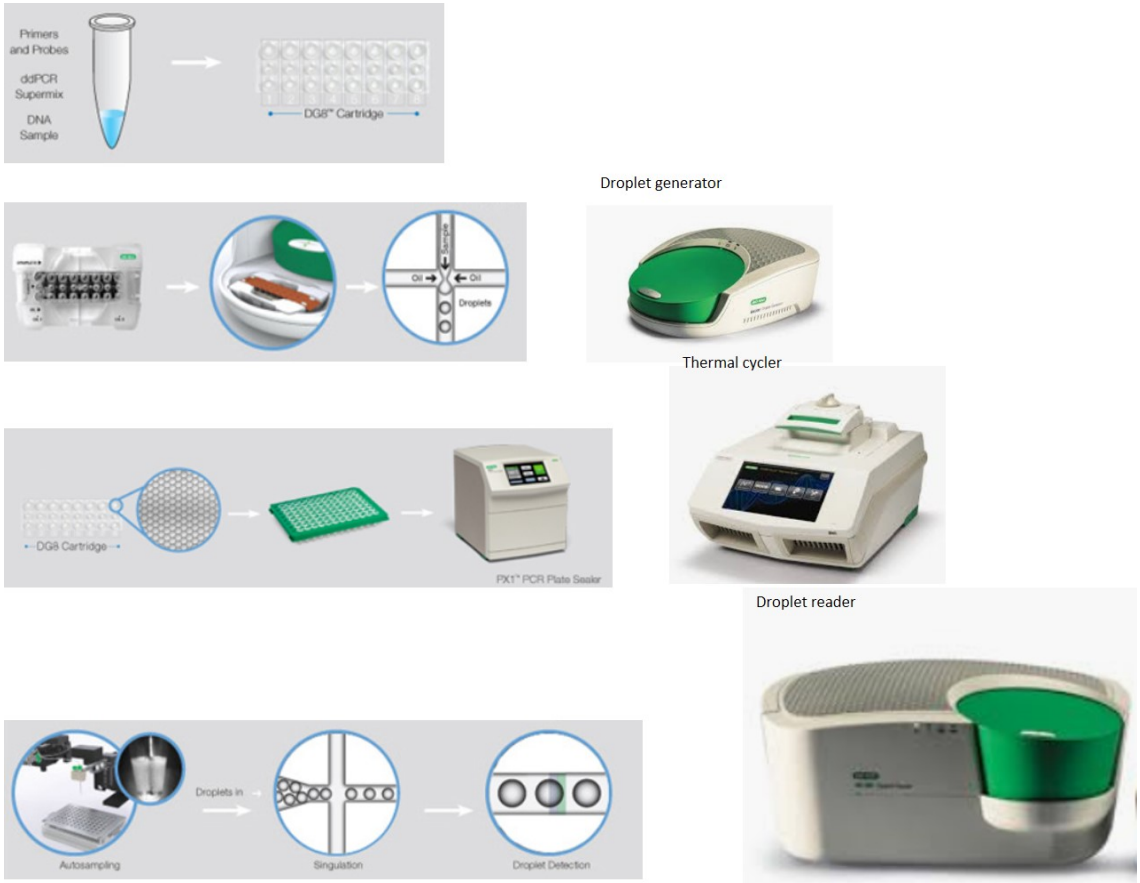


Figure 7. ddPCR workflow [43]

3. MATERIAL and METHODS

3.1 Selection of Patients and Definition

A total of 65 blood samples from prostate cancer patients and 76 blood samples from control groups were collected in the Urology Department of Yeditepe University Hospitals, in Istanbul, Turkey. Before sample collection, written informed consent was obtained from all patients and controls. The study was approved by the local ethics committee of Yeditepe University. 5 mL blood samples were obtained from patients in tubes containing EDTA and blood specimens were stored at +4°C before plasma separation.

3.2 Materials and Devices

3.2.1 Consumables

Commercial kits and other consumables are given in Table 3.

Table 3. Commercial kits and other consumables

Consumable/Kit Name	**Catalog No**	**Brand**
iPrep™ PureLink™ gDNA Blood Kit	IS10005	ThermoFisher Scientific
Human Methylated & Non-methylated DNA Set	D5014-2	Zymo Research
Bisulfite-Converted Universal Methylated Human DNA Standard	D5015	Zymo Research
EZ DNA Methylation- Lightning Kit	D5030	Zymo Research
Light Cycler 480 High Resolution Melting Master	4909631001	Roche
QX200™ ddPCR™ EvaGreen Supermix	1864034	BioRad
ddPCR 96 well PCR plate	12001925	BioRad
DG8™ Cartridges and Gaskets	1864007	BioRad
Droplet reader oil	1863004	BioRad
Eva Green Droplet Generation Oil	1864006	BioRad

3.2.2 Equipment list

Equipment list is given in Table 4.

Table 4. List of equipment and software

Equipment/Software Name	Brand
iPrep PureLink	ThermoFisher Scientific
Thermal Cycler, Veriti	ThermoFisher Scientific
C1000 Touch Thermal Cycler	BioRad - ddPCR System
QX Droplet Generator	BioRad - ddPCR System
QX Droplet Reader	BioRad - ddPCR System
PX1 PCR Plate Sealer	BioRad - ddPCR System
Real Time PCR system	Roche LC480 II Real Time PCR System
HRM Analysis Software	Light Cycler 480 Software release 1.5.0
QuantaSoft, ddPCR software	BioRad
Nanodrop 2000 Spectrophotometer	ThermoFisher Scientific
Laminar Air Flow Cabinets	Holten
Micropipettes (10 µL, 20 µL, 100 µl, 200 µL, 1000 µL)	Gilson
Gel Documentation System	Vilber Lourmat
Vortex	VWR
Quick Spin	Heathrow Scientific
Freezers	Liebherr, Siemens
Power Supply	BioRad
Electrophoresis	BioRad
Eppendorf Tubes	VWR
Centrifuge	Sigma / Beckman Coulter

3.3 Methods

3.3.1 DNA isolation from blood

The venous blood samples taken from the patients and control groups to 5 cc EDTA tubes and stored in the refrigerator until DNA isolation. DNA isolation of the samples was performed using the iPrep Purification Instrument (Thermofisher) and iPrep blood genomic DNA isolation kit (iPrep gDNA Blood kit). The iPrep Purification Instrument is capable of purifying up to 13 samples in a single run and an automated nucleic acid purification instrument which uses ChargeSwitch® technology (CST). ChargeSwitch Technology works based on an ionisable ligand which can change the load according to the pH of the surrounding medium. At low pH levels CST beads are positively charged to bind to the negatively charged nucleic acid backbone. Since other contaminants present in the environment cannot be connected to positively charged CSF beads, they are removed in the wash step together with the wash buffer. To separate the

nucleic acids from the CST beads low saline wash buffer (elution buffer) is used. Thus, the DNA is recovered in 150µL elution buffer. All the DNA samples were aliquoted and maintained in -20°C deep freezer till subsequent analysis.

3.3.2 Measurement of purity and quantity of isolated DNA

Measurement of purity and quantity of the isolated DNA was performed using NanoDrop Instrument (ND2000, Thermofisher). To asses sample purity 260/280 and 260/230 ratios were analysed and expected to get ~1,8 and ~1,8 to 2,2, respectively. To asses sample quantity absorbance at 260nm was used. It is assumed that the absorbance value of 50ng/µL double stranded DNAs at 260nm wavelength is "1 Optic Density" (OD). Using this basic information, the DNA concentration was calculated by placing the measured value at 260 nm into the formula given below:

dsDNA concentration= 50ng/µL x OD260 x Dilution factor

3.3.3 Bisulfite modification of the isolated DNA

The bisulfite modification of the isolated DNA was performed using EZ DNA Methylation- Lightning Kit (Zymo Research). The amount of input DNA was calculated using the concentration information obtained from Nanodrop Instrument. Bisulfite modification was performed using 450 ng of DNA according to the manufacturer's protocol. The DNA was eluted in 20µL elution buffer and quantified using absorption coefficient of RNA because recovered DNA is A, U and T-rich single stranded DNA. 40µg/mL for Ab260=1 was used to determine the concentration of the bisulfite converted DNA. Bisulfite converted DNAs stored at -20°C for later use. Human Methylated & Non-methylated DNA Set from Zymo Research used as bisulfite modification control alongside the clinical samples.

3.3.4 Detection of DNA methylation

Different approaches tested to detect and quantify the DNA methylation of ctDNA:

- MS-HRM analysis were performed to detect methylated tumour DNA using methylation specific primers and bisulfite converted DNA as template.
- Nested PCR was performed using methylation independent primers with the aim of to amplify low copy numbered methylated ctDNA. Bisulfite converted DNA was used as template. Using diluted nested PCR amplicons MS-HRM analysis was performed to detect methylated tumour DNA.

- ddPCR was performed to detect and quantify the methylated tumour DNA using methylation specific primers and bisulfite converted DNA as template.
- ddPCR was performed to detect and quantify the methylated tumour DNA using methylation specific primers and diluted nested PCR amplicons as template.

3.3.4.1 Primer selection and verification

GSTP1 primers used in this study were taken and applied as previously described[45]. The DNA sequences of the GSTP1 gene downloaded from the National Centre for Biotechnology Information (NCBI) website and given in Table 5. The promoter sequence beyond 1 kb of the Exon 1 region of the relevant gene was determined and highlighted in grey in the sequence in Table 5. CpG island content was checked by web-based software Hahn lab utility (http://canna.cau.ac.kr/util/). CpG islands are given in Figure 1. The information of the methylated and unmethylated, bisulfite converted DNA sequences are also given in Table 5. Methylation independent PCR (MI), methylation specific PCR and unmethylation specific PCR primer information are given in Table 6.

Table 5. GSTP1 gene info

Gene Name	Homo sapiens glutathione S-transferase pi 1 (GSTP1), RefSeqGene (LRG_723) on chromosome 11
NCBI Reference Number	NG_012075.1
Selected Region	From 4904 to 5381
Sequence of GSTP1 gene Grey highlighted region is Exon 1	TTCCCCCCGCGATGTCCCGGCGCGCCAGTTCGCTGCGCACACTTCGCTGCGGTCCTCTTCC TGTCTGTTTACTCCCTAGGCCCCGCTGGGGACCTGGGAAAGAGGGAAAGGCTTCCCCGGC CTGCGCGGCGACTCCGGGGACTCCAGGGCGCCCCTCTGCGGCCGACGCCCGGGGTGCAG CGCCGGGGCTGGGGCCGGCGGGAGTCCGCGGGACCCTCCAGAAGAGCGGCCGGCGCCGT TCAGCACTGGGGCGGAGCGGGGCGGGACCACCCTTATAAGGCTCGGAGGCCGCGAGGCC GCTGGAGTTTCGCCGCCGCAGTCTTCGCCACCAGTGAGTACGCGCGGCCCGCGTCCCCGC TGGGGCTCAGAGCTCCCAGCATGGGGCCAACCCGCAGCATCAGGCCCGGGCTCCCGGCA CTCCTCGCCCACCTCGAGACCCGGGACGGGGGCCTAGGG
Bisulfite converted Methylated GSTP1 sequence	TTTTTTTCGCGATGTTTCGGCGCGTTAGTTCGTTGCGTATATTTCGTTGCGGTTTTTTTTTC TTTGTTTATTTTTTAGGTTTCGTTGGGGATTTGGGAAAGAGGGAAAGGTTTTTTCGGTTAC CGCGGCGATTTCGGGGATTTTAGGGCGTTTTTTTGCGGTCGACGTTCGGGGTGTAGCGGT GGGGTTGGGGTCGGCGGGAGTTCGCGGGATTTTTTAGAAGAGCGGTCGGCGTCGTGATTT ATTGGGGCGGAGCGGGGCGGGATTATTTTTATAAGGTTCGGAGGTCGCGAGGTTTTCGTT GTTTCGTCGTCGTAGTTTTCGTTATTAGTGAGTACGCGCGGTTCGCGTTTTCGGGGATGGC TAGAGTTTTTAGTATGGGGTTAATTCGTAGTATTAGGTTCGGGTTTTCGGTAGGGTTTTTC ATTTCGAGATTCGGGACGGGGGTTTAGGG*
Bisulfite converted Unmethylated GSTP1 sequence	TTTTTTTTGTGATGTTTTGGTGTGTTAGTTTGTTGTGTATATTTTGTTGTGGTTTTTTTTTTG TTGTTTATTTTTTAGGTTTTGTTGGGGATTTGGGAAAGAGGGAAAGGTTTTTTTGGTTAGT TGGTGATTTTGGGGATTTTAGGGTGTTTTTTTGTGGTTGATGTTTGGGGTGTAGTGGTTGT GTTGGGGTTGGTGGGAGTTTGTGGGATTTTTTAGAAGAGTGGTTGGTGTTGTGATTTAGTA GGGTGGAGTGGGGTGGGATTATTTTTATAAGGTTTGGAGGTTGTGAGGTTTTTGTTGGAG GTTGTTGTAGTTTTTGTTATTAGTGAGTATGTGTGGTTTGTGTTTTTGGGGATGGGGTTTA TTTTAGTATGGGGTTAATTTGTAGTATTAGGTTTGGGTTTTTGGTAGGGTTTTTTGTTTATT AGATTTGGGATGGGGGTTTAGGG**

*Underlined primers are for methylation independent PCR. Red highlighted primers are for methylated sequence

** Green highlighted primers are for unmethylated sequence

Table 6. Primers Sequences

Primer ID	**Sequence 5'--3'**	**Fragment length, bp**
GSTP1-MI F	GGCGGGATTATTTTTATAAGGTT	124
GSTP1- MI R	CTAAAAACTCTAAACCCCATCC	
GSTP1-MSP F	GGAGGTCGCGAGGTTTTC	52
GSTP1- MSP R	CTAATAACGAAAACTACGACGACGA	
GSTP1-USP F	GTTTGGAGGTTGTGAGGTTTTT	64
GSTP1- USP R	TGTTGTTGTAGTTTTTGTTATTAGTGAGTATG	

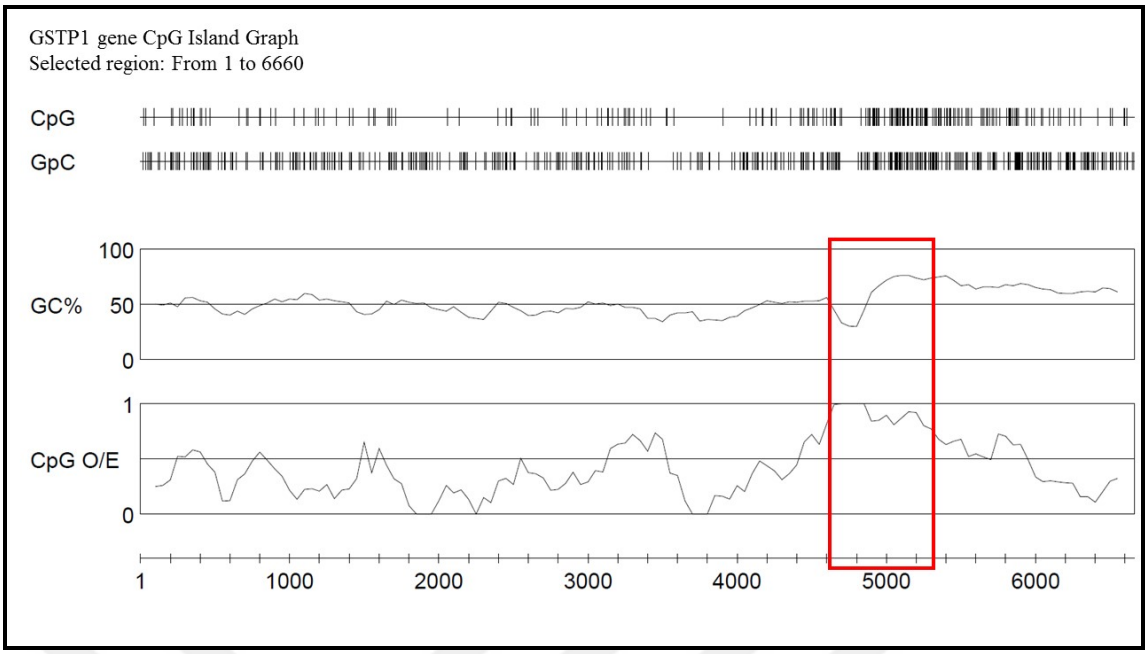


Figure 8. GSTP1 gene CpG Island Graph

3.3.4.2 Nested PCR

DNA sample isolated from peripheral blood contains unmethylated DNA (belongs to leukocytes gDNA and healthy cells cfDNAs) at high concentration and also contains ctDNA comparatively at low concentration depending on the size of the tumour. For this reason, nested PCR was performed using "GSTP1 methylation independent primers" which covers the region that shows different methylation profiles in control and patient groups. The aim of the nested PCR was to amplify the region that carries different methylation patterns and bring the methylated DNA concentration to a detectable concentration. Nested PCR was performed using Veriti Thermal Cycler Instrument, Thermofisher Scientific. Primers are given in the Table 6, cycling conditions and preparation of Nested PCR reaction mix are given in Table 7 and Table 8, respectively. Nested PCR products diluted to 10^8 times and stored at -20°C for later use. Bisulfite-Converted Universal Methylated Human DNA Standard from Zymo Research used to optimise the nested PCR protocol and as a positive control for PCR.

Table 7. Cycling conditions for Veriti thermal cycler for nested PCR

Program	Temp (°C)	Duration (sec)
Pre-Incubation	95	600
	No. Of Cycles:30	
	95	30
3-Step Amplification	57	30
	69	30
Final Extension	69	180

Table 8. Preparation of Nested PCR reaction mix

Component	Volume (µL)	Final Conc.
Tris –HCL, pH:8,8 (1M)	1,675	67mM
$(NH_4)_2SO_4$ (0,5M)	0,8	16mM
$MgCl_2$ (25mM)	6,7	6,7mM
βMercaptoethanol (1M)	0,25	10mM
dNTP's (25mM)	0,8	0,2mM
DNA polymerase (500U)	0,025	0,5U
MI-Primer F (10 µM)	0,5	0,2 µM
MI-Primer R (10 µM)	0,5	0,2 µM
MgCl2, 25mM	2,4	3mM
Bisulfite converted DNA template	10,45	25-50ng

3.3.4.3 MS-HRM analysis

qPCR and High-Resolution Melting analysis were used to amplify and analyse the bisulfite converted samples and the amplicons obtained using nested PCR. Roche Light Cycler 480 II Real Time PCR System, Light Cycler 480 High Resolution Melting Master and Universal Methylated Human DNA Standard from Zymo Research used for qPCR. Light Cycler 480 Software was used to analyse methylation profiles of the samples. Primers to amplify methylated and unmethylated DNA's are given in Table 6, thermal protocol and HRM protocol are given in Table 9. Information about preparation of LC480 qPCR reaction mix is given in Table 10.

Table 9. Cycling conditions for Roche LC480 MS-HRM analysis

Program	Temp (°C)	Duration (sec)	Acquisition Mode
Pre-Incubation	95	600	None
	No. Of Cycles:45		
	95	10	None
3-Step Amplification	62	15	None
	72	20	Single
	95	60	None
High Resolution	40	60	None
Melting	65	1	None
	95	1	Continuous (25 acquisition per /°C)

Table 10. Preparation of Roche HRM reaction mix

Component	Volume (µL)	Final Conc.
Master Mix, 2x conc.	10	1x conc.
Primer F (10 µM)	1	0,5 µM
Primer R (10 µM)	1	0,5 µM
MgCl2, 25mM	2,4	3mM
Water	0,6	
cfDNA (concentration adjusted template)	5	
Total Volume	20	

3.3.4.4 ddPCR

QX 200 droplet Generator used to obtain droplets using the 96 well plate which contains EvaGreen master reaction mix and template DNA. PX1 PCR Plate sealer used to seal the 96 well plate and C1000 touch thermal cycler was used to perform thermal cycling using bisulfite converted samples and the amplicons obtained using nested PCR as template. QX200 Droplet Reader and QuantaSoft Software was used for data acquisition and analysing. Thermal protocol and preparation of ddPCR reaction mix are given in Table 11 and Table 12, respectively. Methylation and Unmethylation specific primers given in Table 6 used in ddPCR.

Table 11. Preparation of ddPCR EvaGreen reaction mix

Component	Volume per Reaction, µL	Final Concentration
2x QX200 ddPCR EvaGreen Supermix	10	1x
Forward Primer	1	100nM
Reverse Primer	1	100nM
DNA template	3	Up to 100ng
ddH_2O	5	-
Total Volume	20	

Table 12. Cycling conditions for BioRad's C1000 Touch Thermal Cycler

Cycling Step	Temperature, °C	Time	Ramp Rate	Number of Cycles
Enzyme activation	95	5 min	2 °C/sec	1
Denaturation	95	30 sec		40
Annealing/extension	62	1 min		40
Signal stabilisation	4	5 min		1
	90	5 min		1

3.3.5 Statistical Analysis

Statistical analysis of this study was performed using licensed SPSS 23.0 and MedCalc software for Receiver Operating Characteristic (ROC) analysis. Statistical significance was taken as $p < 0.05$.

4. RESULTS

4.1 Demographic Data of Working Group

The demographic data of 65 Prostate Cancer Patient Groups and 76 healthy controls participated in our thesis project are summarized in Table 4-1.

Table 13. Demographic characteristics of the study population

Parameter		Prostate Cancer (n=65)	Control (n=76)	p- Value
Age (years),		67.61 ± 7.34	67.53 ±8.77	0.967
Body mass index (kg/m), mean ±SD		27.01±3.71	27.28±3.55	0.773
Smoking (pack year), mean±SD		30.56±18.68	27.75±17.04	0.594
Prostate-specific antigen (ng/ml) mean±SD		25.94±41.14	3.03±2.66	0.006*
Gleason score, mean±SD		7.74±0.88	-	-
Family history of cancer, n (%) ‡	Yes	31 (43.7%)	9 (11.8%)	-
	No	38 (53.5%)	10 (13.2%)	-
	NA	2 (2.8%)	57 (75%)	-
Pathological T-stage, n (%)	T2a	9 (12.7%)	-	-
	T2b	10 (14.1%)	-	-
	T2c	29 (40.8%)	-	-
	T3a	11 (15.5%)	-	-
	T3b	10 (14.1%)	-	-
	NA	2 (2.8%)	-	-
Clinical T-stage, n (%)	Early (T1+T2)	61 (85.9%)	-	-
	Late (T3+T4)	8 (11.3%)	-	-
	NA	2 (2.8%)	-	-

N: Number of individuals; NA: not available * statistically significant difference, ‡first degree

In this study 65 patients with prostate cancer who were treated at the Department of Urology, Yeditepe University Hospital and Göztepe Research and Education Hospital. To confirm the prostate cancer diagnosis clinical, laboratory and pathological examinations were used. Gleason score criteria were used to evaluate tumour differentiation. As a result of clinical examinations, clinical T stage was classified as early (T1 and T2) and late stage (T3 and T4). Pathological T-stage was classified as

T2a, T2b, T2c, T3a and T3b. Prostate cancer negative patients, a total of 76 age-matched controls were selected from the Urology Clinics of the same hospitals. Hospital record were used to collect clinical parameters (body mass index, smoking habit etc) for each participant. Peripheral bloods samples were taken after obtaining informed consent and the study was conducted prospectively. Local Ethical Committee approval was obtained for the study (protocol no: 63/505). The protocol followed was consistent with the World Medical Association Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects).

4.2 qPCR optimisation

qPCR optimisations were performed using Human Methylated & Non-methylated DNA Set. qPCR conditions such as annealing temperature, primer specificity and primer and $MgCl_2$ concentration optimised using LC480 PCR equipment and Light Cycler 480 High Resolution Melting Master.

Figure 9 shows melting curves, melting peaks and melting temperatures of methylated and unmethylated GSTP1 controls after qPCR optimisation.

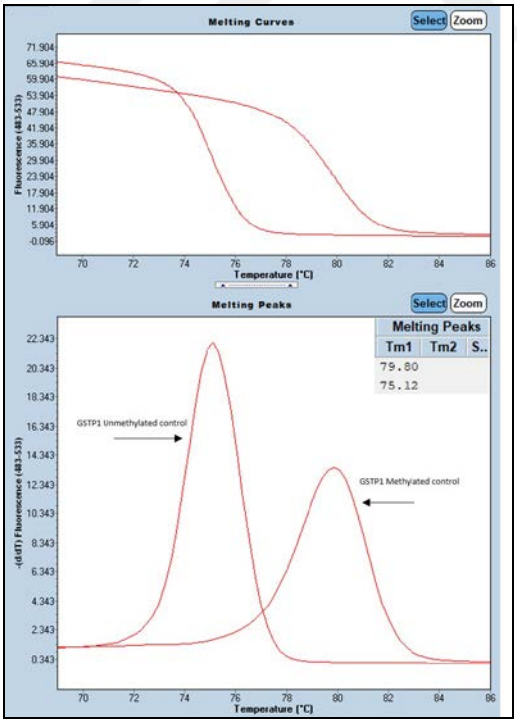


Figure 9. Melting profiles belong to methylated and unmethylated GSTP1 controls

4.2.1 Optimisation of $MgCl_2$ concentration

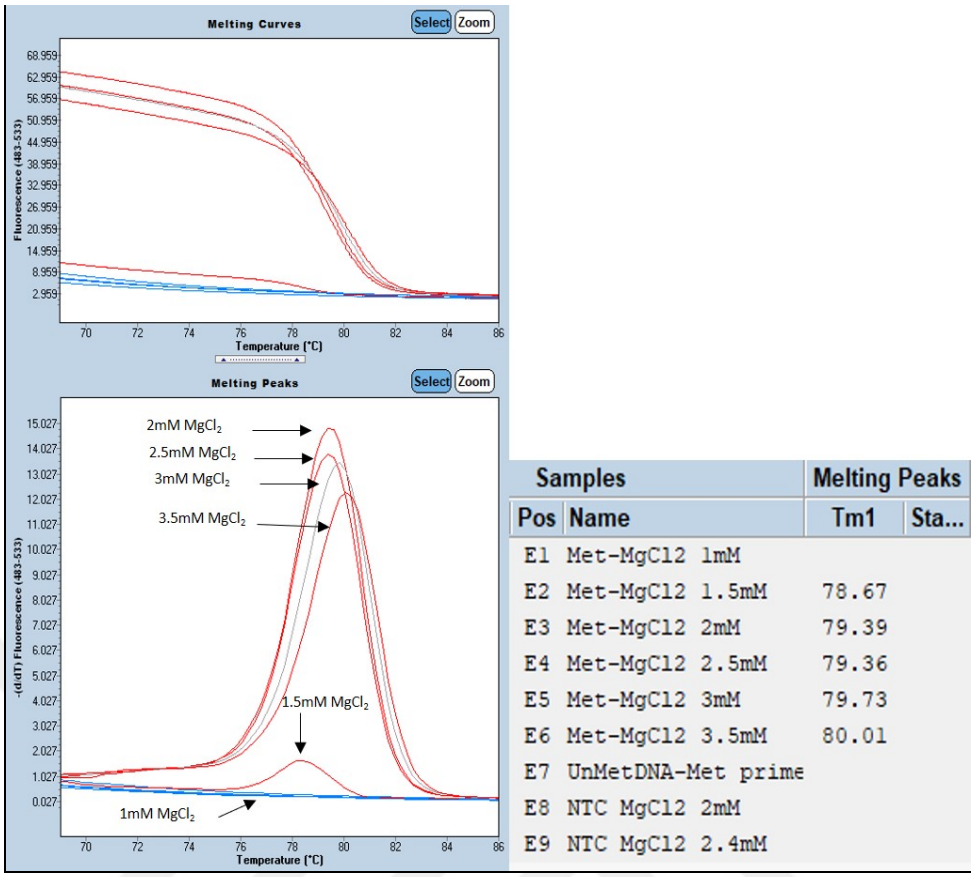
Six different $MgCl_2$ (1mM, 1.5mM, 2mM, 2.5mM, 3mM, 3.5mM) concentration were tested using methylated and unmethylated control DNAs as template.

Between the tested concentrations, the concentration which has the lowest Cq and the highest end point PCR product was determined and 3mM $MgCl_2$ concentration was selected to be used in the subsequent experiments. Figure 10, Figure 11, Figure 12 and Figure 13 shows the melting peaks, melting temperatures, amplification curve and Cq values obtained under different $MgCl_2$ concentrations. It was observed that the melting temperature increased in parallel with the increase in $MgCl_2$ (Table 14).

In this experiment, it was also checked whether there was nonspecific amplification between methylated DNA and unmethylated primers and between unmethylated DNA and methylated primers. Since nonspecific amplification was not detected, both primer groups were found to be specific to their respective sequences.

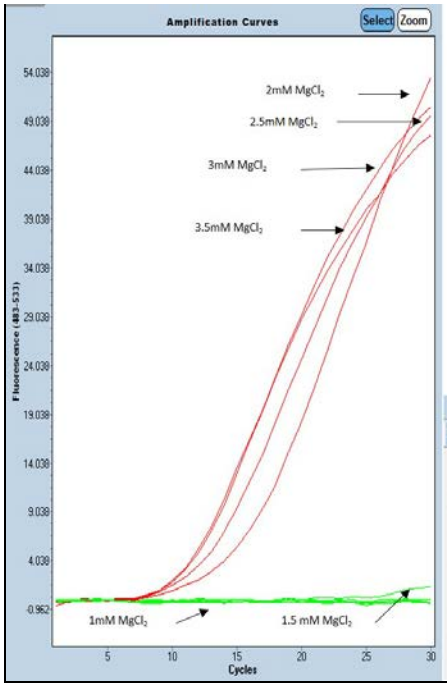
Table 14. $MgCl_2$ optimisation-comparison of Tm and Cq values

$MgCl_2$ concentration	Melting Temperature		Cq	
	Methylated	Unmethylated	Methylated	Unmethylated
1mM	-	-	-	-
1.5 mM	78.67	73.90	-	22.92
2 mM	79.39	73.99	16.56	8.20
2.5 mM	79.36	74.73	13.03	7.96
3 mM	79.73	75.18	11.46	8.04
3.5 mM	80.01	75.55	10.94	8.19



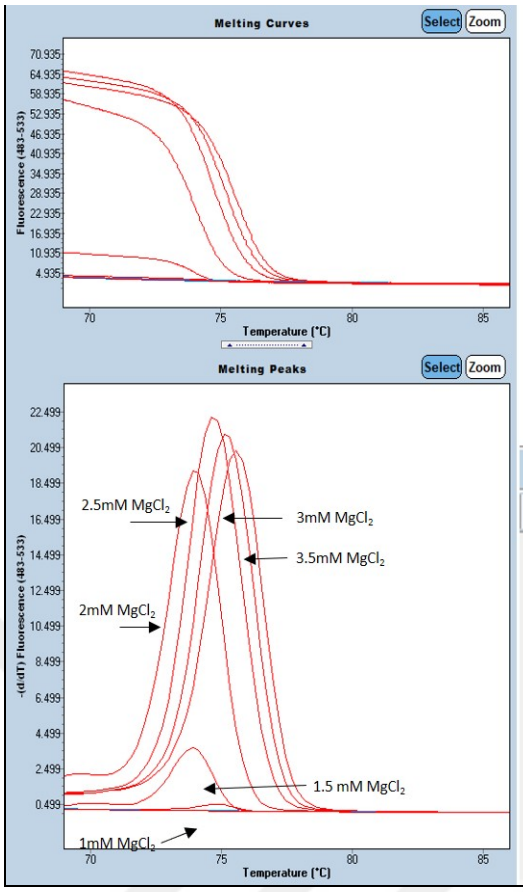
Samples		Melting Peaks	
Pos	Name	Tm1	Sta...
E1	Met-MgCl2 1mM		
E2	Met-MgCl2 1.5mM	78.67	
E3	Met-MgCl2 2mM	79.39	
E4	Met-MgCl2 2.5mM	79.36	
E5	Met-MgCl2 3mM	79.73	
E6	Met-MgCl2 3.5mM	80.01	
E7	UnMetDNA-Met prime		
E8	NTC MgCl2 2mM		
E9	NTC MgCl2 2.4mM		

Figure 10. $MgCl_2$ optimisation for methylated GSTP1 assay-melt curves



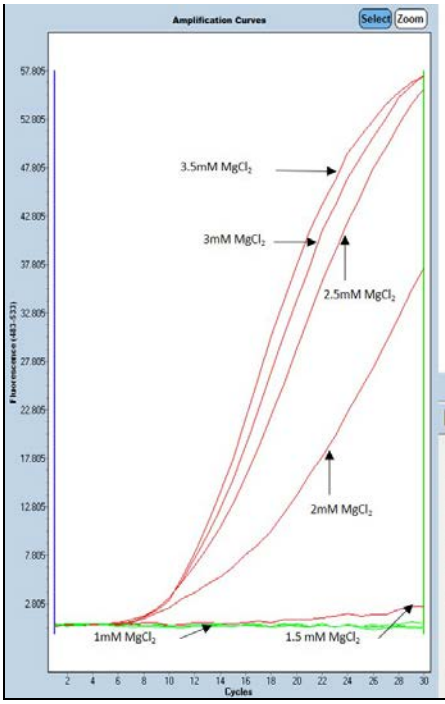
Samples		
Pos	Name	Cp
E1	Met-MgCl2 1mM	
E2	Met-MgCl2 1.5mM	
E3	Met-MgCl2 2mM	16.56
E4	Met-MgCl2 2.5mM	13.03
E5	Met-MgCl2 3mM	11.46
E6	Met-MgCl2 3.5mM	10.94
E7	UnMetDNA-Met prime	
E8	NTC MgCl2 2mM	
E9	NTC MgCl2 2.4mM	

Figure 11. $MgCl_2$ optimisation for methylated GSTP1 assay- amplification curve



Samples		Melting Peaks	
Pos	Name	Tm1	Sta...
F1	UnMet-MgCl2 1mM		
F2	UnMet-MgCl2 1.5mM	73.90	
F3	UnMet-MgCl2 2mM	73.99	
F4	UnMet-MgCl2 2.5mM	74.73	
F5	UnMet-MgCl2 3mM	75.18	
F6	UnMet-MgCl2 3.5mM	75.55	
F7	MetDNA-UnMet prime	74.85	
F8	NTC MgCl2 2mM		
F9	NTC MgCl2 2.4mM	72.99	

Figure 12. MgCl2 optimisation for unmethylated GSTP1 assay-melt curve



Samples		
Pos	Name	Cp
F1	UnMet-MgCl2 1mM	
F2	UnMet-MgCl2 1.5mM	22.92
F3	UnMet-MgCl2 2mM	8.20
F4	UnMet-MgCl2 2.5mM	7.96
F5	UnMet-MgCl2 3mM	8.04
F6	UnMet-MgCl2 3.5mM	8.19
F7	MetDNA-UnMet prime	
F8	NTC MgCl2 2mM	
F9	NTC MgCl2 2.4mM	

Figure 13. $MgCl_2$ optimisation for unmethylated GSTP1 assay- amplification curve

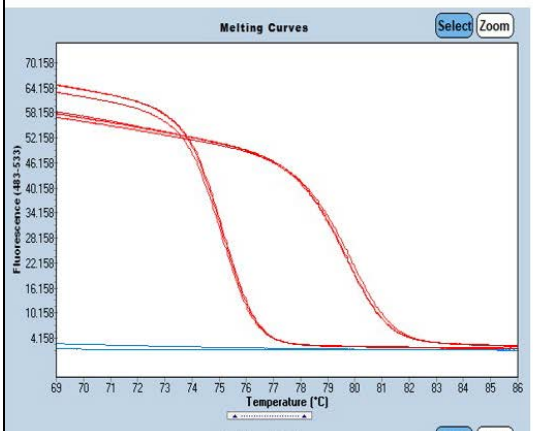
4.2.2 Optimisation of annealing temperature

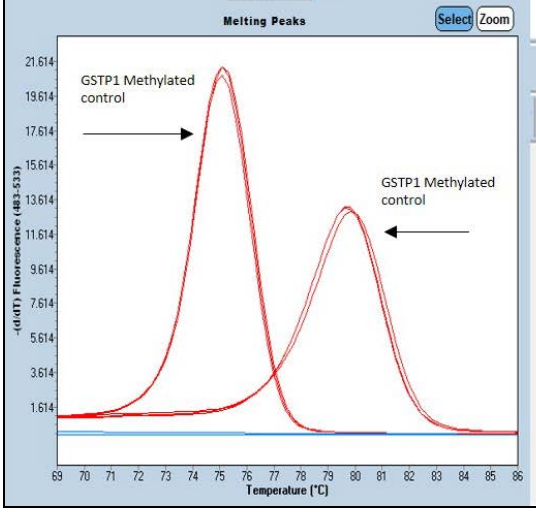
Three different temperature used to optimise PCR conditions: 57°C, 60°C, 62°C. Cq values obtained under different annealing temperatures are given in Table 15. Smallest Cq values were obtained at annealing temperature 62°C and chosen for the following experiments. There is no significant difference observed between melting curves and melting temperatures under different annealing temperature conditions. Melting curve and melting temperatures, amplification curve and Cq values belong to methylated and unmethylated GSTP1 assays under different annealing temperatures are given in Figure 14, Figure 15, Figure 16, Figure 17, Figure 18and Figure 19.

Table 15. Cq values obtained under different annealing temperatures

Annealing Temperature	Melting Temperature		Cq	
	Methylated	Unmethylated	Methylated	Unmethylated
57°C	79.69	75.10	21.35	20.83
60°C	79.53	75.23	21.32	20.66
62°C	79.82	75.19	21.1	20.4

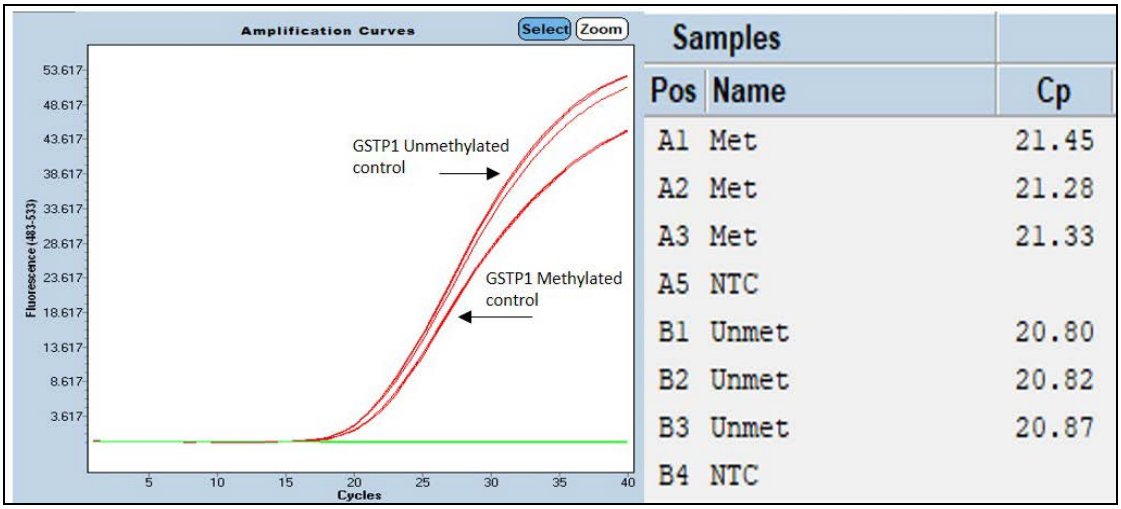
Annealing Temperature: 57°C





Samples		Melting Peaks	
Pos	Name	Tm1	Tm2
A1	Met	79.79	
A2	Met	79.63	
A3	Met	79.64	
A5	NTC		
B1	Unmet	75.14	
B2	Unmet	75.10	
B3	Unmet	75.08	
B4	NTC		

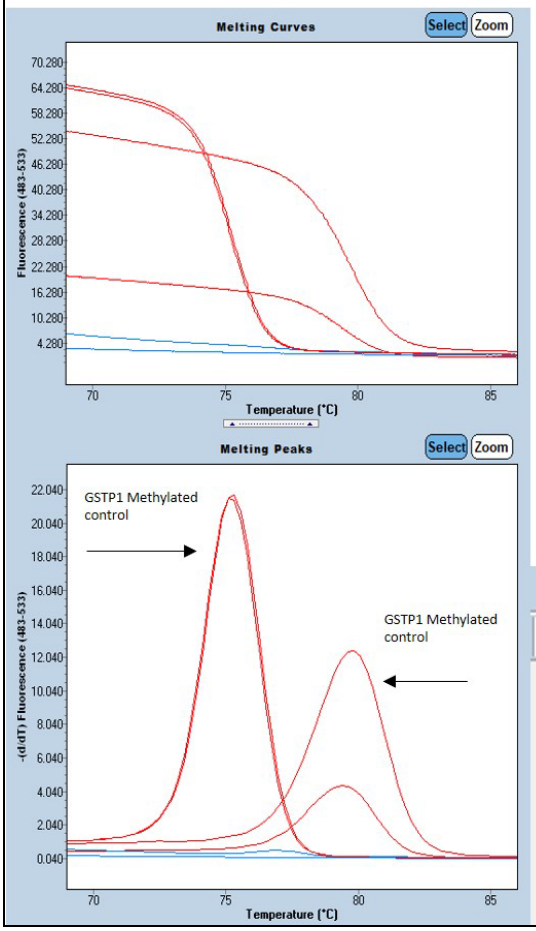
Figure 14.Melting curve and melting temperatures- annealing temperature 57°C



Samples		
Pos	Name	Cp
A1	Met	21.45
A2	Met	21.28
A3	Met	21.33
A5	NTC	
B1	Unmet	20.80
B2	Unmet	20.82
B3	Unmet	20.87
B4	NTC	

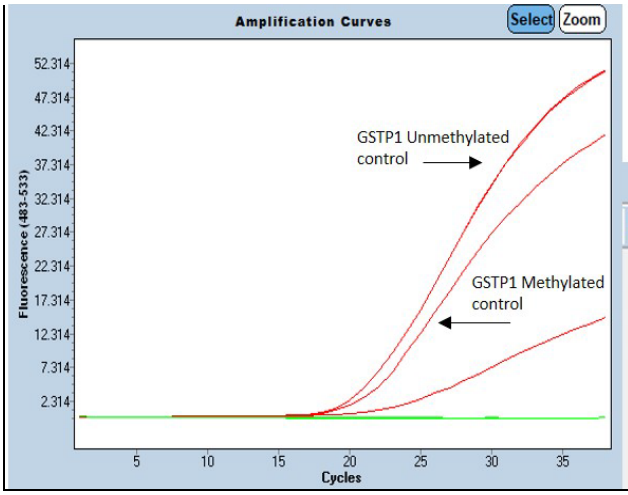
Figure 15. Amplification curve and Cq values - annealing temperature 57°C

Annealing Temperature: 60°C



Samples		Melting Peaks	
Pos	Name	Tm1	Tm2
A1	Met	79.38	
A2	Met	79.68	
A3	NTC		
B1	Unmet	75.25	
B2	Unmet	75.20	
B3	NTC		

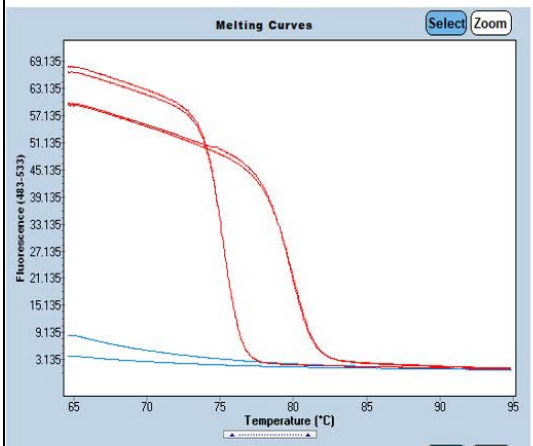
Figure 16. Melting curve and melting temperatures- annealing temperature 60°C

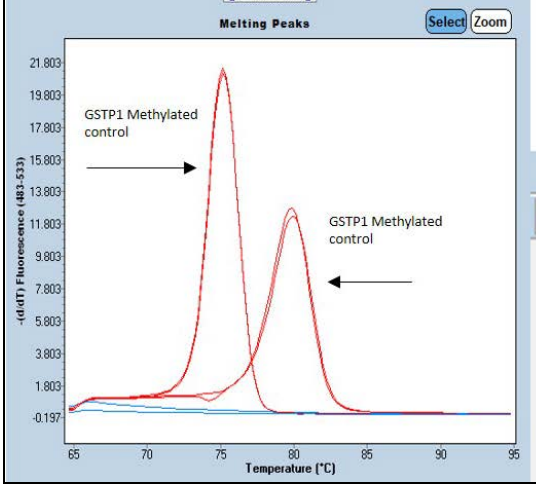


Samples		
Pos	Name	Cp
A1	Met	23.53
A2	Met	21.32
A3	NTC	
B1	Unmet	20.67
B2	Unmet	20.66
B3	NTC	

Figure 17. Amplification curve and Cq values - annealing temperature 60°C

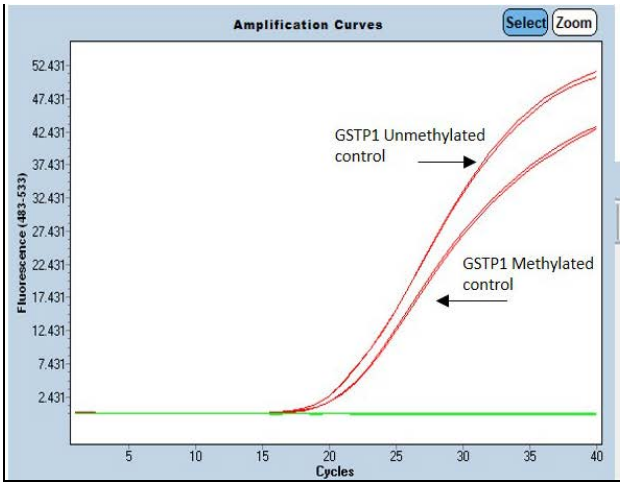
Annealing Temperature: 62°C





Samples		Melting Peaks	
Pos	Name	Tm1	Tm2
A1	Met	79.88	
A2	Met	79.77	
A3	NTC		
B1	Unmet	75.20	
B2	Unmet	75.18	
B4	NTC		

Figure 18. Melting curve and melting temperatures- annealing temperature 62°C



Samples		
Pos	Name	Cp
A1	Met	21.13
A2	Met	21.07
A3	NTC	
B1	Unmet	20.34
B2	Unmet	20.46
B4	NTC	

Figure 19. Amplification curve and Cq values - annealing temperature 62°C

4.2.3 qPCR efficiency

PCR efficiencies for methylated and unmethylated GSTP1 assays were checked using 5-point 1/10 serial dilution of control materials and found as 2.00 and 1.95, respectively. Correlation coefficient ($r^2 > 0,90$) and slope ($-3,2 < slope > -3,6$) values were determined in the range defined in the MİQE guideline [46]. Calibration and amplification curves are given in Figure 20 and Figure 21, efficiency, r^2, slope values are given in Table 16.

Table 16. qPCR efficiency information for GSTP1 assays

Reaction	r^2	slope	efficiency
PCR -methylated	1.987	-3.35	2.00
PCR-unmethylated	1.963	-3.414	1.95

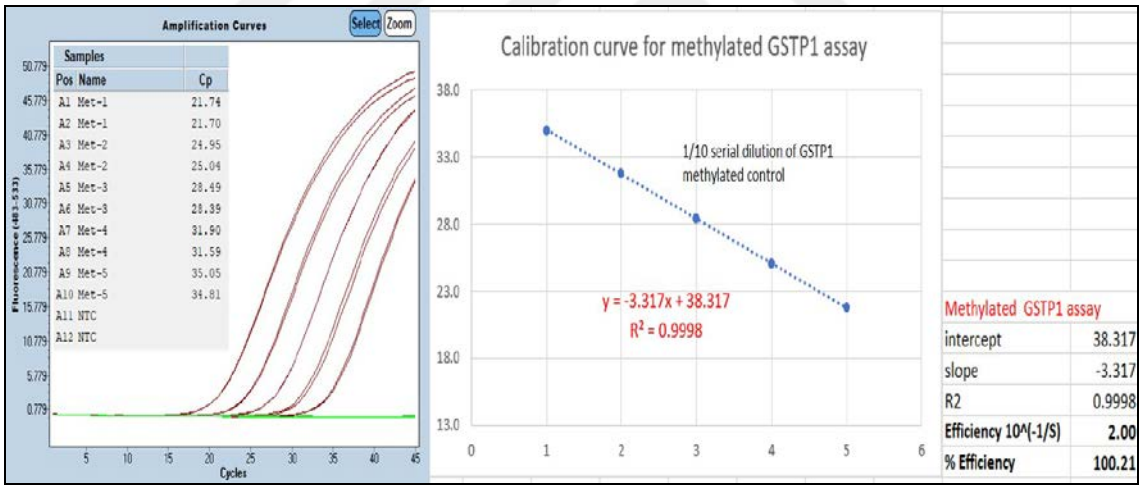


Figure 20. PCR Efficiency of GSTP1 methylated assay

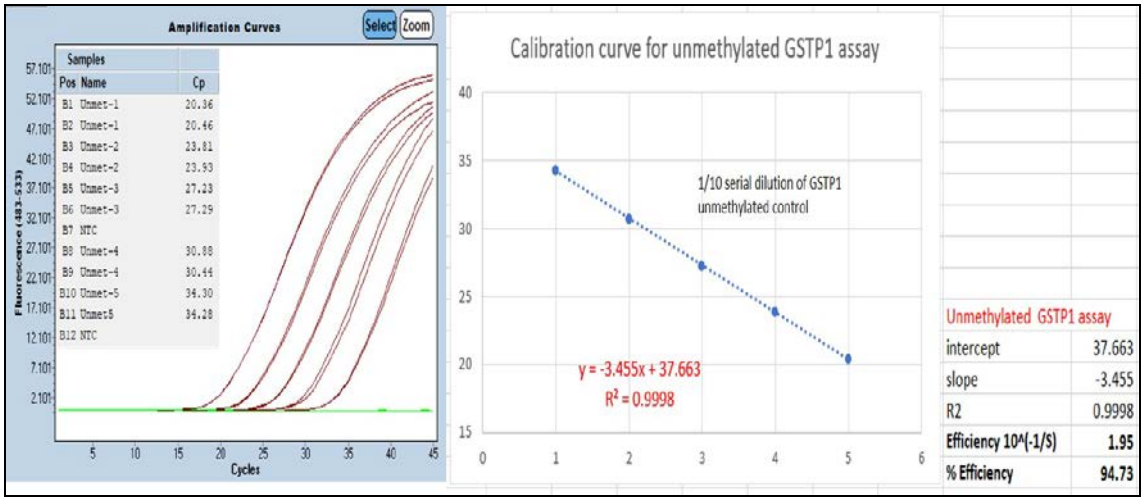
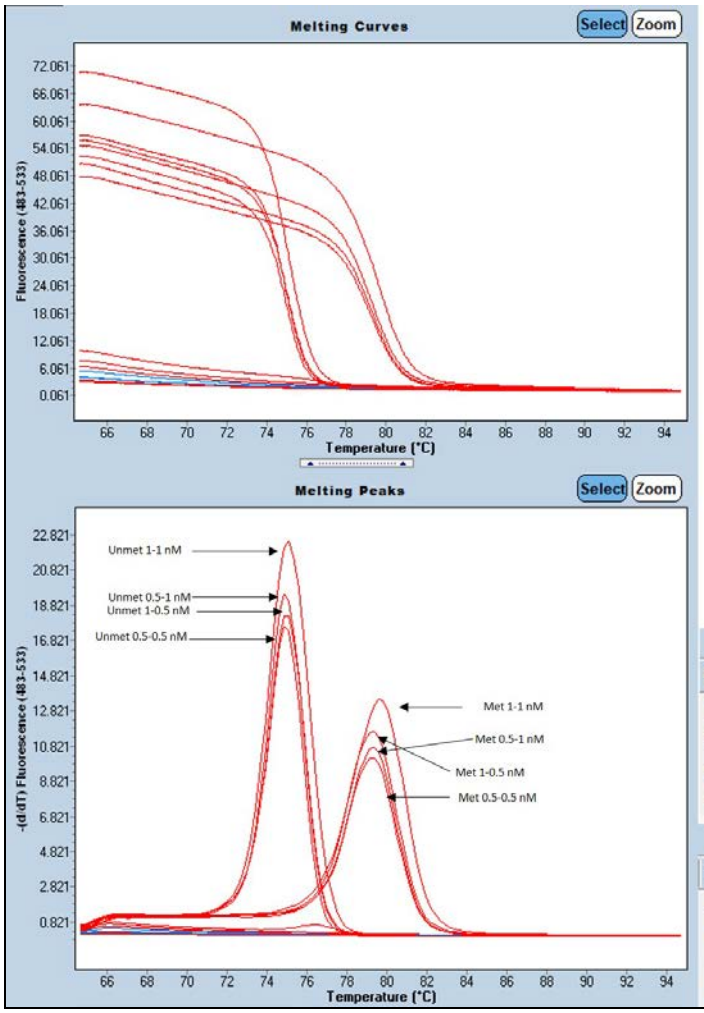


Figure 21. PCR Efficiency of GSTP1 unmethylated assay

Different forward and reverse primer concentrations are used to test robustness of the assays. There was no significant change on the Cq values and melting temperature of the amplicons. A decrease in the melting temperature was observed as the primer concentration decreased. It can be seen at the Figure 1Figure 22.



Samples		Melting Peaks	
Pos	Name	Tm1	Sta...
A1	Met 1-1 nM	79.69	
B1	Met 0.5-1 nM	79.45	
C1	Met 1-0.5 nM	79.41	
D1	Met 0.5-0.5 nM	79.37	

Samples		Melting Peaks	
Pos	Name	Tm1	Sta...
A5	Unmet 1-1 nM	75.09	
B5	Unmet 0.5-1 nM	74.96	
C5	Unmet 1-0.5 nM	75.01	
D5	Unmet 0.5-0.5 nM	74.94	

Figure 22. Primer optimisation-Melting curves and melting temperatures

It was observed that different primer concentrations did not have a significant effect on the Cq value of methylated DNA, whereas the highest Cq value was obtained in forward 1nM, reverse 1 nM in unmethylated DNA. Figure 23 shows the amplification curve and Cq values under different primer concentration.

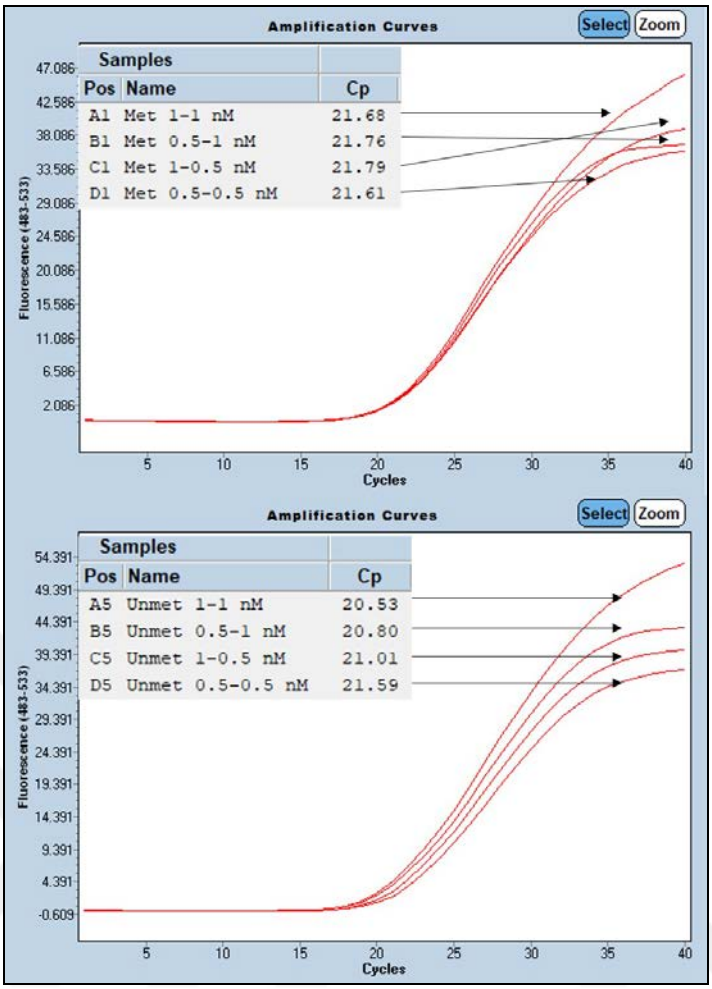
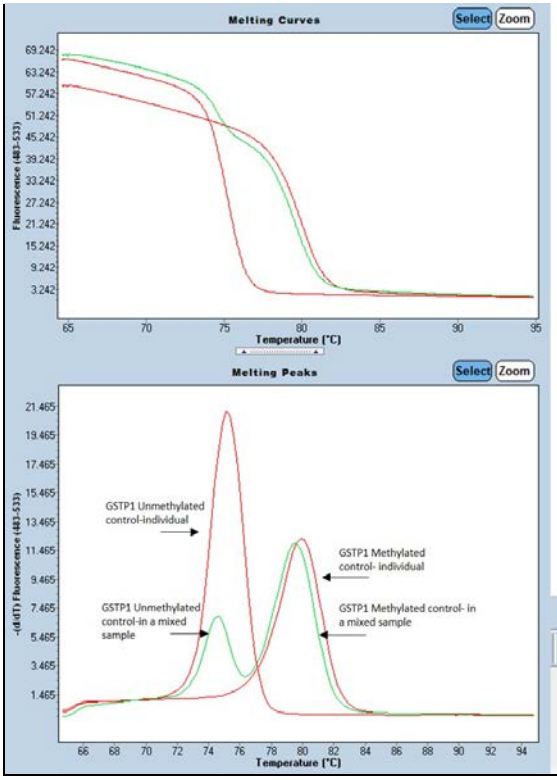


Figure 23. Primer optimisation-Amplification curves and Cq values

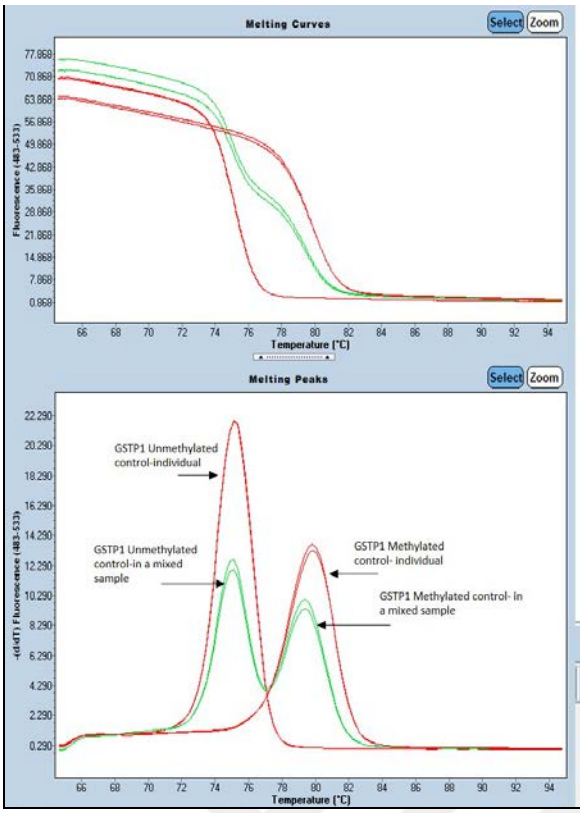
The different primer concentrations were also tested on a representative sample which is prepared mixing the methylated and unmethylated control DNA. When the methylated and unmethylated controls were studied individually, it was observed that they had a melting temperature of about 0.5 degrees higher than the representative mix sample. Also, a PCR bias observed between “unmethylated control” and “unmethylated control in the representative mix”. It was observed that change in the melting peak fluorescence values of the unmethylated control and “the unmethylated control contained in the representative sample" did not change proportionally while the melting peak fluorescence value of the methylated control remained constant. Considering that HRM is an endpoint analysis, we can say that peak heights provide information about the amount of amplicon at the end of PCR. This information shows that PCR efficiency is reduced when multiplex reaction conditions and representative sample (sample contains methylated and unmethylated templates) are used for the unmethylated GSTP1

assay, which indicates that assay needs extra optimization. It is possible to optimise the efficiency of unmethylated GSTP1 assay, changing the primer concentration. Figure 24 shows the PCR bias that occurs when the multiplex PCR and representative sample are used. Figure 25 show how PCR bias can be corrected changing the primer concentrations.



Samples		Melting Peaks		
Pos	Name	Tm1	Tm2	S
A1	Met	79.88		
B1	Unmet	75.20		
C1	Sample 25	74.55	79.49	

Figure 24. PCR bias caused observed in multiplex PCR



Samples		Melting Peaks	
Pos	Name	Tm1	Tm2
H4	Sample 88	75.16	
H7	Sample 91	75.02	79.39
H10	Sample 94	79.79	

Figure 25. PCR bias corrected optimising primer concentration

4.3 MS-HRM analysis using clinical samples

After DNA isolation clinical samples are bisulfite converted. Two different Set of samples prepared for MS-HRM analyses. The first set (Set 1) used for MS-HRM analyses was bisulfite converted clinical samples. The second set (Set 2) consist of nested PCR amplicons which was obtained using bisulfite converted DNAs.

4.3.1 MS-HRM analyses results of Set 1

Set 1 is used as template to detect promotor methylation of clinical samples. GSTP1 methylation was not detected in both control and patient group. All the control group and patient group GSTP1 detected as unmethylated. Melting curves and melting peaks of the GSTP1 methylated control are given in Figure 26.

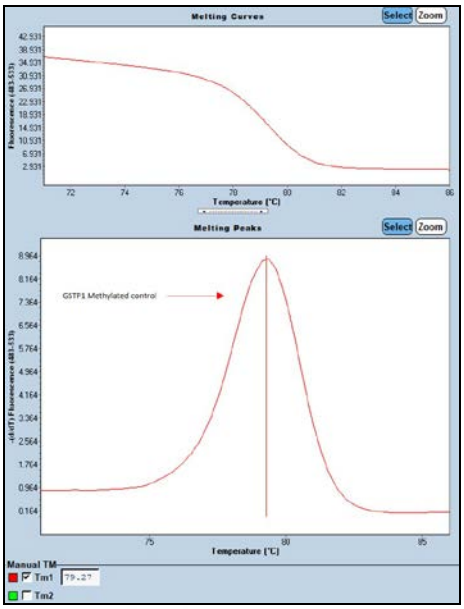


Figure 26. Melting profiles belong to GSTP1 methylated control.

4.3.2 MS-HRM analyses results of Set 2

Set 2 which consist of bisulfite converted and nested PCR performed samples used to detect GSTP1 methylation and of the 65 samples 9 samples found as GSTP1 methylated. Control group and the rest of the patient group found as GSTP1 unmethylated. Melting curves and melting peaks of the methylated GSTP1 assay using Set 2 as templates and their melting temperatures are given in Figure 27.

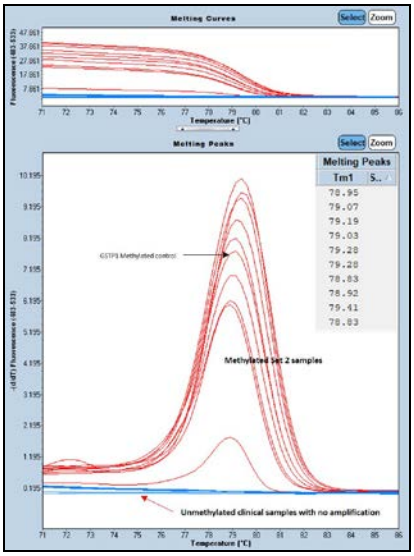


Figure 27. Melting profiles belong to Set 2

Figure 28 shows the melting peak and melting temperature difference between a non-specific amplification and GSTP1 methylated control.

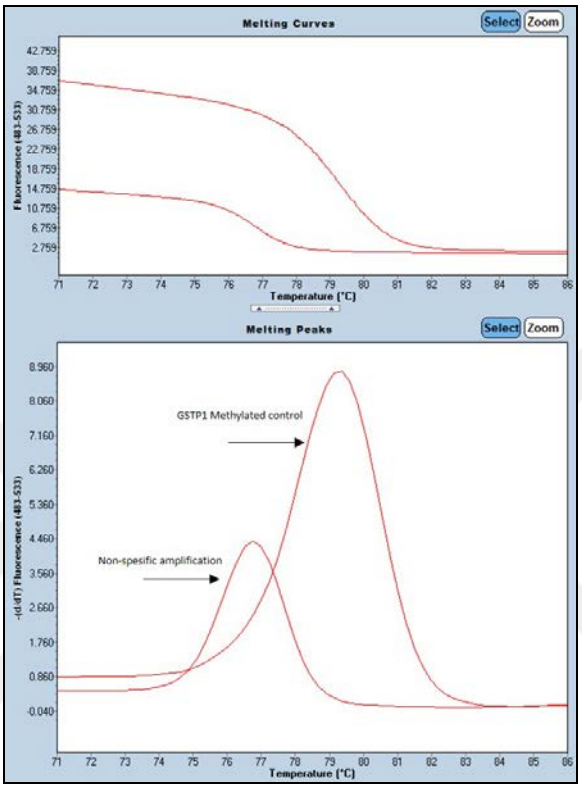


Figure 28. Melting profile of non-specific amplification and GSTP1 methylation

4.4 Optimisation of ddPCR

Relatively lower amount of primer is used in ddPCR. Before starting to work with the ddPCR system primer concentration needs to be optimised and the assays efficiencies needs to be checked.

4.4.1 Optimisation of primer concentration for ddPCR

Primer concentration optimisation were performed using 4 different concentration (0.05mM, 0.1mM, 0.2mM, 0.4mM) of methylated GSTP1 assay and methylated control. In ddPCR it is very important to have clear distinction between positive and negative droplets [44]. In this optimisation experiment we observed best separation for 0.1mM methylated GSTP1 assays. Using this concentration for methylated GSTP1 assay, unmethylated GSTP1 primer concentration optimisation

performed in a multiplex reaction (Figure 30). Figure 29 shows the 1Dimensional (1D) and 2D scatter plots and 1D histogram, belong to the primer optimisation experiment.

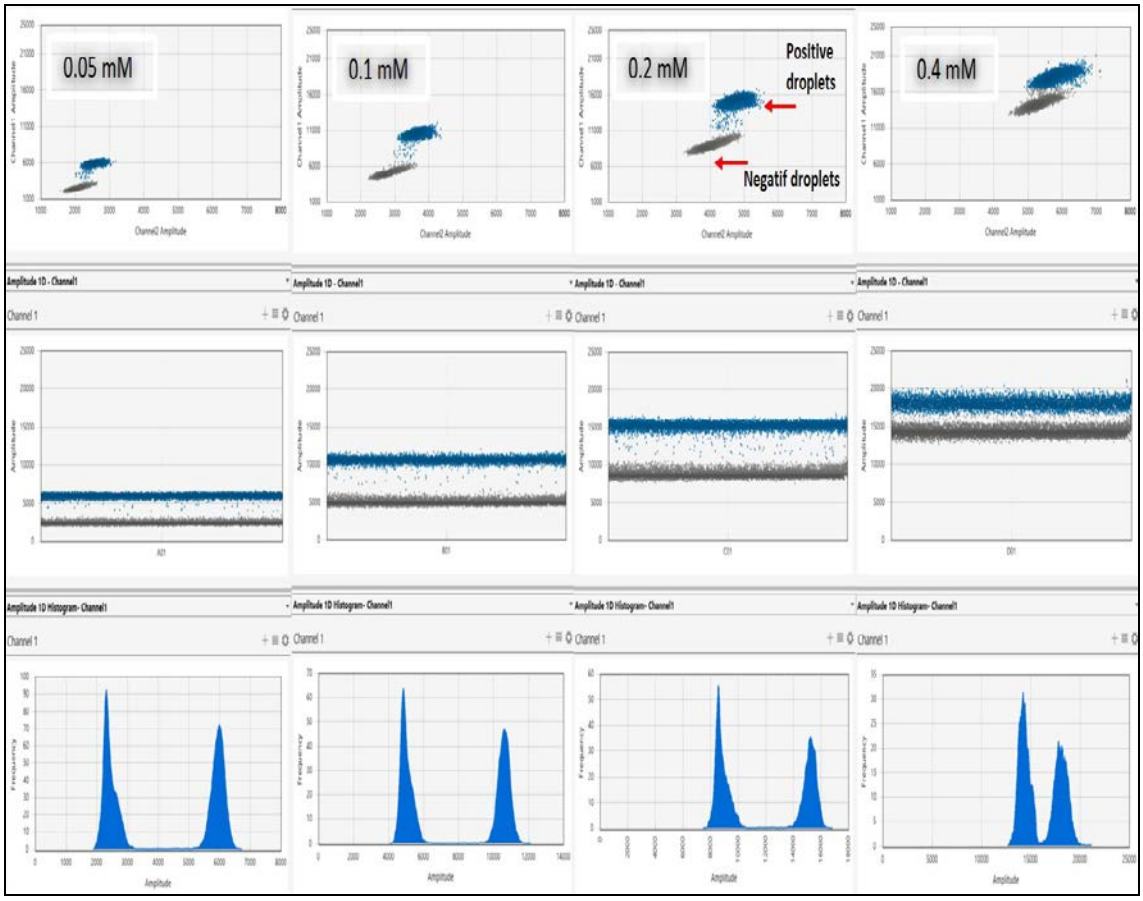


Figure 29. Methylated GSTP1 assay primer optimisation

Representative clinical sample prepared using methylated and unmethylated control DNAs and used as a template for multiplex ddPCR primer optimisation experiment. Related 1D and 2D scatter plots and 1D histogram, belong to the multiplex primer optimisation experiment are given in Figure 30

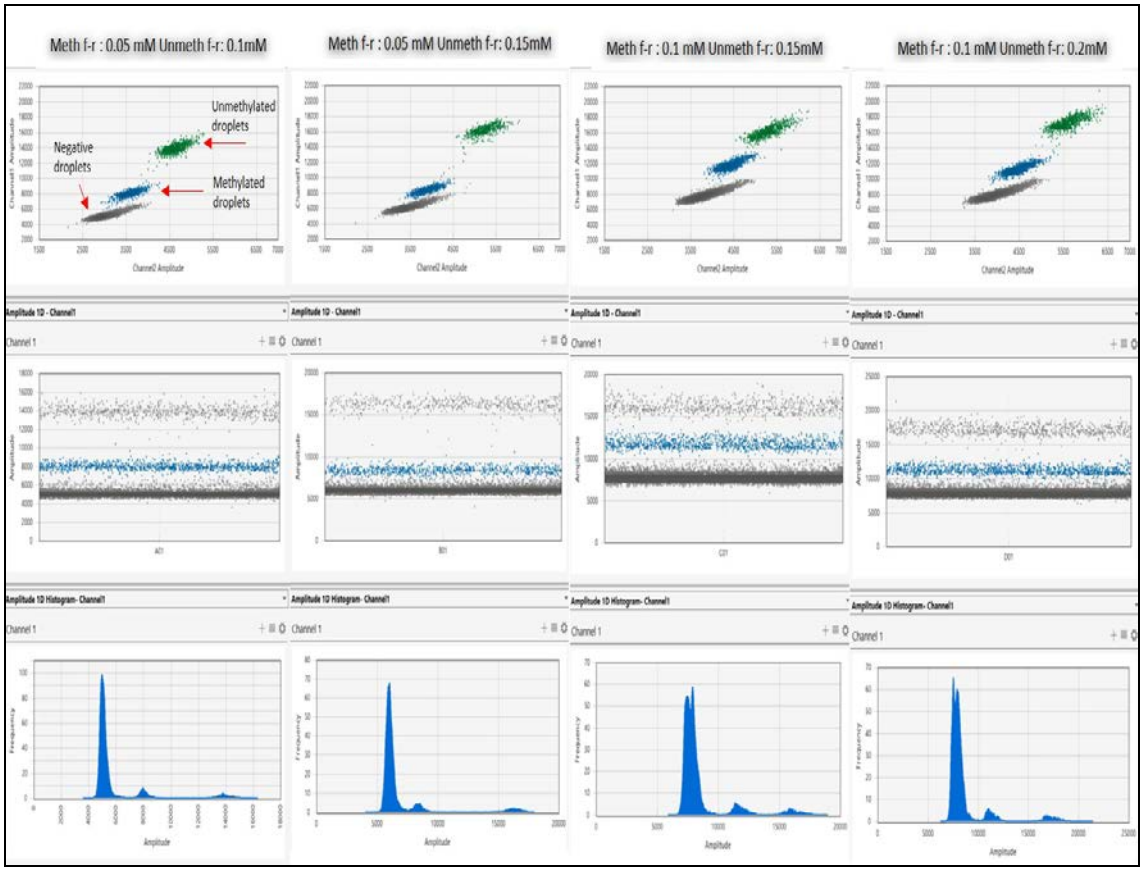


Figure 30. Multiplex ddPCR primer optimisation

4.4.2 ddPCR linearity

ddPCR linearity for unmethylated and methylated GSTP1 assays were checked using 6-point 1/2 serial dilution of unmethylated and methylated controls and linearities were found in the acceptable range ($r^2 > 0{,}90$) according to the MİQE guideline [46]. Plots and histograms are given in Figure 31 for unmethylated GSTP1 assay and Figure 33 for methylated GSTP1 assay.

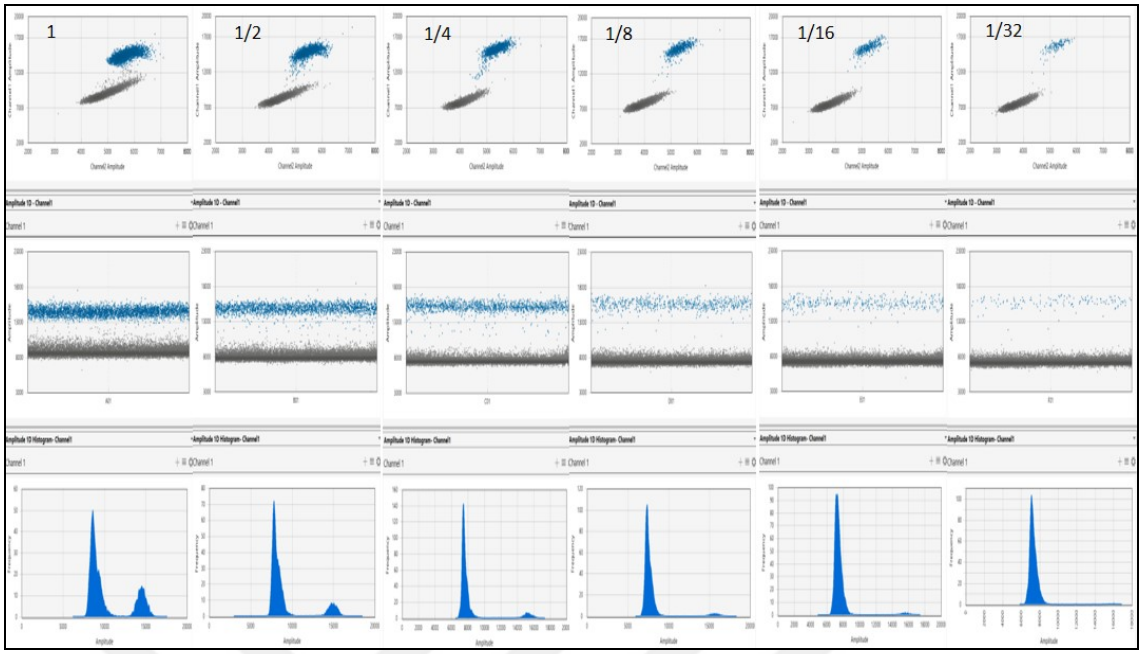


Figure 31. ddPCR efficiency experiment for unmethylated GSTP1 assay

Calibration curves was drawn using the concentration data (final cp/µL) given in Table 17. and Table 18. Calibration curves and r^2 values of the unmethylated and methylated GSTP1 assays are given in Figure 32 and Figure 34.

Table 17. Linearity of unmethylated GSTP1 assay

Exp047 Analysis													
		Reaction volume	20	µl									
		Template volume	3	µl									
		Partition volume	0.00083	µl									
		Droplets/reaction	23980.8	(Assuming all 20 µL is converted into droplets)									
		Template vol/partition	0.00013	µl									
					Imported Data					Calculated Data					
Well	Run #	Sample	Target	Status	Positive Droplets	Negative Droplets	Accepted Droplets	λ	[Final] c/µL	Copies/ 20µL	[Input] (c/µl)	Dilution factor	c/µl Extract
G01		Unmeth Control M.	EVA1	Manual	3670	10491	14161	0.300	359.7	7194	2398	500	1198937
H01		Unmeth Control M 1/2	EVA1	Manual	2049	12364	14413	0.153	183.9	3677	1226	500	612876
A02		Unmeth Control M. 1/4	EVA1	Manual	1362	15749	17111	0.083	99.5	1989	663	500	331513
B02		Unmeth Control M. 1/8	EVA1	Manual	660	16597	17257	0.039	46.8	935	312	500	155859
C02		Unmeth Control M. 1/16	EVA1	Manual	405	19009	19414	0.021	25.3	506	169	500	84260
D02		Unmeth Control M. 1/32	EVA1	Manual	161	17158	17319	0.009	11.2	224	75	500	37329

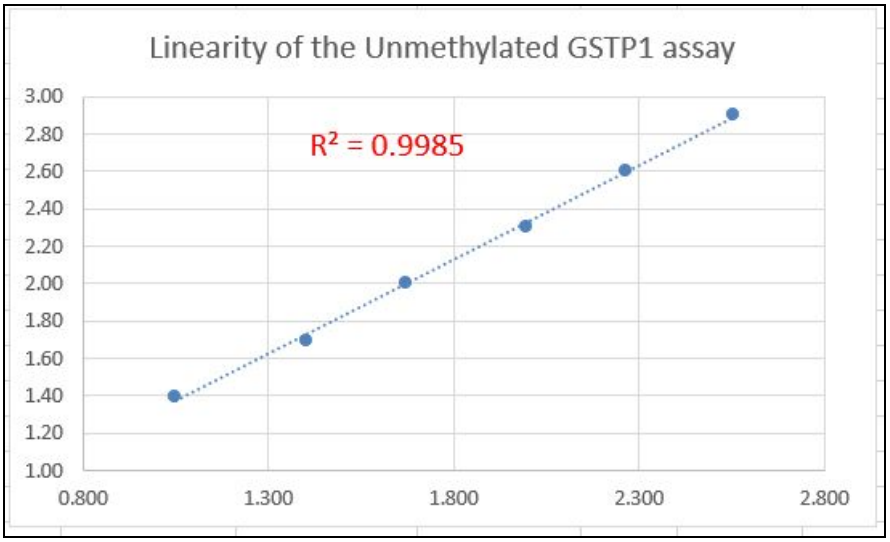


Figure 32. Calibration curve and r^2 of Unmethylated GSTP1 assay

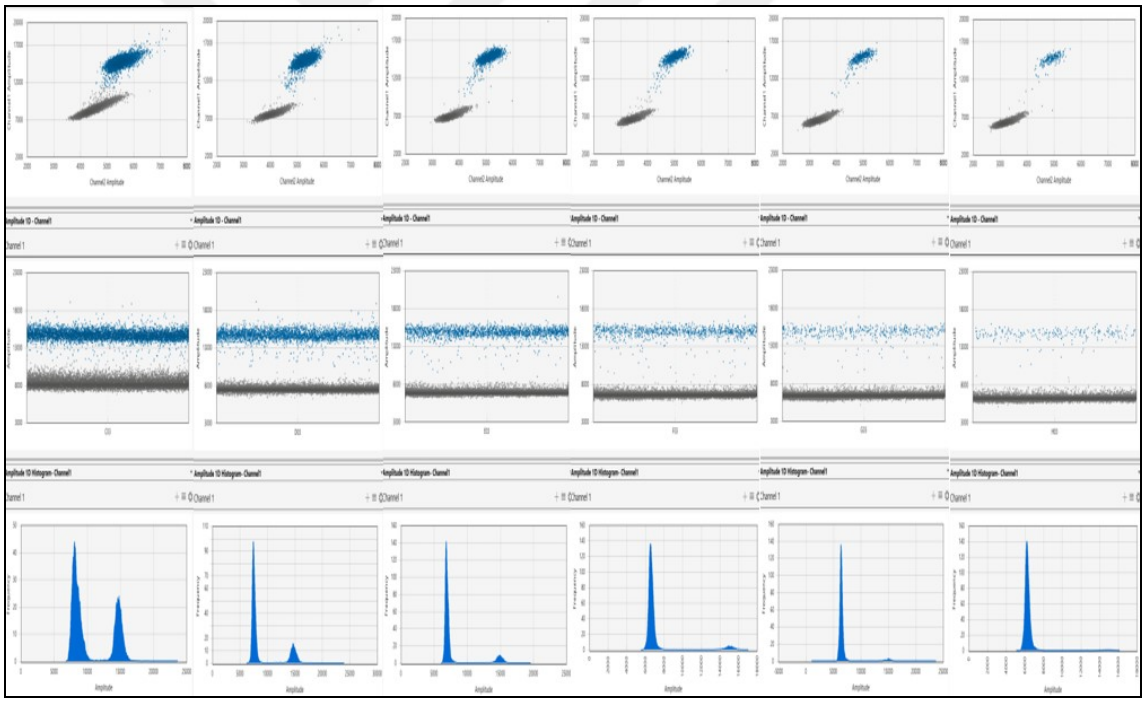


Figure 33. ddPCR efficiency experiment for methylated GSTP1 assay

Table 18. Linearity of methylated GSTP1 assay

Exp047 Analysis														
		Reaction volume	20	µl										
		Template volume	3	µl										
		Partition volume	0.00083	µl										
		Droplets/reaction	23980.8	(Assuming all 20 µL is converted into droplets)										
		Template vol/partition	0.00013	µl										
Imported Data							Calculated Data							
Well	Run #	Sample	Target	Status	Positives	Negatives	Accepted	λ	[Final] c/µL	Copies/ 20µL	[Input] (c/µl)	Dilution factor	c/µl Extract	
C03		Meth Control M.	EVA1	Manual	6272	11260	17532	0.443	530.9	10618	3539	1	3539	
D03		Meth Control M 1/2	EVA1	Manual	3726	12930	16656	0.253	303.6	6072	2024	1	2024	
E03		Meth Control M. 1/4	EVA1	Manual	2002	15225	17227	0.124	148.1	2963	988	1	988	
F03		Meth Control M. 1/8	EVA1	Manual	1009	15429	16438	0.063	76.0	1519	506	1	506	
G03		Meth Control M. 1/16	EVA1	Manual	477	15403	15880	0.030	36.6	731	244	1	244	
H03		Meth Control M. 1/32	EVA1	Manual	230	15309	15539	0.015	17.9	358	119	1	119	

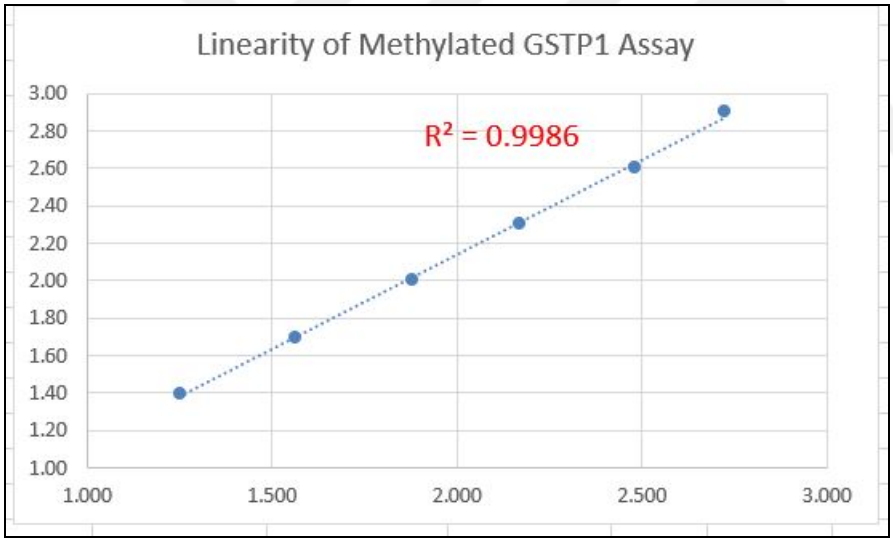


Figure 34. Calibration curve and r^2 of methylated GSTP1 assay

4.5 ddPCR analyses using clinical samples

The first set (Set 1) used for ddPCR analyses was bisulfite converted clinical samples. The second set (Set 2) consist of nested PCR amplicons which was obtained using bisulfite converted DNAs.

4.5.1 ddPCR analyses of Set1

Set 1 is used as template to detect promotor methylation. In control group GSTP1 methylation was not detected and out of the 10 positives detected in the MS-HRM after

nested PCR, only 2 were detected as positive in ddPCR. Figure 35 shows the plots of the GSTP1 methylated samples. Figure 35

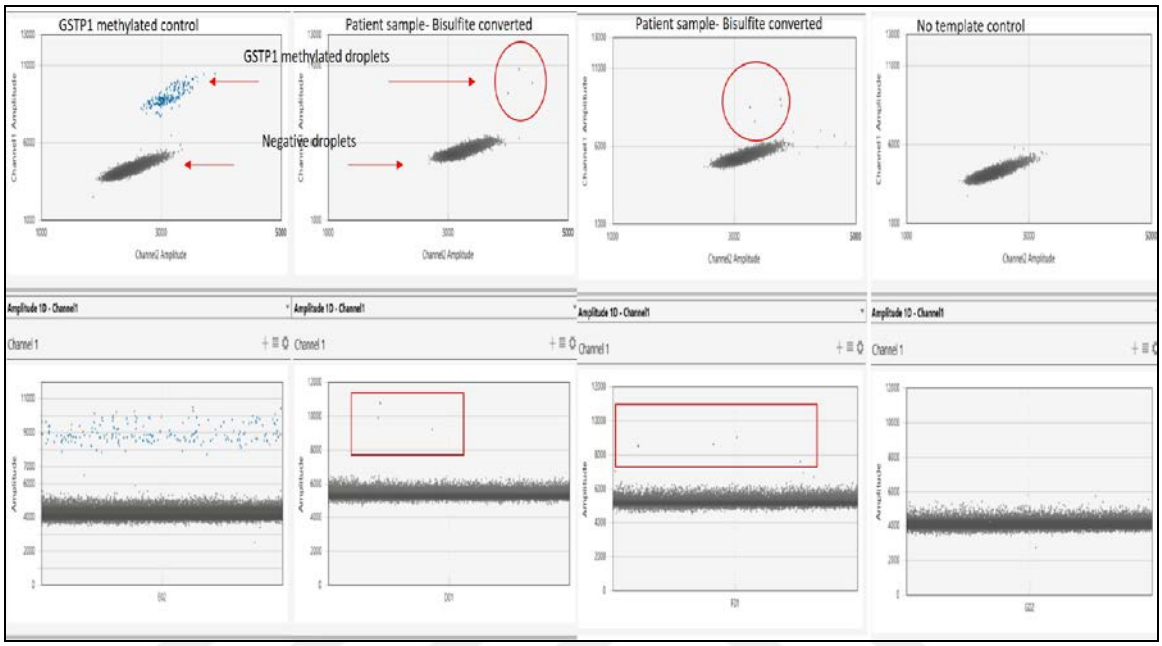


Figure 35. Representative sample for GSTP1 methylated sample from Set1

Table 19. Copy number calculation of samples in Set 1

Exp047 Analysis													
		Reaction volume	20	µl									
		Template volume	5	µl									
		Partition volume	0,00083	µl									
		Droplets/reaction	23980,8	(Assuming all 20 µL is converted into droplets)									
		Template vol/partition	0,00021	µl									
					Imported Data						Calculated Data						
Well	Run #	Sample	Target	Status	Positives	Negatives	Accepted	λ	[Final] c/µL	Copies/ 20µL	[Input] (c/µl)	Dilution factor	c/µl Extract
F01		Unknown	EVA1	Manual	5,000	15580	15585	0,0003	0,4	7,7	1,5	1	1,5
D01		Unknown	EVA1	Manual	3,000	17113	17116	0,0002	0,2	4,2	0,8	1	0,8

4.5.2 ddPCR analyses of Set2

Using bisulfite converted-Nested PCR amplicon ddPCR performed and GSTP1 methylation was not detected in control group. 9 samples which were detected as

methylated by MS-HRM were also found as positive in ddPCR. Copy number calculation and values are given in Table 20.

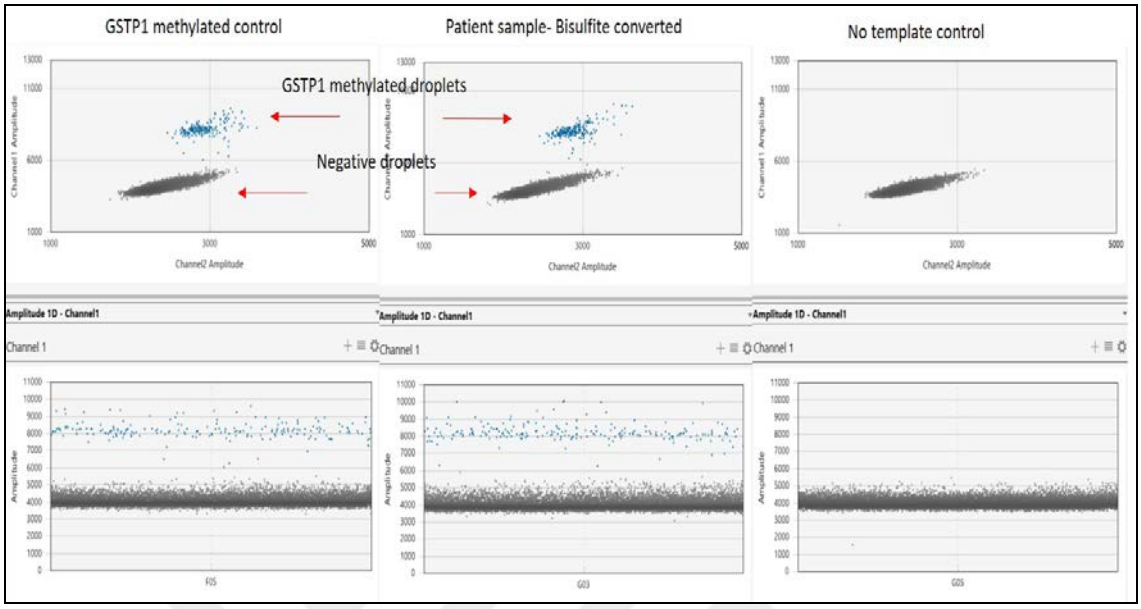


Figure 36. Representative sample for GSTP1 methylated sample from Set2

Table 20. Copy number calculation of samples in Set 2

Exp047 Analysis													
		Reaction volume	20	µl									
		Template volume	5	µl									
		Partition volume	0.00083	µl									
		Droplets/reaction	23980.8	(Assuming all 20 µL is converted into droplets)									
		Template vol/partition	0.00021	µl									
Imported Data								Calculated Data					
Well	Run #	Sample	Target	Status	Positives	Negatives	Accepted	λ	[Final] c/µL	Copies/ 20µL	[Input] (c/µl)	Dilution factor	c/µl Extract of nested PCR
C03		Sample 1	EVA1	Manual	39	13438	13477	0.0029	3.5	69	14	50000	694967
D03		Sample 2	EVA1	Manual	229	12660	12889	0.0179	21.5	430	86	50000	4298997
E03		Sample3	EVA1	Manual	58	14494	14552	0.0040	4.8	96	19	50000	957715
F03		Sample 4	EVA1	Manual	118	13035	13153	0.0090	10.8	216	43	50000	2161108
G03		Sample 5	EVA1	Manual	216	12346	12562	0.0173	20.8	416	83	50000	4159295
H03		Sample 6	EVA1	Manual	252	12658	12910	0.0197	23.6	473	95	50000	4727285
A04		Sample 7	EVA1	Manual	2.00	15826	15828	0.0001	0.152	3.03	0.61	50000	30304
B04		Sample 8	EVA1	Manual	3.00	16186	16189	0.0002	0.222	4.44	0.89	50000	44443
C04		Sample 9	EVA1	Manual	16.00	13919	13935	0.0011	1.378	27.55	5.51	50000	275503

5. DISCUSSION and CONCLUSION

Promotor methylation is a potential biomarker observed in the early stages of tumor formation that can be used for both early diagnosis and follow-up of treatment [15]. In addition, due to being a reversible chemical change, epigenetically silenced drugs can be reactivated using appropriate drugs [32].

There are many promotor methylation studies for many different cancer types. For specific cancer types, different promotor region methylation sites which has high sensitivity and specificity have been identified. CDKN2A gene methylation in lung cancer has been shown to have 100% specificity and 88% analytical sensitivity, GSTP1 gene methylation and 100% specificity and 75% analytical sensitivity in prostate cancer.[21].

Gene methylation studies are performed on invasive biopsy specimens or non-invasive specimens such as plasma, serum, urine or salivary fluid called fluid biopsy. Since biopsy samples are taken by invasive methods, sampling may not always be possible and biopsy may not give precise information about the tumour because it is not homogenous. For these reasons, liquid biopsies have gained great importance in recent studies [25].

As soon as tumour formation begins, the cells separated from the tumour tissue and the DNAs contained in the hydrolysed tumour cells circulate freely in the body fluids. Body fluids contain not only DNA from tumour tissue (ctDNA), but also DNA (cfDNA) from normal cells. When these DNAs were examined in terms of promoter methylation, it was observed that the promoter regions of related genes on ctDNAs were methylated. [2,9]. These DNAs can be obtained by appropriate methods and used in promoter site methylation assays. Based on the knowledge that the amount of ctDNAs circulating in body fluids increases in parallel with the size of the tumour tissue[47], the amount of ctDNA circulating in the early stages of cancer is very small. [9,26]. Therefore, if blood is used as a liquid biopsy sample, leukocytes must be removed from the samples taken within the first half hour to prevent contamination of ctDNAs with leukocyte gDNA. Otherwise, it will not be possible to detect the change in the methylation profile of ctDNA from a sample containing dense leukocyte genomic DNA [48]. Therefore, the most important step of methylation studies is the sample preparation step.

GSTP1 gene is the most commonly used gene in the diagnosis and prognosis of prostate cancer with its high sensitivity and selectivity. [49]. Carmen Jeronimo et al. reported that GSTP1 promoter methylation may be a good biomarker for the diagnosis of prostate cancer patients and PCR-based methods are molecular methods that can be used to detect these biomarkers [50].

Zhang Weijie and colleagues described the GSTP1 gene as an epigenetic biomarker for early diagnosis of prostate cancer [47].

In this study, the methylation profile of GSTP1 gene with high selectivity and sensitivity in the diagnosis and treatment of prostate cancer was investigated in peripheral blood of prostate cancer patients. Studies indicate that serum DNA concentration is higher than plasma DNA concentration [51]. It is also known that analysis of ctDNAs becomes more difficult if leukocytes are not removed during sample preparation [48]. In this study, DNA was isolated from whole blood (leukocytes were not removed) to be used in promoter methylation analysis of the GSTP1 gene. The aim is to show whether methylation analysis can be performed when the patient cannot be re-sampled or using existing samples (contaminated with leukocyte gDNA).

Methylation analysis of the promoter region of DNA isolated from peripheral blood was performed using two different methods. The first method is the MS-HRM method using real-time PCR. When this method was used, GSTP1 methylation could not be detected in any of the patient samples and control group samples. The unmethylated GSTP1 reaction of dense leukocyte gDNA suppressed the methylated GSTP1 reaction and prevented its detection. Digital PCR was used as the second method. By using this method, which divides the prepared reaction mixture into 20000 reactions with approximately nanoliter volume and enables PCR to be carried out in each droplet, 2 of 65 samples could be detected GSTP1 methylated and the concentration of the bisulfite converted samples calculated as 1.5 and 0.8 cp / μL. In the second method used for the analysis of samples, bisulfite converted samples were amplified by nested PCR using methylation independent primers and then analysed by MS-HRM. When this method was applied, 9 of 65 patient samples were found to be GSTP1 methylated, while methylation was not detected in the control group. The same samples were examined by ddPCR and the concentration of the diluted amplicons calculated. In the light of this calculation, 5 of the patients having concentration around 5 cp / μL were evaluated as early stage prostate cancer and 4 of them with higher

number of copies around 20 cp / µL were evaluated as more advanced prostate cancer than the first group.

In this study, it has been shown that GSTP1 promoter methylation can be detected even in the presence of leukocyte gDNA by optimized suitable methods.

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Doctorate	Molecular Medicine	Yeditepe University, Institute of Health Sciences	2019
Master	Clinical Microbiology	Istanbul University, Institute of Health Sciences	2005
University	Biology	Istanbul University, Science Faculty	2002
High school	Science and Mathematics	Pertevniyal High School	1998

	Languages	**Grades**
1	KPDS	72,5

Work Experience (Sort from present to past)

	Position	**Institute**	**Duration (Year - Year)**
1	Senior Researcher	TUBITAK UME - Bioanalysis Laboratory	2016-Ongoing
2	Senior Researcher	TUBITAK UME – Reference Materials Laboratory	2013-2016
3	Researcher	TUBITAK UME - Bioanalysis Laboratory	2001-2013
4	Application Specialist on Life Science Product	Bio-Rad Laboratories	2009-2010
5	Project Moderator	TİBO- Diagnostic Innovative Biotechnology Organization	2005-2007

Computer Skills

Program	**Level**
Microsoft Office Programs	Good

Scientific works

The articles published in the journals indexed by SCI, SSCI, AHCI

Erkan Mozioğlu, Sema Akyürek, Simay Gündüz, Muslum Akgoz, Ahmet C. Gören and Tanıl Kocagöz. Oligomer based real-time detection of microorganisms producing nuclease enzymes. Analyst. 2018, DOI: 10.1039/C8AN02129E
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Others (Projects / Certificates / Rewards)

Novel methods and materials for the detection, traceable monitoring and evaluation of antimicrobial resistance, EMPIR-SRT-h01 AntiMicroResist, 2016-2019, Researcher
Korea-Turkey collaboration on development of an international standard system for measurement of gene methylation, TÜBİTAK-NRF, 2016-2018, Project Coordinator
Traceability for biologically relevant molecules and entities, EMRP-SIB54-BioSITrace, 2013-2016, Researcher
Metrology for monitoring infectious diseases, antimicrobial resistance, and harmful micro-organisms, EMRP-HLT08-INFECT MET, 2012-2015, Researcher
Determination of Mycobacteria species by polymerase chain reaction, TÜBİTAK 1001, 2007-2010, Researcher
Production and certification of hazelnut reference material for trace elements, National Project, 2013-2015, Researcher

International Measurement Comparisons

Relative quantification of genomic DNA fragments extracted from a biological tissue, 2016
An international comparability study on multiple cancer cell biomarker measurement, 2015
Relative quantification of Bt63 in GM Rice matrix sample,2015
Absolute quantification of DNA, 2014
Quantitative analyses of a gene-specific DNA methylation in a genomic DNA matrix, 2013
Relative quantification of Bt63 in GM rice matrix sample, 2013
Measurement of multiple RNA transcripts, 2012
Relative quantification of genomic DNA fragments extracted from a biological tissue, 2010

Guest Researcher Experience

Development of ddPCR Method for Screening Antimicrobial Resistance, LGC (National Metrology Institute of UK), London, United Kingdom, Feb-July 2018
Optimisation of DNA methylation measurements, KRISS (Korea Research Institute of Standards and Science), Deajeon, South Korea, Feb 2017
Optimisation of DNA methylation measurements, KRISS (Korea Research Institute of Standards and Science), Deajeon, South Korea, Feb 2016
Serotyping, phage typing and plasmid profile analyses of Salmonella typhimurium and Salmonella enteritidis strains, BfR (Bundesinstitut für Risikobevertung), Berlin, Germany, Sep-Oct 2004
South Africa & Brazil worked on a project titled “Colorimetric Mycobacterial Culture System” that is supported by the WHO. Within the scope of the project, in Brazil and South Africa two clinical laboratories were visited to provide training of the lab personnel for “Colorimetric Mycobacterial Culture System-TK system”, 14 days, 2006